Research

at the

de Duve Institute

and

Brussels Branch of the Ludwig Institute for Cancer Research

August 2008

The de Duve Institute

THE DE DUVE INSTITUTE: AN INTERNATIONAL BIOMEDICAL RESEARCH INSTITUTE

In 1974, when Christian de Duve founded the Institute of Cellular Pathology (ICP), now renamed the de Duve Institute, he was acutely aware of the constrast between the enormous progress in biological sciences that had occurred in the 20 preceding years and the modesty of the medical advances that had followed. He therefore created a research institution based on the principle that basic research in biology would be pursued by the investigators with complete freedom, but that special attention would be paid to the exploitation of basic advances for medical progress. It was therefore highly appropriate for the Institute to be located on the campus of the Faculty of Medicine of the University of Louvain (UCL). This campus is located in Brussels. The University hospital (Clinique St Luc) is located within walking distance of the Institute.

Benoît Van den Eynde

Emile Van Schaftingen

The main commitment of the members of the de Duve Institute is research. Discovery is the endpoint of their efforts and the only element taken into account for their evaluation. The Institute functions in symbiosis with the Faculty of Medicine and many of its senior members hold a Faculty position and have teaching appointments. The influx of doctoral students and postdoctoral fellows from the University is also a key element in the success of the Institute.

In 1978 the Ludwig Institute for Cancer Research decided to base its Belgian branch within the walls of the de Duve Institute. A fruitful collaboration between the two Institutions has been pursued since that time. Even though the two Institutes are completely independent, the collaboration between the scientists of the de Duve Institute and the Ludwig Institute is extremely close and the sharing of resources is considerable.

The de Duve Institute is managed by a directorate of three scientists, presently composed of Emile Van Schaftingen, Benoît Van den Eynde, and Miikka Vikkula. The directorate is appointed by the Board of directors, which comprises the Rector of the University of Louvain, one of the Pro-rectors, the General Administrator of the University and the Dean of the Faculty of Medicine. Also present in the Board of directors are eminent members of the business community.

About 170 researchers work in the de Duve Institute and in the Ludwig Institute, assisted by a technical and administrative staff of about 80 members. Despite this relatively small size, the de Duve Institute has the ambition of pursuing research projects of high quality under conditions that allow original, long-term projects to be pursued. The Institute has a limited endowment, which is a source of key financing for priority issues, such as the creation of new laboratories for promising young researchers. We expect that the quality of our researchers, supported by sound organisational approaches, will enable the de Duve Institute to stand at the forefront of European Research.

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ACKNOWLEDGMENTS

In 2007, the de Duve Institute has attracted major gifts from several foundations, companies and individuals who have been very generous. These sponsors are providing the resources that enable our scientists to better understand and treat diseases that afflict people around the world. Gifts are the lifeblood of new research initiatives and private resources are crucial in underwriting the costs of new laboratories. On an annual basis, fund-raising from private sources has nearly tripled during the past decade over levels achieved previously and now supports 6 % of the Institute's budget.

The appeal for sponsoring postdoctoral fellowships was also widely followed. In 2007 the Institute has been able to allocate the following fellowships, entirely supported by our donors :

the «Haas-Teichen» fellowship was attributed to Nicolas DIF and Jhansi KOTA

the «Pierre Lacroix» fellowship to Nisha LIMAYE

the «de Visscher» fellowship to Christan PECQUET

two other fellowships were awarded by the Institute to Mariana IGOILLO and Artur CORDEIRO

We express our gratitude to all who contributed to the financing of post-doctoral fellows and state-of-the art research laboratories at the de Duve Institute, ensuring that this institute will remain at the top of the field in biomedical research.

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GENETICS OF HUMAN CARDIOVASCULAR ANOMALIES, CLEFT LIP PALATE AND CEREBRAL TUMORS

The basic aim of our research is to gain insights into the molecular mechanisms underlying a variety of disorders of the cardiovascular and skeletal systems, a well as certain cancers. We are especially interested in evaluating the importance of genetic variation in human disease development. The precise cause of many disorders remains unknown, and current treatments are therefore aimed at alleviating symptoms. Identification of the primary causes as well as modulating factors would allow for the development of treatments that are more "curative" and specific. As this research is based on human DNA extracted from blood and tissue samples obtained from patients, the group works tightly together with several clinicians and multidisciplinary centers worldwide (e.g. Centre des Malformations Vasculaires, Cliniques Universitaires St-Luc; Vascular Anomalies Center, Children's Hospital, Boston, USA, Consultation des Angiomes, Hôpital Lariboisière, Paris, and Centre labio-palatin, Cliniques Universitaires St-Luc).

VENOUS MALFORMATIONS AND GLOMUVENOUS MALFORMATIONS ("GLOMANGIOMAS")

P. Brouillard, V. Wouters, N. Limaye, M. Uebelhoer, V. Aerts, M. Amyere, L.M. Boon, M. Vikkula, in collaboration with B.R. Olsen, Harvard Medical School, Boston, USA; J.B. Mulliken and S. Fishman, Children's Hospital, Boston, USA; O. Enjolras, Hôpital Lariboisière, Paris, France; A. Dompmartin, CHU, Caen, France

Venous malformations (VM) are bluish-purple cutaneous and mucosal vascular lesions. They are often congenital, but can appear later in life. They have a tendency to grow slowly with the growth of the child. Glomuvenous malformations (GVM, "glomangiomas") are a special subtype of venous anomalies (1). They are clinically similar to VMs, yet our clinico-genetic study allowed for their clinical differentiation (2).

We previously discovered that rare, hereditary venous malformations can be caused by

an activating mutation in the endothelial cell receptor tyrosine kinase TIE2/TEK. We employed the DHPLC system, which allows for more efficient and sensitive screening for such mutations, and identified several novel activating mutations amongst affected families. In addition, we hypothesized that as the lesions are localized, a somatic second hit might be needed in the normal allele of the TIE2 gene, for lesions to develop. We have obtained proof for this from one lesion (Wouters et al, Submitted). We have begun to carry out functional analyses of the role of TIE2 in VM-pathogenesis, using a variety of in vitro and in vivo methods. These include the generation of mouse models of the anomaly, by substitution of the normal TIE2 allele with the most frequently mutated form encountered in inherited VMCM.

In contrast to VMs, inherited glomuvenous malformations are caused by mutations in the gene we named "glomulin". Using high-throughput DHPLC mutation-screens, we have discovered that 80% of individuals with GVM show one of eight common glomulin mutations, allowing for efficient genetic diagnosis (Fig. 1). As most of the identified mutations cause premature truncation of the glomulin coding sequence, loss-of-function is the most likely mechanism causing GVM. In four tissues, expression analyses showed significantly reduced levels of the molecule in tissue-derived cDNA, suggesting two-hit mechanism to be true for GUMs, as well (McIntyre et al, In Prep).

Glomulin does not have sequence identities to known proteins, nor does it contain known functional domains. Thus, its molecular function has remained unknown. It is present in almost all tissues, being expressed exclusively in vascular smooth muscle cells. We went on to create glomulin-deficient mice. While homozygous knock-outs are lethal, heterozygotes appear normal (Brouillard et al, Unpublished). We are therefore currently creating conditional knock-out mice, in which to further study the role of glomulin.

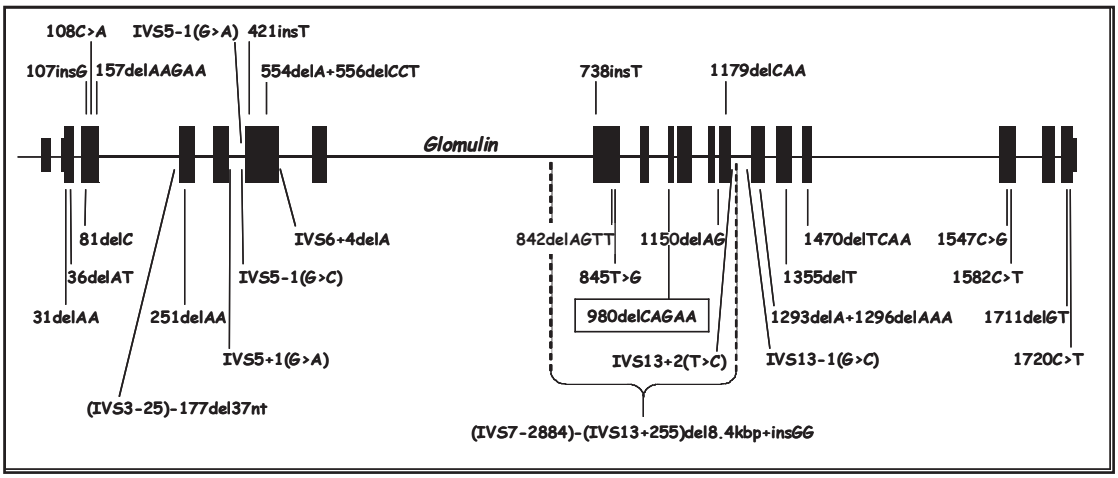

Figure 1. Glomulin gene and mutations. Small boxes: non-coding, and tall boxes: coding exons. The 8 most frequent mutations found amongst 100 families screened, appear above the gene.

LYMPHEDEMA

A. Ghalamkarpour, L.M. Boon, M. Vikkula in collaboration with K. Devriendt, KUL; D. Chitayat, Hospital for Sick Children, Toronto, Canada; K. Alitalo, Haartman Institute and Helsinki University, Finland.

Lymphedema is an external manifestation of lymphatic failure. It may be categorized as primary (idiopathic) or secondary (acquired). Primary hereditary lymphedema can occur at birth (Nonne-Milroy disease) or at puberty (Meige's disease). It is extremely difficult to treat lymphedema, and the patients have a lifetime problem with progressive swelling of extremities. We use genetic approaches to unravel the pathophysiology. In some families with Nonne-Milroy disease, missense inactivating mutations in the VEGFR3 gene were identified. We also recently found that VEGFR3 mutation can cause hydrops fetalis in Nonne-Milroy transmitting families, and that some sporadic congenital primary lymphedemas are explained by a VEGFR3 mutation (3). Moreover, we showed, for the first time, that recessive primary congenital lymphedema can be caused by a particular homozygous VEGFR3 mutation, which has a moderate effect on receptor function and can cause lymphedema only when both alleles are altered (Ghalamkarpour et al, In Prep). In addition, mutations in the transcription factor gene, SOX18, were identified in families with autosomal recessive and dominant hypotrichosis-lymphedema-telangiectasia syndrome.

VASCULAR ANOMALIES AFFECTING CAPILLARIES

N. Revencu, N. Limaye, M. Amyere, L.M. Boon, M. Vikkula in collaboration with J.B. Mulliken, Children's Hospital, Boston, USA; S. Watanabe, Showa University School of Medicine, Tokyo, Japan; A. Dompmartin, CHU de Caen, France; Virginia Sybert, Washington University, Seattle, USA

Capillaries, the smallest blood vessels that connect arterioles to venules, can give rise to various anomalies, two of which are very common: 1) hemangioma, a benign, localized overgrowth of capillary-like vessels, and 2) capillary malformation (CM; commonly known as portwine stain), a localized maldevelopment of capillary like vessels. Hemangiomas have a frequency of up to 12 % in 1-year-old children, and CMs occur in 0.3% of newborns. Whereas hemangiomas usually disappear spontaneously, capillary malformations stay throughout life, if not treated. Other types of cutaneous capillary anomalies also exist. In addition, some can affect other organs, such as the brain, in case of CCMs, cerebral capillary malformations.

As the molecular mechanisms leading to these localized capillary lesions are unknown, we have collected clinical information and samples from families in which more than two individuals are affected. These studies led to the discovery that inherited hyperkeratotic cutaneous capillary-venous malformations (HCCVM) associated with cerebral capillary malformations are caused by a mutation in the KRIT1 (Krev interaction trapped 1) gene. This suggested that KRIT1, a possible intracellular signaling molecule, is important not only for cerebral but also for cutaneous vasculature. In addition, a genome-wide linkage mapping on families with inherited capillary malformations led us to identify a linked locus CMC1. Screening of positional functional candidate genes resulted in the identification of mutations in the RASA1 gene, a modifier of Ras signaling pathway. This implies that RAS signaling pathway modulators may serve as a novel therapy for these patients in the future. Ongoing studies have led to the identification of 54 additional families with RASA1 mutation, accounting for about 30% of those affected. This has allowed for a more precise clinical description of the clinical signs and symptoms associated with this newly recognized disorder that we have named: Capillary Malformation-Arteriovenous Malformation (CM-AVM) (4).

CARDIOPATHIES

I. Gutierrez-Roelens, M Amyere, M. Vikkula, in collaboration with T. Sluysmans, C. Ovaert, St-Luc, UCL and M. Gewillig and K. Devriendt, KUL

The cardiovascular system can encounter developmental problems affecting the heart. These cardiac defects or cardiopathies vary from physiological septal defects to life-threatening complex malformations. To investigate the molecular mechanisms behind these phenotypes, we collect samples from families with possibly hereditary cardiopathies. In three families, in which atrial septal defect is associated with progressive atrioventricular conduction defect, we identified three novel mutations in the CSX/Nkx2.5 gene, an important transcription factor for cardiac development. Identification of mutation carriers is crucial, as in the few studied families the first "symptom" has sometimes been sudden death. Identification of mutations allows for genetic testing in families, enabling tight follow-up and preventive pacemaker implantation.

More recently, we have performed a whole-genome linkage analysis using the 10K Affymetrix SNP-chips and identified a possible locus for a gene causing heterotaxia, situs inversus (5).

CLEFT LIP AND PALATE

M. Ghassibé, L. Desmyter, N. Revencu, M. Vikkula, in collaboration with B. Bayet, R. Vanwijck, N. Deggouj, and Y. Gillerot, St-Luc, UCL

Cleft lip and palate (CLP) is a congenital anomaly of complex etiology. Predisposition is governed by numerous genetic loci, in combination with environmental factors. Clefts have an incidence of 1/700 births.

We have collected DNA samples from a large number of patients affected with van der Woude syndrome, the most common cleft syndrome, and showed that IRF6 is the major causative gene in our Belgian cohort. Moreover, IRF6 is the gene responsible for the popliteal pterygium syndrome. This study in turn led to several collaborations that allowed us to carry out a genotype-phenotype correlation on hundreds of patients from different ethnic backgrounds. Results showed that IRF6 is mutated in 69% of VWS patients and 97% of PPS patients. Interestingly, mutation-distribution is non-random: 80% are localized in IRF6 exons 3, 4, 7 and 9 for VWS, and 72% in exon 4 for PPS patients. These findings are of great importance for clinical diagnosis, mutational screens and genetic counseling. We also demonstrated that IRF6 predisposes to non syndromic clefts in Europe (6).

CEREBRAL TUMORS

T. Palm, M. Vikkula, in collaboration with C. Godfraind, Laboratory of Neuropathology, St-Luc, UCL

Morphological characterization and classification of tumors is not always clear. Thus, better (molecular) criteria are needed. We are especially interested in two types of cerebral tumors: oligodendrogliomas and ependymomas. Using array-CGH, we recognized a subgroup of supratentorial ependymomas affecting young adults, which are characterized by trisomy of chromosome 19 (7). To even better understand the molecular alterations leading to ependymomal oncogenesis, we performed microarray-based expression profiling on a series of 34 frozen ependymomas. Results of our profiling study are in concordance with the "oncology recapitulates ontology" hypothesis, in which genes implicated in stem cell fate decisions may be important for supporting cancer stem cells as well. Pathways activated in high grade ependymomas were consistent with the histological appearance of a more aggressive tumor phenotype (Palm et al, In Prep).

NEUROENDOCRINE TUMORS

A. Persu, Division of Cardiology, Saint-Luc, UCL; M. Amyere, A. Van Egeren, M. Vikkula, in collaboration with P. Rustin, INSERM U676, Hôpital Robert Debré, Paris, France.

Pheochromocytomas and head and neck paragangliomas are neuroendocrine tumours derived from the neural crest. Paragangliomas are associated with parasympathetic ganglia and are usually non-secreting. By contrast, pheochromocytomas are derived from paraganglia associated with the orthosympathetic system and are characterized by increased secretion of catecholamines and paroxystic hypertension.

We are studying the nature and frequency of mutations in the known predisposing genes in pheochromocytoma and paraganglioma from Belgium, to detect possible genotype-phenotype correlations. A multicentric collaboration including the main academic centers from Belgium has therefore been established.

The SDH genes code for the subunits of succinate deshydrogenase, at the crossroad of the mitochondrial respiratory chain and Krebs cycle. Three of the four subunits of succinate deshydrogenase, i.e. SDHD, SDHB, and more rarely SDHC, have been associated with paraganglioma and pheochromocytoma. Furthermore, SDHB mutations have been associated with an increased risk of recurrent and malignancy in several European series. In our study, the prevalence of SDHB was unexpectedly high in head and neck paraganglioma. Surprisingly, tumours associated with such mutations were mainly late-onset unilateral tumours without evidence of recurrence or malignancy (8). We also described a family with a very rare presentation of severe head and neck paraganglioma with liver and spine localization. No evidence of mutation was found in the known predisposing genes by dHPLC and/or SSCP. An in depth search for the genetic abnormality underlying this unusual form of paraganglioma is currently under way.

ESSENTIAL HYPERTENSION

A. Persu (Division of Cardiology, Saint-Luc, UCL), N. Limaye, and M. Vikkula.

High blood pressure - commonly called hypertension - is found in almost 20 % of the adult population worldwide and affects 2 million Belgians. Despite the wide range of antihypertensive therapies available, blood pressure is adequately controlled in only 30-40% of hypertensive patients. In a large majority of cases, no specific cause is found ("essential hypertension") and high blood pressure is thought to reflect the interplay of lifestyle (obesity, high salt intake) and genetic factors; 30-50 % of blood pressure level is thought to be genetically determined. Despite this, conventional linkage and association studies have failed to establish the role of genetic variants in susceptibility.

In an effort to find genetic variations that account for a significant proportion of blood pressure heritability, and to study the interactions between known variants with mild to moderate effects, we set up a multicentric national genome wide association study (HYPERGEN) with the support of the Belgian Hypertension Committee. We are recruiting at least 1000 hypertensive patients and 1000 normotensive subjects. Detailed phenotyping including renin and aldosterone dosages are obtained in all patients, and genotyping will be performed using SNP chip technology.

HEMATOLOGICAL MALIGNANCIES

H. Antoine-Poirel, V. Havelange, F. Duhoux, M. Herman, G. Ameye, K. Bahloula, Human Genetics Center, St.Luc, UCL; with M. Vikkula

The genetic nature of hematological malignancies (mainly leukemias, myelodysplasias, chronic myeloproliferative disorders, lympho-

mas, myelomas) has been clearly established. Despite this, we lack biomarkers for diagnosis, prognosis and treatment, for clinical management as well as for a better understanding of the genetic and epigenetic processes leading to tumorigenesis. Towards this end, we use a variety of techniques including conventional and molecular cytogenetics or FISH, molecular biology, and microarray approaches.

In an international therapeutic trial of children with mature B-cell lymphomas, we showed the adverse prognostic impact of chromosomal alterations of 13q and 7q, detected by cytogenetics. Furthermore, using genome-wide SNP array technology, we found that most 13q alterations lead to an amplification of the microRNA 17-92 cluster, known to interact with the MYC oncogene, a finding confirmed with mature miRNA expression profiling. In addition, we detected numerous cryptic genomic alterations including partial uniparental disomies. Their prognostic value is currently under study in collaborations within different therapeutic trials across Europe.

SNP-CHIP PLATFORM

M. Amyere and M. Vikkula

We host the DNA-oligonucleotide microarray platform of the Medical Faculty of UCL (Affymetrix). This platform is currently used by several groups in the de Duve Institute and UCL for expression profiling as well as genotyping. In collaboration with Dr Jüppner from Harvard Medical School, Boston, we genotyped a large family with a new form of hypophosphatemia and mapped this autosomal recessive form (designated ARHP) to chromosome 4q21. This allowed for the identification of causative homozygous mutations in DMP1 (dentin matrix protein 1), which encodes a non-collagenous bone matrix protein expressed in osteoblasts and osteocytes (9). In collaboration with Dr Vermeesch from K U Leuven, we genotyped a large family with autosomal-dominant microtia. Copy number analysis led to the identification of five tandem copies of a copy number-variable region at chromosome 4p16, linked to the disease (10). Moreover, in collaboration with G Matthijs from KULeuven, we have been able to combine genotyping and expression profiling in one consanguineous family with a congenital glycosylation disorder (CDG). Autozygosity mapping along with expression profile analysis allowed us to identify a new gene for CDG (unpublished).

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LIVER AND PANCREAS DEVELOPMENT

The group studies the molecular and cellular mechanisms that govern development of the liver and pancreas, two organs which play essential metabolic roles and which derive from the endoderm (primitive gut of the embryo). The fundamental knowledge gained by this work is essential for improving cell therapy of liver and pancreatic diseases, such as liver deficiencies (metabolic disease, acute hepatitis, cirrhosis) and diabetes, and for understanding the pathophysiology of organ malformations.

LIVER DEVELOPMENT

A. Antoniou, I. Laudadio, S. Margagliotti, P. Raynaud

The main cell types of the liver are the hepatocytes, which exert the metabolic functions of the organ, and the biliary cells which delineate the bile ducts. We study how the hepatocytes and biliary cells differentiate and how bile ducts are formed in the embryo. Our preferred model organism to investigate liver development is the mouse, and this includes generation and analysis of transgenic mouse lines.

The biliary tract is constituted by intrahepatic bile ducts which collect the bile produced by the hepatocytes, and by extrahepatic ducts which drain the bile from the liver to the intestine. Biliary cells, also called cholangiocytes, delineate the lumen of the bile ducts and modify the composition of the bile. These cells, like hepatocytes, derive from liver progenitor cells called hepatoblasts. Our discovery of the Onecut transcription factors Onecut-1 (OC-1/HNF-6), OC-2 and OC-3, and the subsequent phenotypic characterization of HNF-6 and OC-2 knockout mice lead to the identification of the first transcriptional network regulating bile duct development. Current efforts are de-

voted to the characterization of the transcription factors and signal transduction pathways that control bile duct development.

We have shown that the Transforming Growth Factor-beta (TGF-beta) signaling stimulates differentiation of biliary cells. TGF-beta signaling is detectable in the liver as a gradient, with high signaling activity near the portal vein, where biliary cells differentiate, and lower signaling activity in the parenchyma, where hepatocytes differentiate. The results of this research are now used by collaborating teams who attempt to program in vitro differentiation of stem cells to hepatocytes for cell therapy of liver disease. Our efforts in 2007 were concentrated on the understanding of the control of TGF-beta signaling, and on how this signaling influences the formation of bile ducts.

In collaboration with clinical centers, we investigate how the knowledge gained from our fundamental studies can be translated in the understanding of the pathophysiology of human biliary diseases. The latter include biliary atresia associated with ductal plate malformations. They also include ciliopathies, i.e. diseases that are characterized by dysfunction of a small organelle called cilium whose dysregulation leads to polycystic malformations. In 2007, we showed that fetuses affected with Meckel syndrome - a polymalformative disease with cilium and biliary anomalies - display an unexpected variability in the presence or absence of cilia on the biliary cells. Moreover, we found that the livers have abnormal hepatocytes. In contrast to current beliefs, our findings indicate that the liver deficiency in Meckel syndrome patients is not restricted to bile ducts, and most likely results from an early defect affecting the differentiation of liver cells at the progenitor cell stage.

Our previous work on Onecut transcription factors has also uncovered that they control the initiation of liver development, i.e. the budding of the organ out of the primitive gut. In 2007 we further investigated the role of the Onecut factors HNF-6 and OC-2 and found that in their absence, the onset of liver growth is retarded as a result from deficient cell migration. In the latter process, metalloproteases are involved and HNF-6 and OC-2 control a network of genes required for cell adhesion and migration. This network comprises osteopontin, thrombospondin-4 and E-cadherin. Since hepatic cancer cell invasion and metastasis are associated with E-cadherin repression and expression of osteopontin, our findings in deve-

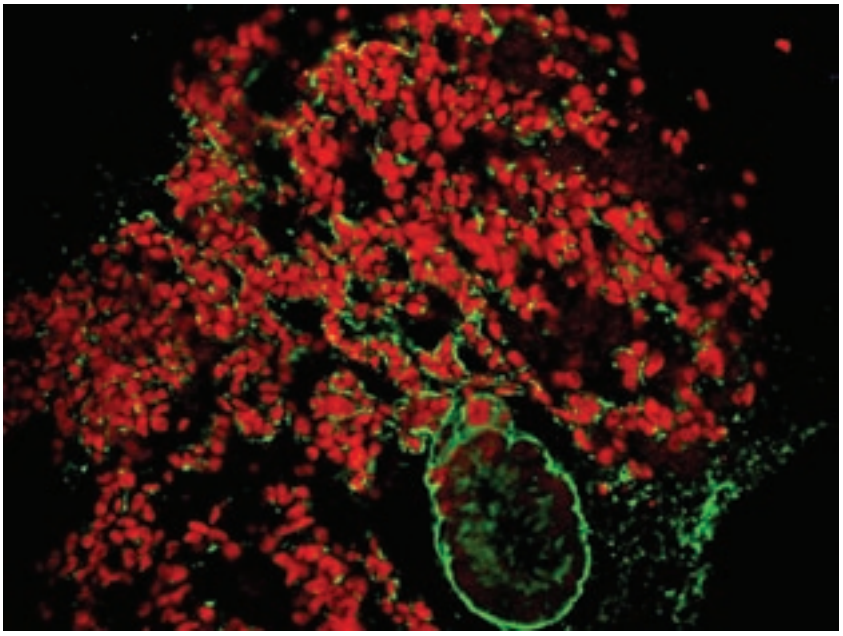

Fig. 1. At the onset of liver development the hepatoblasts, stained in red for expression of the transcription factor HNF-4, migrate out of the primitive gut. The latter is delineated by a layer of laminin (green).

loping liver raise the possibility that a network regulated by HNF-6 and OC-2 plays a role in hepatic cancer.

PANCREAS DEVELOPMENT

E. Heinen, A. Simion, C. Pierreux

In the embryo, the pancreas develops as an outgrowth of the endoderm which is the cell layer that delineates the primitive gut. Pancreatic progenitors derived from the endoderm form two buds (dorsal and ventral) which later fuse to form a single organ. Within these buds the progenitor cells give rise, through a stepwise process, to endocrine cells that produce insulin, glucagon, somatostatin, pancreatic polypeptide or ghrelin. The endocrine cells associate to form the islets of Langerhans. Our group investigates the transcriptional mechanisms and signal transduction pathways that control how endoderm cells become pancreatic progenitors and how the latter develop into endocrine precursor cells. This research is currently pursued using the mouse, including transgenic mice, as a model organism.

In 2007 we investigated how the Onecut transcription factors and FGF-10 signaling control development of pancreatic progenitors from the endoderm, and the subsequent generation of endocrine precursor cells. The Onecut transcription factors HNF-6 and OC-2 exert redundant roles in pancreas morphogenesis and in differentiation of endocrine cells. FGF-10 is produced by the mesenchyme surrounding the developing pancreas and sustains the development of the pancreas. The results of this research are being translated in the design of cell therapy of diabetes by collaborating teams who attempt to differentiate stem cells to insulin-producing cells. These collaborations include our participation to the EU-sponsored network BetaCellTherapy.

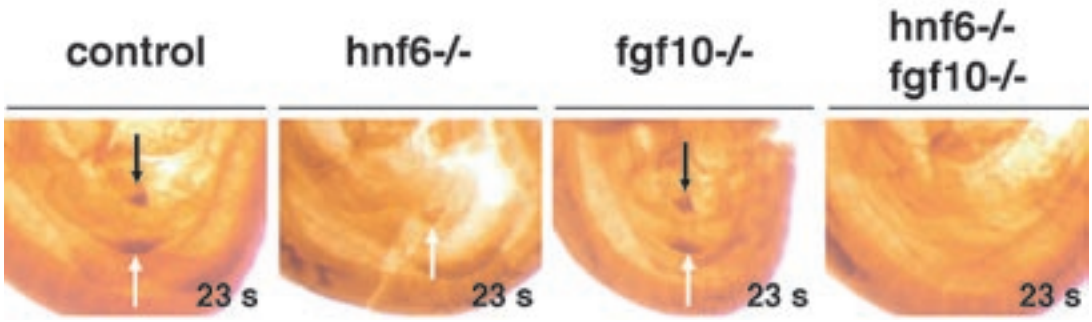

Fig. 2. Embryos that are wild-type or knockout for HNF-6 and/or FGF-10 were immunostained for the pancreatic marker Pdx1 (brown staining). The embryos were analysed at the onset of pancreas development and a blow-up of the abdominal region is shown with arrows pointing to the pancreatic buds. The size of the pancreatic buds is reduced in hnf6-/- and fgf10-/- embryos; in double knockouts the pancreas does not develop.

During pancreas development, the progenitor cells also give rise to exocrine and ductal cells. Our work addresses how pancreatic ducts are generated. These ducts drain the secretions from the pancreatic exocrine cells to the intestine and are delineated by ductal cells. We have shown that the Onecut factor HNF-6 controls a network of genes that is required for the formation of cilia at the apical pole of the ductal cells and for normal develo-

pment of the ducts. In the absence of HNF-6, the ducts form cysts, much like in human polycystic diseases.

Work by our and other teams has provided evidence that ductal cells can transdifferentiate to pancreatic endocrine cells. Current work investigates the transcriptional mechanisms that govern this transdifferentiation process.

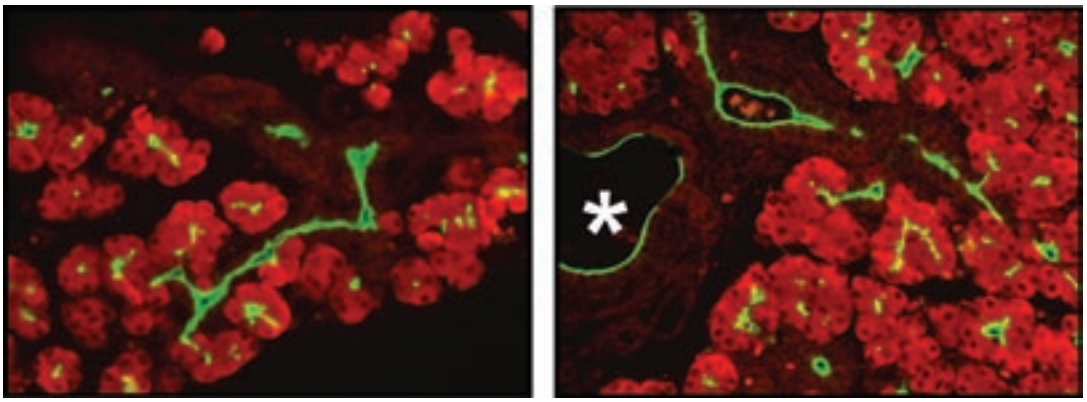

Fig. 3. In wild-type embryos (left panel), the lumen of ducts are delineated by Mucin-1 (green) expressed at the apical pole of the ductal cells. In HNF6 knockout embryos (right panel), the pancreatic ducts form cysts (asterisk). The cells are stained for expression of carboxypeptidase (red).

CONCLUSIONS

Our work on the signaling pathways and transcription factors in developing liver and pancreas opens perspectives for understanding the pathophysiology of liver and pancreatic congenital diseases. The application of our findings to the programmed differentiation of cultured stem cells should help developing cell therapy of hepatic deficiencies and of pancreatic diseases such as diabetes.

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DNA DAMAGE REPAIR AND TELOMERE MAINTENANCE

Cellular DNA is constantly subjected to damage. Genotoxic lesions include DNA double-strand breaks (DSBs) which can be caused either by external agents such as ionizing radiations or by physiological cellular processes such as V(D)J recombination in the immune system or meiosis. In dividing cells, DNA replication provides another major source of DSBs. Cells from all organisms have evolved several mechanisms to re-seal DSBs as proper repair of chromosome breaks is necessary to prevent genomic rearrangements, a hallmark of cancer cells, or cell death. DNA repair mechanisms have been well conserved throughout evolution and yeast has proven to be a good model for their study.
In the absence of any protection, extremities of linear chromosomes, like human chromosomes, would also be recognized as DSBs by the cell and subjected to DNA repair, resulting in chromosomal fusions. To circumvent this problem, chromosomal extremities are protected by specialized DNA-protein complexes, the telomeres. Synthesis of telomeric DNA repeats requires the ribonucleoprotein enzyme telomerase in replicating cells. In most adult cells, telomerase gene expression is switched off as cells have stopped dividing. Hence, proliferation of cancer cells requires the re-establishment of a telomere maintenance mechanism. In about 80 % of the cases, this is achieved by re-activation of the telomerase gene during tumorigenesis while 20 % of cancer cells rely on telomerase-independent mechanism(s) to maintain their telomeres.

DNA DAMAGE REPAIR IN FISSION YEAST SCHIZOSACCHAROMYCES POMBE

A. Decottignies

Chromosomal and extrachromosomal DSBs can be induced experimentally in virtually any kind of cell. Such systems led to the dissection of the two major mechanisms of DNA repair: homologous recombination (HR) and non-homologous end-joining (NHEJ). In the lab, fission yeast (Fig. 1) was used to investigate genetic requirements for microhomology-mediated end- joining (MMEJ), a third DNA repair process poorly characterized so far. Construction of a series of yeast mutants revealed that MMEJ is a DNA repair pathway related to HR [1]. Specifically, MMEJ was found to require *rad22* and *exo1*, two genes implicated in the single-strand annealing mechanism of HR. Mismatch repair genes are also involved in the process. Finally,

Figure 1. *Schizosaccharomyces pombe.* **(Image courtesy of Prof. Rosa Aligue Alemany, University of Barcelona, Spain)**

the study investigated the critical number of microhomologous nucleotides required for efficient MMEJ as well as the distance between DSB end and the microhomologous region [1].

From yeast to mammals, different studies reported the insertion of DNA fragments of various sources at experimentally-induced DSBs, including mitochondrial DNA (mtDNA) in budding yeast and repetitive DNA in mammalian cells. Interestingly, recent studies reported the association of human genetic diseases with *de novo* insertions of mtDNA in the nuclear genome, including a patient exposed to Chernobyl radiations. A new simple extrachromosomal DSB repair assay in fission yeast revealed that DSB repair is associated with the capture of endogenously produced mtDNA fragments in nearly 30 % of the events [2], supporting the hypothesis that DSB repair is a universal mutagenic mechanism responsible for the insertion of linear mtDNA molecules into chromosome breaks, providing a novel mechanism of human inherited disease. Moreover, systematic sequencing of budding yeast and human nuclear genomes revealed the presence of nuclear sequences of mitochondrial origin (NUMTs) in chromosomes. Similarly, the *S. pombe* nuclear genome comprises 33 NUMTs (22-358 bp-long) [2], a value close to that reported in budding yeast. For comparison, it has been reported that the human nuclear genome comprises between 211 and 612 NUMTs, depending on the threshold values used for BLAST analysis. Hence, it appears that capture of mtDNA fragments at naturally occurring DSBs took place during evolution in eukaryotic cells, remodeling the nuclear genome. Careful analysis of NUMT distribution in both budding and fission yeast nuclear genomes was carried out and revealed a preferential insertion of NUMTs into intergenic regions, with no preference for promoters versus 3' intergenic regions. Analysis of the impact of NUMT insertion into promoter region on gene expression level is currently under investigation.

TELOMERASE AND ALTERNATIVE MECHANISM(S) OF TELOMERE MAINTENANCE

Influence of telomere maintenance mechanism on gene expression in human fibroblasts

G. Tilman, M. Mattiussi, A. Decottignies

Activation of a telomere maintenance mechanism is indispensable for the immortalization of human cells. Most cancer cells maintain their telomeres via telomerase activation. In some cancers, however, telomeres are maintained in the absence of telomerase activity by one or more mechanisms that are

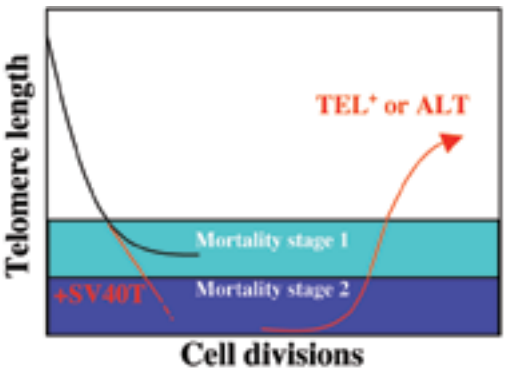

Figure 2. Telomere length in the course of cellular transformation. In telomerase-negative cells, telomere length decreases with continuous replication. At a critical telomere length, cells enter M1 through the action of p53/pRb and eventually die. Upon inactivation of p53/pRb, cells continue to divide and enter M2 characterized by huge genomic instability. A small percentage of cells survive by re-activation of a telomere maintenance mechanism.

known as alternative lengthening of telomeres (ALT). ALT cell lines can also be obtained after *in vitro* immortalization of telomerase-negative human fibroblasts with SV40 T antigen. Such a procedure has indeed been reported to give an average of 40 % ALT and 60 % TEL+ survivors following crisis which occurs after about 80 population doublings, when telomeres have reached a critical length (Fig. 2). These two pathways of telomere maintenance are very distinct phenotypically (Fig. 3). In telomerase-expressing cells (TEL+), telomere length is very homogenous (around 5 kb) and telomeres are found at the end of all chromosomes. However, in ALT cells, telomeres are very heterogeneous, ranging from 0 to 50 kb and some chromatids lack telomeres (Fig. 3).

In addition to its role in telomere length maintenance, hTERT has been reported to play non-canonical roles in the cell, including modulation of expression of genes implicated in tumorigenesis, through mechanisms that are still largely unknown.

Our study aims to identify genes that are distinctly regulated in ALT and TEL+ cells. To achieve this goal, we compared the global gene expression profiles of ALT and matching TEL+ human immortalized fibroblasts by RDA (Representational Difference Analysis). We identified a series of genes showing distinct expression levels in ALT and TEL+ cell lines. Genes with higher expression in TEL+ cells included periostin (*POSTN*), *COL6A3,* fibronectin *(FN1)* and *ACTG2*. On the opposite, the expression levels of tumor-suppressor gene *DAL1* and *IGSF4C* were higher in ALT cells. We are currently investigating the effect of telomerase (hTERT and/or hTR subunits) on the expression of these genes in both normal and immortalized human fibroblasts. We showed that hTERT expression in fibroblasts partially rescues the senescence-associated decrease of expression of a subset of genes like *COL6A3*. On the other hand, we test the effect of telomerase on TGF-β signaling in human fibroblasts. We are also currently investigating the impact of hTERT on TGF-β-induced cell cycle arrest.

Our work also focused on the distribution of *POSTN* periostin gene expression in both normal and tumoral tissues. *POSTN* encodes a secreted protein which, through binding to αVβ3 or αVβ5 integrins, promotes metastatic growth, angiogenesis and cell motility. Our results suggest that periostin expression may be

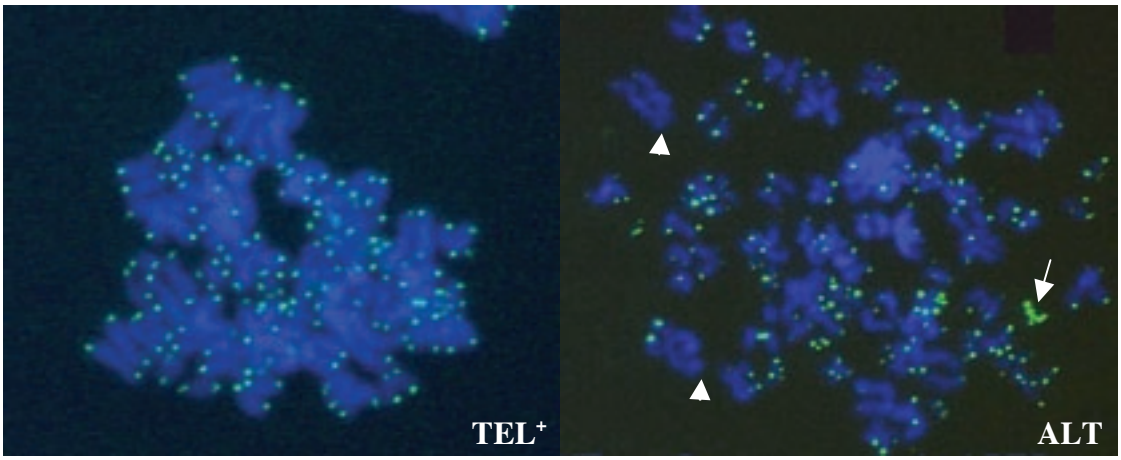

Figure 3. Telomere-specific fluorescence in situ hybridization (FISH) on metaphase chromosomes of telomerase-positive and ALT cancer cells. Telomeres are hybridized with a fluorescent telomeric probe (green) and DNA is stained with DAPI (blue). In ALT cells, telomeres are very heterogeneous and not always present at chromosome ends (arrowheads). ALT cells are further characterized by the presence of extrachromosomal telomeric DNA (arrow).

mediated almost exclusively by stromal fibroblasts [3]. We are currently investigating the factors that modulate periostin gene expression in fibroblasts. Our data indicate that POSTN expression in human fibroblasts is modulated by TGF-β and conditioned media from tumor cell line cultures.

Telomere maintenance and subtelomeric DNA methylation

G. Tilman, A. Van Beneden, A. Decottignies

Previous work from the group of M. Blasco (Madrid, Spain) revealed that subtelomeric DNA methylation status affects telomeric sister chromatid exchange (T-SCE) in mouse. As ALT cells rely on T-SCE to maintain their telomeres, we started to analyze the subtelomeric DNA methylation status in matching ALT and TEL+ SV40-immortalized fibroblasts. The same analysis is carried out on U2OS osteosarcoma ALT cells and hTERT/hTR–retrovirally infected U2OS cells that express active telomerase.

Our preliminary results revealed that reduced subtelomeric DNA methylation may not be required for maintenance of ALT mechanism in SV40-immortalized human fibroblasts. On the other hand, our data suggest that telomerase may modulate subtelomeric DNA methylation level. We are currently investigating this hypothesis.

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EPIGENETIC ALTERATIONS IN CANCER

Deregulation of normal gene expression patterns can give rise to cellular dysfunctions, which can lead to malignancy. Chromatin modifications are key to gene regulation. Such modifications are qualified as "epigenetic" because they induce heritable changes without change in the DNA sequence. DNA methylation, which is associated with transcriptional repression, is an essential component of the epigenetic machinery. Cancer cells often show widespread loss of DNA methylation. This appears to promote tumor development by inducing genome instability. Studies from our group, have shown that hypomethylation in tumors is also associated with aberrant activation of a group of germline-specific genes, which we termed "cancer-germline" genes. We are currently investigating the mechanisms that target DNA demethylation towards these "cancer-germline" genes in tumor cells.

To date, ~50 cancer-germline genes or gene families have been identified. Some of these have been isolated by us (1,2). Cancer-germline genes are activated in a wide variety of tumors. Importantly, their activation results in the production of tumor-specific antigens, and clinical trials of therapeutic vaccination of cancer patients against these antigens are underway. It is not yet clear if the expression of cancer-germline genes in tumors is a functionally irrelevant by-product of cellular transformation or if it corresponds to the activation of a "gametogenic program" that favors tumor development.

Cancer-germline genes tend to be co-expressed in tumors, suggesting that they share, at least in part, a common mechanism of regulation. Past work from our group showed that DNA methylation is an essential component of the repression of cancer-germline genes in normal somatic tissues (3). Cancer-germline gene promoters are methylated in normal somatic tissues, and unmethylated in testicular germ cells. They become demethylated in a number of tumors, and this is associated with transcriptional activation. Hypomethylation of cancer-germline gene promoters in tumors appears to be a consequence of the global demethylation process commonly observed in cancer.

MECHANISMS OF DEMETHYLATION OF CANCER-GERMLINE GENES IN TUMOR CELLS

The process leading to hypomethylation of DNA sequences in tumors remains obscure. We undertook to address this issue by using MAGEA1, the founding member of the cancer-germline group of genes, as a model. Detailed methylation analyses of the MAGEA1 genomic locus in expressing tumor cells, revealed preferential hypomethylation within

the 5' region of the gene (4). Furthermore, transfection experiments with in vitro methylated MAGEA1 constructs, indicated that this site-specific hypomethylation relies on a historical event of DNA demethylation, and on the presence of appropriate transcription factors to protect the region against subsequent remethylation (4,5). The proposed model of MAGEA1 demethylation and activation during tumor development is illustrated in Figure 1. The factors that are responsible for the initial DNA demethylation process and for maintaining cancer-germline gene promoters unmethylated remain to be identified.

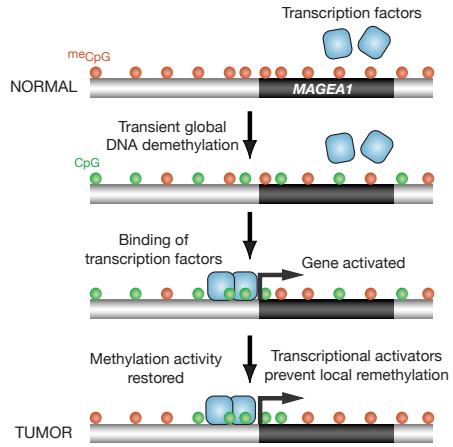

Figure 1. Model for the stable activation of MAGEA1 in tumors.

BORIS: AN ACTIVATOR OF CANCER-GERMLINE GENES ?

In collaboration with O. Kholmanskikh, F. Brasseur, and E. De Plaen, Ludwig Institute for Cancer Research, Brussels

The germline-specific gene BORIS (Brother Of the Regulator of Imprinted Sites), which encodes an 11-zinc-fingers transcriptional regulator, was recently qualified as a new cancer-germline gene, as it was found to be activated in a variety of tumor samples.

Moreover, it was suggested that BORIS might be responsible for the activation of most other cancer-germline genes, including gene MAGE-A1, in tumors.

We evaluated the frequency of BORIS activation in human melanoma by quantitative RT-PCR. BORIS activation was detected in a significant proportion of melanoma tissue samples (27%, n=63). Surprisingly, many melanoma samples expressed MAGE-A1 and other cancer-germline genes in the absence of BORIS activation, suggesting that BORIS is not an obligate factor for activation of these genes in melanoma. Consistently, forced expression of BORIS in melanoma cell lines did not induce expression of MAGE-A1. Similar results were obtained when inducing BORIS expression in other human cell types, including immortalized keratinocytes and normal fibroblasts. We conclude that, whereas BORIS may serve as a useful target for immunotherapy of melanoma, it is neither necessary nor sufficient for the activation of other cancer-germline genes (6).

MOUSE EMBRYONIC STEM CELLS AS A MODEL TO STUDY MAGEA1 DEMETHYLATION

In collaboration with O. De Backer, Molecular Physiology Research Unit, FUNDP, Namur

Studies aiming at understanding the process of DNA demethylation in tumors require the identification of cell lines that possess ongoing DNA demethylation activity. Our studies suggest that this activity does not persist in most tumor cell lines. We therefore turned to mouse embryonic stem (mES) cells as a potential system to study the DNA demethylation process, because these cells appear to be characterized by a high level of DNA methylation plasticity. It has been shown that mES cells have both

demethylating and de novo methylating activities. Each of these opposing activities appears to be targeted to selected DNA sequences. Interestingly, in vitro methylated human MAGEA1 transgenes became demethylated following transfection into mES cells (7). Demethylation was targeted to the 5' region of MAGEA1 and was strongly reduced on mutated MAGEA1 transgenes exhibiting impaired promoter activity. Mouse ES cells appear therefore as a valuable experimental system to study the mechanisms of DNA demethylation within MAGEA1 sequences.

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PROTEIN REPAIR AND INBORN ERRORS OF METABOLISM

Our laboratory has a longstanding interest in the metabolism of carbohydrates and related compounds. The study of the mechanism of formation of an intriguing phosphate ester, fructose 3-phosphate, led us to identify fructosamine 3-kinase [2], an enzyme which proved to be a repair enzyme permitting the removal of sugar adducts from proteins (deglycation). More recent work has led us to identify additional enzymes that are potentially also involved in deglycation, but if and why protein deglycation is important is still an open question. Our laboratory aims also at identifying the enzymatic defects underlying 'new' inborn errors of metabolism.

PROTEIN DEGLYCATION

Y. Achouri, F. Collard, J. Drozak, J. Fortpied, R. Gemayel, A. Preumont, K. Peel, T. Sokolova, M. Veiga-da-Cunha, E. Van Schaftingen in collaboration with M.H. Rider and D. Vertommen, Horm Unit

Fructosamine 3-kinase

Chronic elevation of the blood glucose concentration in diabetes appears to be responsible for the long-term complications of this disease. The link between the elevated concentration of glucose and the development of these complications is not yet clear. One of the theories on this link emphasizes the role of fructosamines. These are formed through a spontaneous reaction (known as 'glycation') of glucose with primary amines, followed by an Amadori rearrangement. Fructosamine 3-kinase (FN3K) is a recently identified enzyme [3] that phosphorylates both low-molecular-weight and protein-bound fructosamines. Fructosamine 3-phosphates are unstable, brea-

king down spontaneously to 3-deoxyglucosone, inorganic phosphate and the amino compound that originally reacted with glucose (Fig. 1).

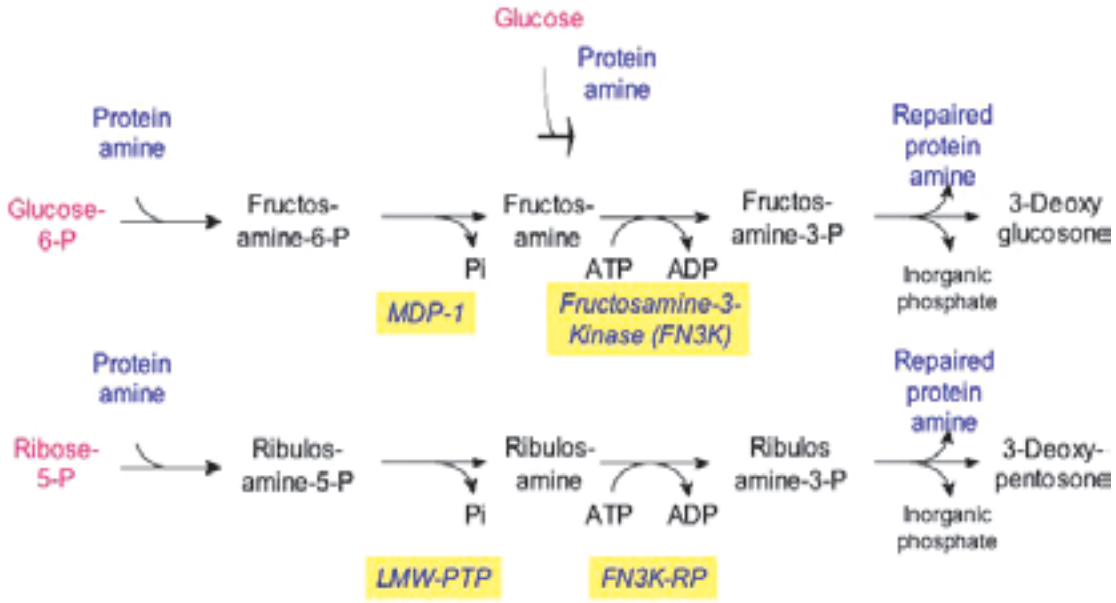

Figure 1. Role of fructosamine 3-kinase and other enzymes in the deglycation of proteins

The role of FN3K as a protein-repair enzyme is studied on a mouse knock-out model obtained by targeted gene inactivation of the FN3K gene. The level of haemoglobin-bound fructosamines is about 2.5-fold higher in $FN3K^{-/-}$ mice than in control ($Fn3k^{+/+}$) mice. Other cytosolic proteins (FN3K is a cytosolic enzyme) are also significantly more glycated in FN3K-deficient mice than in control mice and this applies to all investigated tissues. These findings indicate that FN3K is indeed able to remove fructosamines from proteins in vivo, though it is unable to remove all of them. In vitro studies on purified proteins indicate that this is due to a lack of accessibility of this enzyme to 'buried' fructosamines. As $FN3K^{-/-}$ mice are apparently healthy, we still need to understand why it is important to repair protein glycation. One of our aims is to identify physiological or biochemical processes that are affected by FN3K deficiency.

An intriguing observation made in these studies is that the glycation level of cytosolic muscle proteins is only slightly lower than that found in liver and erythrocytes despite a 100-fold lower cytosolic concentration of glucose. The explanation for this paradoxical observation is that fructosamines may also be formed from glucose 6-phosphate, which is more abundant than glucose in muscle cytosol and which is intrinsically 5-fold more reactive than glucose. We have identified an enzyme that dephosphorylates fructosamine 6-phosphates to fructosamines [4]. Our hypothesis is that this enzyme, for which protein-bound fructosamine 6-phosphates appear to be the best substrates, teams up with FN3K to free proteins from the glycation products that are formed from glucose 6-phosphate.

Fructosamine-3-kinase related protein

Fructosamine-3-kinase-related protein (FN3K-RP) is an enzyme that shares about 65 % sequence identity with FN3K and is encoded by a gene that neighbours the FN3K gene on human chromosome 17 [1]. Intriguingly, FN3K-RP does not phosphorylate fructosamines, but well ribulosamines and erythrulosamines. The ribulosamines 3-phosphates and erythrulosamine 3-phosphates that are so formed are unstable (even more so than fructosamine 3-phosphates) and their spontaneous breakdown also leads to the regeneration of a free amino group. Remarkably, plant and bacterial homologues of FN3K also phosphorylate ribulosamines and erythrulosamines, but not fructosamines indicating that functional homologues of FN3K-RP are more widely distributed than functional homologues of FN3K.

How are the substrates of FN3K-RP formed ? It is unlikely that they arise through a reaction of amines with free ribose or erythrose, because these sugars are present at very low concentration (< 10 μM) in tissues. Our present hypothesis is that substrates of FN3K-RP are formed through a reaction of proteins with ribose 5-phosphate or erythrose 4-phosphate, two extremely potent glycating agents that react ≈ 80 and 500-fold more rapidly than glucose. This view is consistent with the observation that FN3K-RP, which is rather evenly distributed in mammalian tissues, is poorly active in skeletal muscle, a tissue known for its particularly low pentose phosphate cycle activity.

The ribulosamine 5-phosphates and erythrulosamine 4-phosphates that are formed from phosphorylated intermediates need to be dephosphorylated before being phosphorylated on their third carbon by FN3K-RP, and thereby destabilized and removed from proteins. A phosphatase that catalyses the dephosphorylation of ribulosamine 5-phosphates has recently been purified from human erythrocytes and identified as 'low-molecular weight tyrosine phosphatase A' (LMW-PTP-A) [3]. The ribulosamine-5-phosphatase activity of LMW-PTP-A was found to be higher than its protein-tyrosine-phosphatase activity. Furthermore, several bacterial genomes contain an operon encoding both a FN3K homologue and a LMW-PTP homologue, suggesting that this type of phosphatase is involved in the repair of ribulosamine 5-phosphates and/or erythrulosamine 4-phosphates. The mechanism of formation of the substrates for FN3K-RP is one of the main questions we are presently trying to answer.

As a 'side-product' of our studies on the metabolism of fructosamines in eukaryotes, we have also identified several enzymes that metabolise glycation products in bacteria. These enzymes allow the metabolism of glycation products formed from different hexoses, namely D-glucose, D-fructose and D-allose, and from different amino acids. Typically, the glycation products are phosphorylated on the sixth carbon of their sugar moiety by an ATP-dependent kinase or a 'PTS' (phospho-transfer system, which uses phosphoenolpyruvate as phosphoryl donor) and the 6-phosphoderivative is then used by an enzyme catalysing an Amadori rearrangement. As only few bacteria possess the enzymatic arsenal to metabolise these compounds, glycation products offer 'nutrient niches' for selected bacteria.

DISORDERS OF L-SERINE BIOSYNTHESIS

Y. Achouri, E. Wiame, E. Van Schaftingen

L-Serine, one of the twenty 'standard' amino acids, is important not only for protein synthesis, but also for the formation of several other biomolecules including phospholipids, sphingolipids, nucleotides, and two other amino acids, glycine and cysteine. L-serine is a 'non-essential' amino acid, which means that humans and other mammals have the enzymatic equipment necessary for its synthesis, i.e., a

three step pathway branching from glycolysis at the level of 3-phosphoglycerate (Fig. 2). Although substantial amounts of serine are present in the food or result from the endogenous degradation of proteins, it is now clear that *de novo* serine biosynthesis is essential for the proper development of the brain. This conclusion derives from the identification of serine deficiency disorders.

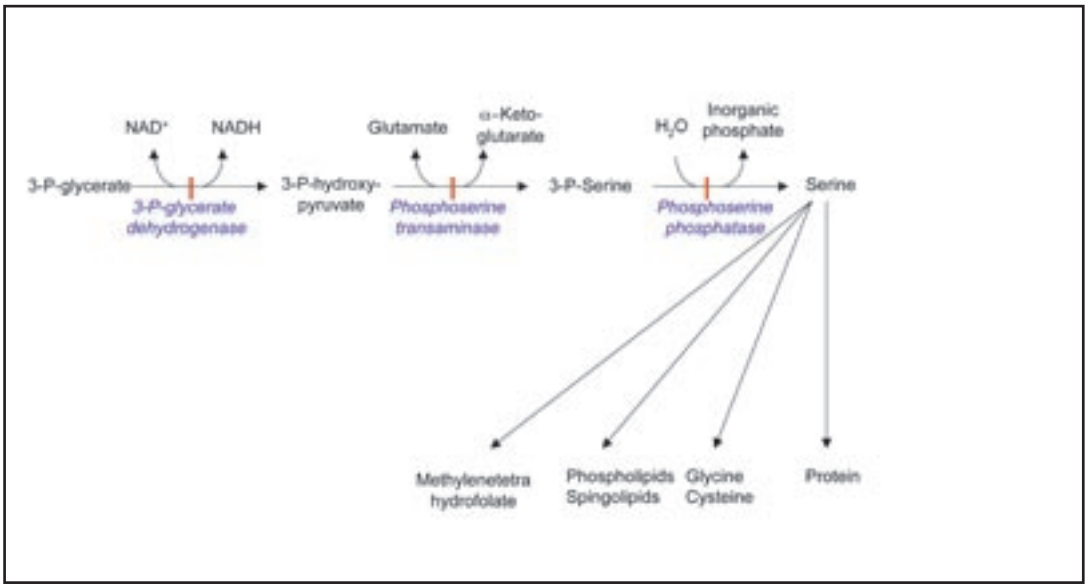

Figure 2. The pathway of serine biosynthesis and its defects.

About ten years ago, our group identified, in collaboration with Prof. J. Jaeken (KULeuven), 3-phosphoglycerate dehydrogenase deficiency in patients with a severe neurological disorder. Patients with this deficiency have been treated with supplemental serine and glycine with varying degrees of success. Phosphoserine phosphatase deficiency has also been identified in one patient.

We have recently identified the first cases of phosphoserine aminotransferase deficiency (in collaboration with colleagues in Newcastle and Leuven) [5]. This new disorder of serine biosynthesis has been discovered in two siblings showing low concentrations of serine and glycine in plasma and CSF. The index case showed microcephaly, hypertonia and psychomotor retardation, and died at seven months of age despite supplementation with serine and glycine from 11 weeks of age. The younger sibling was treated from birth leading to a normal outcome at 3 years of age. Mutational analysis of the three genes encoding the enzymes of serine biosynthesis revealed compound heterozygosity for two mutations in the phosphoserine aminotransferase gene. One mutation is frameshifting whereas the other is a point mutation that result in a marked decrease in the enzymatic activity. These findings indicate that phosphoserine aminotransferase deficiency is a new form of serine deficiency that can be treated successfully if the diagnosis is made soon after birth.

2-HYDROXYGLUTARIC ACIDURIAS

Y. Achouri, T. Kardon, R. Rzem, G. Connerotte, G. Noël, Th. De Barsy, M. Veiga-da-Cunha, E. Van Schaftingen

D- and L-2-hydroxyglutaric acidurias are distinct neurometabolic diseases characterized by the accumulation of abnormal amounts of either D- or L-2-hydroxyglutarate in cerebrospinal fluid, blood and urine. Work in our lab has led to the elucidation of the metabolism of

these compounds (Fig. 3). Both of them are converted to alpha-ketoglutarate by distinct FAD-linked dehydrogenases. The dehydrogenase acting on L-2-hydroxyglutarate is bound to mitochondrial membranes and mutations in its gene are responsible for L-2-hydroxyglutaric aciduria [9]. The dehydrogenase acting on D-2-hydroxyglutarate is in the mitochondrial matrix and most likely transfers its electrons to the respiratory chain via electron-transfer-flavoprotein. It is mutated in a significant fraction of the patients with D-2-hydroxyglutaric aciduria. One of our aims is to identify the other cause(s) of D-2-hydroxyglutaric aciduria.

Formation of D-2-hydroxyglutarate is catalysed by an enzyme that metabolizes 4-hydroxybutyrate (which is produced endogenously but is also a therapeutic agent - and an abuse drug). Unlike other alcohol dehydrogenases this enzyme does not tranfer electrons

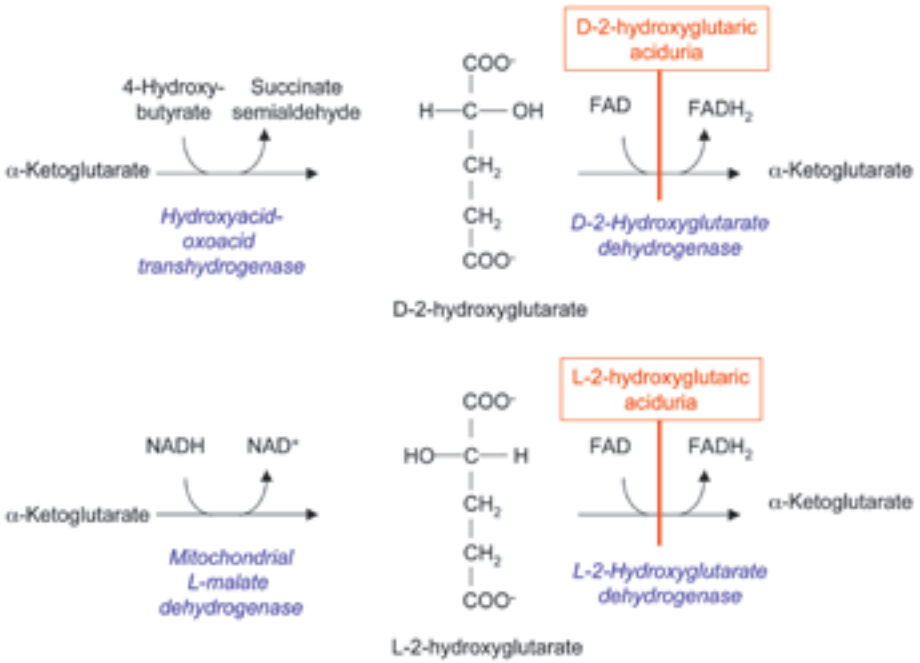

Figure 3. Formation and degradation of D- and L-2-hydroxyglutarate and metabolic defects in 2-hydroxyglutaric acidurias. See Text.

onto free NAD or NADP, but onto alpha-ketoglutarate (Kaufman et al. 1988). We recently cloned this enzyme and showed that it corresponds to a protein designated as 'iron-dependent alcohol dehydrogenase', the enzyme that metabolizes 4-hydroxybutyrate [6]. This enzyme comprises binding sites for NAD (which is tightly bound, serving as an intermediary electron acceptor) and for a divalent cation. The cloning of this enzyme opens perspectives for the study of the mechanism of action of 4-hydroxybutyrate.

Formation of L-2-hydroxyglutarate appears to be catalysed by mitochondrial L-malate dehydrogenase. This enzyme is not entirely specific for oxaloacetate, since it can also reduce alpha-ketoglutarate with a 10^7 lower catalytic efficiency. Though very low, this activity is sufficient to account for the daily formation of L-2-hydroxyglutarate. Since L-2-hydroxyglutarate does not appear to have any physiolo-

gical role but to have only toxic effects, L-2-hydroxyglutarate dehydrogenase is a 'repair enzyme' and L-2-hydroxyglutaric aciduria is a disorder of metabolite repair.

OTHER INBORN ERRORS OF METABOLISM

P. Maliekal, G. Connerotte, G. Noël, T. de Barsy, E. Van Schaftingen

Our group is also involved in the enzymatic diagnosis of glycogen storage diseases and of congenital disorders of glycosylation (as a member of the Euroglycanet network). In the latter context, one of our aims is to identify enzymes in the glycosylation pathway that have not yet been molecularly identified. This led us to report recently the identification of the genes encoding N-acetylneuraminate-9-phosphate phosphatase, phosphopentomutase and glucose 1,6-bisphosphate synthase [8].

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NUCLEOSIDE ANALOGUES IN LEUKAEMIA

Our group had a long-standing interest in purine metabolism, particularly adenine nucleotide metabolism, and its genetic defects. More recently, we expended our investigations on two therapeutic purine nucleoside analogues, 2-chlorodeoxyadenosine and fludarabine, which have revolutionized the treatment of indolent lymphoproliferative disorders. Despite this activity, clinical resistance to these drugs is frequently observed. The main objectives of our present studies are to unravel the mechanisms leading to resistance to nucleoside analogues and to find novel therapeutic strategies to counteract them, particularly in B-cell chronic lymphocytic leukaemia.

In 1997, a collaborative study of the antileukaemic nucleoside analogues, 2-chlorodeoxyadenosine (CdA) and fludarabine (Fig. 1), was started with the Department of Haematology of the University Hospital Saint-Luc. These adenosine deaminase-resistant deoxyadenosine analogues display remarkable therapeutic properties in indolent lymphoid malignancies including hairy cell leukaemia and B-cell chronic lymphocytic leukaemia (B-CLL). Nevertheless, resistance is also observed, and nucleoside analogues do not confer a survival advantage when compared to more conventional therapies such as alkylating agents. The aims of the project are to understand the mechanisms that lead to resistance to nucleoside analogues, and to improve their therapeutic efficacy by searching for synergisms with other compounds.

CdA and fludarabine are prodrugs. To exert their antileukaemic effect, they have to be phosphorylated by deoxycytidine kinase (dCK) into nucleoside analogue monophosphates, followed by conversion into di- and triphosphate derivatives. The latter are the active metabolites. They have been shown to inhibit various processes involved in DNA and RNA synthesis. Moreover, they can be incorporated into newly synthesised DNA, causing chain termination. Together, these actions result in arrest of DNA synthesis and in the progressive accumulation of DNA strand breaks, leading to apoptosis by mechanisms which are not yet entirely clear (1, for a review).

MECHANISMS OF ACTION

F. Bontemps, C. Smal, L. Bastin-Coyette, E. de Viron, A. Aerts, E. Van Den Neste

To improve our understanding of the me-

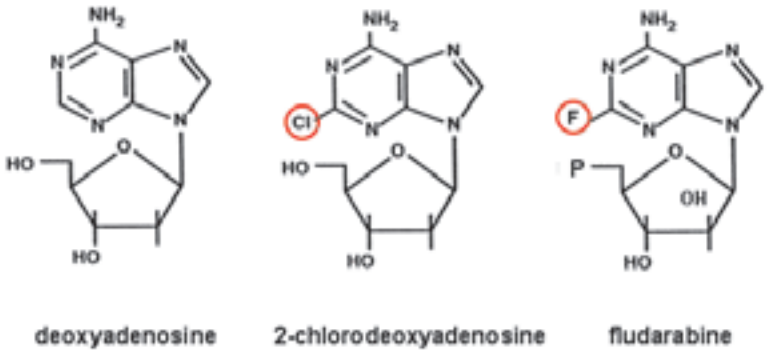

Fig. 1. Structures of deoxyadenosine and analogues.

chanisms by which CdA induces apoptosis in B-CLL cells, we study its effects in EHEB cells, a continuous cell line derived from a patient with B-CLL. The EHEB cell line was found to be less sensitive (10- to 100-fold) to the nucleoside analogue CdA than other human lymphoblastic cell lines. This can be explained by a lower intracellular accumulation of CdATP, the cytotoxic metabolite of CdA, due to a reduced dCK activity. Unexpectedly, DNA synthesis, measured by thymidine incorporation into DNA, was found to be increased, up to 2-fold, after a 24 h-incubation with CdA at concentrations close to the IC_{50} (5 µM) (2). Analysis by flow cytometry, using double labelling with propidium iodide and bromodeoxyuridine, has shown that CdA, in EHEB cells, provokes an increase in the proportion of cells in S phase, synthesising actively DNA. These results contrast with those reported in other leukaemic cell lines, like CCRF-CEM cells, in which CdA inhibits DNA synthesis. Kinetics and synchronisation experiments have shown that the increase in the proportion of cells in S-phase induced by CdA in EHEB cells was due to a stimulation of the progression of cells from G1 to S phase. Accordingly, we found that CdA enhances the activity of cyclin-dependent kinase 2 (Cdk2), a kinase that plays a major role in the progression of cells from G1 to S phase. This unexpected result has led us to examine the effects of CdA on proteins involved in the regulation of cell cycle, and to investigate whether Cdk2 activation contributes to CdA-induced apoptosis in EHEB cells. Our further aim will be to elucidate how CdA and possibly other nucleoside analogues activate Cdk2.

In parallel, we have analysed the mechanisms of cell death induced by 2-chloroadenosine (2-CAdo) in various types of leukaemic B-cells. This analogue is not activated by dCK, but by adenosine kinase. We found that 2-CAdo was efficiently converted into 2-chloroATP, resulting in ATP depletion, as well as in inhibition of DNA, RNA and protein synthesis. 2-CAdo also caused activation of the intrinsic pathway of apoptosis, which was associated with a decline in Mcl-1 protein level, and p53-independent. Prevention of ATP loss by inhibition of AMP deaminase reduced apoptosis, indicating that ATP depletion plays an important role in 2-CAdo-induced apoptosis (3).

Finally, we have recently initiated microarray analyses to identify survival or death pathways that are activated in response to nucleoside analogue treatment. We intended to compare genes induced or repressed by nucleoside analogues in sensitive and refractory B-CLL patients. We found that some genes were systematically induced in sensitive patients, and not in refractory patients. We are now analysing whether the induction of one or more of these genes play(s) a role in nu-

cleoside analogue-induced cell death. This study is performed in collaboration with Dr L. Knoops from the Ludwig Institute at the de Duve Institute.

SEARCH FOR POTENTIATION OF ANTILEUKAEMIC EFFECT OF CdA

E. Van Den Neste, C. Smal, F. Bontemps

In recent years, we have shown that combination of CdA with DNA-damaging agents, such as cyclophosphamide (CP) derivatives (4) or UV-light (5), resulted in synergistic cytotoxicity in B-CLL lymphocytes, due to inhibition of DNA repair. The *in vitro* synergy between CdA and CP derivatives has provided the rationale for a clinical trial of this combination, which gives encouraging results (6).

Lately, we have explored the possibility that CdA interacts with the mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway. We have shown that CdA, at concentrations close to the IC_{50}, activated the ERK pathway in EHEB cells. Because activation of this pathway is assumed to exert anti-apoptotic effect, we combined CdA with inhibitors of the ERK pathway. The latter were found to enhance CdA-induced apoptosis. These results suggest that the efficacy of CdA could be strengthened by combination with inhibitors of the ERK pathway (7).

REGULATION OF DEOXYCYTIDINE KINASE ACTIVITY

C. Smal, A. Aerts, E. Van Den Neste, F. Bontemps

Since deoxycytidine kinase (dCk) activates numerous nucleoside analogues used in anticancer and antiviral therapy, knowledge of its regulation can be expected to allow optimization of the activation of these analogues. Recently, it has been shown by others and by us that dCK activity can be increased by various genotoxic agents, including CdA, aphidicolin, etoposide, and UV-C irradiation. This activation could not be explained by an allosteric effect or by an increase of the protein amount. Therefore, a post-translational activation of dCK by intracellular signalling pathways was suggested. To unravel the mechanism of the activation of dCK, we first investigated the effect of a variety of activators and inhibitors of protein kinases on dCK activity. We found that some of them could modify dCK activity in several types of leukaemic cells, strengthening the hypothesis of a phosphorylation/dephosphorylation process as a mechanism of regulation of dCK activity. Most particularly, our results indicated that dCK could be downregulated by the JAK/MAPK pathway and that it might be an *in vivo* target of protein phosphatase 2A (8).

We then overexpressed dCK in HEK 293T cells and observed that the enzyme was labelled after incubation with [^{32}P]orthophosphate, confirming that dCK is a phosphoprotein. Tandem mass spectrometry performed by Dr D. Vertommen and Prof. M.H. Rider from the Horm unit allowed the identification of four *in vivo* phosphorylation sites, Thr-3, Ser-11, Ser-15 and Ser-74 (Fig. 2). Site-directed mutagenesis demonstrated that Ser-74 phosphorylation was crucial for dCK activity in HEK 293T cells, whereas phosphorylation of other identified sites did not seem essential (9). Phosphorylation of Ser-74 was also observed on endogenous dCK in CCRF-CEM cells and in lymphocytes from B-CLL patients, in which the Ser-74 phosphorylation state was increased by several genotoxic agents (UV, etoposide, …) that enhanced dCK activity, and decreased by sorbitol that diminished dCK activity. Moreover, interindividual variability in dCK activity in B-CLL lymphocytes could be related to its phosphorylation level on Ser-74 (10). To conclude, our work has demonstra-

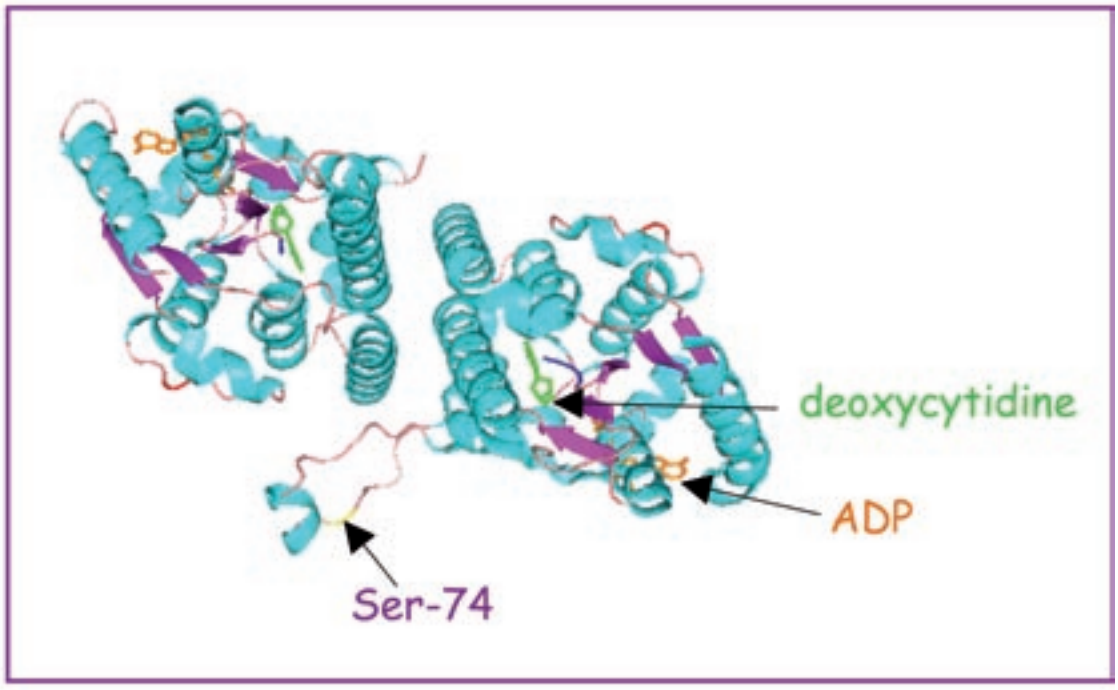

Figure 2. Three-dimensional structure of deoxycytidine kinase in complex with ADP and deoxycytidine

The Ser-74 phosphorylation site is located at a very flexible 15-residue insert (Ser-63-Asn-77) easily accessible to cellular protein kinases. The N-terminal extremity of dCK (residues 1-19) was flexible and could not be solved. Therefore, the Thr-3, Ser-11 and Ser-15 phosphorylation sites cannot be exactly located.

ted that dCK activity largely depends on the phosphorylation state of Ser-74 in human leukaemic lymphocytes. We are now attempting to identify the protein kinase(s) responsible for the phosphorylation of dCK on Ser-74 and the signalling pathways that lead to dCK activation following treatment with DNA damaging agents.

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PROTEIN FOLDING IN BACTERIA

To be active and stable, newly synthesized proteins have to fold correctly. However, reaching a proper three-dimensional structure is a real challenge for most proteins. This is especially true for proteins that are secreted to the cell envelope of Gram-negative bacteria. These proteins are assembled in the cytoplasm, are then transported across the inner-membrane and finally fold within the periplasm or the outer-membrane, in an environment devoid of any obvious energy source. Moreover, the correct folding of many of these proteins requires the formation of a disulfide bond between two cysteine residues, which is a rate-limiting step of the folding process. Failure to fold secreted proteins properly affects the integrity of the bacterial cell envelope, which increases the sensitivity of bacteria to antimicrobial drugs.
In our group, we study the pathways that govern disulfide bond formation in the periplasm of Escherichia coli and the assembling of the bacterial outer-membrane.

OXIDATIVE PROTEIN FOLDING IN THE PERIPLASM

G. Connerotte, M. Depuydt, P. Leverrier, J.-F. Collet

In the gram-negative bacterium *Escherichia coli*, disulfide bonds are introduced in the periplasm by the Dsb (Disulfide bond) proteins family [4].

The primary oxidant is the soluble protein DsbA. DsbA has a CXXC catalytic site motif present within a thioredoxin fold. The cysteine residues of this motif are found oxidized *in vivo*. The disulfide bond of DsbA is very unstable and is rapidly transferred to secreted unfolded proteins. DsbA is then re-oxidized by the inner-membrane protein DsbB that transfers electrons from DsbA to the electron transport chain (Figure 1).

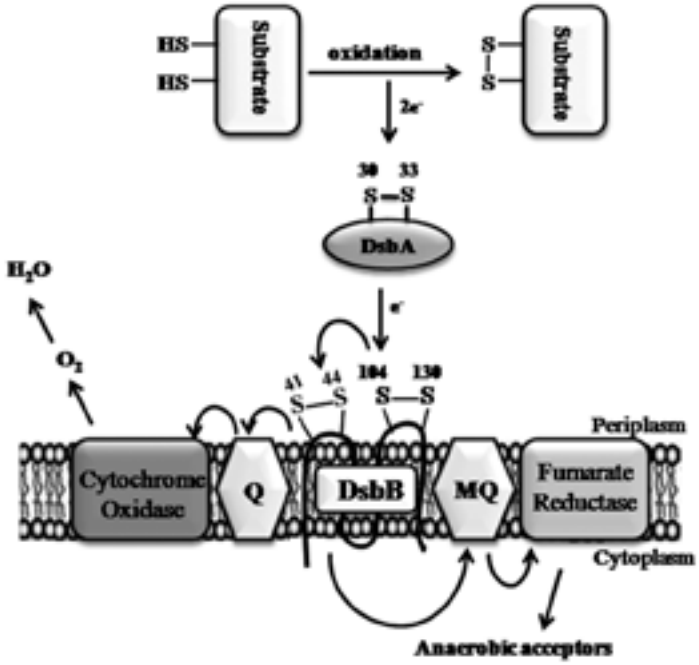

Figure 1. Disulfide bond formation in the E. coli periplasm. Disulfides are introduced into folding proteins by DsbA. DsbA is re-oxidized by DsbB, an inner-membrane protein that generates disulfides de novo from quinone (Q) or menaquinone (MQ) reduction. Electrons flow from the reduced proteins to the CXXC motif of DsbA and then to cysteine residues of DsbB.

DsbA is a very powerful oxidant that apparently lacks proofreading activity. If the native disulfide bond pattern involves cysteine residues that are non-consecutive in the amino-acid sequence, DsbA can form incorrect disulfides. The correction of these non-native disulfides is the role of a disulfide isomerization system (Figure 2). This system involves a soluble periplasmic protein disulfide isomerase, DsbC. Like DsbA, DsbC presents a thioredoxin fold and a CXXC catalytic site motif. In contrast to DsbA, the CXXC motif of DsbC is kept reduced in the periplasm. This allows DsbC to attack non-native disulfides, a necessary step in the isomerization reaction. The protein that keeps DsbC reduced is the inner-membrane protein DsbD. DsbD transfers reducing equivalents from the cytoplasmic thioredoxin system to the periplasm via a succession of disulfide exchange reactions.

A revised model for the formation of protein disulfide bonds in the *E. coli* periplasm

In *E. coli*, the current view is that the distinct disulfide catalytic pathways have well defined roles and are isolated from each other: the DsbA/DsbB system is important in oxidizing disulfide bonds, whereas the DsbC/DsbD system is important in isomerizing them. Our recent findings show that this view needs to be reinterpreted [7].

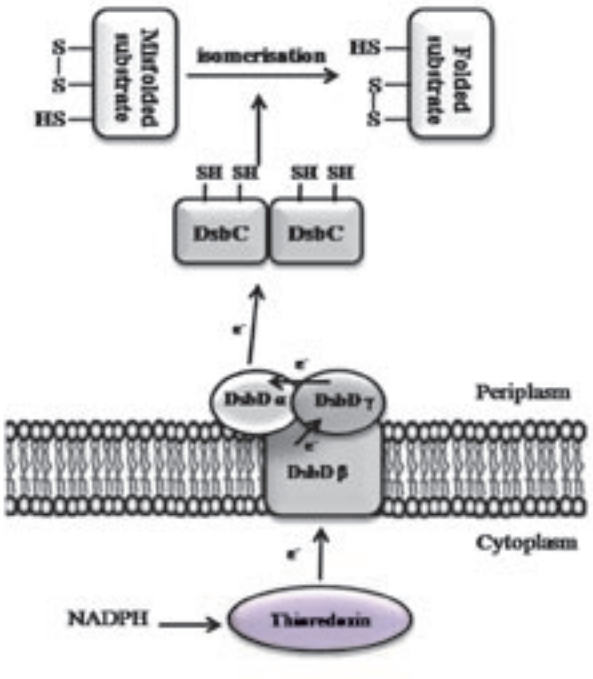

Figure 2. Disulfide bond isomerization in the *E. coli* periplasm. DsbC is a protein disulfide isomerase that corrects non-native disulfides. DsbC is recycled by DsbD, a transmembrane protein that transfers electrons from the cytoplasmic thioredoxin system to the periplasm.

First, we showed that the simultaneous absence of DsbA and DsbC has severe consequences on *E. coli*'s viability and outer membrane integrity. For instance, we found that *dsbA*$^-$*dsbC*$^-$ mutants are resistant to the Φ80 bacteriophage and seem unable to fold the trimeric porin LamB. These phenotypes are not exhibited by either *dsbA*$^-$, *dsbC*$^-$, or *dsbA*$^-$*dsbD*$^-$ mutations. Second, in collaboration with Dr. D. Vertommen (HORM Unit), we developed a proteomic approach based on 2D-LC-MS/MS that allowed us to show that the *dsbA*$^-$*dsbC*$^-$ mutants exhibit unique changes at the protein level that are not exhibited by *dsbA*$^-$*dsbD*$^-$ mutants. Altogether, these data indicate that DsbC can assist DsbA in a DsbD-independent manner to oxidatively fold envelope proteins. DsbC's function is therefore not limited to the framework of the disulfide isomerization pathway.

On the basis of our results, we have adapted the model of disulfide bond formation in the *E. coli* periplasm [7]. We propose that when DsbC gets oxidized upon reduction of a non-native disulfide, it is either reduced by DsbD or by transferring its disulfide to a reduced protein. DsbC may possibly be acting as a stand-alone protein folding catalyst that is able to cycle from the reduced to the oxidized state upon substrate oxidation and substrate reduction, respectively. This activity of DsbC seems important to maintain the integrity of the cell envelope and is not restricted to the correction of non-consecutive disulfides.

Our proteomic data also highlighted some dramatic changes that occur in all strains impaired in disulfide bond formation, including the induction of several stress proteins belonging to the SigmaE regulon and other uncharacterized proteins. We also reported the identification of new DsbA and DsbC substrates [7].

In collaboration with Dr. J. Messens (Vrij Universiteit Brussel), we showed that the oxidase DsbA can fold a protein with a nonconsecutive disulfide, suggesting that DsbA is more specific than generally assumed [8].

OUTER-MEMBRANE BIOGENESIS

G. Connerotte, C. Bouillot, C. Recour, P. Leverrier, J.-F. Collet

We recently started to study the mechanisms of outer-membrane biogenesis in *E. coli*. The outer-membrane (OM) is a permeability barrier that is essential for the viability of Gram-bacteria and protects them against various antibiotics. OM components are synthesized in the cytoplasm or in the inner-membrane. The mechanisms by which unfolded proteins and lipids are then transported through the hydrophilic periplasm and are inserted in the OM are obscure. Our overall goal is to solve the fascinating problem of how such a complex macromolecular structure is assembled in a compartment devoid of energy. The proteins that are involved in OM biogenesis are also attractive targets for the design of new antibiotics and anti-inflammatory drugs.

First, in order to advance our understanding of the mechanisms that govern OM biogenesis, we have adapted our 2D-LC-MS/MS proteomic technique to the study of the OM proteome. In particular, we have characterized the function of the *E. coli* periplasmic chaperone SurA. SurA has been proposed to escort β-barrel proteins en route to the OM. However, our understanding of SurA's function is based on studies performed on a small number of OM proteins and its role on the global OM proteome had never been determined. Using 2D-LC-MS/MS, we determined the impact of *surA* deletion on the relative abundance of 67 OM proteins, including 26 β-barrels proteins. This is the first time that the abundance of so many OM proteins has been determined using a single approach. We found that the loss of SurA only affects the levels of certain proteins, including the major β-barrel proteins that account for most of the OM protein mass. We observed that levels of the essential protein Imp, which is encoded in a conserved operon with *surA*, are dramatically reduced in the absence of SurA, despite its increased synthesis. This suggests that Imp is a true SurA substrate. On the basis of our results, we have proposed an improved model for SurA's function in β-barrel transport and assembly. We are currently using our powerful proteomic technique to characterize other mutants that present defects in OM integrity.

Second, in order to identify new genes that are involved in OM biogenesis, we screened a collection of *E. coli* deletion mutants for sensitivity to antibiotics. This approach allowed us to identify 15 genes with unknown function that seem to play a major role in maintaining OM integrity. We are currently characterizing some of them.

CHARACTERIZATION OF *E. COLI* THIOREDOXIN 2

H. El Hajjaji, J.-F. Collet

Thioredoxins are small redox proteins present in many eukaryotic and prokaryotic genomes. They share a similar 3-dimensional structure and possess a conserved WCGPC catalytic motif. They reduce disulfide bonds formed between cysteine residues as a result of either a catalytic activity or oxidative stress. In the course of the reaction, the two catalytic cysteine residues of the WCGPC motif are oxidized. They are then converted back to the reduced form by thioredoxin reductase, a NADPH-dependent protein.

Two thioredoxins have been described in *E. coli*. The first one, Trx1, is a 12 kDa protein that has been identified 40 years ago and is well characterized. The second thioredoxin, Trx2, coded by the gene trxC, has been discovered more recently. The sequence of this 15 kDa protein presents 28 % of identity with TrxA. Trx2 has two striking characteristics that differentiate it

from TrxA and suggest that it has a specific role to play: First, Trx2 is under OxyR control, a transcription factor that induces the expression of several antioxidant genes. Second, Trx2 contains an additional amino-terminal domain of 32 residues in which two CXXC motifs are present. We have shown that these additional cysteine residues tightly bind zinc. Upon oxidation with hydrogen peroxide *in vitro*, the zinc is released, which triggers a conformational change. Our goal is to find the function of this unique zinc binding thioredoxin.

In collaboration with Joris Messens (VUB) and André Matagne (ULg), we have characterized Trx2 *in vitro*. We have determined the redox potential, the pKa of the active site nucleophilic cysteine, and the protein stability of Trx2. We found that Trx2 (-222 mV) is more oxidizing than Trx1 (-270 mV). Not surprisingly for a reducing enzyme, the oxidized form of Trx2 is more stable than its reduced form. Zn^{2+} was removed by mutating the four cysteines of the Zn^{2+}-binding domain to serine. The redox potential of this mutant becomes more reducing (-254 mV) compared to wild type Trx2 and its overall stability decreases. These data indicate that the Zn^{2+}-center of Trx2 fine-tunes the properties of this thioredoxin.

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SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

Research in our group focuses on the role of protein phosphorylation in the control of cell function, with special emphasis on the control of metabolism by nutrients, hormones and various stresses. As a model system, we studied 6-phosphofructo-2-kianse (PFK2)/fructose-2,6-bisphosphatase (FBPase-2) and the control of its activity by various protein kinases. This bifunctional enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis that was discovered in this Institute by Van Schaftingen, Hue and Hers in 1980. Our previous work on the regulation of heart PFK-2 activity by phosphorylation led to the study of the insulin and AMP-activated protein kinase (AMPK) signalling cascades, which are now our main research interests.

INSULIN SIGNALLING

L. Toussaint, D. Vertommen, L. Hue, M.H. Rider, in collaboration with B. Hemmings, Basel and D. Alessi, Dundee

Activation of heart PFK-2 by insulin

Insulin stimulates heart glycolysis by increasing glucose transport and by activating PFK-2. This in turn leads to a rise in fructose 2,6-bisphosphate. The mechanism involved in this insulin-induced activation of heart PFK-2 is being studied both in vitro and in intact cells. Recombinant heart PFK-2 isozyme is a substrate of several protein kinases, especially protein kinases of the insulin signalling pathways, such as protein kinase B (PKB), also known as Akt, which is believed to mediate most metabolic effects of insulin. Our previous studies suggested that PFK-2 activation by insulin was dependent on PDK-1, a protein kinase upstream of PKB. Therefore, the potential role of PKB has been thoroughly investigated. Our first results using a dominant-negative PKB transfected in HEK293 cells suggested that PKB did not participate in heart PFK-2 activation by insulin. However, in HEK293 cells co-transfected with heart PFK-2 and total PKB siRNA, insulin-induced PFK-2 activation was abrogated. Our results with PKB beta-knockout mice indicated that this isoform is not required for heart PFK-2 activation by insulin and so we are currently focusing on the role of PKB alpha using the siRNA approach.

We studied the potential role of other insulin-stimulated kinases in PFK-2 activation by insulin. We purified a wortmannin-sensitive and insulin-stimulated protein kinase (WISK). WISK phosphorylates heart PFK-2 on Ser466 leading to its activation. We showed that WISK contains protein kinase C zeta (PKCzeta). However, PKCzeta is probably not involved in PFK-2 activation by insulin, because PKCzeta was not activated by insulin in this organ. This conclusion was confirmed by experiments in cells transfected with a kinase-inactive PKCzeta construct, which failed to prevent insulin-induced PFK-2 activation (1). We also studied the role of SGK3 (serum- and glucocorticoid-regulated protein kinase-3) in insulin action in perfused rat hearts and in HEK 293T cells. PFK-2 can be phosphorylated by SGK3 in vitro leading to its activation. However, co-transfection of HEK 293T cells with SGK3 siRNA did not affect PFK-2 activation, suggesting that this protein kinase was not required for PFK-2 activation by insulin.

Figure 1 summarizes the protein kinases from different signalling pathways that phosphorylate heart PFK-2.

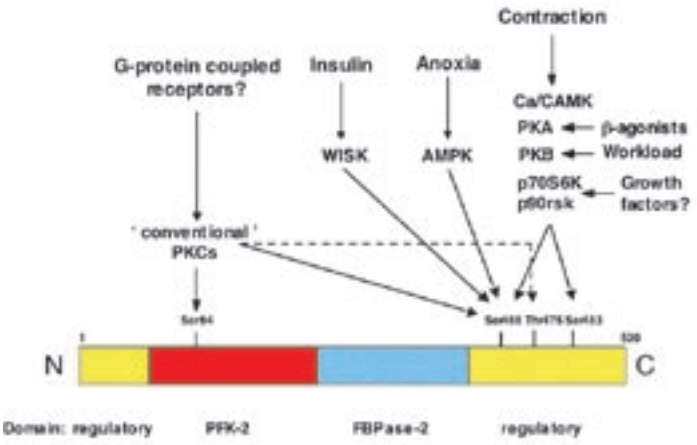

Fig. 1. Protein kinases of signalling pathways that converge on heart PFK-2. The numbering of residues refers to the bovine H1 isoform (2).

AMP-ACTIVATED PROTEIN KINASE

S. Horman, D. Vertommen , L. Miranda, L. Bultot, L. Hue, M.H. Rider, in collaboration with N. Morel and C. Beauloye, UCL, Brussels, D. Carling, London, D.G. Hardie, Dundee, T. Walliman, D. Neumann and U. Schlattner, Zurich, J. Jenssen, Oslo, K. Storey, Ottawa, B. Viollet, Paris, C. Forcet, Lyon and M.P. Walsh, Calgary

The AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status. AMPK is activated by an increase in the AMP/ATP ratio as it occurs during hypoxia or after exposure of cells to inhibitors of the mitochondrial respiratory chain, such as oligomycin. The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways and inhibiting energy-consuming biosynthetic pathways. We contributed to the development of this concept by the discovery of new substrates of AMPK. We demonstrated that the activation of PFK-2 by AMPK participates in the stimulation of heart glycolysis by ischaemia. Similarly, we showed that the inactivation of elongation factor 2 (eEF2) by AMPK explains at least in part the inhibition of protein synthesis by anoxia (3 and see below). We are currently engaged in identifying new substrates of AMPK.

AMPK activation inhibits protein synthesis

Protein synthesis, in particular peptide chain elongation, consumes a large proportion of intracellular ATP. We previously showed that in anoxic rat hepatocytes, AMPK activation was associated with protein synthesis inhibition. This was due to eEF2 inactivation via

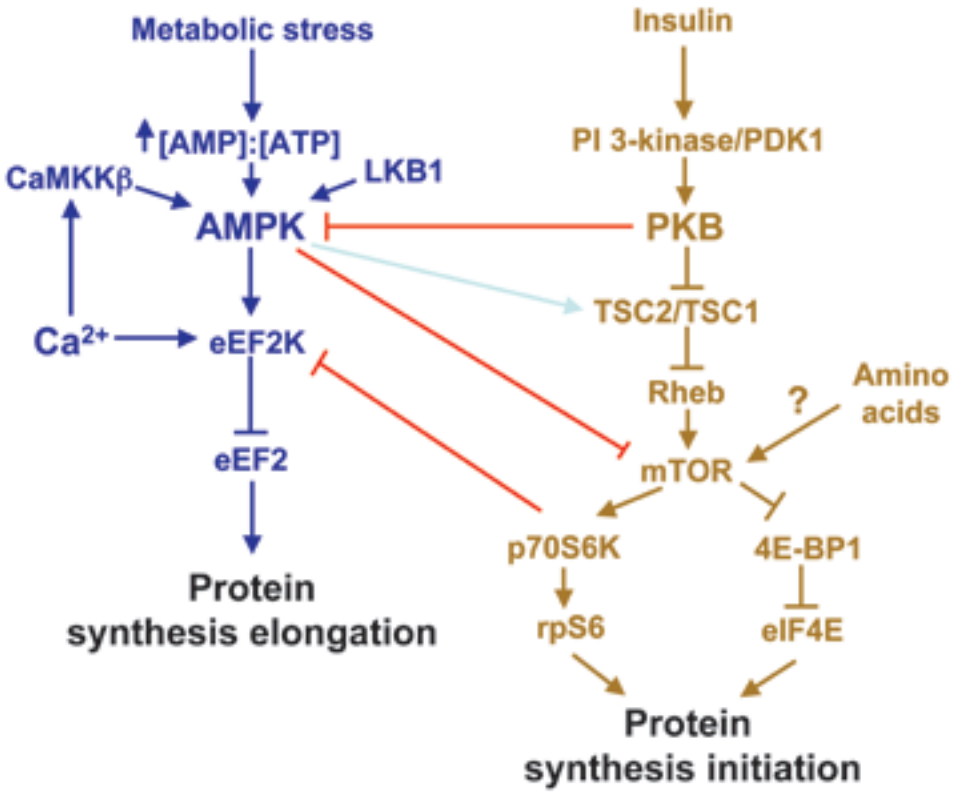

Fig. 2. Cross talk between the insulin/PKB/mTOR and AMPK signalling pathways with respect to the control of protein synthesis.

AMPK-induced phosphorylation and activation of eEF2 kinase (eEF2K), the kinase that phosphorylates eEF2 at its inactivating Thr56 site, providing a novel mechanism for the inhibition of protein synthesis (3). In a model of electrical stimulation of rat epitrochlearis skeletal muscles, protein synthesis was inhibited by 75% during contraction. In this model, Thr56 eEF2 phosphorylation increased within 1 min, but was not related to AMPK activation, in spite of the fact that AMPK was activated 5-fold (4). Moreover, the inhibition of protein synthesis during contraction could not be explained by inhibition of the mammalian target of rapamycin (mTOR)/p70 ribosomal S6 kinase (p70S6K)/4E-BP1 pathway (see Fig. 2). Therefore, the inhibition of protein synthesis by contraction might be explained by an increase in eEF2 phosphorylation at the onset of contraction, probably secondary to a rise in calcium, but does not result from a decease in mTOR signalling (4).

AMPK activation in animals adapting to extreme energy stress (7,8)

Freezing is one of most serious environmental stresses faced by living organisms. We investigated whether AMPK could play a role in metabolic re-sculpting that occurs in animals that adapt to extreme energy situation. Although AMPK does not appear to play a major role in shutting down energy consumption in tissues during mammalian hibernation, it could play an important role in freeze-tolerance (5).

Inhibition of cell respiration by AICAR

AICA (5-aminoimidazole-4-carboxamide) riboside, often referred to as AICAR, has been extensively used in certain cells to activate AMPK, since it enters these cells to be phosphorylated into ZMP, an analogue of AMP. We investigated the effects of AICA riboside on mitochondrial oxidative phosphorylation in hepatocytes. AICA riboside was found to inhibit cell respiration and to decrease inorganic phosphate, ATP, AMP and total adenine nucleotide contents. This effect persisted in hepatocytes from mice lacking both isoforms of the catalytic subunits of AMPK, thus demonstrating that the AICA riboside effects were not mediated by AMPK. In in vitro experiments, ZMP directly inhibited the mitochondrial respiratory-chain complex 1. In addition, inhibition of respiration by AICA riboside was potentiated in cells incubated with fructose to deplete their adenine nucleotide and inorganic phosphate contents. We conclude that AICA-riboside inhibits cellular respiration by an AMPK-independent mechanism that likely results from a combination of phosphate depletion and ZMP accumulation (6). Our results also demonstrate that the effects of AICA riboside are not necessarily caused by AMPK activation and that their interpretation should be taken with caution.

AMPK phosphorylates and desensitizes smooth muscle myosin light chain kinase

Smooth muscle contraction is initiated by a rise in intracellular calcium leading to activation of smooth muscle myosin light-chain kinase (smMLCK) via calcium/calmodulin (CaM). Activated smMLCK then phosphorylates the regulatory myosin light chains (MLC), triggering cross-bridge cycling and contraction. We showed that smMLCK is a substrate of AMPK (7). The phosphorylation site in chicken smMLCK was identified by mass spectrometry to be located in the calmodulin-binding domain at Ser815. Phosphorylation by AMPK desensitized smMLCK by increasing the concentration of CaM required for half-maximal activa-

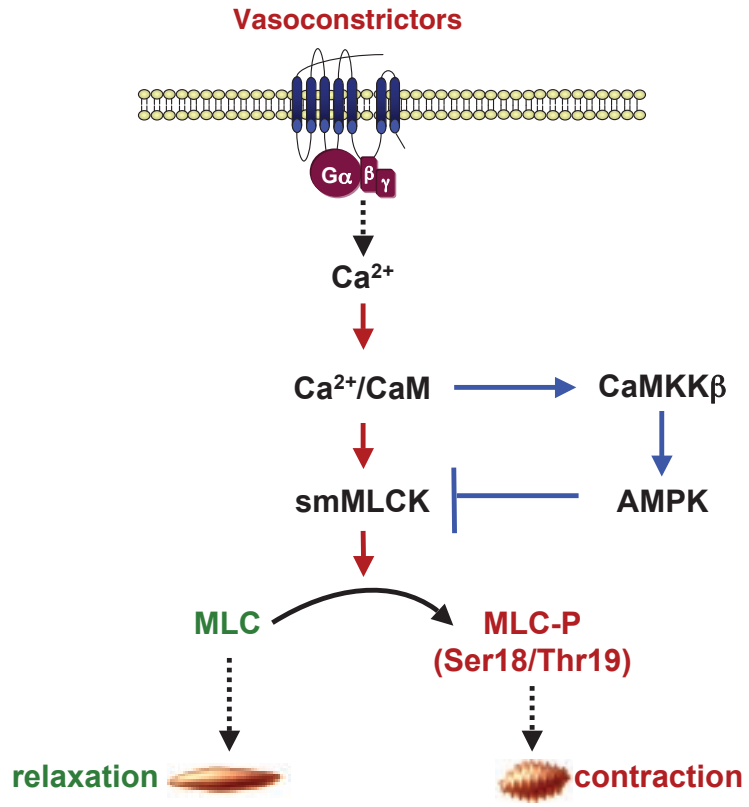

Fig. 3 AMPK activation is induced by vasoconstrictors acting through Gq-coupled receptors, via the calmodulin kinase kinase–beta (CaMKKbeta) pathway in smooth muscle cells. AMPK attenuates contraction by phosphorylating and desensitizing smooth muscle myosin light chain kinase (smMLCK), thereby contributing to reduced ATP turnover in the tonic phase of contraction.

tion. In primary cultures of rat aortic smooth muscle cells, vasoconstrictors activated AMPK in a calcium-dependent manner via CaM-dependent protein kinase kinase-beta (CaMKKbeta), a known upstream kinase of AMPK. Indeed, vasoconstrictor-induced AMPK activation was abrogated by STO-609, the CaMKKbeta inhibitor. MLC phosphorylation was increased under these conditions, suggesting that contraction would be potentiated by ablation of AMPK. Indeed in aortic rings from mice in which alpha1, the major catalytic subunit isoform of AMPK in arterial smooth muscle, had been deleted, KCl- or phenylephrine-induced contraction was increased. The findings suggest that AMPK attenuates contraction by phosphorylating and inactivating smMLCK (Fig. 3). This might contribute to reduced ATP turnover in the tonic phase of smooth muscle contraction.

MASS SPECTROMETRY

The development of mass spectrometry facilities within our laboratory has been an enormous asset to our group and institution. Since the acquisition of our first electrospray mass spectrometer in 1997, the application of mass spectrometry techniques to protein identification, identification of sites of covalent modification and quantification of changes in protein expression has led to well over 30 publications. In our own research, it enabled us to identify new phosphorylation sites in several targets of protein kinase and in the AMPK complex. In January 2008, we updated our electrospray machine to the Finnigan LTQ linear ion trap equipped with electron-induced transfer dissociation (ETD) fragmentation.

We are continuing our efforts to develop new techniques for the mass spectrometric analysis of proteins. These include methods for the enrichment of phosphopeptides with a view to identify new AMPK substrates and establishing a 2D-LC/MS label-free proteomics approach for differential protein expression studies. We pursue our collaboration with other laboratories within the Institute and from other universities. Label-free proteomics has been applied to study periplasmic oxidative protein folding in E. Coli in collaboration with J-F Collet. We estimated the abundance of 130 secreted proteins in various strains which allowed us to propose a revised model of disulfide bonds formation in the periplasm of E. Coli (8). With F. Opperdoes and P. Michels, we studied differential protein expression in glycosomes and mitochondria from the bloodstream and insect stages of *Trypanosoma brucei*, the sleeping sickness parasite. The abundance of glycosomal marker proteins identified in the two life-cycle stages corresponded well with the relative importance of biochemical pathways present in the glycosomes of the two stages. Several proteins involved in oxidative stress protection, sugar-nucleotide synthesis, purine salvage, nucleotide-monophosphate formation and purine-nucleotide cycle were identified as glycosomal proteins (9). Along with the group of E. Van Schaftingen we identified several new enzymes. With the team of F. Bontemps we identified in vivo phosphorylation sites in human deoxycytidine kinase. Lastly, in collaboration with the group of F. Dequiedt (FUSA-Gembloux) we identified new phosphorylation sites in class IIa histone deacetylases and studied dephosphorylation of HDAC7 by protein phosphatase-2A (10).

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METABOLIC COMPARTMENTATION IN TRYPANOSOMES

Trypanosomatidae are protozoan parasites which cause sleeping sickness, Chagas' disease and leismaniasis in man and which are responsible for the deaths of millions of people each year. For these diseases better drugs are urgently needed. By studying the biochemistry of these organisms we try to provide a basis for the development of new and better drugs. Trypanosomes rely on glycolysis for their ATP supply and are characterized by a unique form of glycolytic compartmentation where the majority of the enzymes are sequestered inside peroxisome-like organelles called glycosomes. Since many years, we study the functional and structural properties of the glycosomal enzymes and have validated many of them as potential drug targets. Structural information about these enzymes is used for the design of effective and selective inhibitors. Glycosome assembly and degradation, taking place during cell differentiation, are being studied as well. Many so called «peroxins», proteins involved in glycosome biogenesis, have already been identified and validated as excellent drug targets. Finally, the availability of the genome sequences of three trypanosomatids in combination with various proteomic appraoches has allowed to make a comprehensive inventory of the metabolic capacities of these organisms and to identify essential differences between the respective parasites and their host.

ENZYMES AND PATHWAYS

Glycosomal metabolism

F. Opperdoes, J.-P. Szikora, P. Michels, in collaboration with D. Vertommen and M. Rider, Horm Unit

A proteomics approach was used to compare the glycosomal and mitochondrial proteomes of the bloodstream- and insect-form of *Trypanosoma brucei*. This approach proved extremely useful for the generation of large scale proteomics data for the comparison of different life-cycle stages. Many proteins involved in oxidative stress protection, the hexose-monophosphate pathway and sugar-nucleotide synthesis were identified as glycosomal proteins. Also many enzymes of purine salvage, nucleotide-monophosphate synthesis and purine-nucleotide metabolism were found to be associated with glycosomes (Figure 1). The presence of such enzymes inside glycosomes suggests that they may be endowed with properties other than their soluble orthologues from the host. This together with the availability of thousands of nucleotide analogues, developed as anti-tumour agents, may open interesting new avenues for drug screening.

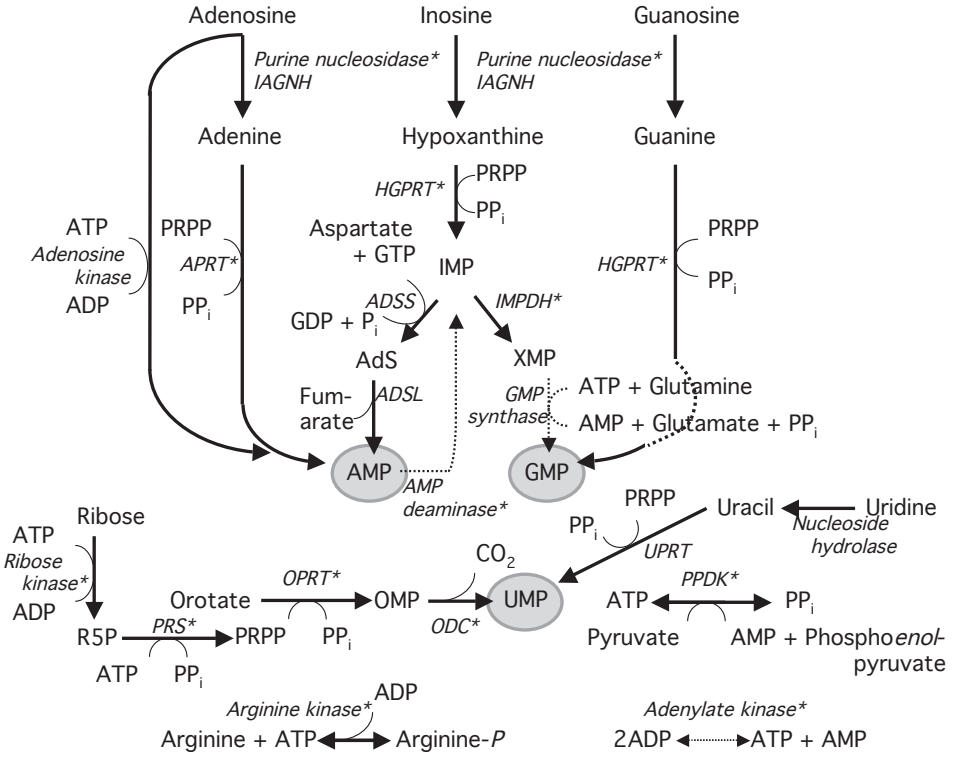

Figure 1. Reactions of nucleotide metabolism which are associated with glycosomes.

Phosphofructokinase and pyruvate kinase

V. Hannaert, P. Michels in collaboration with M. Walkinshaw and L. Gilmore, University of Edinburgh, Scotland

Two enzymes that received our particular attention in 2007 were phosphofructokinase (PFK) and pyuvate kinase (PYK). Both trypanosomatid enzymes are allosteric; they show cooperative binding of their substrate fructose6-phosphateandphosphoenolpyruvate, respectively. Moreover, PYK can uniquely be activated by fructose 2,6-bisphosphate. ATP-dependent trypanosomatid PFK (Fig. 2) is evolutionary more related to the PPi-dependent PFKs of some bacteria and other protozoa than to the ATP-dependent PFKs found in human, offering great promise for drug design. The resolution of the crystal structure of *T. brucei* PFK without any bound ligands has been reported last year. Now also a structure with ATP bound in the active site has been determined. A comparison of the apo- and holo-enzyme structures revealed that they have quite distinct conformations. The unliganded enzyme would be in the inactive T-state, whereas binding of ATP seems to have caused the transition of PFK to the active R-state or an intermediate activated state. The conformational transitions observed are quite distinct from those reported for bacterial and mammalian ATP-dependent PFKs. These observations give further encouragement to the possibility of structure-based drug discovery.

Previously, our coworkers at the University of Edinburgh determined the crystal structure of *Leishmania mexicana* PYK without any li-

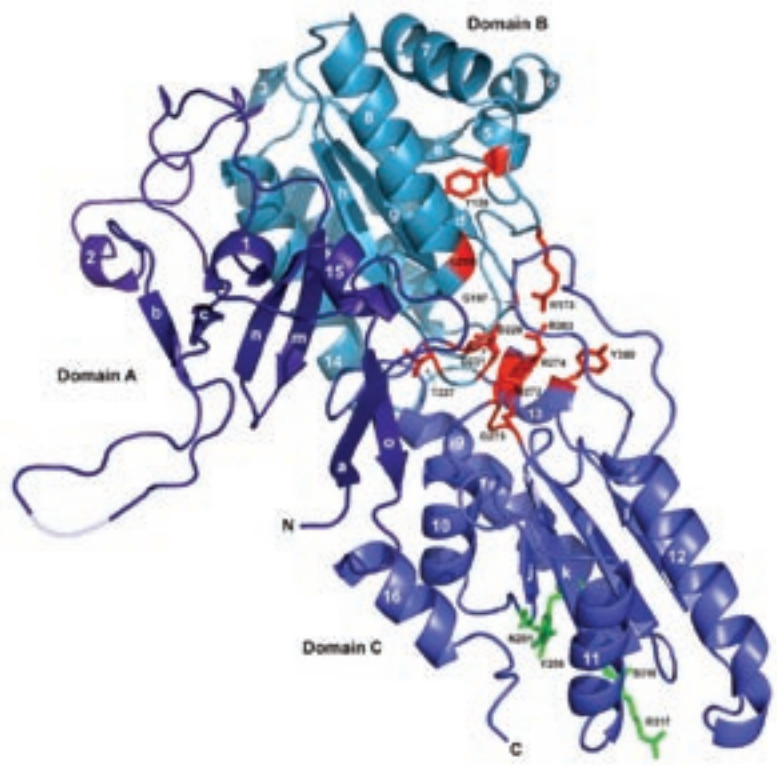

Figure 2. Structure of *T. brucei* phosphofructokinase apoenzyme subunit.

gands, supposedly in the T-state. Now additional crystal structures of the enzymes have been obtained, showing different conformations, with sulphates occupying positions similar to the phosphates of ligands bound to active (R-state) PYK from other organisms, and two sulphate ions mimicking the phosphates of fructose 2,6-bisphosphate in the effector site. Also in this case, the new information is being used for drug discovery.

A wide range of compounds have been prepared by structure-based design and stepwise library synthesis, starting from the N-substituted-1-amino-2,5-anhydro-1-deoxy-D-mannitol scaffold that has structural similarity to fructosephosphates, thus to the PFK substrate and the PYK effector. This has led to the identification of furanose sugar amino amides as a novel class of inhibitors with IC50 values of 23 µM and 26 µM against trypanosomatid PFK and PYK, respectively. These compounds will be further optimized by detailed structure-activity relationship analysis for their further development into anti-parasite drugs.

Trypanosomatid glucokinases

A. Cordeiro, P. Michels in collaboration with A. Cáceres and J Concepción, Universidad de Los Andes, Mérida, Venezuela, and W. Versees, Vrije Universiteit Brussel

Trypanosoma cruzi and *Leishmania major*, but not *T. brucei*, have in addition to a hexokinase gene, a glucokinase gene. The latter codes for an enzyme with a peroxisome-targeting signal and the enzyme was localized to glycosomes. Both trypanosomatid enzymes were overexpressed, purified and characterized. They are only able to phosphorylate glucose indicating that they are true glucokinases. These glucokinases present highest, albeit still a moderate 24% sequence identity with their counterpart from *Trichomonas vaginalis*, which has been classified into group A of the hexokinase family. The *T. cruzi* glucokinase was crystallized in complex with glucose and ADP and the structure solved. In contrast to hexokinases, which show a moderate preference for the alpha anomer of glucose, the electron density clearly shows the D-glucose bound in the beta configuration in the *T. cruzi* glucokinase. Kinetic assays with alpha and beta-D-glucose further confirmed a moderate preference of the *T. cruzi* glucokinase for the beta anomer. Structural comparison of the glucokinase and hexokinases permitted the identification of a possible mechanism for anomer selectivity in these hexose-phosphorylating enzymes. The preference for distinct anomers suggests that in *T. cruzi* hexokinase and glucokinase are not directly competing for the same substrate and are probably both present, because they exert distinct physiological functions.

GENOME DATABASE MINING

Metabolism of trypanosomes

F. Opperdoes in collaboration with M. Ginger, University of Oxford, UK and A.H. Fairlamb, University of Dundee, Scotland.

The genes required for various pathways of carbohydrate metabolism, including glycolysis and the hexose-monophosphate pathway, are all present, but a comparison between African and American trypanosomes indicates that only *T. cruzi* contains a considerable number of genes encoding bacterial-type kinases with predicted specificity for various sugars, other than glucose. The enzymes encoded by these genes all contain targeting signals for import into the glycosomes, suggesting that in American trypanosomes the glycosomes have adapted to the breakdown of a wide variety of sugars. In *T. brucei* the carbohydrates that can provide carbon for energy metabolism are likely to be limited to glucose, fructose and mannose. No evidence in either trypanosome species was found for the

presence of either a functional glyoxylate cycle or uric acid cycle. Both *Trypanosoma* species are capable of synthesising or oxidising fatty acids. Consistent with previous biochemical studies, the capacity of trypanosomes for general lipid synthesis is, in comparison with many parasitic protozoa, impressive. Most amino acids, apart from the aromatic ones, can be oxidised by the two organisms, but amino-acid synthesis is generally limited to the so-called non-essential amino acids. *T. cruzi*, but not *T. brucei*, is able to utilise histidine as an energy source. The assembly of the mitochondrial respiratory chain, which is required for oxidative phosphorylation and efficient metabolism of amino-acid and fatty acid carbon sources, appears to be a balance between conserved and unique biochemical processes. Finally, the presence of numerous genes of bacterial ancestry indicates that horizontal gene transfer has played an important role in shaping the trypanosomatids' metabolic capacities.

Metabolism of *Leishmania*

F. Opperdoes, P. Michels, in collaboration with G.H. Coombs, University of Glasgow, Scotland.

The complete analysis of the *Leishmania* genome has facilitated the prediction of its metabolic capabilities. Gene predictions were in agreement with the results from experimental studies. Moreover, the available information allowed to interprete a number of key metabolic adaptations of *Leishmania* and why they differ from *T. brucei* and *T. cruzi*. These differences could be related to the special adaptations required for the viability of *Leishmania* inside the macrophage's phagosomal compartment and for its virulence. Numerous interesting drug targets could be identified, such as NADP-dependent fumarate reductase, acetate:succinate CoA transferase, dihydroorotate dehydrogenase and arginase. Genomic information has also provided a possible explanation for the mode of action of the newly introduced anti-leishmanial drug Miltefosine.

GLYCOSOME BIOGENESIS

N. Galland, E. Verplaetse, M. Gualdron, P. Michels, in collaboration with D. Vertommen (HORM), P. Van Der Smissen, P. Courtoy (CELL) and W. Hol, (University of Washington, Seattle, USA)

We have identified and characterized 10 *T. brucei* orthologues of peroxins (PEX proteins) directly involved in the import of proteins into the matrix of yeast and mammalian peroxisomes. Peroxisomal and glycosomal proteins utilize different types of targeting signals to be targeted from their site of synthesis, the cytosol, to the organelles' matrix: (i) the C-terminal peroxisome-targeting signal type 1 or PTS1 that mediates binding to the receptor PEX5, (ii) the N-terminal motif PTS2 recognized by PEX7 or (iii) sorting information that does not conform to an identifiable consensus sequence and may be present in a polypeptide-internal segment (Fig. 3). Depletion of either PEX5 or PEX7 from the trypanosome by RNA interference led to partial mislocalization of glycosomal enzymes to the cytosol resulting in death of the parasites. Similar results were obtained by knocking down the expression of any of the genes coding for peroxins acting downstream of the receptors and which form a pathway through which, by a cascade of PEX-PEX interactions, both PTS1 and PTS2-bearing proteins are translocated across the membrane and the discharged receptors recycled to the cytosol. These data prove that all these peroxins are essential for the trypanosomes and might be interesting drug targets. Drugs to be developed should interfere with the interactions between the different peroxins of the parasites without interfering with the functioning of human peroxins. The very low sequence conservation of peroxins offer promise for the feasibility of this approach. Interactions between different peroxins and between receptors and proteins or peptides containing PTS motifs have been studied by a variety of techniques and crucial residues, motifs or peptides involved in such

interactions have been identified for several *T. brucei* peroxins. Crystal structures of the parasite's PEX5 PTS1-binding domain, complexed with a variety of PTS1 peptides have been solved and compared with the available structure of the human receptor. The trypanosome's PEX5 structure revealed a unique hydrophobic pocket in the interface of the two subdomains between which the PTS1 peptides are bound and that might be exploited to obtain compounds which interfere selectively with the PTS-protein binding or release in trypanosomatids and so disrupt glycosome biogenesis and prevent parasite growth.

GLYCOSOME DEGRADATION

M. Herman, A. Brennand, P. Michels, in collaboration with N. Schtickzelle (BIOL) and D. Perez-Morga, E. Pays (Université Libre de Bruxelles)

Trypanosomes have a complicated life cycle, involving two major, distinct stages living in, respectively, the mammalian bloodstream and the tsetse fly. Previous studies on non-differentiating trypanosomes have shown that the metabolism and enzymatic contents of glycosomes in bloodstream-form and insect stages, differ considerably. We have now followed the morphology of glycosomes and their position relative to the lysosome, organelles responsible for degradation of macromolecules and other

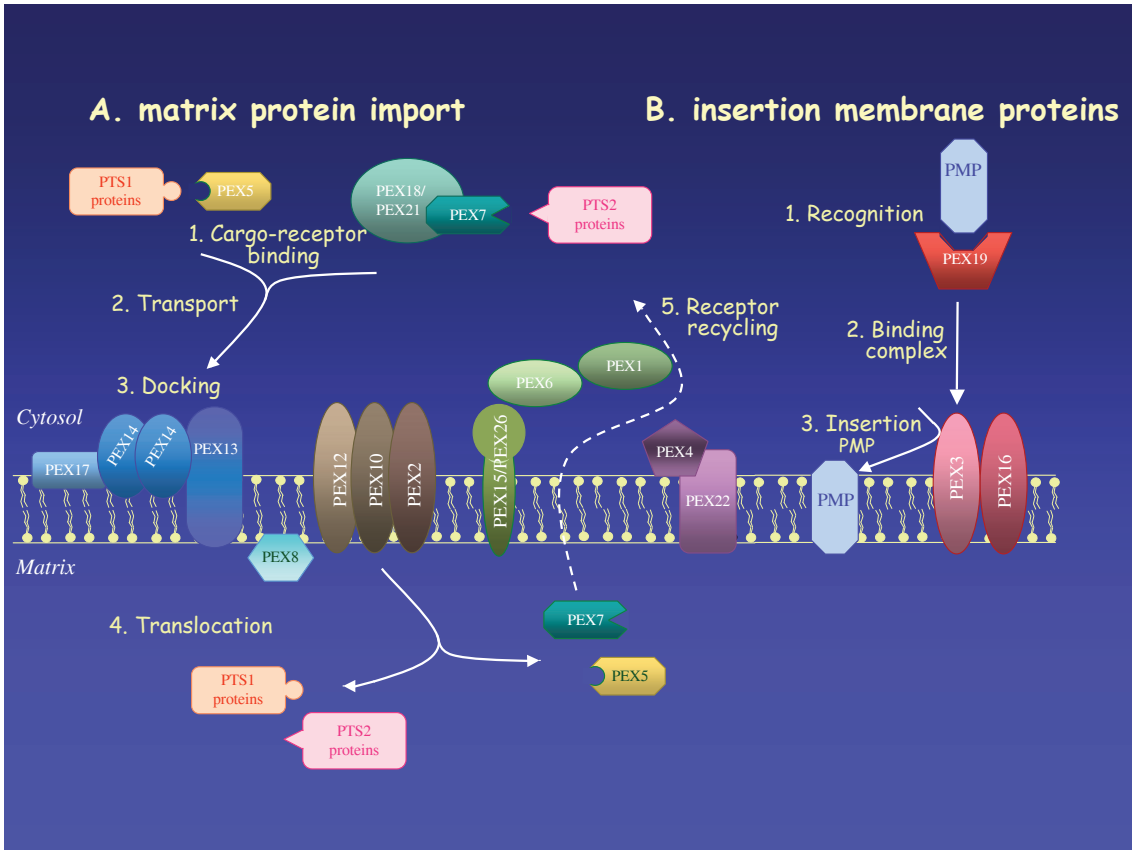

Figure 3. Schematic representation of proteins involved in the biogenesis of peroxisomes

organelles, as well as the levels of some glycosomal enzymes and markers for other subcellular compartments, during the differentiation of trypanosomes from the bloodstream to the procyclic form. These studies revealed a small tendency of glycosomes to associate with the lysosome, when a population of long-slender bloodstream forms differentiated into short-stumpy forms which are pre-adapted to live in the fly. The same phenomenon was observed during the short-stumpy to procyclic transformation, but then the process was fast and almost all glycosomes were associated with the dramatically enlarged degradation organelle. The observations suggested an efficient glycosome turnover involving autophagy. Changes observed in the levels of marker enzymes for different cell compartments are consistent with the notion that, during differentiation, glycosomes with enzymatic contents specific for the old life-cycle stage are degraded and new glycosomes with different contents are synthesized. This way the metabolic repertoire of trypanosomes is, at each stage, optimally adapted to the environmental conditions encountered. The genes of various proteins probably involved in the autophagy of glycosomes have been cloned and their precise function is under study.

GLYCOSOME SOLUTE TRANSPORTERS

M. Igoillo-Esteve, P. Michels

We have previously reported the identification of three ABC transporters in the glycosomal membrane of *T. brucei*. They have been designated GAT1 - GAT3 for Glycosomal ABC Transporters. They are so-called half ABC transporters, comprising only one transmembrane domain and a single nucleotide-binding domain. For GAT1 and GAT2 it was shown by immunofluorecence studies of trypanosomes transfected with deletion constructs fused to GFP that a short sequence motif adjacent to a transmembrane segment comprises the glycosome-targeting determinant. Recent protease protection assays indicated that the nucleotide-binding domain is at the cytosolic face of the membrane, suggesting that the transporters function as importers of substrates into the glycosome. GAT1 and GAT3 are expressed in both bloodstream-form and insect *T. brucei* while GAT2 is only found in bloodstream forms. Complementation studies with yeast mutants and phenotypic analysis of RNAi mutants suggest that the GATs transport fatty acids. Knocking down the expression of GAT1 resulted in overexpression of GAT3. Nevertheless this could not counteract the deleterious effect of the RNAi in insect stages grown in the absence of glucose, suggesting that both transporters have different substrate specificities.

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ENDOCYTOSIS AND EPITHELIAL DIFFERENTIATION

Endocytosis, a central activity of all eukaryotic cells, allows for cell nutrition, regulates the composition of the cell surface and controls transfer of macromolecules across epithelial barriers. The role of endocytosis in signalling, and, conversely, signalling to endocytosis, are fields of intense investigations. This research group has made significant contributions in the dissection of endocytic pathways (see Fig. 1) and in unravelling the contribution of endocytosis to physiopathology (3,4,6,8,9), parasitology (2) and pharmacology. We are currently addressing the molecular machineries controlling the endocytic activity at the apical surface of epithelial cells and upon malignant transformation. Recent achievements include: (i), the elucidation of the signalling cascade (10) whereby the paradigmatic oncogenes, v -Src and K-Ras, control the actin cytocortex (1), so as to perturb motility (7) and to induce macropinocytosis (1), specifically at the apical domain of polarized epithelial cells (5), like enteropathogens; (ii) the fine tuning of apical endocytosis leading to regulated production of thyroid hormones (3,9); (iii) the elucidation of a deficit of apical endocytosis in a genetic form of kidney stones (4); and (iv) the demonstration of a major role of apical endocytosis in the (mannose 6-phosphate-independent) biogenesis of kidney lysosomes (6). A strong asset of our group is a versatile cellular and tissular imaging platform including live cell imaging, confocal, multiphotons, transmission and scanning electron microscopes. Dr. C. Pierreux recently joined our Unit to develop a new project on tubulogenesis in the developing pancreas and salivary glands, as complementary models of controlled interconversion between multilayered cell masses and polarized monolayers.

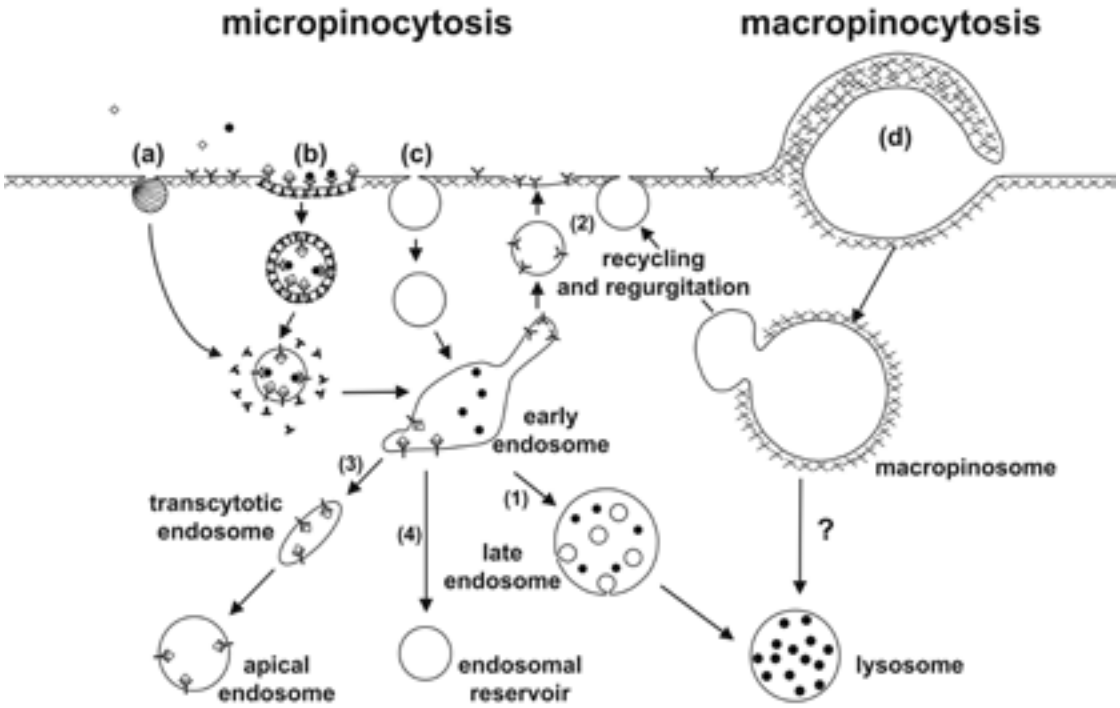

Fig. 1. Pathways of endocytosis. This scheme represents four possible modes of vesicular entry of solutes into cells, with the basolateral membrane represented above : (a) caveolae; (b) clathrin- and dynamin-associated pits, or «coated pits»; (c) clathrin- and dynamin-independent micropinocytic pits; and (d) macropinocytosis. Crosses represent cortical actin. It further emphasises the endosomes as sorting organelles after micropinocytosis and outlines the four endocytic routes inside the cell : (1) the degradative pathway to lysosomes; (2) the recycling pathway, back to the plasma membrane; (3) transcytosis to the opposite membrane domain (here illustrated from basolateral to apical); and (4) sequestration into slowly recycling endosomes.

REGULATION OF ENDOCYTOSIS BY V-SRC IN POLARIZED CELLS

M. Mettlen, Ph. de Diesbach, T. Medts, S. Carpentier, P. Van Der Smissen, D. Tyteca, P.J. Courtoy

Oncogenic transformation is well known to accelerate the endocytic activity, but the underlying mechanisms remained poorly understood. We have originally reported that v-Src and K-Ras cause a profound remodelling of actin cytoskeleton in Rat-1 fibroblasts, resulting in stress fiber disappearance, cortical actin polymerisation, ruffling and macropinocytosis (2). Since most cancers are of epithelial origin, and since apical endocytosis depends on actin, we examined whether v-Src would similarly trigger fluid-phase endocytosis in MDCK cells and whether apical endocytosis would be selectively affected. Because stable cell transformation abolishes epithelial polarity due to epithelio-mesenchymatous transition, we resorted to MDCK cells bearing a thermosensitive (ts) v-Src kinase. When MDCK/tsLA31 cells were plated at high density on a permeable support and cultured at the non-permissive temperature (40°C), a polarized epithelial monolayer could be established, with a 2-fold faster rate of fluid-phase endocytosis at the basolateral than at the apical surface. Shifting to the permissive temperature (34°C) rapidly activated v-Src kinase but preserved a tight monolayer for at least 6 h, allowing to study the effect of Src on polarized endocytosis. During this interval, Src kinase induced apical circular ruffling and selectively accelerated apical fluid-

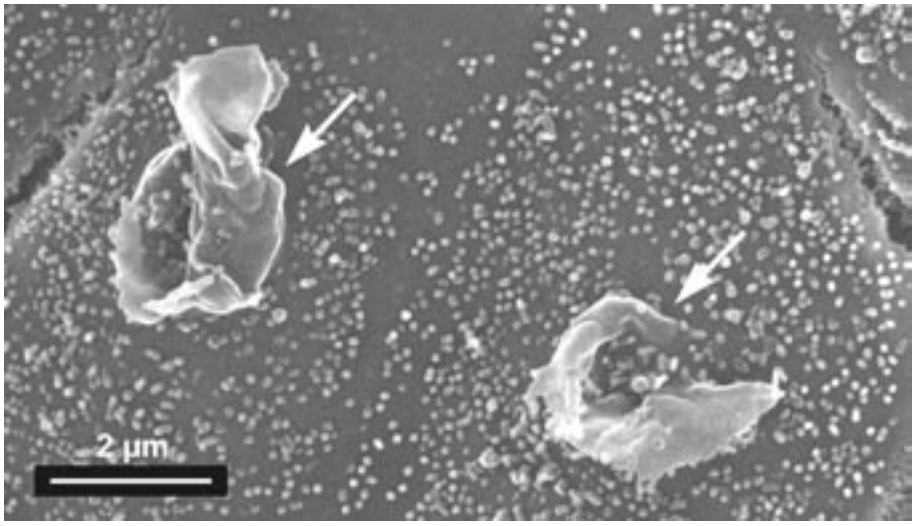

Fig. 2. Src causes circular apical ruffling (scanning electron microscopy).

phase endocytosis (up to 6-fold) (Fig. 2). This was accompanied by the induction of macropinosomes, merging into a huge (> 5 µm) apical endocytic vacuole, generated by swelling of the apical recycling compartment (ARE). Preservation of ARE tubulation and of apical polarity indicated that function of this essential compartment was not affected. Macropinosomes and the ARE were labeled for v-Src, Rab11, and rabankyrin-5, but not for early endosome antigen-1, thus distinguishing two separate Rab5-dependent pathways. Ruffling and macropinocytosis depended on an amplification cascade involving PI3K, PLC and PLD, as shown by inhibition by wortmannin, NCDC and 1-butanol, respectively. These data show that v-Src selectively affects the dynamics of the apical plasma membrane, where microdomains known as "lipid rafts" are abundant. The mechanisms of Src-induced apical ruffling and macropinocytosis could shed light on the apical entry into enterocytes triggered by enteroinvasive pathogens and on the apical differentiation of osteoclasts (5).

DIFFERENTIAL MEMBRANE RECRUITMENT OF SRC MAY SPECIFY ITS DOWNSTREAM SIGNALLING TO MAPK AND PI3K

Ph. de Diesbach, T. Medts, S. Carpentier, L. D'Auria, P. Van Der Smissen, A. Platek, M. Mettlen, A. Caplanusi, M.F. van den Hove, D. Tyteca, P.J. Courtoy

Most Src family members are diacylated and constitutively associate with membrane "lipid rafts" that coordinate signalling. Whether the monoacylated Src, frequently hyperactive in carcinomas, also localizes at "rafts" remains controversial. Using polarized MDCK cells expressing the thermosensitive v-Src/tsLA31 variant, we here addressed how Src tyrosine-kinase activation may impact on its (i) membrane recruitment, in particular to "lipid rafts"; (ii) subcellular localization; and (iii) signalling. The kinetics of Src-kinase thermoactivation correlated with its recruitment from the cytosol to sedimentable membranes where Src largely resisted solubilisation by non-ionic detergents at 4 °C, and floated into sucrose density gradients like caveolin-1 and flotillin-2, i.e. "lipid

rafts". By immunofluorescence, activated Src showed a dual localization, at apical endosomes/macropinosomes and at the apical plasma membrane. The plasma membrane Src pool did not colocalize with caveolin-1 and flotillin-2, but extensively overlapped GM1 labelling by cholera toxin. Severe (~70%) cholesterol extraction with methyl-β-cyclodextrin (MβCD) did not abolish "rafts" floatation, but strongly decreased Src association with floating "rafts" and abolished its localization at the apical plasma membrane. Src activation independently activated first the MAP-kinase-ERK1/2 pathway, then the PI3-kinase-Akt pathway. MAP-kinase-ERK1/2 activation was insensitive to MβCD, which suppressed Akt phosphorylation and apical endocytosis induced by Src, both depending on the PI3-kinase pathway. We therefore suggest that activated Src is recruited at two membrane compartments, allowing differential signalling, first via ERK1/2 at "non-raft" domains on endosomes, then via PI3-kinase-Akt on a distinct set of "rafts" at the apical plasma membrane. Whether this model is applicable to c-Src remains to be examined (10).

RELATION BETWEEN ENDOCYTOSIS AND CELL MOTILITY

A. Platek, M. Mettlen, D. Tyteca, Ph. de Diesbach, P.J. Courtoy

Endocytosis may contribute to cell motility by selective removal of plasma membrane constituents from the trailing edge and their recycling to the leading edge. Since v-Src accelerates both motility and endocytosis, we examined whether these two processes are linked and depend on the same regulatory machinery. To this aim, we made use of fibroblasts and epithelial cells harbouring thermosensitive v-Src kinase (Rat-1/tsLA29 and MDCK/tsLA31 cells). In both cell lines, v-Src activation accelerated cell motility by ~ 2-fold, as evidenced by the population-based wound healing assay and by single cell recording in Dunn chambers. Accelerated motility was selectively abrogated by PI3K, PLC and PLD inhibitors. These observations indeed suggest a link between accelerated motility and endocytosis.

In addition, v-Src activation abrogated directionality of cell motility in response to chemotactic growth factor (GF) gradients. v-Src down-regulated GF-receptors by about 2-fold, but directional motility of non-transformed cells was maintained at half receptor occupancy. The loss of chemotactic response to platelet-derived growth factor (PDGF) gradient was further examined in Rat-1/tsLA29 fibroblasts. At non-permissive temperature, PDGF receptor (PDGFR) signalling, assessed by phosphoY751-specific Western blotting (a docking site for PI3K), was not detected without PDGF and was induced by low PDGF concentrations. Both PI3K immunolabeling and a live cell imaging probe of its phosphatidylinositol 3,4,5 tris-phosphate product showed polarized translocation at lamellipodia towards a PDGF gradient, with the concomitant orientation of centrosomes and of PDGFR- and Src-bearing endosomes. Src thermoactivation without PDGF caused Y751 phosphorylation, which further increased with PDGF. Src and PDGFR activation were reciprocal and synergistic. As a consequence, PI3K was recruited and activated along the entire plasma membrane without PDGF and did not polarize in response to a PDGF gradient. Src activation also abrogated polarization of centrosomes and of PDGFR-bearing endosomes. Thus, PDGFR signalling persists despite strong Src kinase activity, but diffuse activation of PI3K by Src abrogates cell polarization and chemotaxis: "signalling requires silence" (7).

A FINE CONTROL OF THE RATE OF APICAL ENDOCYTOSIS REGULATES THYROID HORMONE PRODUCTION

P.J. Courtoy, K. Croizet, D. Tyteca, Ph. de Diesbach, M.F. van den Hove

The production of thyroid hormones by thyrocytes is achieved by apical endocytosis of thyroglobulin (Tg) stored in the colloid and intracellular proteolysis, but the cellular mechanisms responsible for its fine regulation are not clear. Since both substrates and hydrolytic enzymes are in vast excess, we hypothesised that the production of thyroid hormones is regulated by their encounter, i.e. depends on rate-limiting endocytic catalysts, such as Rab5 and Rab7. This hypothesis was tested by two approaches. First, we analysed clinical samples of autonomous hyperactive adenomas that result from activating mutations of the TSH receptor/cAMP cascade. The expression of Rab5a and Rab7 was increased up to 6-fold in adenomas by reference to perinodular quiescent tissues. Increased Rab5a and Rab7 expression was selective, coordinate, and correlated with a decrease of the residual Tg content. Second, regulation by TSH of the rate of Tg endocytosis and thyroid hormone production was tested in vitro, using primary cultures of normal thyrocytes in polarized monolayers that closely mimic apical Tg secretion then endocytosis, and basolateral release of thyroid hormones. TSH, or forskolin to mimic downstream activation of the cAMP cascade, increased Rab5a and Rab7 expression, apical Tg endocytosis and basolateral thyroid hormone secretion by 2-fold (3).

Since the activity of Rab catalysts depends on their GTP-bound state, we further addressed whether TSH also controls Rab5a activity by promoting GDP/GTP exchange factor(s) (GEF). In hyperactive autonomous adenomas, Rab5a-GEF total activity (availability) and sedimentability (engagement) were both increased by reference to quiescent perinodular tissue. Comparing all in vivo samples, GEF activity correlated with Tg depletion and expression of Rab5a and Rabex-5 (the classical Rab5-GEF). In polarized human thyrocyte monolayers, a 2-hours TSH exposure did not affect Rab5a-GEF but promoted its membrane recruitment; after 4 days, TSH increased total and sedimentable Rab5a-GEF, and Rabex-5 expression. Thus, the TSH receptor/cAMP cascade stimulates endocytosis by a two-stage

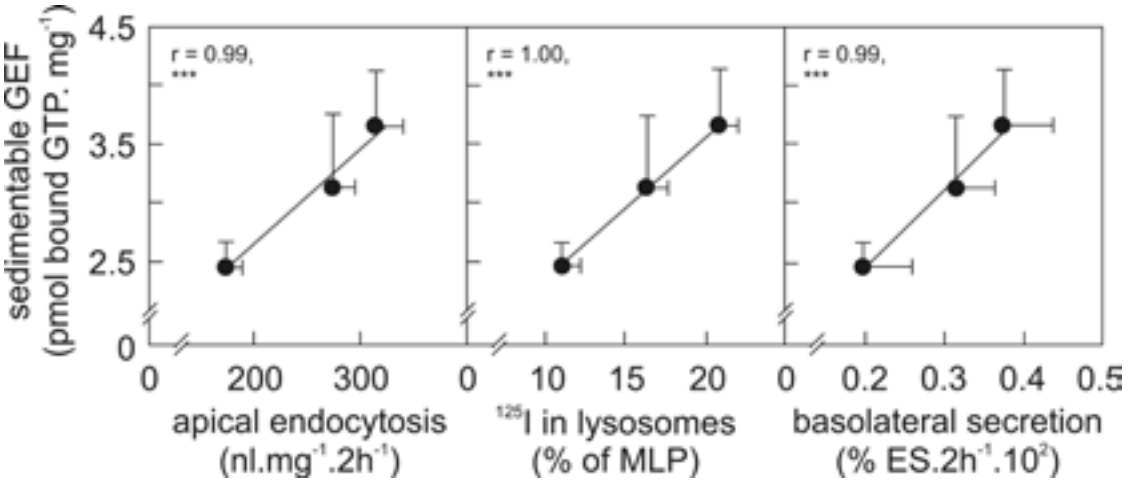

Fig. 3. Fine regulation of Rab5a activity by association with its GEF strongly correlates with apical endocytosis and lysosomal transfer of Tg, and with basolateral secretion of derived hormones.

mechanism: (i) rapid membrane recruitment of Rab5a-GEF; then (ii) coordinate increased expression of Rab5a and Rab5a-GEF. Comparing all in vitro experiments, sedimentable Rab5a-GEF strongly correlated with apical endocytosis and lysosomal transfer of Tg, and with basolateral secretion of derived hormones (Fig. 3). In conclusion, we have provided the first clinical and experimental evidence that control of the expression and activity of a rate-limiting endocytic catalyst finely tunes a normal cellular function, ultimately controlling whole-body metabolism (9).

ROLE OF APICAL ENDOCYTOSIS IN THE BIOGENESIS OF LYSOSOMES IN KIDNEY PROXIMAL TUBULAR CELLS

P.J. Courtoy, G. Dom, W. Rezende Lima, in collaboration with E.I. Christensen and his colleagues (Aarhus University, Denmark), O. Devuyst (NEFR, UCL) and M. Jadot (FUNDP, Belgium)

Recruitment of acid hydrolases to lysosomes generally occurs by intracellular sorting based on recognition of a common mannose 6-phosphate signal in the trans-Golgi network and selective transport to late endosomes/lysosomes. We have obtained direct evidence for an alternative, efficient secretion-recapture pathway, mediated by megalin, and exemplified by cathepsin B in kidney proximal tubular cells (PTC). We found that in mouse kidneys with defective megalin expression [megalin knockout (KO)] or apical PTC trafficking (ClC-5 KO, see below), the (pro)cathepsin B mRNA level was essentially preserved, but the protein content was greatly decreased and the enzyme was excreted in the urine as mannose 6-phosphate-devoid species. In polarized PTC monolayers, purified cathepsin B was avidly and selectively taken up at the apical membrane, and uptake was abolished by the megalin competitor, receptor-associated protein. Direct interaction of cathepsin B with megalin was demonstrated by surface plasmon resonance. Procathepsin B was detected in normal mouse serum. Purified cathepsin B injected into mice was preferentially taken up by kidneys and targeted to lysosomes where it remained active, as shown by autoradiography and subcellular fractionation. A single cathepsin B injection into cathepsin B KO mice could reconstitute full lysosomal enzyme activity in the kidneys. These findings demonstrate a pathway whereby circulating lysosomal enzymes are continuously filtered in glomeruli, reabsorbed by megalin-mediated endocytosis, and transferred into lysosomes to exert their function, providing a major source of enzymes to PTC. These results also extend the significance of megalin in PTC and have several physiopathological and clinical implications (6).

MOLECULAR MEDICINE: ROLE OF AN ENDOCYTIC TRAFFICKING DEFECT OF KIDNEY PROXIMAL TUBULAR CELLS IN HEREDITARY KIDNEY STONES

C. Auzanneau, W. Rezende Lima, S. Carpentier, P.J. Courtoy, in collaboration with O. Devuyst (NEFR)

Kidney proximal tubular cells (PTC) show one of the highest endocytic activities in the body, to recapture all ultrafiltrated low-molecular weight (LMW) proteins. Conversely, defective PTC function in inherited or acquired diseases, collectively referred to as Fanconi syndromes, leads to LMW proteinuria. A paradigm of Fanconi syndromes is Dent's disease, or X-linked nephrolithiasis, due to inactivating mutations of ClC-5. To understand the pathophysiology of Dent's disease, we have studied ClC-5 KO mice, in collaboration with E.I. Christensen (Aarhus, DK), O. Devuyst (NEFR, UCL) and W.B. Guggino (Johns Hopkins, Baltimore, MD, USA). These mice showed a severe deficit in PTC endocytosis of a variety

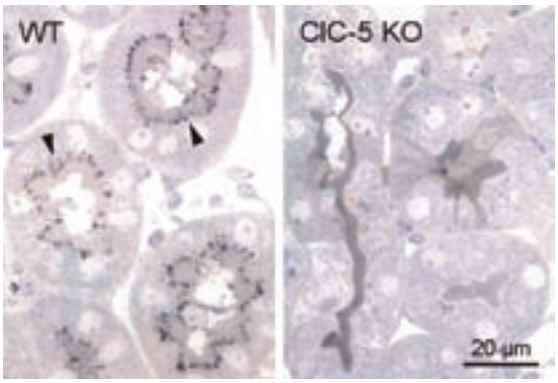

Fig. 4. Evidence for a deficit of apical endocytosis of low-molecular weight proteins in kidney proximal tubular cells of ClC-5 KO mice. Arrows indicate cytochemical detection of injected, ultrafiltrated peroxidase; note the strong labelling by peroxidase of multiple apical PTC endosomes in WT mice, contrasting with poor apical uptake and luminal retention in ClC-5 KO mice.

of ligands of megalin and cubilin, acting as high-capacity tandem receptors at the apical membrane (brush border) (Fig. 4). In contrast, fluid-phase endocytosis was better preserved, pointing to a role of megalin and cubilin trafficking. By the combination of analytical subcellular fractionation and ultrastructural immunogold staining, we could demonstrate that both receptors failed to reach the apical membrane, as part of a general membrane trafficking defect (4).

ClC-5, being primary located at PTC endosomes, was proposed to provide the inward chloride conductance necessary to neutralize the membrane potential generated by the electrogenic vacuolar ATPase, in order to support endosomal acidification. We addressed this hypothesis by measuring in vitro acidification of early and late endosomes labeled by apical fluid-phase endocytosis of FITC-dextran. ATP-dependent fluorescence quenching depended on chloride, but the acidification rate was undistinguishable between wild-type and ClC-5 KO mice. This indicates that another chloride channel plays a key role in normal PTC endosome acidification, or can compensate for ClC-5 absence. The trafficking defect in ClC-5 KO mice points to a role of ClC-5 in another compartment (ARE ?), or to another function of this channel. We currently address the molecular mechanisms regulating apical receptor-mediated endocytosis in polarized PTC monolayers.

COLLABORATIONS ON MEMBRANE TRAFFICKING AND CELL IMAGING

We have pursued our long-term commitment to promote collaborations by sharing expertise in endocytosis and cellular imaging. For the recent years, we may cite our contribution to the study of the endocytic trafficking of syndecans and its role in signalling by its heparin-sulfate ligands (Zimmermann et al., 2005, Dev. Cell 9:377-88); of the biogenesis of vacuolar H+-ATPase and the role of CFTR in kidney (Jouret et al., 2005, J. Am. Soc. Nephrol. 16:3235-46); of the subcellular trafficking of thrombopoietin receptor (Royer et al., 2005, J. Biol. Chem. 280:27251-61) and of the amyloid precursor protein, APP (Feyt et al., 2005, J. Biol. Chem. 280:33220-7; 2007, Biochem. Biophys. Res. Commun. 357:1004-10); the ultrastructural analysis of differentiating hepatoblasts (Clotman et al., 2005, Genes Dev. 19:1849-54) and the biogenesis of glycosomes in Trypanosoma brucei (Galland et al., 2007, Biochim. Biophys. Acta Mol. Cell. Res. 1773:521-35), or the morphological evidence by FRET of the interaction between key players of CTL that is interrupted during anergy (Demotte et al., 2008, Immunity 28:414-24).

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EXTRACELLULAR MATRIX BREAKDOWN

The extracellular matrix (ECM) plays a central role in the structural and functional organization of tissues and organs. ECM constituents, in particular fibrillar collagens, are the most abundant proteins of the human body. Physiological and pathological breakdown of ECM is predominantly achieved by a family of enzymes called matrix metalloproteinases (MMPs; see Fig. 1). Our laboratory was the first to demonstrate that menstrual bleeding is due to a dramatic change in the focal expression and/or activation of MMPs (1). This seminal observation led us to : (i) use this system as a human model to study the regulation of MMPs, in particular cellular interactions that integrate overall hormonal impregnation with local environmental changes; and (ii) explore whether this basic knowledge can lead to a better understanding and a rational treatment of abnormal uterine bleeding, a major health problem (5). We also investigate the control by individual cells of local MMP activity, which can be either increased by recruitment and retention to the plasma membrane, or down-regulated by receptor-mediated endocytosis and degradation.

DIFFERENTIAL CONTROL OF ENDOMETRIAL MMPS AND CYTOKINES BY OVARIAN STEROIDS, IN VIVO AND IN EXPLANTS

H. Gaide Chevronnay, C. Pretto, P.J. Courtoy, E. Marbaix, P. Henriet

Both endocrine and paracrine factors participate in controlling the expression and activity of MMPs involved in menstrual breakdown of the human endometrium. Several genes encoding MMPs and cytokines show maximal endometrial mRNA concentrations around menstruation. However, their expression profiles substantially diverge during the other phases of the cycle, indicating a differential response to estradiol and progesterone. Differential expression patterns could be classified according to the time frame, sharpness and magnitude of the changes. The differential responses to the ovarian steroids were faithfully reproduced in explant culture. Altogether, our observations suggest that, in vivo, local regulators finely tune in time, space and amplitude the global control by estradiol and progeste-

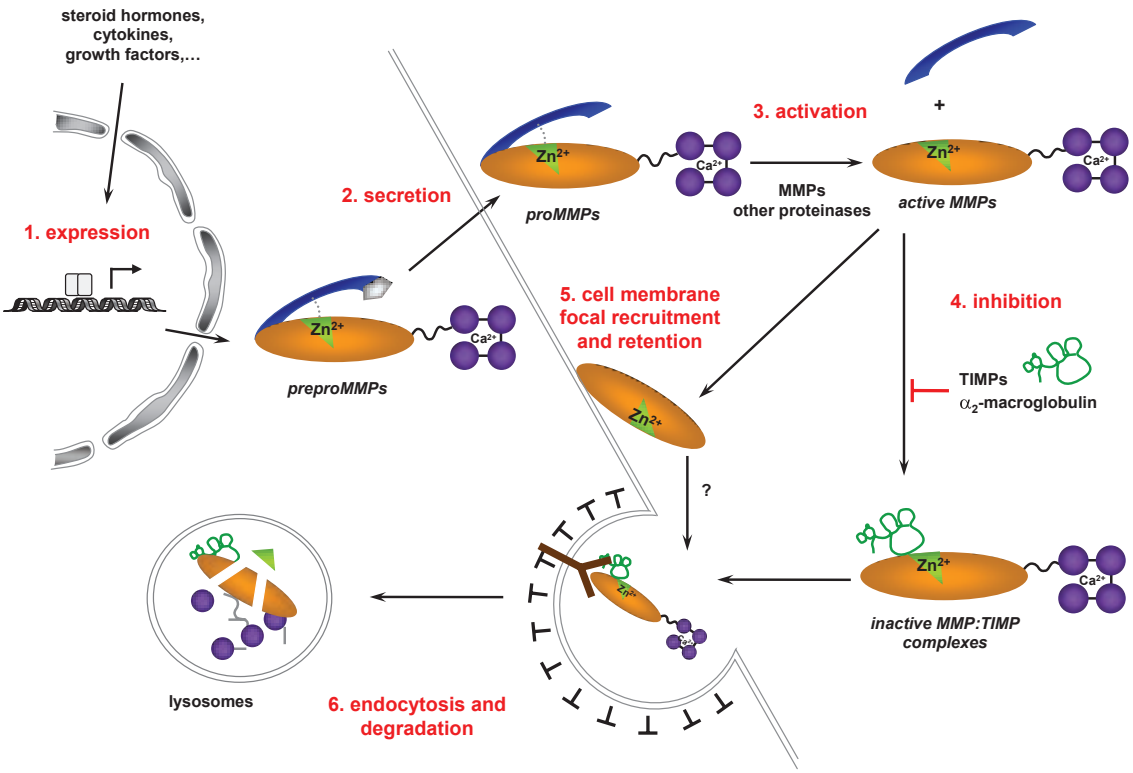

Figure 1. Regulation of soluble MMPs activity in the human endometrium: current model.

MMPs, the major actors of extracellular proteolysis, share a common intramolecular control due to masking by a N-terminal prodomain (blue, here shown with the signal peptide in grey) of the Zn^{2+}-dependent catalytic site (green) within the catalytic domain (orange). All but MMP-7 and -26 (matrilysins, the "mini MMPs"), contain an additional, variable C-terminal hemopexin-like domain stabilized by calcium (mauve) responsible for substrate specificity, linked by a hinge domain (black). The overall activity of MMPs can be controlled at 6 different levels: (1) expression, (2) secretion (regulated in a limited number of cell types such as neutrophils), (3) zymogen activation upon prodomain excision, (4) inhibition of active forms by physiological inhibitors such as TIMPs (represented with their tertiary structure) and α_2-macroglobulin, (5) secondary membrane recruitment increasing pericellular activity, and (6) down-regulation by endocytosis. In the human endometrium, MMPs activity is tightly regulated to remodel the extracellular matrix both for blastocyst implantation and, in its absence, for menstrual breakdown of an irreversibly specialized tissue. In particular : (1) ovarian steroids and their intracellular receptors as well as cytokines, growth factors and downstream signaling pathways interact to form an integrated system that differentially controls the focal expression of endometrial MMPs and TIMPs. (2) Neutrophils are numerous at menstruation and could contribute to an abundant secretion of MMPs. (3) MMPs can be activated by other MMPs, by plasmin, itself activated during menstruation, or by as yet unidentified proteinases. (4) TIMPs are particularly abundant in the human endometrium; like MMPs, the level of TIMPs is regulated by ovarian steroids and cytokines. (5) MMP-7 binds to membrane receptors in cholesterol-rich domains, a mechanism which enhances pericellular MMP activity. (6) Endometrial LRP-1 (brown) binds and internalizes MMP-2 and MMP-2: TIMP-2 complexes, leading to lysosomal degradation. Our research has unraveled, and is focused on, levels (1), (3), (4), (5) and (6).

rone of the expression of genes required for menstrual ECM breakdown.

Among local regulators, transforming growth factors-β (TGF-βs) have been reported to mediate the repression by progesterone of several MMPs in the human endometrium, thereby preventing menstrual breakdown but reports were conflicting on the expression profiles, source and regulation of the TGF-β system in this tissue. We therefore readdressed by real-time RT-PCR and ELISA the expression of the three TGF-βs (total and mature forms) and their two receptors throughout the menstrual cycle, as well as their regulation by ovarian steroids in cultured explants, including in microdissected epithelial and stromal compartments (Fig. 2, adapted from ref. 10). This comprehensive study on a large collection of endometrial samples evidenced a differential regulation of TGF-β isoforms expression. TGF-β2 and -β3 are differentially expressed during the menstrual cycle and differentially regulated by progesterone in epithelial vs stromal cells. The study also disclosed an opposite regulation of TGF-β2 and -β3 by cAMP and MAPK, which could account for their distinct expression *in vivo*.

BINDING OF ACTIVE MMP-7 TO THE PLASMA MEMBRANE ENHANCES ITS PERICELLULAR ACTIVITY AND PREVENTS ITS INHIBITION BY TIMPS

A. Berton, C. Selvais, P. Henriet, P.J. Courtoy, E. Marbaix, H. Emonard (in collaboration with CNRS, Reims, France)

Matrix metalloproteinase-7 (matrilysin-1) is specifically expressed by epithelial cells and modulates crucial biological events by processing a variety of epithelial cell surface-associated effectors. We therefore addressed how MMP-7, a "mini MMP" almost restricted to its catalytic domain, can interact with human epithelial cells while keeping its activity. We found that active MMP-7, but not its latent proform, is preferentially retained in cultured endometrial explants. By immunohistochemistry, active MMP-7 was localized at the periphery of glandular epithelial cells, contrasting with a diffuse intracellular pattern of the latent proenzyme. Incubation of recombinant latent and active ^{125}I-MMP-7 with Ishikawa cells (derived from a human endometrial carcinoma) confirmed that only the active species binds to the epi-

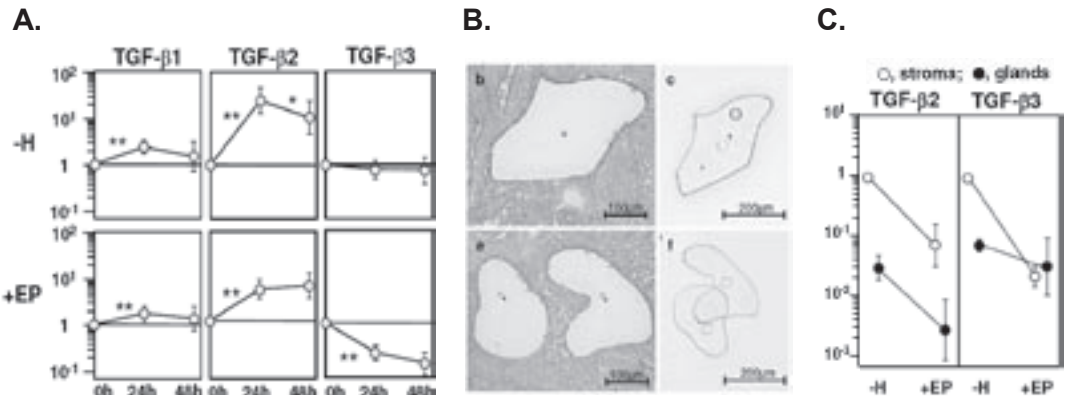

Figure 2. Differential expression of TGF-β2 and -β3 by endometrial explants. A. Explants were cultured in the absence (-H) or presence (+EP) of estradiol and progesterone for 24h or 48h. TGF-β mRNA levels were quantified by RT-PCR and normalized according to β-actin mRNA (geometric means with 95% confidence interval by reference to the non-cultured tissue, 0h). B,C. Laser capture microdissection of stroma (b,c) and glands (e,f) collected after 24h of culture highlights a differential regulation of TGF-β2 and -β3 by EP specifically in glands (mRNA levels in C presented by comparison to levels in stroma -H ; from ref. 10).

thelial cell surface, with saturable, specific and high-affinity binding (K_D ~ 2.5 nM). Cholesterol depletion by β-methyl cyclodextrin or competition by heparin independently decreased MMP-7 binding. Complexing with either natural MMPs inhibitors (large proteins) or synthetic inhibitors (small drugs) impeded MMP-7 cell surface binding, suggesting interaction via its catalytic domain. However, once membrane-bound, MMP-7 efficiently degraded a natural substrate, casein, and became resistant towards its physiological inhibitor, TIMP-2. In conclusion, active MMP-7 focalizes at the cell surface where it not only remains functional but even becomes resistant to natural inhibition (9).

LRP-MEDIATED ENDOCYTIC CLEARANCE OF PROMMP-2-:TIMP-2 COMPLEXES AND FREE TIMP-2

H. Emonard, C. Selvais, P. Henriet, E. Marbaix, P.J. Courtoy (in collaboration with CNRS, Reims, France)

Looking at mechanisms of MMPs clearance, we found that a strong accumulation of MMP-2 and -9 occurs in the conditioned medium of cultured endometrial explants upon addition of RAP, a natural antagonist of the low-density lipoprotein receptor-related protein (LRP-1). This observation suggested that LRP-mediated endocytosis of MMPs could represent a potent additional regulatory mechanism of MMPs activity during the menstrual cycle. We indeed demonstrated that proMMP2:TIMP-2 complexes and free TIMP-2 are efficiently cleared by endocytosis via LRP-1 in the HT1080 human fibrosarcoma cell line, a process requiring prior binding of TIMP-2 to another, as yet unidentified receptor (4).

ROLE OF MATRIX METALLOPROTEINASES IN ABNORMAL ENDOMETRIAL BLEEDING

C. Galant, P.J. Courtoy, P. Henriet, E. Marbaix

Since MMPs play a key role in initiating normal menstrual breakdown, we looked for their contribution in metrorrhagia. This abnormal endometrial bleeding is frequent but poorly understood. Using cultured explants from patients on long-term progesterone contraception, we previously reported that metrorrhagia involves MMPs (2). In collaboration with Dr. J. Donnez (UCL), we have investigated another cause of metrorrhagia referred to as "irregular dysfunctional bleeding", a spontaneous condition that cannot be accounted for by primary organic lesions, such as uterine myomas, endometrial polyps or cancer. We found that irregular dysfunctional bleeding is also associated with menstrual-like stromal breakdown, particularly in foci containing low levels of ovarian steroids receptors, and correlating with increased expression and activity of several MMPs, together with decreased production of TIMP-1 (Fig. 3). Neutrophils are further recruited to the same foci, release abundant proMMP-8 and -9, and thereby increase ECM proteolysis (5).

In collaboration with the laboratory of Dr. J.M. Foidart (ULg), we are currently developing xenografts in immunodeficient mice. This new experimental model should enable us

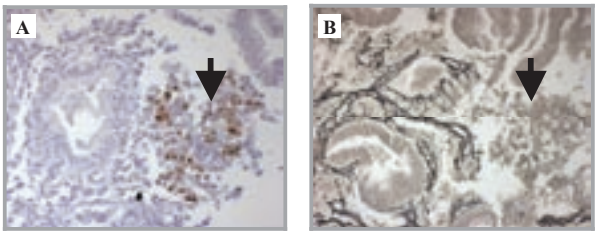

Fig. 3. Focal expression of MMPs correlates with ECM breakdown.
A. MMP-3 immunostaining.
B. Silver staining. Arrows show that MMP-3 expression is restricted to the area showing disappearance of collagen-rich argyrophilic fibers of the endometrial stroma during menstruation.

to inhibit in vivo the activity of MMPs so as to directly address their role in physiological and abnormal endometrial bleeding, endometrial angiogenesis and vessel maturation through pericyte recruitment (8).

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SIGNAL TRANSDUCTION BY GROWTH FACTOR RECEPTORS

Growth factors are soluble proteins that stimulate cell proliferation and migration through receptor tyrosine kinases. Our group is mainly interested in Platelet-derived growth factors (PDGFs) and Fibroblast growth factors (FGFs), which play an important role in wound healing and in embryonic development. The activated receptors phosphorylate a large number of substrates, including themselves, thereby initiating a complex network of signaling cascades (Fig. 1). Uncontrolled activation of these receptor tyrosine kinases is associated with brain, stomach, skin and hematological cancers. Our goal is to understand in detail (a) how receptor tyrosine kinases are activated in tumors, and (b) how signal transduction pathways regulate gene expression and, ultimately, cell growth.

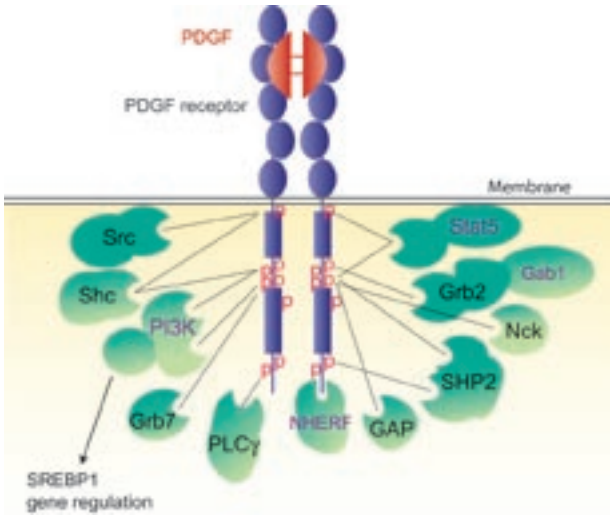

Fig. 1. Signal transduction by the PDGF receptors. Signaling molecules of special interest for our group are indicated in purple. The receptor (blue) contains five extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (boxes). p, phosphorylated tyrosine residue.

PDGF RECEPTOR ACTIVATION

A. Nzokirantevye, J.B. Demoulin.

PDGF receptors have an unusually long C-terminal domain. We isolated proteins binding to a peptide corresponding to the PDGF receptor β C-terminus. Using mass spectrometry, these proteins were identified as the PDZ-domain containing adaptors NHERF and NHERF-2. NHERFs binding did not modify the receptor activity, but regulated the reorganization of the cytoskeleton by PDGF (1).

Using a panel of PDGF receptor b mutants with progressive C-terminal truncations, we observed that deletion of the last 46 residues, which contain a Pro/Glu-rich motif, increased the activity of the receptor in the absence of ligand, compared to wild-type receptors (2). By contrast, the kinase activity of mutant and wild-type receptors that were pre-activated by treatment with PDGF was comparable. Using a conformation-sensitive antibody, we found that truncated receptors exhibited an active conformation even in the absence of PDGF. A soluble peptide containing the Pro/Glu-rich motif specifically inhibited the PDGF receptor β kinase activity. Deletion of this motif enhanced ligand-independent transforming ability of the receptor. These findings indicate that allosteric inhibition of the PDGF receptor β by its C-terminal tail is one of the mechanisms involved in keeping the receptor inactive in the absence of ligand.

Interestingly, NHERF is now considered as a tumor suppressor, being inactivated in a number of cancer. A PDGF receptor α mutant that has also been identified recently in glioblastoma has lost the C-terminal tail, and therefore the ability to bind NHERF.

In conclusion, we identified two distinct motifs in the PDGF receptor C-terminus : one binding to NHERF and one inhibiting the kinase domain.

GROWTH FACTORS STIMULATE LIPID SYNTHESIS BY ACTIVATING THE SREBP TRANSCRIPTION FACTORS

N. Dif, A. Essaghi, J.B. Demoulin.

We analyzed the transcriptional program elicited by stimulation of normal human fibroblasts with PDGF using cDNA microarrays produced by the Sanger/Ludwig/CRUK consortium. We identified 103 significantly regulated transcripts that had not previously been linked to PDGF signaling. Among them, a cluster of genes involved in fatty acid and cholesterol biosynthesis, including stearoyl-CoA desaturase (SCD), fatty acid synthase and hydroxy-methylglutaryl-CoA synthase (HMGCS), was up-regulated by PDGF after 24 h of treatment. Their expression correlated with an increase in membrane lipid biosynthesis. All these genes are known to be controlled by sterol regulatory element-binding proteins (SREBP). PDGF increased the amount of mature SREBP-1, and regulated the promoters of SCD and HMGCS in a SREBP-dependent manner. In line with these results, blocking SREBP processing by addition of 25-hydroxycholesterol blunted the effects of PDGF on lipogenic enzymes and PDGF-driven proliferation. SREBP activation was dependent on the phosphatidylinositol 3-kinase (PI3K) pathway, as judged from the effects of the inhibitor LY294002 and mutation of the PDGF receptor β tyrosine residues that bind the regulatory PI3K subunit p85. In conclusion, our results suggest that PDGF induces membrane lipid synthesis via PI3K and the activation of SREBP (3). The role of SREBP in PDGF and tumor development will be further analyzed.

Using Affymetric arrays, we obtained a more complete picture of gene regulation by PDGF. We are now focusing on genes regulated by transcription factors of the STAT, AP1 and forkhead families. We are also comparing gene regulation by PDGF receptors in normal cells and in tumor cells (5).

REARRANGEMENTS OF THE RECEPTOR TYROSINE KINASE GENES ASSOCIATED WITH CHRONIC LEUKEMIA

F. Toffalini, A. Nzokirantevye, C. Montano, J.B. Demoulin.

Although PDGF receptors are expressed on platelets and macrophages, deficient mice show no obvious hematopoietic or immune defect. In vitro, PDGF is a poor mitogen for hematopoietic cells.

However, modifications of PDGF receptor genes as a result of chromosomal translocation or deletion causes chronic malignant hemopathies, such as chronic eosinophilic leukemia, atypical chronic myeloid leukemia and chronic myelomonocytic leukemia. In all cases, the rearranged gene produces a hybrid protein comprising the PDGF receptor tyrosine kinase domain and an oligomerisation domain. Transfection of these constructs in Ba/F3 cells stimulated their proliferation, by contrast to wild type receptors. We and others have observed that PDGF receptor hybrids activate STAT5, an important transcription factor for Ba/F3 proliferation (6), whereas the wild-type PDGF receptors do not.

TEL-PDGFRβ (TPβ, also called ETV6-PDGFRB) is a hybrid protein produced by the t(5;12) translocation in chronic myelomonocytic leukemia (CMML). FIP1L1-PDGFRα (FPα) results from a deletion on chromosome 4q12 in patients with chronic eosinophilic leukemia (CEL). In contrast to wild-type PDGF receptors α and β, which are quickly degraded upon activation, we observed that TPβ and FPα escaped down-regulation resulting in stabilization of the proteins in Ba/F3 cells. High stability of FPα was confirmed in leukocytes from a patient with eosinophilic leukemia. Similar data were obtained in cells expressing ZNF198-FGFR1, another fusion protein associated with the 8p11 myeloproliferative syndrome. Cbl-mediated monoubiquitination of receptor lysines targets them for lysosomal degradation. Ubiquitination of TPβ and FPα was much reduced compared to wild-type receptors, despite marked Cbl phosphorylation in cells expressing hybrid receptors. Treatment with proteasome inhibitors slightly increased TPβ stability and revealed a modest TPβ polyubiquitination. Deletion of the pointed (PNT) domain, impairing TPβ polymerization, strongly destabilized the protein, suggesting that TPβ clustering prevents its degradation. In conclusion, chimeric receptor tyrosine kinases escape efficient down-regulation through lysosomes and proteasomes, by a mechanism that may involve altered ubiquitination and protein clustering.

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VIRAL IMMUNITY AND PATHOGENESIS

The possibility for evoluted organisms to survive viral infections depends on the ability of their immune system to eliminate the infectious agent. Therefore, numerous mechanisms, involving different types of immune cells such as cytolytic lymphocytes, T helper and B lymphocytes and macrophages, the molecules that allow those cells to communicate, namely the lymphokines, and the products of those interactions, including antibodies, have been elaborated. On the other hand, viruses have developed strategies to escape the immune system of their hosts, such as large frequencies of mutations or latency, or even to im- pair this system, which often leads to diseases such as autoimmunity or immunodeficiencies. Our project is to analyse, in murine models, some aspects of these relations between viruses and the immune system.

VIRAL INFECTIONS RESULT IN A DRAMATIC INCREASE IN THE PROPORTION OF IGG2A

Of particular interest is the fact that all antibody responses are not equal. Indeed, depending on their isotype, immunoglobulins display various properties. For example, IgG1, one of the major IgG subclass in mice, cannot fix the complement, contrary to IgG2a, another major component of murine immunoglobulins. Such a difference may lead to dramatic variations in the functional effect of antibodies, as their ability to lyse cells they have bound. During the last few years, we found that the isotype of antibody responses was influenced by concomitant viral infections. The effect of the virus resulted in a dramatic increase in the proportion of IgG2a, not only in antiviral antibodies, but also in immunoglobulins with an antigenic target unrelated to viral proteins. A dual regulation of antibody responses by gamma-interferon (IFN-γ) and interleukin-6 explains this isotypic bias (1, 2). In the case of antiviral antibodies, a possible explanation for this phenomenon could be the selection by the infected host of the most appropriate response against the virus. Using a model of infection with lactate dehydrogenase-elevating virus (LDV) (3), we could demonstrate that IgG2a antiviral antibodies are indeed more efficient than other isotypes to protect mice against a fatal polioencephalomyelitis induced by the virus (4, 5). The advantage for the host to select IgG2a in non-antiviral responses is more difficult to understand. In addition, the modification of the isotype of antibodies reacting with self antigens could potentially lead to more deleterious autoimmune reactions. This property of viruses to enhance selectively the production of one immunoglobulin isotype could depend on the preferential activation

of a subset of T helper lymphocytes. Indeed, different subpopulations of those cells, called Th1 and Th2, respectively, are distinguished in particular by their capability of producing selectively IFN-γ or interleukin-4, which can selectively trigger B lymphocytes to produce IgG2a or IgG1, respectively.

ACTIVATION OF NATURAL KILLER CELLS

Many of the influences that viruses may have on diverse immune responses can be explained by the production of pro-inflammatory cytokines, including IFN-γ. Therefore, our analysis of the relationship between viruses and the immune system has focused on the activation, by LDV, of cells from the innate immune system that are able to secrete this cytokine, namely the natural killer (NK) cells.

Within a few days after infection, a strong and transient NK cell activation, characterized by accumulation of this cell population in the spleen, by enhanced IFN-γ message expression and production, as well as by cytolysis of target cell lines was observed. Two pathways of IFN-γ production have been observed that both involve NK cells. The first pathway, found in normal mice, is independent from type I IFN and from interleukin-12. The second pathway involves interleukin-12, but is suppressed by type I IFN. Because NK cells and IFN-γ may participate in the defense against viral infection, we analyzed their possible role in the control of LDV titers, with a new agglutination assay. Our results indicate that neither the cytolytic activity of NK cells nor the IFN-γ secretion affect the early and rapid viral replication that follows LDV inoculation (6).

Similarly, infection with mouse hepatitis virus (MHV) is followed by NK cell activation and leads to IFN-γ production by those activated cells. In contrast to LDV, MHV replication is controlled by this cytokine and animals unresponsive to this molecule quickly die after infection. The protective effect of IFN-γ appears to target infected cells rather than lymphocytes.

ACTIVATION OF MACROPHAGES AND AUTOIMMUNE DISEASES

Activation of cells of the innate immune system includes also macrophages and leads to an enhanced phagocytic activity, with potential detrimental consequences for ongoing autoimmune diseases. Our analysis has been focused on autoantibody-mediated blood autoimmune diseases. A new experimental model of anti-platelet response was developed in the mouse (7). Immunization of CBA/Ht mice with rat platelets was followed by a transient thrombocytopenia and production of autoantibodies that react with epitope(s) shared by rat and mouse platelets. In contrast, BALB/C mice similarly immunized with rat platelets did not develop thrombocytopenia. The specificity of the antibody response elicited in these two mouse strains differed markedly, with platelet glycoprotein Ib recognized in CBA/Ht, but not in BALB/C animals. We have analysed whether a viral infection could modulate such an autoantibody-mediated autoimmune disease. In mice treated with anti-platelet antibodies at a dose insufficient to induce clinical disease by themselves, infection with LDV or mouse hepatitis virus was followed by severe thrombocytopenia (8). Similarly, administration of anti-erythrocyte monoclonal autoantibody to mice resulted in the development of a transient hemolytic anemia that was dramatically enhanced by a simultaneous infection with LDV, leading to the death of most animals. This viral infection induced an increase in the ability of macrophages to phagocytose in vitro autoantibody-coated red cells, and an enhancement of erythrophagocytosis in the liver (9). Treatment of thrombopenic or anemic mice with clodronate-containing liposomes and with total IgG indicated that opsonized platelets and erythrocytes were cleared by macrophages. Administration of clodronate-containing liposo-

mes decreased also the in vitro phagocytosis of autoantibody-coated red cells by macrophages from LDV-infected animals. The increase of thrombocytopenia triggered by LDV after administration of anti-platelet antibodies was largely suppressed in animals deficient for IFN-γ receptor. Together, these results suggest that viruses may exacerbate autoantibody-mediated thrombocytopenia and anemia by activating macrophages through IFN-γ production, a mechanism that may account for the pathogenic similarities of multiple infectious agents. Regulation of macrophage activation results in modulation of autoantibody-mediated cell destruction and may be considered as a possible treatment for autoimmune diseases that involve phagocytosis as a pathogenic mechanism. Finally, the Docile strain of lymphocytic choriomeningitis virus (LCMV) induces also anemia in a number of inbred strains of mice, including C3HeB/FeJ and CBA/Ht animals. A difference in the kinetics of anemia and of compensatory reticulocytosis suggested that impaired erythropoiesis was the major pathogenic mechanism involved in CBA/Ht, but not in C3HeB/FeJ mice. In both mouse strains an anti-erythrocyte autoantibody production that depended on the presence of functional CD4+ T lymphocytes was observed. Although depletion of T helper lymphocytes prevented anemia in C3HeB/FeJ mice, this treatment largely failed to inhibit the development of the disease in CBA/Ht animals. This observation indicated that the anti-erythrocyte autoimmune response induced by the infection was at least partly responsible for the anemia of C3HeB/ FeJ, but not of CBA/Ht mice. Erythropha- gocytosis was enhanced in both mouse strains after LCMV infection, but did not appear to be a major cause of anemia. These data clearly indicate that similar disease profiles induced by the same virus in two different host strains can be the result of distinctly different mechanisms. Macrophage activation by LDV led also to an enhanced response to lipopolysaccharide (LPS), and to an exacerbate susceptibility to endotoxin shock (10). A synergistic effect of LDV and LPS triggered dramatic production of tumor necrosis factor (TNF) and IFN-γ. Susceptibility to LPS shock was completely mediated by TNF, and partially by IFN-γ.

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VIRAL PERSISTENCE AND INTERFERON RESPONSE

Our work focuses on the interplay between viral infections and the immune response of the host. On one hand, we analyze the model infection of the central nervous system by Theiler's virus. This virus can escape the immune defenses of the host and provoke a persistent infection of the central nervous system which can lead to a chronic demyelinating disease considered as a model of multiple sclerosis. On the other hand, we analyze the innate immune response against viral pathogens in the particular context of the central nervous system. Our work focuses on the analysis of the type I (IFN-α/β) and type III (IFN-λ) interferon responses which are critically important to control viral infections and to modulate the acquired immune responses.

THEILER'S VIRUS

Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a murine pico-rnavirus showing a striking ability to persist in the central nervous system of the host in spite of a specific cellular and humoral immune response. Persistence of the virus is associated with a strong inflammatory response

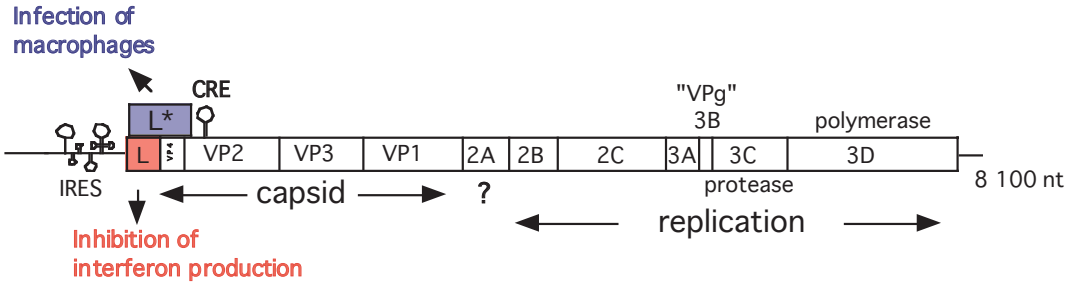

Fig. 1. Genome of Theiler's virus. A large open reading frame encodes a 2000 amino acid-long polyprotein that is cleaved, by autoproteolytic activity, into 12 mature proteins. Proteins VP1, VP2, VP3 and VP4 form the viral capsid. Proteins 2B, 2C ... 3D participate in the complex invoved in viral RNA replication. A replication signal has been discovered in the VP2 coding sequence and is denoted CRE for «cis-acting replication element» (1) Our analysis focuses on 2 proteins L and L* that interfere with the host innate immune responses and therefore facilitate the establishment of a persistent infection. Protein L inhibits type-I interferon production and affects nucleo-cytoplasmic trafficking of host proteins (2, 4). Protein L* is encoded by an alternative open reading frame. It was shown to facilitate the infection of macrophages (3) and to interfere with the IFN response.

and with lesions of primary demyelination reminiscent of those found in human multiple sclerosis. Our work aims at understanding how a virus can persist in the central nervous system (CNS) of an immunocompetent host, thus evading the immune response.

ANALYSIS OF VIRAL PROTEINS INVOLVED IN THEILER'S VIRUS ESCAPE OF THE HOST IMMUNE RESPONSE.

C. Ricour, F. Sorgeloos, T. Michiels

Two viral proteins, namely L and L* were found to be crucial for persistence of the virus in the central nervous system though they are not required for replication of the virus in cell culture. Hence, these proteins are believed to interact with host factors in vivo and to counteract the host immune defenses.

Inhibition of type-I IFN production and alteration of nucleocytoplasmic trafficking by the L protein

The leader (L) protein encoded by Theiler's virus is a 76 amino acid-long peptide containing a zinc-binding motif. We showed previously that the L protein could inhibit production of type-I interferons (IFNs) by infected cells (2). Mutation of the zinc-finger is sufficient to abolish the anti-IFN activity of the L protein in vitro and to dramatically impair viral persistence in the central nervous system of mice.

We also showed that the leader protein interferes with nucleo-cytoplasmic trafficking of host cell proteins (4). Perturbation of nucleocytoplasmic trafficking can constitute a effective way to inhibit early cell defense mechanisms. Indeed, the induction of many genes involved in early host defense, such as genes coding cytokines and chemokines, depends on the nuclear translocation of transcription factors such as NFκB or IRF-3 into the nucleus.

Infection of mice deficient for the type-I interferon receptor (IFNAR-/-) indicates that IFN production inhibition by the L protein also occurs in vivo. However, the inhibition of IFN production is not absolute. If it were, the DA virus would cause a fatal encephalomyelitis in wild-type mice analogous to the disease it causes in IFNAR-/- mice (6).

The L protein encoded by Encephalomyocarditis (EMCV), a related Cardiovirus, shares about 35% amino acid identity with that of Theiler's virus. It differs from the latter by lacking a serine/threonine-rich C-terminal domain and by carrying phosphorylated residues not conserved in Theiler's virus L protein. Nevertheless, in spite of these differences, the L protein of EMCV shares, with that of Theiler's virus, the ability to inhibit the transcription of type I interferon, cytokine and chemokine genes and to interfere with nucleo-cytoplasmic trafficking of host-cell proteins (7). Recent work performed in collaboration with S. Hato and F. van Kuppeveld (NCLMS, Nijmegen, The Netherlands) confirmed the importance of the L protein of EMCV in counteracting the IFN response in vivo.

Interestingly, analysis of viral RNA replication of chimeric viruses constructed between TMEV and EMCV raised the hypothesis that L proteins of TMEV and EMCV diverged during evolution to adapt to the different replication fitness of these viruses (7).

Influence of the L* protein on macrophage infection and viral persistence

Persistent strains of Theiler's virus produce an 18 KDal protein called L*, which is encoded by an open reading frame (ORF) overlapping the ORF coding the viral polyprotein (see Fig. 1). This protein was shown to enhance the infection of macrophage cell lines and to be critical for persistence of the virus in the central nervous system of the mouse (3).

Current efforts aim at characterizing the function of the L* protein in the infection of macrophages. Macrophages are indeed key players in the demyelinating disease induced by Theiler's virus, being simultaneously effectors of the immune response and targets of viral infection. Our recent data suggest that the L* protein antagonizes an important effector pathway of the interferon response. Thus Theiler's virus would interfere both with the production of IFN by infected cells and with the response of cells to this cytokine. This outlines the major importance of the IFN system in the defense of the host against viral infections.

TYPE-I AND TYPE-III INTERFERONS

Interferons were the first cytokines to be identified. They were discovered about 50 years ago by Isaacs and Lindemann who observed that chick chorio-allantoic membranes developped resistance to viral infection after exposure to heat-inactivated influenza virus. Interferons are typically secreted by cells that are infected by a virus. They alert neighbouring cells about the presence of a viral infection and allow these cells to develop an anti-viral state. The interferon system represents a critical protection mechanism of the body against viral infections. In addition, interferons have anticancer properties and modulate the acquired immune response of the host.

Type I IFNs

C. Sommereyns, P. Hermant, T. Michiels

Mouse and human genomes carry more than a dozen of genes coding for closely related interferon-alpha (IFN-α) subtypes. These interferons and other interferons, like IFN-β, IFN-κ, IFN-ε, IFN-ω, and limitin, form the type-I IFN family. In spite of important sequence divergences, all these IFNs bind the same receptor, raising the question of whether they possess specific functions.

We previously characterized the murine IFN-α family. 14 IFN-α genes were detected in the mouse genome, two of which, IFN-α13 and IFN-α14, were previously undescribed. In addition the mouse genome contains three IFN-α pseudogenes (5).

Some IFN-α subtypes are N-glycosylated and others are not. However, we failed to detect a clear difference in the activity of glycosylated and non-glycosylated IFNs in vitro and in vivo (10). The various IFN-α subtypes displayed a good correlation between their antiviral and antiproliferative potencies, suggesting that IFN-α subtypes did not diverge primarily to acquire specific biological activities, but probably evolved to acquire specific expression patterns.

Type-III Interferons (IFN-I)

C. Sommereyns, S. Paul, T. Michiels

Recently, another IFN family was described and called «type-III IFN» or «IFN-λ». Although type III IFNs bind a receptor distinct from the type-I IFN receptor, they activate the same signal transduction pathway and upregulate the same set of genes. Thus, they are expected to exert very similar biological activities as type I IFNs. Our work addresses the question of the redundancy of these two IFN systems in vivo.

We recently observed that the relative expression of IFN-λ over that of IFN-α/β exhibited some extent of tissue specificity and was low in the brain. We also used a strategy based on in vivo expression of cloned IFN genes to compare the responses of different tissues to IFN-α and IFN-λ. Interestingly, response to IFN-λ appeared to be specific to epithelial cells, unlike response to IFN-α which occurred in most cell types and was particularly prominent in endothelial cells. Accordingly, tissues with a high epithelial content such as intestine, skin or lungs were the most responsive to IFN-λ and expressed the higher amounts of IFN-λ receptor (10).

Our data suggest that the IFN-λ system evolved as a specific protection of epithelia and that it might contribute to prevent viral invasion through skin and mucosal surfaces. Future work will address the physiolgical importance of the IFN-λ system.

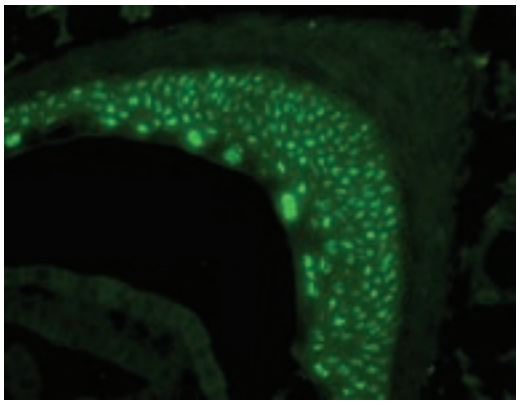

Fig. 2. Reponse to IFN-λ. Section of the urinary epithelium showing a strong response of epithelial cells to IFN-λ. Shown is a fluorescent labelling of the nuclear Mx1 protein, the expression of which is typically induced in response to IFN.

Production of IFN-α/β by neurons

S. Paul, T. Michiels

In peripheral organs, a specialized population of dendritic cells known as «plasmacytoid» dendritic cells (pDCs) was reported to account for most of the type I IFN production. Constitutive expression of the IRF-7 transcription factor enables pDCs to rapidly synthesize large amounts of IFNs after viral infection. In the central nervous system, however, these cells are reportedly absent or very rare.

In collaboration with the teams of Peter Staeheli and Friedemann Weber (Univ. Freiburg, Germany) we analyzed the cells that are responsible for type I IFN production in the central nervous system, during acute encephalitis caused by two neurotropic viruses: Theiler's virus (picornavirus) or La Crosse virus (bunyavirus).

IFN-α/β turned out to be produced by many scattered parenchymal cells and less by cells of the inflammatory foci. Interestingly, besides some macrophages and ependymal cells, neurons turned out to be important producers of both IFN-α and IFN-β. However, not more than 3% of infected neurons expressed IFN, suggesting that some restriction to IFN production might occur in these cells (9).

All CNS cell types analyzed, including neurons, were able to respond to type I IFN by producing Mx or IRF-7. Our data show that, in vivo, in spite of the relative immune privilege of the CNS parenchyma, neurons take an active part to the antiviral defense by being both IFN producers and responders (8, 9).

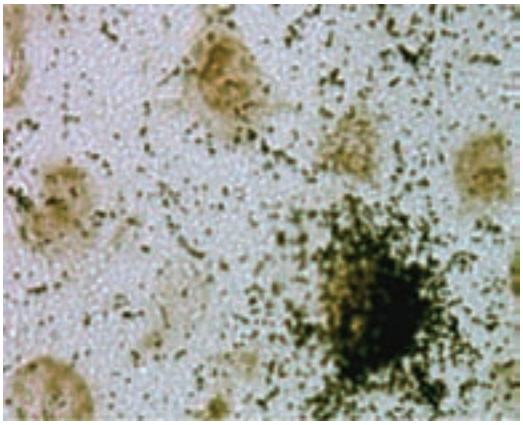

Fig. 3. Neurons can produce IFNα/β. Neuron (immunostained in brown for NeuN) producing IFN-β (detected by in situ hybridization: black dots) after infection with La Crosse virus.

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HUMAN TUMOR IMMUNOLOGY

Tumor-specific antigens, such as those encoded by the MAGE genes, have been used to vaccinate melanoma patients with detectable disease. About 20 % of the vaccinated patients display a tumor regression, a frequency that appears well above the level reported for spontaneous melanoma regressions. Nevertheless, the treatment fails in most patients, and this can probably only be improved by a better understanding of the anti-tumor immune responses of the patients and of the mechanisms of tumor resistance to immune attack.

Along this line, a first objective is to assess the immunogenicity of tumor vaccines and to compare various vaccine modalities. We have developed very sensitive methods for the detection of anti-tumor T lymphocytes, and now apply them to patients included in cancer vaccination trials conducted by Dr. Jean-François Baurain at the Cliniques Universitaires St Luc and by the clinical team of the Ludwig Institute (1, 2, 3).

A second objective is to understand the mechanism of the tumor regressions that occasionally occur in vaccinated patients. The detailed analysis of one such patient indicated that, surprisingly, the anti-vaccine T lymphocytes are largely outnumbered by other tumor anti-T cells, which recognize tumor-specific antigens different from the vaccine antigens. These anti-tumor T cells represent most of the T cells present in a regressing tumor, and they probably play a major role in the rejection process (4, 5). Why these anti-tumor T cells become activated following vaccination with antigens that they do not recognize is not clear.

That local immunosuppression could be involved in preventing tumor rejection is compatible with the observation that the anti-tumor T cells mentioned above are systematically present in tumors already before vaccination. Considering that suppressive or so-called regulatory T cells are recognized as important attenuators of immune responses, we have initiated an analyzis of their role in the vaccinated patients. We have also started to explore the functional status of the T lymphocytes that are infiltrating melanoma tumors, with an in situ genetic approach.

ANTI-VACCINE T CELL RESPONSES IN MELANOMA PATIENTS VACCINATED WITH DEFINED TUMOR-SPECIFIC ANTIGENS

G. Hames, V. Corbière, P.G. Coulie, in collaboration with A.-M. Feyens and J.-F. Baurain, Department of Medical Oncology, Cliniques Universitaires St Luc, and N. van Baren, Brussels branch of the Ludwig Institute for Cancer Research.

We focused on the analysis of CD8 T cell responses to antigenic peptides presented by HLA-A1 or A2 molecules. Several small clinical trials have been performed with the MAGE-A3 antigenic peptide EVDPIGHLY, presented by HLA-A1 (7). Table 1 presents a summary of the results obtained in patients who showed evidence of tumor regression and in patients who did not, after vaccination with either ALVAC-MAGE, a recombinant poxvirus containing a minigene encoding the MAGE-3. A1 peptide, or dendritic cells loaded with the peptide (G. Schuler, Erlangen and K. Thielemans, Vrije Universiteit van Brussel), or peptide MAGE-3.A1 alone. The observed correlation between CTL responses and tumor regression supports the notion that the tumor regressions are caused by the vaccines (3). We then analyzed melanoma patients vaccinated with 8 antigenic peptides, all encoded by genes displaying a tumor-specific pattern of expression and all presented by HLA-A2 molecules. The peptides were co-administered with the immunological adjuvant CpG7909, a ligand for Toll-like receptor 9. Monitoring the frequencies of blood T cells against each individual peptide indicated a hierarchy in the immunogenicity of these peptides, with proportions of patients with a detectable T cell response ranging from 0% for several peptides to 50% for one peptide. As expected, the adjuvant participates in the immunogenicity of these peptidic vaccines, as T cell responses were much rarer in patients who received the same set up of 8 peptides without adjuvant. We also compared CpG7909 with another immunological adjuvant that can be used in humans : Montanide ISA51, a clinical grade incomplete Freund's adjuvant. The results were similar.

Despite the higher immunogenicity of the CpG + 8 peptides combination, as compared to peptide MAGE-3.A1 alone, we have not observed a significant difference in the clinical results obtained in the two groups of vaccinated patients. This suggests that the main limitation to the clinical efficacy of the MAGE vaccines is not their immunogenicity.

Vaccination mode	CTL response in patients with	
	evidence of tumor regression	no evidence of tumor regression
ALVAC-MAGE	3/4	1/11
Dendritic cells + peptide MAGE-3.A1	3/3	0/3
Peptide MAGE-3.A1 alone	1/7	0/13
	7/14	1/27

Table 1. Summary of anti-MAGE-3.A1 CTL responses in vaccinated melanoma patients.

TUMOR REGRESSIONS OBSERVED AFTER VACCINATION: A ROLE FOR TUMOR-SPECIFIC CYTOLYTIC T LYMPHOCYTES THAT DO NOT RECOGNIZE THE VACCINE ANTIGENS

V. Corbière, T. Connerotte, T. Aerts, C. Muller, P.G. Coulie, in collaboration with C. Lurquin, B. Lethé, Brussels branch of the Ludwig Institute for Cancer Research.

It is clear from Table 1 that several vaccinated patients displayed tumor regression in the absence of a detectable anti-vaccine CTL response. In addition, even among those vaccinated patients who showed a CTL response, most had a low frequency of anti-MAGE-3.A1 CTL in the blood, ranging between 10^{-6} and 10^{-5} of CD8 T cells. Because such a level of CTL might be insufficient to produce on its own the observed tumor regressions, we examined the possibility that CTL directed against other antigens present on the tumor might contribute to the regression. For seven vaccinated melanoma patients, selected because it had been possible to derive a permanent cell line from their tumor, we estimated the blood frequencies of CTL directed against any antigen present on the tumor cells. For all seven patients, anti-tumor CTL were found at high frequencies, i.e. from 10^{-4} to 3×10^{-3} of the CD8 T cells, in the blood after vaccination. Unexpectedly, they were already present at similar high frequencies before vaccination. The frequency of anti-tumor CTL observed after vaccination was considerably higher than that of the anti-vaccine CTL, ranging from 12 fold to 20,000 fold higher (4). Because T cells directed at other tumor antigens than the vaccine antigen could make an important contribution to the tumor regressions, we felt that it was necessary to define the precise nature of their target antigens. We focused our effort on patient EB81, who had shown complete regression of a large number of cutaneous metastases following vaccination with ALVAC-MAGE. A majority of anti-tumor CTL clones recognized antigens encoded by MAGE-C2, a cancer-germline gene (4). Others recognized an antigen encoded by gp100, a melanocytic differentiation gene. In conclusion we are facing a paradoxical situation where the melanoma patients that are being vaccinated, have already mounted a high spontaneous response against the types of antigens used in the vaccines. At the time of vaccination this spontaneous T cell response is clearly ineffective in halting tumor progression. To evaluate the potential contribution of the "anti-tumor" T cells to the tumor rejection that occured following vaccination, we measured the frequency of the anti-vaccine and anti-tumor T cells in metastases of patient EB81. The frequency of anti-MAGE-3.A1 T cells was 2.5×10^{-6} of CD8 T cells in the blood and it was 6-fold higher in a metastasis. An anti-tumor CTL recognizing an antigen encoded by MAGE-C2 showed a considerably higher enrichment: its blood frequency was 9×10^{-5}, and it was about 1,000 times higher in the tumor. Several other anti-tumor T cell clonotypes also had frequencies above 1% and appeared to constitute the majority of the T cells present in metastases (5). These results suggest that the anti-vaccine CTL may not be the principal effectors that kill the bulk of the tumor cells. They may exert their effect mainly by an interaction with the tumor that creates conditions enabling the stimulation of large numbers of CTL directed against other tumor antigens, which then proceed to destroy the tumor cells.

FUNCTIONAL ANALYSIS OF TUMOR-SPECIFIC T CELL CLONES

T. Connerotte, T. Aerts, P.G. Coulie

The results summarized above suggested that, at least in some vaccinated patients, a surprisingly low number of anti-MAGE-3.A1 T cells sufficed to trigger a tumor rejection response. Among other possibilities, these rare anti-vaccine T cells could do so as a result of a particularly high affinity for the MAGE-3.A1

antigen, or a very high lytic activity against melanoma cells. We therefore conducted functional analyses on these anti-MAGE-3.A1 T cells. Our first objective was to find explanations for their putative anti-tumor activity in vivo, keeping in mind their low frequencies in the vaccinated patients. Our second objective was to examine whether different vaccination modalities with the same antigen resulted in different functions being exerted by the anti-vaccine T cells. The very low number of anti-MAGE-3.A1 T cells in most of our vaccinated patients prevents robust ex vivo functional analyses, and we resorted on analysing a representative collection of 16 anti-MAGE-3.A1 CTL clones, derived from 9 vaccinated melanoma patients who showed tumor regression following vaccination. The CTL clones were screened for their lytic activity, functional avidity, cytokine secretion and gene expression profiles. The functional avidities of these CTL clones were surprisingly low, suggesting that high avidity was not part of the putative capability of these CTL to trigger tumor rejection. Most anti-MAGE-3.A1 CTL clones obtained after vaccination with dendritic cells, but not with peptide or recombinant ALVAC poxviruses, produced IL-10 (9). Transcript profiling confirmed this result and indicated that about 20 genes, including *CD40L, prostaglandin D2 synthase, granzyme K* and *granzyme H*, were highly differentially expressed between the anti-MAGE-3.A1 CTL clones derived from patients vaccinated with either peptide-ALVAC or peptide-pulsed DC. These results indicate that the modality of vaccination with a tumor-specific antigen influences the differentiation pathway of the anti-vaccine CD8 T cells, which may have an impact on their capacity to trigger a tumor rejection response.

TUMOR INFILTRATING LYMPHOCYTES

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As indicated above, our analysis of the complete T-cell response that some melanoma patients mount against their own tumor indicated that spontaneous responses occured prior to any kind of vaccination, and demonstrated that some of these tumor-specific T cells could be present in the tumors. The reasons for this seemingly pacific co-existence of tumor cells and tumor-specific T lymphocytes remain unclear. It is known from histological analyses that some melanoma tumors are infiltrated by T cells, and for primary tumors this infiltration is correlated with a better clinical prognosis. But the reason for the T cell infiltration of only some tumors is unknown. Over the last year, we have set up and combined several methods to analyze melanoma-infiltrating T cells in situ. Our objective is to be able to characterize the activation status and T cell Receptor (TCR) repertoire of these T cells and to compare these results with the exact localization of the T cells within the tumor. Nicolas van Baren observed that, very often, the so-called tumor-infiltrating lymphocytes are actually clustered around the tumor nodules. In some tumors they are present both in these margins and within the nodules. We would like to know whether these different types of T cells are activated, if this is the case what is their proportion and exact localization, and ultimately whether or not they are tumor-specific and whether or not they are immunosuppressed. The methodological approach that we follow comprises laser microdissection of small numbers (±100) of cells, guided by histochemistry. The very low amount of starting material imposes a preliminary amplification of the cDNA prior to real-time PCR analysis of many genes of interest, or to complete gene expression profiling. To this end we have adapted methods proposed for the amplification of genetic material from single cells.

CLONAL ANALYSIS OF REGULATORY T CELLS FROM CANCER PATIENTS

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Regulatory T cells, or T_{regs}, are a subset of CD4+ lymphocytes specialized in the suppression of immune responses. Their existence was initially revealed by their ability to prevent the development of auto-immune diseases in mouse models. Transcription factor *FOXP3* is specifically expressed in murine T_{regs} and is indispensable for their differentiation, maintenance and function. *FOXP3* is also highly expressed in human $CD4^+CD25^+$ T cells with suppressor function. However, in contrast to mouse cells, *FOXP3* is also expressed in other activated human T cells. In mice, T_{regs} were shown to contribute to cancer progression by inhibiting anti-tumor immune responses. It has long been proposed that T_{regs} could play a negative role in cancer patients, but this has remained difficult to verify due to the lack of a T_{reg}-specific marker, and to an incomplete understanding of their suppressive function. Our long term objective is to develop tools to test whether anti-tumor immune responses in cancer patients are under the negative influence of T_{reg} cells.

We succeeded in obtaining stable human Treg clones, namely clones that expressed high surface CD25 at rest, were anergic in vitro, and suppressed the proliferation of co-cultured $CD4^+$ cells. *FOXP3* mRNA and protein were high in these clones, but were also detected in CTL and in non-suppressive $CD4^+$ Thelper clones. In contrast, as was previously described for polyclonal T cells, demethylation of a conserved region of *FOXP3* intron 1 was only observed in the Treg clones. We used a set of clones defined by this stable epigenetic mark to gain insight into human T_{reg} cell function. Microarray analysis of T_{reg} and T_{helper} clones indicated that the transcriptional profile that is specific of activated T_{reg} clones includes a TGFβ signature (10). A TGFβ signature was also displayed by a T_{helper} clone "suppressed" by a T_{reg} clone. Finally, activation of T_{reg} but not Thelper clones resulted in the cleavage of the inactive pro-TGFβ precursor protein into mature TGFβ. Altogether, these results provide a rigorous demonstration that a hallmark of activated human T_{reg} cells is to produce bioactive TGFβ which has autocrine and paracrine actions on neighboring T cells (10).

We are currently attempting to identify the mechanisms by which activated T_{regs} cleave the pro-TGFβ precursor. We will also analyze the consequences of TGFβ signaling on the effector function of human T_{helper} or cytolytic T cells. Finally, we will try to define the mechanisms of resistance to TGFβ that characterizes some T_{helper} lymphocytes. The latter aspect seems to us of a particular interest. Indeed, we made the unexpected observation that some human lymphocyte populations are resistant to the cytostatic effect of TGFβ, in contrast to what is observed with other types of lymphocytes and as it is generally described in the literature. The lymphocytes that we analyze are derived from non-leukemic patients, implying that resistance is not a consequence of tumoral transformation. We observed that sensitive and resistant lymphocytes express similar levels of the TGFβ receptor and phosphorylate SMAD factors to comparable levels. However, TGFβ signal transduction is interrupted in resistant lymphocytes, which do not induce nor repress genes that are regulated by TGFβ in sensitive lymphocytes. We will further dissect the molecular causes of resistance to the cytostatic effects of TGFβ in human T lymphocytes.

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LUDWIG INSTITUTE FOR CANCER RESEARCH

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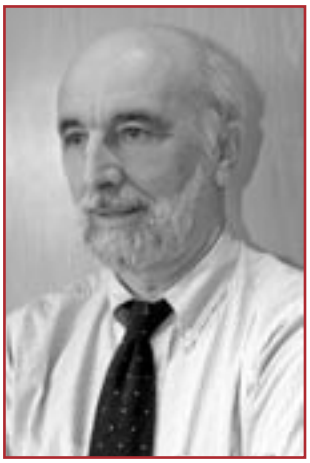
Cancer is a major concern in human health. The prospects for bringing cancer under control require linked innovative basic and clinical research. In this view, Daniel K. Ludwig created in 1971 the Ludwig Institute for Cancer Research, an international organization bringing together scientists and clinicians from around the world. Many Ludwig investigators are leaders in many areas of science, involving genetics, bioinformatics, immunology, virology, cell biology and signal transduction.



Faithful to the organizing principles laid down by Mr Ludwig, the Institute conducts its research through nine Branches, located in seven countries. The Branch structure allows the Institute to interact with a number of different research and clinical environments. Each Branch is focused on a research program defined by the Branch Director in relation with the overall objectives of the Institute. The Branches are established in association with University Hospitals, to stimulate close collaborations between research laboratories and the clinic. By organizing and controlling its own clinical trials programs, the Institute has indeed created a continuum that integrates laboratory and clinical research.

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TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

Building up on the molecular definition of tumor antigens recognized by T cells, our group mainly focuses on two aspects of tumor immunology, namely the processing of tumor antigens and the study of animal models to optimize cancer immunotherapy and evaluate tumor resistance mechanisms.

Tumor antigens recognized by Cytolytic T Lymphocytes (CTL) consist of peptides that are presented by MHC molecules at the cell surface and derive from intracellular proteins that are degraded by the proteasome. The intracellular pathway leading from the protein to the peptide/MHC complex is known as "antigen processing". Our group focuses on the proteasome and recently described a new mode of production of antigenic peptides by the proteasome, based on cutting and pasting peptide fragments to form a new spliced peptide.
The first example was a peptide derived from human melanocyte protein gp100. This antigenic peptide is nine-amino acid long and is produced by the splicing of two fragments that were initially non-contiguous in the parental protein. The splicing is made by the proteasome, is tightly coupled to the proteolytic reaction, and appears to occur by transpeptidation involving an acyl-enzyme intermediate. We also described a second example of spliced peptide, which is a minor histocompatibility antigen, and where the two fragments are rearranged before splicing. We are currently working on additional spliced peptides. We are also studying the processing differences between the standard proteasome, which is present in most cells, and the immunoproteasome which is found in dendritic cells and in cells exposed to interferon-gamma. Several tumor antigens - including spliced peptides - were found to be processed differently by the two proteasome types, usually because of a preferential

cleavage made by one or the other proteasome within the antigenic peptide itself. We also study the splicing capacity of the two proteasome types.

Translation of knowledge on tumor antigens into efficient cancer immunotherapy requires additional studies on the various strategies that can be used. Some of these studies can be done in preclinical animal models. The study of such a model allowed us to uncover a powerful mechanism of tumor resistance, which is based on tryptophan catabolism by indoleamine-2,3 dioxygenase, an enzyme that we found to be frequently expressed in tumors. The resulting local tryptophan shortage appears to prevent the proliferation of lymphocytes at the tumor site. Inhibitors of indoleamine-2,3 dioxygenase can be used in vivo to counteract this tumor resistance mechanism. We are searching for new IDO inhibitors that could be developed clinically. We also study additional tumor resistance mechanisms.

The currently available murine models are limited by the fact that they are based on transplantation of tumor cells grown in vitro into a healthy animal. This does not recapitulate the long-term host/tumor relationship that occurs in humans when a tumor slowly develops within a normal tissue. To circumvent this limitation and obtain more relevant information from such preclinical models, we have build a new mouse melanoma model where tumors expressing a given antigen can be induced, using a transgenic system based on Cre-lox recombination.

DIFFERENTIAL PROCESSING OF TUMOR ANTIGENS BY STANDARD PROTEASOMES, IMMUNOPROTEASOMES AND INTERMEDIATE PROTEASOMES

B. Guillaume, V. Stroobant, W. Ma

Antigens recognized by cytolytic T lymphocytes (CTL), such as viral or tumor antigens, usually consist of peptides of 8-10 amino acids in length, which are presented by MHC class I molecules at the cell surface. Because such peptides derive from intracellular proteins, a processing step is required before they can be exposed to the cell surface in association with MHC molecules. Firstly, the peptide is produced through degradation of the parental protein by the proteasome. Secondly, it is taken up by a dedicated transporter named TAP and translocated inside the endoplasmic reticulum where it meets and associates with newly synthesized MHC molecules. The first step of cleavage by the proteasome is crucial in that cleavage location determines the precise sequence of the final antigenic peptide. We have observed that this cleavage may occur differently in some cells, depending on their proteasome content. The proteasome comes in two forms: the standard proteasome, which is found in most cells, and the immunoproteasome, which is expressed by mature dendritic cells and by cells exposed to interferon-gamma (IFNg).

We previously reported that a class-I restricted antigenic peptide derived from an ubiquitous human protein was processed efficiently by the standard proteasome but not by the immunoproteasome. As a result, the relevant epitope is not presented efficiently by mature dendritic cells, which contain immunoproteasomes (1). We have now extended those observations to several antigenic peptides of interest for cancer immunotherapy, including HLA-A2-restricted epitopes derived from tyrosinase, Melan-AMART1 and gp100.

On the contrary, we showed that other tumor epitopes, which are derived from MAGE-3 and MAGE-C2, are processed by the immunoproteasome but not by the standard proteasome and therefore are presented to CTL only by tumor cells pre-treated with IFNg (2). By analyzing the peptidic fragments produced after in vitro digestion with the two proteasome types, we found that the differential processing can result from two mechanisms. In some cases, one of the proteasome types predominantly cleaves within the sequence of the epitope, resulting in its destruction (2). In other cases, the difference lies in the efficiency of cleavage at the C-terminal end of the antigenic peptide. These observations may have major implications for cancer immunotherapy, as they imply that the peptide repertoire presented by tumor cells may differ from the repertoire presented by antigen-presenting cells (3). The peptide repertoire of tumor cells themselves may vary according to the localization of the tumor (e.g. primary tumor versus lymph node metastasis) and its level of exposure to IFNg. It is therefore essential to study those processing differences in detail, so as to define the most effective vaccination strategy for each epitope and to use the appropriate combination of antigens in order to minimize the risk of tumor escape by proteasome switching. We have also observed the presence in many tumor lines of proteasome types that are intermediate between the standard proteasome and the immunoproteasome. These intermediate proteasomes contain only some of the three catalytic subunits of the immunoproteasome, i.e. only ß5i or ß1i and ß5i. In terms of production of antigenic peptides, these intermediate proteasomes produce more or less the same peptides as the immunoproteasome, and therefore explain the recognition of tumor cells by CTL directed against these peptides in the absence of IFNg. However, we have also identified two antigenic peptides that are produced exclusively by intermediate proteasomes.

ANTIGENIC PEPTIDES PRODUCED BY PEPTIDE SPLICING IN THE PROTEASOME

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By studying the antigen recognized by a CTL clone isolated from a melanoma patient, we identified an antigenic peptide composed of two non-contiguous fragments of the same protein, namely the melanocytic protein gp100. The production of this peptide requires the excision of an intervening fragment of 4 amino acids and the splicing of a fragment of 3 residues with a fragment of 6 residues. We have shown that this splicing is exerted by the proteasome and can be reproduced in vitro by incubating a precursor peptide with purified proteasomes. Splicing is coupled directly to peptide bond cleavage by the proteasome and appears to occur by transpeptidation involving an acyl-enzyme intermediate (Fig. 1) (4). The splicing reaction appears not to involve a particular motif, but rather to result from a low-efficiency reversal of the proteolysis reaction. Its occurrence is depending only on the occurrence of peptide cleavage.

We have also identified a second antigenic peptide produced by peptide splicing in the proteasome (5). This peptide is recognized by CTL directed against a minor histocompatibility antigen. The CTL was isolated from a multiple myeloma patient treated with HLA-identical bone marrow transplantation. The peptide is encoded by the polymorphic region of a gene ubiquitously expressed. Again it is made by the joining of two fragments that are initially non-contiguous in the parental protein. In addition, the two fragments are inverted in the spliced peptide, i.e. the fragment that was more N-terminal in the parental protein ends up at the C-terminal side of the spliced peptide, and vice-versa. We showed that splicing

and transposition could be reproduced in vitro with purified proteasomes. The splicing mechanism based on transpeptidation immediately after peptide bond cleavage is compatible with a transposition of the fragments prior to splicing. Together with the previous description of a peptide produced by protein splicing of FGF-5, this is the third example of antigenic peptide produced by splicing. These results indicate that spliced peptides are not uncommon and may represent a significant part of the peptide repertoire presented by MHC class I molecules.

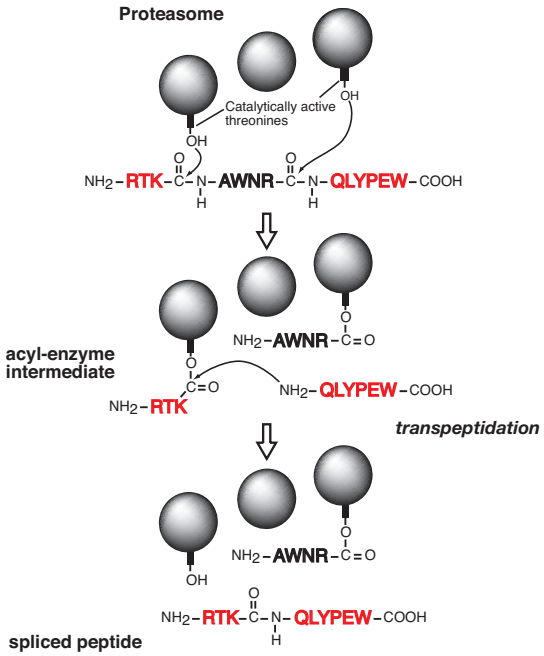

Figure 1. Model of the peptide-splicing reaction in the proteasome. The active site of the catalytic subunits of the proteasome is made up of the side-chain of a threonine residue, which initiates proteolysis by performing a nucleophilic attack on the carbonyl group of the peptide bond. An acyl- enzyme intermediate is formed, which is then liberated by hydrolysis. In the peptide-splicing reaction, a second peptide fragment appears to compete with water molecules for performing a nucleophilic attack on the acyl-enzyme intermediate, resulting in a transpeptidation reaction producing the spliced peptide. Experimental support for this model of reverse proteolysis includes evidence that the energy required to create the new peptide bond is recovered from the peptide bond that is cleaved at the amino-terminus of the excised fragment, and that the amino-terminus of the other fragment needs to be free for transpeptidation to occur.

IDENTIFICATION OF NEW ANTIGENS RECOGNIZED BY AUTOLOGOUS CTL ON HUMAN MELANOMA

W. Ma, N. Vigneron (in collaboration with P. Coulie)

Melanoma patient EB81 was vaccinated with a MAGE-type antigen and showed regression of all cutaneous metastases. Blood lymphocytes collected after the regression were stimulated with autologous tumor cells, and CTL clones were obtained. Surprisingly, none of these clones was directed against the antigen used for vaccination. Using a cDNA expression cloning approach, we identified the antigens recognized by three of them. These antigens correspond to three distinct peptides all derived from MAGE-C2, a gene with a cancer-germline expression pattern, which is expressed in about 40% of melanomas and 30% of bladder carcinomas. Two peptides are presented by HLA-A2, and one by HLA-B57 (6). Because of their strict tumor-specificity and their wide expression in tumors, these new antigens represent promising targets for cancer immunotherapy. The processing of two of these peptides is dependent on the immunoproteasome (2).

A NOVEL TUMOR IMMUNE ESCAPE MECHANISM BASED ON TRYPTOPHAN DEGRADATION BY INDOLEAMINE 2,3 DIOXYGENASE

C. Uyttenhove, L. Pilotte, I. Théate, D. Donckers, N. Parmentier, V. Stroobant, D. Colau

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that catalyses rapid tryptophan degradation. Because tryptophan can freely cross the plasma membrane, IDO expression results in a local depletion of tryptophan in the extracellular medium surrounding the expressing cell. Tryptophan depletion was shown to impair T lymphocyte proliferation, and therefore IDO expression represents a powerful immunosuppressive mechanism that accounts, for example, for maternal tolerance to allogeneic fetuses, where IDO expression by placenta was found to play an essential role. Expression of IDO can be induced by interferon-gamma in many cellular types, including macrophages and dendritic cells, and appears to play a prominent role in immune regulation.

We have observed that many human tumors express IDO in a constitutive manner (7). To determine whether IDO expression provides tumor cells with a survival advantage by allowing their escape from immune rejection in vivo, we used the well-characterized model system of mouse tumor P815, where the antigen encoded by gene P1A is the major target of the rejection response. We observed that expression of IDO by P815 tumor cells prevents their rejection by pre-immunized mice.

This effect can be partly reverted by systemic treatment of mice with an inhibitor of IDO, in the absence of noticeable toxicity (7). These results suggest that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor. We are currently searching for new IDO inhibitors that could be developed at the clinical level. We also study the expression of IDO in normal mature dendritic cells, making use of a novel monoclonal antibody we have raised against human IDO (in collaboration with the group of Jean-Christophe Renauld).

AN INDUCIBLE MOUSE MODEL OF MELANOMA EXPRESSING A DEFINED TUMOR ANTIGEN

C. Powis de Tenbossche, C. Hervé, S. Goriely (in collaboration with A.-M. Schmitt-Verhulst, CIML, Marseille)

Cancer immunotherapy based on vaccination with defined tumor antigens has not yet shown strong clinical efficacy, despite promising results in preclinical models. This discrepancy might result from the fact that available preclinical models rely on transplantable tumors, which do not recapitulate the long-term host-tumor interplay that occurs in patients during progressive tumor development and results in tumor tolerance. To create a faithful preclinical model for cancer immunotherapy, we generated a transgenic mouse strain developing autologous melanomas expressing a defined tumor antigen recognized by T cells (8). We chose the antigen encoded by P1A, a well-characterized murine cancer germline gene. To transform melanocytes, we aimed at simultaneously activating the Ras pathway and inactivating tumorsuppressor Ink4a/Arf, thereby reproducing two genetic events frequently observed in human melanoma. The melanomas are induced by s.c. injection of 4-OH-tamoxifen (OHT). By activating a CreER recombinase expressed from a melanocyte-specific promoter, this treatment induces the loss of the conditional Ink4a/Arf gene in melanocytes. Because the CreER gene itself is also flanked by loxP sites, the activation of CreER also induces the deletion of its own coding sequence and thereby allows melanocyte-specific expression of genes H-ras and P1A, which are located downstream on the same transgene. All melanomas induced in those mice with OHT show activation of the Ras pathway and deletion of gene Ink4a/

Arf. In addition, these melanomas express P1A and are recognized by P1A-specific T lymphocytes. This model will allow to characterize the interactions between the immune system and naturally occurring tumors, and thereby to optimize immunotherapy approaches targeting a defined tumor antigen. We have now optimized the induction of melanoma in this model, and we reach an incidence of 70-80% tumors. In many tumor-bearing mice, we observed an accumulation of immature myeloid cells bearing both the CD11b and Gr1 markers. We are characterizing those cells, which might correspond to the myeloid-derived suppressor cells described in other models. In parallel, we have developed a strain of mice transgenic for the P1A-specific T cell receptor, which will be useful for such studies.

PHYSIOPATHOLOGY OF SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

B. Lauwerys, I. Gutierrez-Roelens, V. Badot, A.-L. Maudoux (in collaboration with F. Houssiau, Unité de Rhumatologie)

SLE is a systemic autoimmune disorder of unknown etiology. From a biological point of view, the disease is characterized by overt polyclonal B cell activation and CD4 T cell-driven production of specific autoantibodies directed against constituents of the chromatin. These antibodies (in particular the double-stranded DNA antibodies) are pathogenic and associated with the most severe manifestations of the disease. In order to better understand the underlying molecular pathways, we performed analyses of global gene expression on sorted CD4 T and B cells from SLE patients as compared to controls and patients with rheumatoid arthritis (RA), using Genechip U133 Plus 2.0 arrays. We also performed similar experiments on synovial tissue from SLE patients with arthritis. We found the presence of a strong type 1 interferon signature in SLE samples, i.e. the presence of numerous interferon-induced genes, as previously observed in SLE PBMC by other groups. We are currently investigating the physiopathological pathways that are dysregulated by the over-expression of these genes, using PBMC from patients and animal models of the disease.

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TUMOR GENETICS GROUP

Human tumors express specific antigens arising from the activation of genes, such as MAGE, BAGE, GAGE and LAGE/NY-ESO1, that are normally expressed only in germ cells. As germ cells are not subject to scrutiny by the immune system, antigens encoded by these genes are strictly tumor-specific. Our group has developed methods to identify genes that are specifically expressed in tumors and germ cells (1, 2). Most of these genes have their normal site of expression in spermatogonia, the pre-meiotic stage of sperm development, and are located on the X chromosome (3). Efforts are now devoted to determining the function of «cancer-germline» genes and deciphering the mechanism leading to their activation in tumor cells.

To analyze the functions of a MAGE protein, MAGE-A1, we searched to identify binding partners of this protein. Using yeast two-hybrid screening, we found an interaction between MAGE-A1 and transcriptional regulator SKIP (4). SKIP is an adaptor protein that connects DNA-binding proteins to proteins that activate or repress transcription. Results obtained by transient transfection in HeLa cells indicate that by binding to SKIP and by recruiting histone deacetylase 1, protein MAGE-A1 present in the nucleus can act as a potent transcriptional repressor. In addition, we have observed an interaction between MAGE-A1 and DNA methyltransferases (DNMT). Since recruitment of DNMT3A by the Myc transcription factor has been shown to repress the p21Cip1 promoter (5), we are now trying to evaluate whether promoters could be repressed by MAGE-A1 in the presence of DNMT.

It has been recently published that the germline-specific gene BORIS (Brother Of the Regulator of Imprinted Sites) might be responsible for the activation of most cancer-germline genes, including MAGE-A1 (6). In collaboration with Charles De Smet and his group, we have evaluated the frequency of BORIS activation in melanoma by quantitative RT-PCR (7). BORIS activation was detected in 27% (n = 63) of melanoma tissue samples. Surprisingly, many melanoma samples expressed MAGE-A1 and other cancer-germline genes in the absence of BORIS activation, suggesting that BORIS is not an obligate factor for activation of these genes in melanoma. Moreover, we could not induce expression of MAGE-A1 by forced expression of BORIS in two melanoma cell lines, one sarcoma cell line, immortalized human keratinocytes and normal human fibroblasts. It appears therefore that BORIS is neither necessary nor sufficient for the activation of cancer-germline genes.

Finally, in collaboration with Nicolas Van Baren and Francis Brasseur, we are analyzing the molecular mechanisms by which IFN-γ, TGF-β, IL1-β and TNF-α reduces the expression of melanocyte differentiation genes. We are also evaluating, in collaboration with Pierre van der Bruggen, the consequences of cytokine exposure for recognition of melanoma cells by cytolytic T lymphocytes.

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REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

We identified in the early 1990s the first gene coding for a human tumor antigen recognized by cytolytic T lymphocytes (CTL). It was named MAGE-A1. We spent several years to define antigenic peptides encoded by genes such as those of the MAGE gene family. These peptides have been used in therapeutic vaccination trials of cancer patients. Efforts have been devoted to set up assays that accurately monitor T cell responses to cancer vaccines, including regulatory T cells.

We further analyzed the properties of anti-tumor CTL clones. The differentiation of naive T cells into memory and effector cells is marked by changes in the expression of surface molecules such as CCR7 and CD45. We found that the expression of CD45RA on CCR7$^-$ CD8$^+$ T cells is indicative of the time elapsed since the last antigenic stimulation rather than the signature of a terminally differentiated status with an incapacity to proliferate.

Anti-tumor T cells are present in tumor metastases that are progressing. This spontaneous anti-tumor T cell response must become ineffective at one point, possibly because the effector cells have become unable to exert their function, a state known as anergy. This anergy could result from inhibitory processes elicited by tumor cells. The group is currently involved in the study of T cell anergy. We have identified a novel mechanism causing anergy of human tumor-infiltrating lymphocytes, and established a new approach to correct this anergy in vitro. We observed that exhausted CTL clones and anergic tumor-infiltrating lymphocytes had lost the colocalization of T cell receptor (TCR) and CD8. Effector function and TCR-CD8 colocalization were restored with competitive galectin binders, such as sugars, suggesting that the binding of TCR to galectin plays a role in the distancing of TCR from CD8. Administration of competitive galectin binders may be a therapeutic option to induce a more efficient and long-lasting anti-tumor immune response.

In the 1970s it became clear that T lymphocytes, a subset of the white blood cells, were the major effectors of tumor rejection in mice. In the 1980s, human anti-tumor cytolytic T lymphocytes (CTL) were isolated in vitro from the blood lymphocytes of cancer patients, mainly those who had melanoma. Most of these CTL were specific, i.e. they did not kill non-tumor cells. This suggested that they target a marker, or antigen, which is expressed exclusively on tumor cells. We started to study the anti-tumor CTL response of a metastatic melanoma patient and contributed to the definition of several distinct tumor antigens recognized by autologous CTL. In the early 1990s, we identified the gene coding for one of these antigens, and defined the antigenic peptide (1). This was the first description of a gene, MAGE-A1, coding for a human tumor antigen recognized by T lymphocytes.

Genes such as those of the MAGE family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They are therefore referred to as "cancer-germline genes". They encode tumor specific antigens, which have been used in therapeutic vaccination trials of cancer patients (2). A large set of additional cancer-germline genes have now been identified by different approaches, including purely genetic approaches. As a result, a vast number of sequences are known that can code for tumor-specific shared antigens. The identification of a larger set of antigenic peptides, which are presented by HLA class I and class II molecules and recognized on tumors by T lymphocytes, could be important for therapeutic vaccination trials of cancer patients and serve as tools for a reliable monitoring of the immune response of vaccinated patients. To that purpose, we have used various approaches that we have loosely named "reverse immunology" , because they use gene sequences as starting point (3).

The group of Stefan Constantinescu found that a mutation in the kinase JAK2 is present in a majority of *Polycythemia vera* patients. An abnormal peptide, resulting from the JAK2 mutation, could be expressed on the cell surface of the malignant clone present in the bone marrow of *Polycythemia vera* patients. This would correspond to a new tumor antigen, able to be targeted by cytolytic T lymphocytes. The research project of Vijay Singh in our group, in collaboration with N. Van Baren and S. Constantinescu, is to evaluate whether such an abnormal peptide exists on *Polycythemia vera* cells, to characterize it, and to isolate cytolytic T lymphocytes that recognize this peptide and kill abnormal cells.

Human tumor antigens recognized by $CD4^+$ or $CD8^+$ T cells are being defined at a regular pace worldwide. Together with colleagues at the de Duve Institute, we read the new publications and incorporate the newly defined antigens in a database accessible at <http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm>

ANALYSIS OF THE T CELL RESPONSES OF CANCER PATIENTS

$CD8^+$ T cells

J. Carrasco (till end 2005), D. Godelaine, V. Ha Thi, in collaboration with A. Van Pel, K. Thielemans (Vrije Universiteit Brussel) and B. Neyns (Vrije Universiteit Brussel)

We analyzed the T cell response in one patient who regressed upon vaccination with autologous dendritic cells pulsed with MAGE-3.A1 and MAGE-3.DP4 peptides. This patient developed a mixed tumor response, with disappearance/reduction of metastases and appearance of new metastases (Figure 1). In many MAGE-vaccinated patients who show tumor regression, the frequency of anti-vaccine T cells is very low. We wondered how such a low number of T cells could provide the main component of the specific effectors destroying the tumor cells. This led us to evaluate the frequency of all the T cells that recognized the

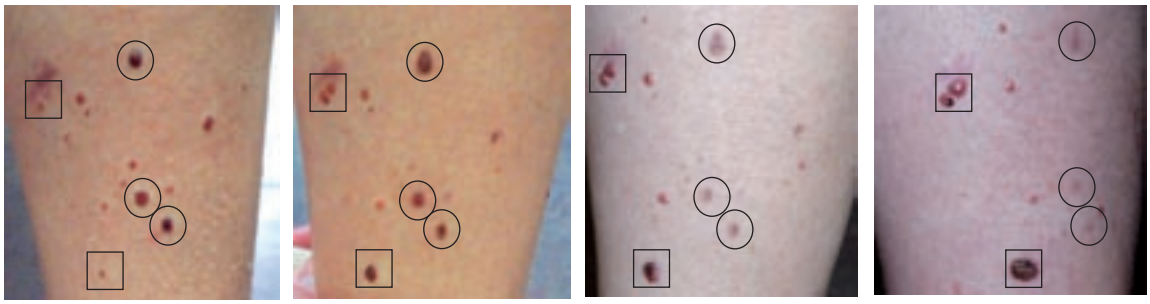

Figure 1. Development of a mixed tumor response in patient LB2586. Regressing lesions on the posterior face of the right leg are encircled. The squares frame lesions that progressed over the same period of time.

autologous tumor cell line. We performed an in-depth longitudinal analysis of the anti-vaccine and anti-tumor responses, in the blood and in tumor sites of this patient (4). Before vaccination, the patient already had high frequencies of anti-tumor T cells in the blood. Skin metastases contained T cells but they were apparently inactive in destroying tumor cells. Upon vaccination, a modest anti-vaccine response was observed and this response lasted over the observation period of three years, with no evident concentration of anti-vaccine T cells at tumor sites. Interestingly, a new anti-tumor CTL clone appeared in the blood after vaccination and was found to be enriched by more than 1,000-fold in the metastases. This anti-tumor CTL was directed against a previously unknown antigen, a MAGE-C2-derived peptide presented by HLA-B44 (Figure 2) (5).

Three other MAGE-C2 antigenic peptide were identified with CTL isolated from melanoma patients who displayed impressive CTL responses against these antigens, reaching frequencies in a tumor metastasis of several percent of CD8 T cells. Whether this makes MAGE-C2 derived antigens particularly useful for anti-tumoral vaccines will have to be determined by clinical experimentation.

The CD8 T cell response observed in this patient reinforce an hypothesis proposed by T. Boon and P. Coulie: anti-vaccine CTL are not the effectors that kill the tumor cells but their arrival at the tumor site containing exhausted anti-tumor CTL, generates conditions allowing

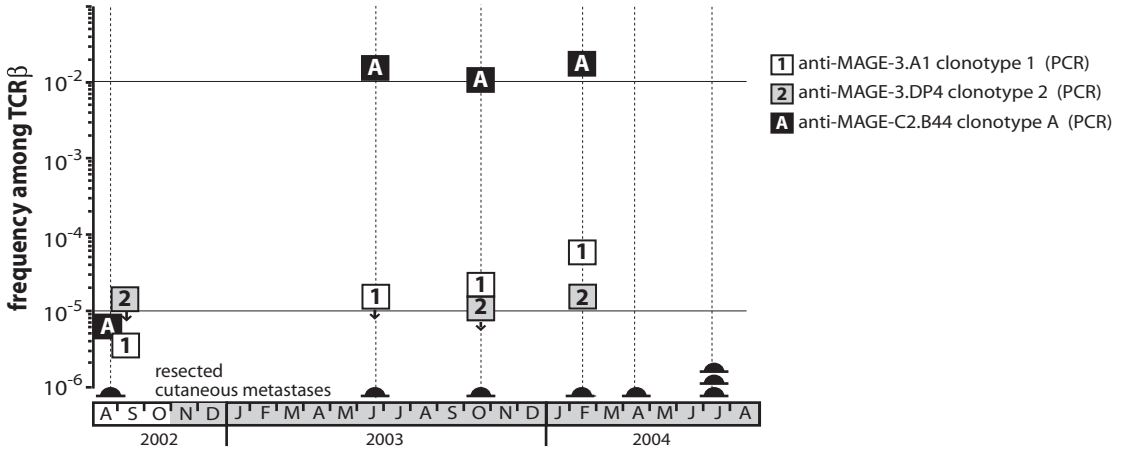

Figure 2. Frequency and diversity of anti-vaccine and anti-tumor T cells in various skin metastases of patient LB2586. The frequencies were estimated by PCR using primers specific for the CD3/TCRβ region of each clonotype.

the reawakening of the exhausted CTL and/or activation of new anti-tumor CTL clones, some of them contributing directly to tumor destruction.

CD4⁺ T cells

V. François, S. Ottaviani, N. Renkvist (till end 2005), D. Colau, P. van der Bruggen, in collaboration with S. Lucas, J. Stockis, G. Schuler (University of Erlangen, Germany), K. Thielemans (Vrije Universiteit Brussel) and B. Neyns (Vrije Universiteit Brussel)

We have produced HLA-DP4 fluorescent multimers loaded with the MAGE-A3243-258 peptide and designed a method to analyze low-frequency specific CD4 T cell responses in vaccinated cancer patients, by ex vivo staining of the blood cells with the fluorescent multimers and amplification of the sorted multimer⁺ CD4 clones. The specificity of the clones was assessed by their ability to secrete cytokines or to upregulate activation markers upon contact with the MAGE-3 antigen (6). Using this approach, low frequencies of about 1 out of 1 million CD4 T cells could be detected (Figure 3). One advantage of the multimer approach is that antigen-specific CD4 T cells are detected independently of their effector functions. It allows therefore the detection of anti-vaccine

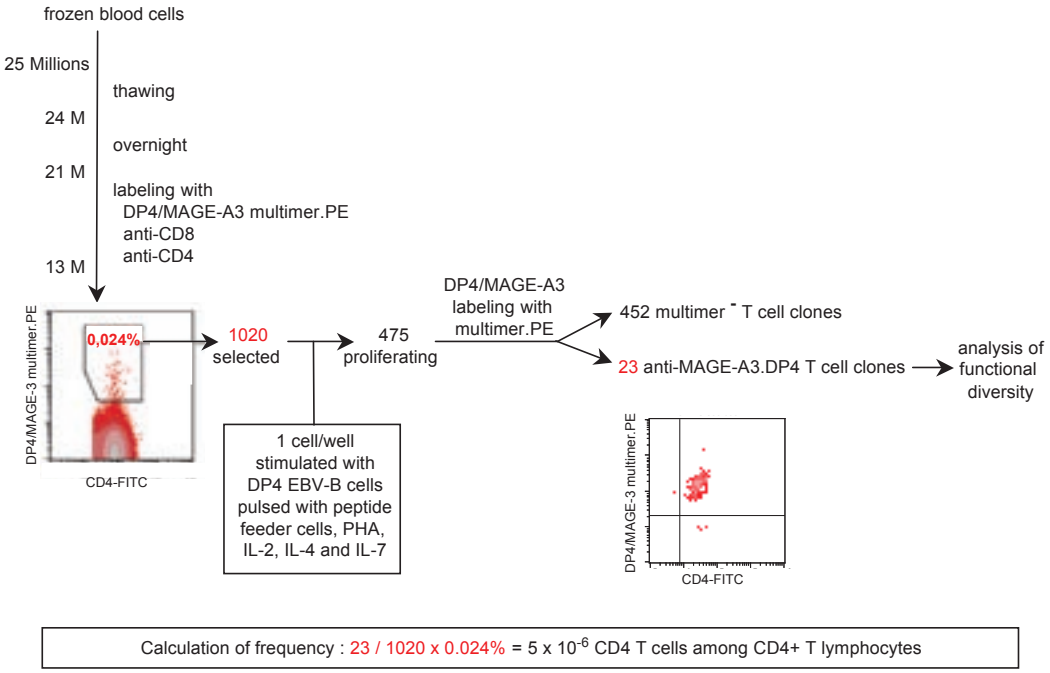

Figure 3. Overview of the procedure used to isolate anti-MAGE-A3.DP4 T cell clones. The numbers indicated correspond to an experiment performed with blood cells of a vaccinated patient. Frozen blood cells were thawed and kept overnight in culture medium. Cells were collected and labeled with DP4 fluorescent multimers folded with a MAGE-A3 peptide (DP4/MAGE-A3), anti-CD8 and anti-CD4 antibodies. Multimer⁺ CD8⁻ CD4⁺ cells were selected by flow cytometry and distributed at one cell per microwell. After three weekly stimulations, cloned T cells that had proliferated were screened with the multimer. Functional diversity of the clones was assessed by their ability to release cytokines IFN-γ, IL-10 and IL-2 upon contact with the antigen. We calculated the frequency by multiplying the fraction of cloned cells that yielded anti-MAGE-A3.DP4 clones with the fraction of the CD4 T cells that were considered multimer⁺ by flow cytometry analysis.

CD4 T cells secreting no cytokines, including for instance regulatory T cells. We have analyzed the anti-vaccine CD4 T cell response of 14 patients enrolled in four different clinical trials. The patients were injected either with peptides, with or without adjuvant, or dendritic cells pulsed with peptides. All the vaccines contained at least the synthetic MAGE-A3243-258 peptide presented to CD4 T cells by HLA-DP4 molecules. We wondered if the type of vaccine had any influence on the frequency and functional phenotype of anti-MAGE-3.DP4 CD4 T cells.

The anti-MAGE-A3.DP4 T cell responses were polyclonal with frequencies ranging from 1/500,000 to 1/500 CD4 blood T lymphocytes. Their functional diversity was high, but we found no correlation between the type of vaccine and the functional phenotype of the anti-vaccine T cells. Twelve out of 196 clones expressed CD25 in resting state, upregulated CD25 upon stimulation but released no cytokine, suggesting that they are MAGE-A3-specific regulatory T (T_{reg}) cell clones. We searched for functions or markers that would identify T_{reg} clones. A suppression assay based on peptide stimulation was designed and used to screen a set of clones, which were also analyzed for cytokine secretion, *FOXP3* protein expression and for the demethylation of the first intron of *FOXP3*. All but one clone that had an in vitro suppressive activity expressed CD25 in resting state, stained positive for *FOXP3*, released no cytokine upon stimulation, and had a demethylated *FOXP3*. This is the first demonstration that vaccination can induce vaccine-specific T cells with in vitro suppressive activity.

SIGNIFICANCE OF THE CD45RA EXPRESSION ON MEMORY AND EFFECTOR CD8$^+$ T CELLS

J. Carrasco (till end 2005), D. Godelaine, P. van der Bruggen, in collaboration with A. Van Pel

It was reported that the differentiation of naive T cells into memory and effector cells is marked by changes in the expression of surface molecules such as CCR7 and CD45. The quality of the immune response to viruses and tumors was often evaluated with the concept that the CD45RA$^+$CCR7$^-$ cells are the terminally differentiated, most effective CD8$^+$ T cells for the destruction of tumor cells and virus-infected cells (Figure 4). We re-examined this issue by studying, after T-cell receptor stimulation, the

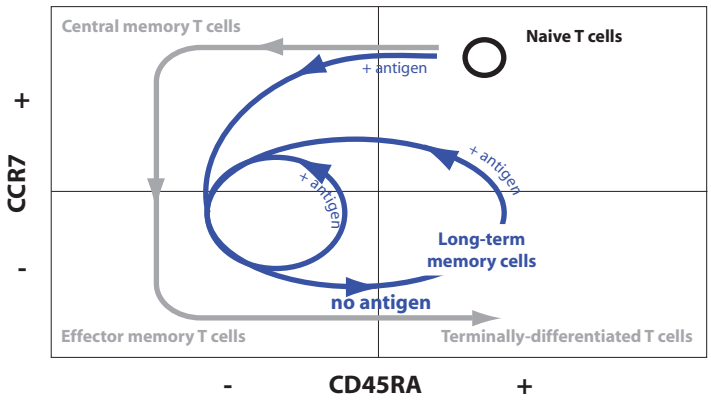

Figure 4. Model for the significance of CCR7 and CD45RA expression on CD8 T cells. The linear differentiation model proposed by Lanzavecchia & Sallusto (Nature, 1999), and Champagne & Pantaleao (Nature, 2001), is indicated in grey. The pathways proposed on the basis of our observations are indicated in blue.

time course of CD45RA and CCR7 expression both on blood CD8 T cells and on CTL clones directed against the MAGE-3 antigen (7). Our results indicate that $CD45RA^+CCR7^-$ $CD8^+$ T cells are resting memory cells which, upon antigenic stimulation and during the next 10 days, can proliferate, lose CD45RA and transiently acquire CCR7. In the absence of further antigenic stimulation, they progressively re-express CD45RA during the 10 following weeks and become $CD45RA^+CCR7^-$. We conluded that the expression of CD45RA on $CCR7^-CD8^+$ T cells is indicative of the time elapsed since the last antigenic stimulation rather than a terminally differentiated status with incapacity to proliferate. This observation leads to a re-interpretation of the significance of the presence of $CD45RA^+CD8^+$ T cells in patients with viral infections or cancer.

A MECHANISM CAUSING ANERGY OF $CD8^+$ T LYMPHOCYTES

N. Demotte, D. Colau, S. Ottaviani, D. Godelaine, C. Wildmann, I. Jacquemart, G. Wieërs, V. Singh, P. van der Bruggen, in collaboration with V. Stroobant, P. Courtoy (ICP, Cell Unit), P. Van Der Smissen (ICP, Cell Unit), I.F. Luescher (Ludwig Institute for Cancer Research, Lausanne Branch), C. Hivroz (Institut Curie, Paris), J.-L. Squifflet (Cliniques Universitaires Saint-Luc) and M. Mourad (Cliniques Universitaires Saint-Luc)

We have observed that human CTL clones lose their specific cytolytic activity and cytokine production under certain stimulation conditions, while retaining an antigen-dependent growth pattern. The labeling of these inactive CTL by an HLA-peptide tetramer was strongly reduced, even though the amount of T cell receptor (TCR) at their surface is similar (8).

We have identified a novel mechanism causing anergy of T lymphocytes, including human tumor-infiltrating lymphocytes, and established new approaches to correct this anergy in vitro. This type of anergy appears to be a transient status during the normal stimulation cycle of T lymphocytes. We observed that from day 1 after antigen stimulation, cytolytic T lymphocytes clones (CTL) lose the capacity to secrete cytokines and in some case their cytolytic activity. These functions are recovered gradually and are usually completely restored by day 14. We noticed that the TCR and the CD8 co-receptor were co-localized at the cell surface of functional CTL but, on the contrary, distant at the cell surface of non-functional CTL.

Human CD8 tumor-infiltrating T lymphocytes (TIL) were isolated from tumor ascites or solid tumors and compared with T lymphocytes from blood donors. TCR were distant from CD8 on the cell surface of TIL, whereas TCR and CD8 were co-localized on blood T lymphocytes. We observed that these TIL were anergic, being unable to secrete INF-γ or other cytokines after non-specific stimulation with anti-CD3 and anti-CD28 antibodies (Figure 5).

On the basis of several publications, we hypothesized that the absence of TCR-CD8 co-localization at the cell surface of non-functional CTL and anergic TIL is due to the loss of TCR mobility trapped into a lattice of glycoproteins (including TCR) clustered by extracellular galectin-3. To test this hypothesis, we incubated anergic CTL and TIL with a disaccharide ligand of galectin-3, N-acetyllactosamine. Treated CTL and TIL recovered the TCR-CD8 co-localization and the capacity to secrete IFN-γ and other cytokines after stimulation. These results were recently published (Figure 6) (9).

The overexpression of galectin-3 by tumor cell lines and the presence of galectin-3 in ascites and tumors have been shown in many studies and confirmed in our laboratory. Our observations indicate that TIL can recover ex vivo their effector functions with galectin li-

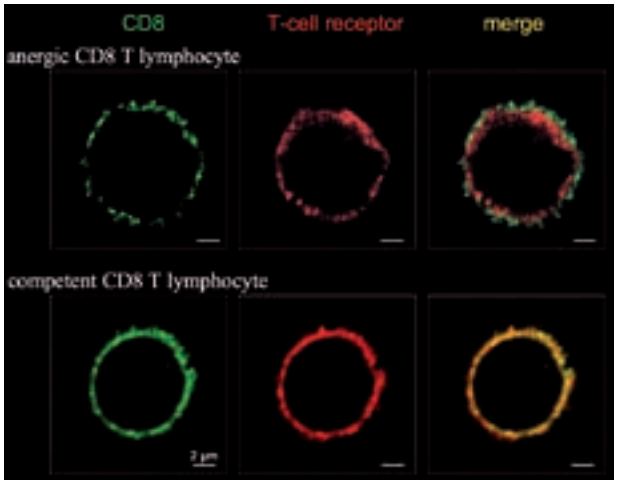

Figure 5. Absence of colocalization of the T cell receptor and CD8 co-receptors at the surface of anergic CD8 T lymphocytes.

gands and suggest that treatment of cancer patients with galectin ligands could correct TIL anergy. It is possible that peptide vaccination combined with galectin ligands induce a more efficient and long-lasting anti-tumoral immune response. It is also possible that treatment of cancer patients with galectin ligands, without peptide vaccination, will also trigger a broad activation of existing anti-tumor T cells, which could destroy the tumor. Examining whether correcting anergy of tumor infiltrating lymphocytes with drugs as simple as oligosaccharides can be translated into a clinical application will be our next challenging goal.

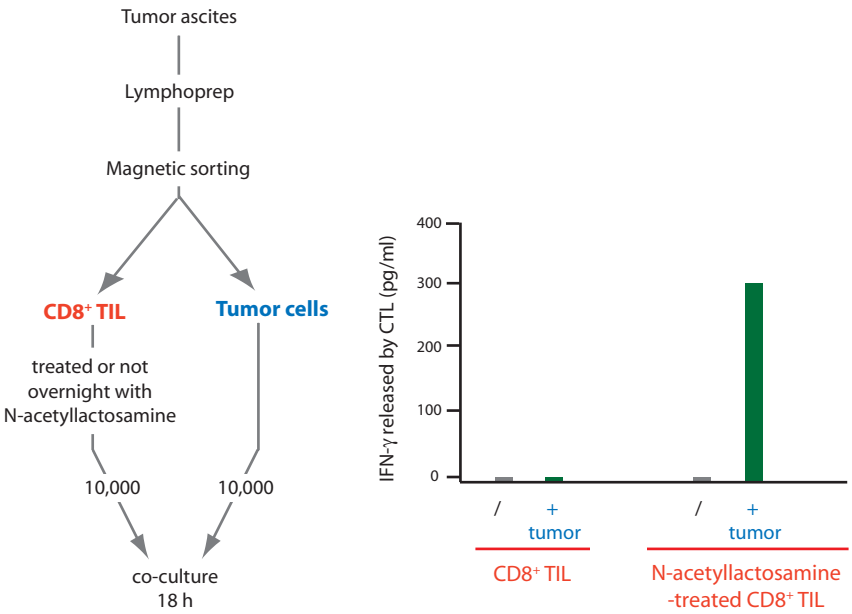

Figure 6. Recovery of effector function by «sugar-treated» tumor infiltrating T lymphocytes.

THEILERIA PARVA CANDIDATE VACCINE ANTIGENS RECOGNIZED BY IMMUNE BOVINE CYTOTOXIC T LYMPHOCYTES

A Collaboration between The International Livestock Research Institute (ILRI), Nairobi, Kenya, The Institute for Genomic Research (TIGR), Rockville, USA, Merial, Lyon, France, The Wellcome Trust Centre for Human Genetics, Oxford, United Kingdom, The Centre for Tropical Veterinary Medicine, Edinburgh, United Kingdom, The Ludwig institute for Cancer Research, Brussels, Belgium, D. Colau, C. Wildmann, and P. van der Bruggen

East Coast fever, caused by the tick-borne intracellular apicomplexan parasite *Theileria parva*, is a highly fatal lymphoproliferative disease of cattle. The pathogenic schizont-induced lymphocyte transformation is a unique cancer-like condition that is reversible with parasite removal. Schizont-infected cell-directed $CD8^+$ CTL constitute the dominant protective bovine immune response after a single exposure to infection. However, the schizont antigens targeted by *T. parva*-specific CTL are undefined (Figure 7).

To identify antigens, cells transiently transfected with schizont cDNA were screened with CTL from live vaccine-immunized cattle of diverse bovine leukocyte antigen (BoLA) MHC class I genotypes (10). In a first approach, a cDNA library was constructed in Brussels with RNA extracted from schizont. Pools of cDNA were transfected either in immortalized bovine skin fibroblasts or in monkey COS cells. The first screening of this library was performed in Brussels with CTL imported from Kenya and the other screening were performed at ILRI, Kenya. In a second approach, genes that were predicted to contain a secretion signal, by using preliminary sequence data of the *T. parva* chromosome, were cloned, transiently transfected in antigen-presenting cells and tested for recognition by CTL. The approach was based on the observation that the schizont lies free in the host cell cytoplasm whereby secreted parasite proteins would directly access the host cell MHC class I antigen processing and presentation pathway. Five candidate vaccine antigens were identified. $CD8^+$ T cell responses to these antigens were boosted in *T. parva*-immune cattle resolving a challenge infection and, when used to immunize naive cattle, induced CTL responses that correlated with survival from a lethal parasite challenge. These data provide a

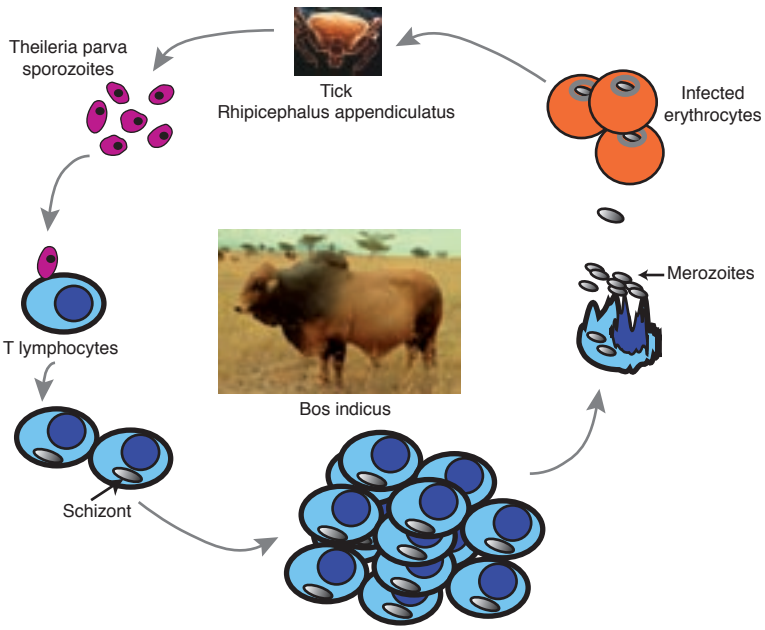

Figure 7. Life cycle of *Theileria parva*, a parasite responsible of the East Coast fever.

basis for developing an anti-East Coast fever subunit vaccine. To monitor anti-vaccine T cell responses in immunized animals, bovine MHC-peptide multimers have been constructed and produced in Brussels. The specificity of these multimers was validated by staining of relevant and non-relevant CTL clones. Experiments are in progress to define the optimal conditions to detect CTL in PBL from immunized animals.

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THERAPEUTIC VACCINATION AND TUMOR EXPRESSION PROFILING GROUP

Tumor cells carry antigens such as MAGE antigens that are absent from normal tissues, and that can be targeted by cytolytic T lymphocytes (CTL). Whilst it is possible to make such CTL recognize and kill autologous tumor cells in vitro, the precise way to induce an effective CTL response against a MAGE antigen in cancer patients is not known yet. In clinical vaccination trials, patients with a MAGE-expressing cancer, often melanoma, are treated repeatedly with a MAGE vaccine. These trials have two main objectives. First, the effectiveness of various vaccination modalities can be assessed by following the clinical evolution of the tumor, by analyzing whether a specific CTL response to the vaccine antigen occurred, and by determining whether immunological and clinical responses are correlated. Secondly, these trials allow crucial biological material to be collected from vaccinated patients. Blood samples provide anti-tumoral CTL clones, which can be functionally characterized. Tumor samples can be analyzed by expression microarrays and immunohistology, which allows to study the interaction between the tumor environment and the immune cells at the transcriptional level. New vaccination modalities can then be defined based on the knowledge acquired from these analyses.

THERAPEUTIC VACCINATION WITH MAGE TUMOR ANTIGENS

In collaboration with J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc) and the group of P. Coulie (Cellular Genetics Unit, de Duve Institute). The clinical trial program was set up and a large part of it was carried out by M. Marchand.

We have set up small-scale clinical trials aimed at evaluating the toxicity, the antitumoral effectiveness and the immunological response in cancer patients immunized with MAGE vaccines involving either peptides, a recombinant protein or a recombinant viral vector. A total of about 380 patients have been included in these multicenter trials.

Clinical trials with the MAGE-3.A1 peptide

In a pilot study, the synthetic Mage-3.A1 peptide was administered to 45 HLA-A1 patients with MAGE-3 expressing melanoma, by subcutaneous (s.c.) and intradermal (i.d.) injections of 100 or 300 µg of peptide on three occasions at monthly intervals. No significant toxicity was reported. Of the 25 melanoma pa-

tients with measurable disease who received all 3 immunizations, seven displayed tumor regressions. We observed 3 complete responses (CR), 1 partial response (PR) and 3 mixed responses i.e., a regression of some metastases while others appear, progress, or stabilize (MxR).

Other vaccination modalities involving the same peptide were investigated in melanoma patients with measurable disease. This peptide was mixed with the immunological adjuvant MPL + QS21 and injected intramuscularly at 4-week intervals to 5 patients, without any evidence of tumor regression. A combination of the MAGE-3.A1 and MAGE-1.A1 peptides was administered s.c. and i.d. every 3 weeks to 11 patients. Two of them experienced tumor regression (1 CR, 1 MxR). MAGE-3.A1 was injected s.c. and i.d. every 10-11 days instead of every 3-4 weeks to analyze whether vaccination at higher frequency could improve the clinical response rate. Among 21 patients treated, three had regressions of tumor lesions (3 MxR). The same peptide was associated with the HLA class II-restricted MAGE-3.DP4 peptide, in order to induce both CD8 and CD4 T cell responses, hoping for an improved immunological and antitumoral effectiveness. None of the 7 patients evaluable after 9 i.d. and s.c. vaccinations given every 10-11 days had tumor regression.

Initial assessment of the CTL responses induced by vaccination with the MAGE-3.A1 peptide was hampered by the lack of sensitivity of available CTL monitoring techniques.

More recently, a new approach with improved sensitivity, involving lymphocyte-peptide culture and the use of HLA/peptide tetramers, was used to document a significant increase in CTLp frequency in a patient who showed tumor regression following vaccination with this peptide at high frequency. This method also showed that the CTL response was monoclonal. It was extended to 19 other patients who received this peptide without adjuvant. None had a detectable CTL response, indicating that this vaccine is weakly immunogenic.

In another study, patients with completely resected primary or regional metastatic melanoma with a high risk of relapse have been vaccinated with the MAGE-3.A1 peptide injected i.d. and s.c. every 2 weeks on 6 occasions. The purpose was to analyze whether vaccination of melanoma patients with less advanced disease in the adjuvant setting would improve the immunological response to a peptide vaccine. No CTL response was detected by our tetramer assay in the 6 patients who have received the complete treatment, including 3 patients with a resected tumor that did not express the appropriate antigen and who are assumed to be immunologically naive.

An ongoing study tests a cocktail of 8 different HLA-A2 restricted peptides mixed with an immunological adjuvant, either CpG7909 or Montanide ISA51. CpG7909 is an immunostimulatory CpG-containing oligonucleotide, which activates antigen presenting cells after binding to Toll-like receptor 9, and is thought to enhance CTL responses. Montanide is similar to incomplete Freund's adjuvant. It forms a water-in-oil emulsion with the peptides in solution, which allows to administer the vaccine mix as a long-lasting depot into the skin. The vaccine is injected on 6 occasions by i.d. and s.c. routes, at 2-week intervals. Fourteen patients are planned in each treatment arm. The purpose is to determine whether the adjuvanted multipeptide vaccine increases the CTL responses, and whether improved tumor response rates will be achieved. Fourteen patients have already received the peptides + CpG7909 vaccine, which was well tolerated. Three of them have shown evidence of tumor regression (all MxR). CTL responses against at least one of the 8 tumor antigens were detected in 6 patients, none of whom had a tumor response. A majority of these CTL responses were directed at the NY-ESO-1.A2 antigen. The second treatment arm in which Montanide is combined with the peptides is ongoing. Seven patients have already been included.

Clinical trials with the MAGE-3 protein

In a phase I/II trial, the recombinant Mage-3 protein was tested as a vaccine in patients with MAGE-3 expressing cancer, mainly melanoma. The patients received either 30, 100 or 300 µg of the protein, with or without the immunological adjuvants MPL and QS21, repeatedly by intramuscular injection. No severe toxicity was reported. Among 33 evaluable melanoma patients, four experienced regressions of metastatic lesions, 2 partial and 2 mixed responses. A partial response was also observed in a patient with metastatic bladder cancer.

The clinical efficacy of the MAGE-3 protein injected i.d. and s.c. without adjuvant in non-visceral melanoma patients was tested in another study. Patients received 300 µg of MAGE-3 protein on 6 occasions at 3-week intervals. Five out of 26 evaluable patients have shown regressions, including 1 partial response and 4 mixed responses. Thus this vaccine does not seem to induce more regressions than the MAGE-3.A1 peptide, but it does not require that the patient carries a specific HLA type. We then mixed this recombinant protein with adjuvant AS15 containing CpG 7909 in addition to MPL and QS21, and combined these i.m. injections with the administration of selected class I or class II peptides by i.d. and s.c. routes, which may result in the simultaneous activation of both CD8+ and CD4+ specific T lymphocytes. 11 patients were included in that study before its early closure. Three of them had a mixed response.

Clinical trial with an ALVAC-MAGE virus.

40 patients with advanced cancer, including 37 with melanoma, were vaccinated with a recombinant canarypox (ALVAC) virus containing a minigene that encodes the MAGE-1. A1 and MAGE-3.A1 antigens, followed by booster vaccinations with the 2 corresponding peptides. The treatment comprised 4 ALVAC injections followed by 3 peptides injections, all i.d. and s.c., separated by 3 weeks each. Local inflammatory reactions at the sites of ALVAC injection were common, but were moderate in intensity and transient in duration. Among the 30 melanoma patients who received at least 4 ALVAC vaccinations, six experienced regression of one or more melanoma metastases. Significant CTL responses were detected in 3 of 4 patients with regressions, and in only one of 11 patients with disease progression, which indicates a significant correlation between immune and antitumor responses.

Summary of relevant observations and perspectives

Immunization with peptides, or with the MAGE-3 recombinant protein, both with or without adjuvant, or with the ALVAC recombinant viral vector, is devoid of significant toxicity. A minority of vaccinated melanoma patients (about 10 to 20%) show regression of metastatic lesions. This frequency is far beyond the reported incidence of spontaneous regressions of melanoma metastases, estimated at 0.2-0.3%, indicating that these regressions are linked to the vaccinations. However, only 5% of the patients experience a true clinical benefit. Some of the remissions have lasted for several years. There is no evidence that one of the vaccines tested is more effective against the tumors than the others. CTL responses were detected in a minority of patients vaccinated either with peptides or with the ALVAC virus. These responses were often weak, and, in the case of the MAGE-3.A1 antigen, were observed mostly in patients who had tumor regressions.

The most likely explanation for the poor effectiveness of cancer vaccines shown until now is the fact that tumors have acquired the ability to resist destruction by antitumoral T cells, through unknown mechanisms (see « Analysis of T cell responses of vaccinated cancer patients

» below). Future strategies aimed at improving cancer immunotherapy will undoubtedly rely on the characterization of these resistance mechanisms, which should define new important therapeutic targets. Vaccination at earlier stages, when the patient has no more detectable tumor after surgery but has a high risk of relapse, is another strategy that is being developed.

EXPRESSION PROFILING OF TUMOR SAMPLES FROM VACCINATED PATIENTS

In collaboration with the group of P. Coulie (Cellular Genetics Unit, de Duve Institute)

Using the microarray technology, we have established the gene expression profile of a series of tumor samples, mainly cutaneous metastases, obtained from melanoma patients, usually before the vaccine treatment was started. It is expected that a comparative analysis between samples from patients who experienced either tumor regressions or no regression at all will help us to identify genes whose expression is predictive of tumor response to cancer vaccines. Such genes might provide clues about the mechanisms by which tumors can resist destruction by immune cells.

We also use the microarray data to characterize the inflammatory events that take place inside those metastases, and to understand the interaction between the tumor cells and the inflammatory cells at the tumor site. This approach is combined with systematic immunohistological analysis of adjacent cryosections, using antibodies directed against tumor cells, T and B cells, macrophages, blood vessels, and various molecules involved in inflammatory reactions. In addition, selected tumors can be further analyzed by laser capture microdissection of small groups of cells with defined immunohistological characteristics, followed by quantitative RT-PCR analysis.

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CYTOKINES IN IMMUNITY AND INFLAMMATION

The cytokine group studies the biology of Interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is a TH2 cytokine that plays a role in immune responses against intestinal parasites and asthma. IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, upregulates the production of acute phase reagents in the liver. Its activity in inflammatory responses is modulated by a specific antagonist, the IL-22 binding protein (IL-22BP). The role of IL-9 and IL-22 in inflammation is currently being investigated using transgenic and gene-targeted mice for these cytokines and their receptors.

INTERLEUKIN 9

J.-C. Renauld, J. Van Snick, L. Knoops, V. Steenwinckel, M. Stevens

Interleukin-9 (IL-9) was discovered in our group in 1989, through its ability to sustain antigen-independent growth of certain murine T helper clones. We further identified human IL-9 by cross-hybridization with the mouse gene. Although IL-9 did not turn out to be a T cell growth factor for freshly isolated T cells, it was found particularly potent on T cell lymphomas, as an anti-apoptotic agent. To determine the biological activities of this factor, we generated transgenic mice overexpressing this cytokine. Analysis of these animals disclosed three essential properties of IL-9: its tumorigenic potential in T lymphocytes, its stimulatory activity on a particular subset of B lymphocytes, and its activity on mast cells and eosinophils with consecutive implications in asthma.

IL-9-transgenic mice : T cell lymphomas

IL-9 transgenic animals showed normal T cell development and T cell numbers but spontaneously developed thymic lymphomas at low frequency (5%) when maintained in a conventional environment. Two lines of evidence indicate that IL-9 is not a conventional oncogene but rather favors tumor development in response to exogenous stimuli. First, the tumor incidence was significantly lower when mice were maintained under pathogen-free conditions. Secondly, all IL-9 transgenic mice developed T cell lymphomas when exposed to subliminal doses of a chemical carcinogen or to irradiation, that were innocuous in wild type mice. The above mentioned anti-apoptotic activity of IL-9 provides an attractive explanation for these observations, namely that IL-9 could lead to increased survival of abnormal cells ge-

nerated by exposure to minimal doses of oncogenic stimuli. The potential implication of IL-9 in oncology was also confirmed in human systems by its constitutive expression in Hodgkin lymphomas.

IL-9-transgenic mice : B1 cell expansion

Further analysis of these IL-9-transgenic mice showed that a particular B lymphocyte population, called B-1 lymphocytes and usually restricted to the peritoneal and pleuropericardial cavities, were dramatically expanded in response to IL-9 overproduction. In addition, such cells were also found in the blood and in the lungs of IL-9 transgenic mice. This observation is reminiscent of mice that are prone to the development of diseases that are characterized by the production of autoantibodies, such as Systemic Lupus Erythematosus, and suggests that IL-9 might play a role in some autoimmune processes.

IL-9-transgenic mice : parasite infections and asthma

In addition, IL-9 transgenic mice were found to harbor increased numbers of mast cells in the intestinal and respiratory epithelia, and were also characterized by a general hypereosinophilia. This phenotypic characteristic was found to increase the capacity of these animals to expel nematodes like Trichinella spiralis or Trichuris muris., suggesting that IL-9 administration could protect susceptible hosts against these parasites. This was confirmed by taking advantage of a new strategy of anti-cytokine vaccination: mice vaccinated against their own IL-9 failed to expel T.muris parasites and had a decreased eosinophilic response against the parasite (1).

The other side of the coin was the discovery that IL-9 overexpression such as that characterizing the IL-9 transgenic animals resulted in bronchial hyperresponsiveness upon exposure to various allergens. Recent studies indicated that IL-9 promotes asthma through both IL-13 -dependent and IL-13-independent pathways (2). The potential aggravating role of IL-9 in asthma was confirmed by genetic analyses performed by others and pointing to both IL-9 and the IL-9 receptor genes as major candidate genes for human asthma. In addition, we found that asthma patients produce increased amounts of IL-9. Phase I clinical trials using anti-IL-9 antibodies produced in our laboratory have been initiated in collaboration with Medimmune.

IL-9 RECEPTOR AND SIGNAL TRANSDUCTION

Jean-Christophe Renauld, Laurent Knoops, Tekla Hornakova, Monique Stevens

Analysis of the mode of action of IL-9 at the molecular level was initiated in 1992 by the cloning of the murine and human IL-9 receptor (IL-9R) cDNAs. By further dissecting the signal transduction cascade triggered by IL-9, we showed that, upon IL-9 binding, the IL-9R associates with a co-receptor protein called γc. This induces the phosphorylation of the JAK1 and JAK3 tyrosine kinases, which are associated with IL-9R and γc, respectively. A single tyrosine residue of the IL-9R is then phosphorylated and acts as a docking site for 3 transcription factors of the STAT family, STAT-1, -3 and -5, which become phosphorylated and migrate to the nucleus, where they activate the transcription of a number of genes. This pathway is common to many cytokines but is often dispensable for their biological activities. For IL-9, our group demonstrated that activation of the STAT transcription factors is crucial for all the effects of IL-9 studied on various cell lines, including positive and negative regulation of cell proliferation, as well as inhibition of corticoid-induced apoptosis in T cell lymphomas. Further analysis demonstrated that STAT-1, -3 and -5 play specific, redundant and synergistic roles in the different activities of IL-9 in vitro (3).

The pathways responsible for IL-9-induced proliferation were studied in details, and this process was found to depend mainly on the activation of STAT-5, on the recruitment of the IRS-1 adaptor, and on the activation of the Erk MAP-Kinase pathway.

The signal transduction pathway downstream the IL-9 receptor is illustrated in Fig. 1.

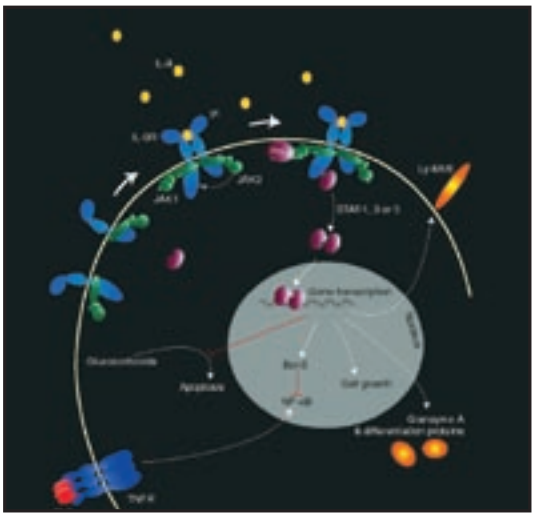

Figure 1. IL-9 receptor signalling.

ANTI-APOPTOTIC ACTIVITY OF I-309RECEPTOR AND SIGNAL TRANSDUCTION

J.-C. Renauld, A. Tounsi, J. Van Snick

Incidentally, our studies of this particular model of the regulation of cell death by cytokines, lead them to purify another protein called I-309, originally described as a human chemotactic factor, and that turned out to exert a significant anti-apoptotic activity for thymic lymphomas (4). However, I-309 and IL-9 trigger completely different pathways and it was shown that the I-309 anti-apoptotic activity was dependent on the activation of G-proteins and the Ras/MAPKinase pathway, whereas the IL-9-mediated effect was not. More recently, we showed that a viral protein related to human chemotactic factors (vMIP-I), and isolated from Herpes viruses that induce T cell tumors, has the same anti-apoptotic activity by binding to the I-309 receptor.

ROLE OF JAK OVEREXPRESSION IN TUMOR CELL TRANSFORMATION

J.-C. Renauld, L. Knoops, T. Hornakova, M. Stevens

Constitutive activation of the JAK-STAT pathway is frequent in cancer and contributes to oncogenesis. Some of our recent data indicate that JAK overexpression plays a role in such processes. Using a murine proB cell line that strictly depends on IL-3 for growth in vitro, cytokine-independent and tumorigenic clones were derived from a two-step selection process. Cells transfected with a defective IL-9 receptor acquired IL-9 responsiveness during a first step of selection, and progressed after a second selection step to autonomously growing tumorigenic cells. Microarray analysis pointed to JAK1 overexpression as a key genetic event in this transformation. Overexpression of JAK1 not only increased the sensitivity to IL-9 but most importantly allowed a second selection step towards cytokine-independent growth with constitutive STAT activation. This progression was dependent on a functional FERM and kinase JAK1 domain. Similar results were observed after JAK2, JAK3 and TYK2 overexpression. All autonomous cell lines showed an activation of STAT5, ERK1-2 and AKT but only TYK2-overexpressing cell lines showed a constitutive activation of STAT3. Thus, JAK overexpression can be considered as one of the oncogenic events leading to the constitutive activation of the JAK-STAT pathway. (5).

IL-TIF/IL-22 : A NEW CYTOKINE STRUCTURALLY RELATED TO IL-10

L. Dumoutier, M. de Heusch, J.-C. Renauld

Searches for genes specifically regulated by IL-9 in lymphomas lead to the cloning of a gene that turned out to encode a 179 amino acid long protein, including a potential signal peptide, and showing a weak but significant sequence homology with IL-10. This protein, originally designated IL-TIF for IL-10-related T-cell derived Inducible Factor, was later renamed IL-22. Recombinant human IL-22 was produced (with D. Colau, LICR) and its crystallograhic structure solved. Despite its structural homology with IL-10, IL-22 fails to recapitulate any of IL-10 biological activities. Biological activities of IL- 22 include the induction of acute phase proteins in liver (6) and protection against experimental hepatitis and colitis (L. Dumoutier, unpublished results). Among the different T cell subset, IL-22 was found to be preferentialy produced by TH17 cells raising some speculations about its potential role in autoimmune processes.

Analysis of genomic databases lead to the identification of a new receptor belonging to the IL-10 receptor family (7). This gene is located in the chromosome 6q24, at 24 kb from the IFNGR1 gene and at 152 kb from the IL-20R. It encodes a protein of 231 amino acid, showing 33 % and 34 % amino acid identity with the extracellular domains of the IL-22R and the IL-20R, respectively, but no cytoplasmic nor transmembrane domains were found. IL-22BP is highly expressed in the placenta, in the breast, in the mammary gland and in the skin. A specific interaction was demonstrated between insolubilized IL-22 and an IL-22-BP-Ig fusion protein. Moreover, recombinant IL-22BP could block IL-22 biological activity demonstrating that this protein can act as an IL-22 antagonist.

Although IL-22 does not share any biological activity with IL-10, these 2 cytokines share a common component of their respective receptor complex, IL-10Rß. Anti-IL-10Rß antibodies indeed block the IL-22-induced acute phase response in HepG2 cells (6). All receptor complexes for IL-10-related cytokines include a long chain and a short chain, based on the length of the cytoplasmic domain of these transmembrane proteins. IL-10Rß is a typical short chain component, with only 76 amino acids in the cytoplasmic domain, whose main function seems to consist in recruiting the Tyk2 tyrosine kinase. In addition to IL-10R ß, IL-22 signalling requires the expression of a long chain protein, called IL-22R and comprising a 319 amino acid long cytoplasmic domain. This chain associates with Jak1, and is responsible for the activation of cytoplasmic signalling cascades such as the JAK/STAT, ERK, JNK and p38 MAP kinase pathways. An unexpected feature of the IL-22R chain is the fact that the C-terminal domain of this receptor is constitutively associated with STAT3, and that STAT3 activation by this receptor does not require the phosphorylation of the receptor, in contrast to the mechanism of STAT activation by most other cytokine receptors (Dumoutier et al., submitted for publication).

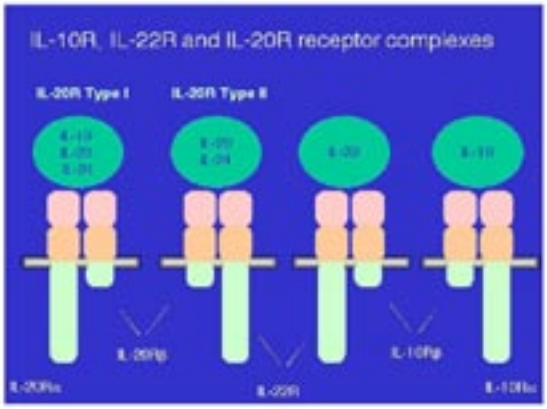

Figure 2. Receptor complexes for IL-10, IL-20 and IL-22

In addition to its role in IL-22 binding and signalling, the IL-22R chain also forms a functional heterodimeric receptor complex by associating with IL-20R ß, the second short chain member of the IL-10R-related receptor family. This complex mediates STAT-1 and –3 activation by IL-20 and IL-24, but not by IL-22 (8). In addition, IL-20 and IL-24 can also bind to other complexes consisting of IL-20Rα and IL-20Rß. This promiscuity in cytokine receptor usage is illustrated in Fig 2 (see also ref. 9 for a review of this cytokine family).

LICR2: A NEW CYTOKINE RECEPTOR MEDIATING ANTIVIRAL ACTIVITIES

J.-C. Renauld, L. Dumoutier

Type II cytokine receptors include receptors for type I and Ii interferons (IFNs) and for IL-10-related cytokines. These transmembrane proteins are almost exclusively related by their extracellular part, which consists of tandem fibronectin type II domains, whereas the cytoplasmic domain is associated with a tyrosine kinase of the Janus Kinase (JAK family). By screening genomic databases for similarity with the extracellular domain of these receptors, we identified a new receptor that we called LICR2 (Likely Interleukin or Cytokine rceptor 2). This receptor binds new cytokines designated IFN-λ1-3, and mediates the same activities as those mediated by the receptors for IFN-α and ß, including antiviral and antiproliferative activities (10), raising the possibility of therapeutic applications in viral infections and cancer.

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SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY GROUP

STRUCTURE AND FUNCTION OF CYTOKINE RECEPTORS

Cytokines and their receptors are critical for the formation of mature blood cells and for the function of the immune system. We study the structure and function of receptors for erythropoietin (Epo), thrombopoietin (Tpo), interleukins 2 and 9 (IL-2, IL-9) and Granulocyte Colony Stimulating Factor (G-CSF). Activation of these receptors is triggered by cytokine-induced changes in receptor dimerization/oligomerization, which lead to the activation of cytosolic Janus tyrosine kinases (JAKs). Regulation by JAK kinases of receptor traffic, the assembly of cell-surface receptor complexes, the mechanisms of dimerization of receptor transmembrane (TM) and cytosolic juxtamembrane (JM) domains, and mechanisms of JAK catalytic activation are major points of interest. The laboratory identified constitutively active mutants of JAK2 and of thrombopoietin receptor and is actively investigating the mechanisms by which JAK2 V617F and thrombopoietin receptor W515 mutants induce, in humans, Myeloproliferative Neoplasms, such as Polycythemia Vera or Primary Myelofibrosis.

THE MECHANISMS BY WHICH A MUTANT JAK2 INDUCES POLYCYTHEMIA VERA AND MYELOPROLIFERATIVE DISEASES IN HUMANS

C. Pecquet, J.-M. Heine

The JAK-STAT pathway is emerging as a key player in cancer, with several mutations in genes coding for JAKs being identified in the past three years (1). Janus kinases possess two kinase domains, one active and the other, denoted as the pseudokinase domain, inactive. JAK2, one of the four known JAKs (JAK1, JAK2, JAK3 and Tyk2) is crucial for signaling by several cytokine receptors, such as the erythropoietin receptor (EpoR), the thrombopoietin receptor (TpoR), the interleukin 3 receptor and the growth hormone receptor. JAKs are appended to the cytoplasmic juxtamembrane domains of receptors and are switched-on upon ligand binding to the receptors' extracellular domains.

Polycythemia Vera (PV), or the Vaquez disease, is characterized by excessive production of mature red cells and sometimes of platelets and granulocytes. Erythroid progenitors in PV are hypersensitive to or independent of erythropoietin (Epo) for proliferation and differentiation. Strikingly, the traffic of TpoR is defective in myeloid progenitors from PV. A hint that JAK2 or JAK2-binding proteins may be involved in PV came when we showed that the wild type JAK2 strongly promotes the maturation and cell-surface localization of TpoR, the very process that is defective in PV (2).

In collaboration with Prof. William Vainchenker and his INSERM unit at the Institut Gustave Roussy in Paris, we have been involved in the discovery of the *JAK2 V617F* mutation in a majority of *Polycythemia Vera* patients (3, 4). The mutation in the pseudokinase domain alters a physiologic inhibition exerted by the pseudokinase domain on the kinase domain. This mutant is found in >95% of PV patients and in 50% of Essential Thrombocythemia and Primary Myelofibrosis (PMF), two other diseases that belong to the myeloproliferative neoplasms (4). Strikingly, the homologous mutations in JAK1 and Tyk2 also enable these kinases to be activated without ligand-binding to cytokine receptors (5). These results suggested that point mutations in JAK proteins might be involved in different forms of cancers (1).

INVOLVEMENT OF TPOR IN MYELOPROLIFERATIVE DISEASES

C. Pecquet, M. Girardot

When the protein sequences of TpoR and the closely related EpoR were aligned, we realized that the TpoR contains a unique amphipathic motif (RWQFP) at the junction between the transmembrane and cytosolic domains. Deletion of this motif (delta5TpoR) results in constitutive activation of the receptor (6), suggesting that these residues maintain the receptor inactive in the absence of TpoR. Further stimulation with Tpo of the delta5TpoR leads to an excess of immature erythroid progenitors at the expense of megakaryocyte differentiation (6). In vivo, in reconstituted mice, the delta5TpoR induces massive expansion of platelets, neutrophils and immature erythroid progenitors and eventually myelofibrosis (Staerk et al., in preparation). Within the KWQFP motif (RWQFP in the human), the key residues that maintain the receptor inactive are the K/R and W residues; mutation of either of the two residues to alanine activates the receptor. We predicted that such mutations may exist in patients with myelofibrosis (6). Indeed, residue W515 has been found to be mutated to either leucine or lysine by the groups of D. G. Gilliland and A. Tefferi. Why the phenotype induced by TpoR W515 mutants is much more severe than that of JAK2 V617F is under investigation in our group.

At present, our laboratory is performing under the auspices of an ARC grant (Action de Recherche Concertée of the Université catholique de Louvain) with the St Luc Hospital departments of Hematology (Prof. Augustin Ferrant) and Clinical Biology (Prof. Dominique Latinne) a large study on the presence of JAK2 and TpoR mutations in patients with myeloproliferative neoplasms. Close collaborations with Drs. Laurent Knoops and Jean-Baptiste Demoulin are supported by the ARC project.

DETERMINATION OF THE INTERFACE AND ORIENTATION OF THE ACTIVATED EPOR, TPOR AND G-CSFR DIMERS

A. Dusa, J.-M. Heine, N. Caceres

Epo binding to the erythropoietin receptor (EpoR) results in survival, proliferation and differentiation of erythroid progenitors into mature red blood cells. In the absence of Epo, the cell-surface EpoR is dimerized in an inactive conformation, which is stabilized by inte-

ractions between the TM sequences. Epo binding to the extracellular EpoR domain induces a conformational change of the receptor, which results in the activation of cytosolic JAK2 proteins.

To identify the residues that form the interface between the receptor monomers in the activated EpoR dimer we have replaced the EpoR extracellular domain with a coiled-coil dimer of α-helices (7). Because coiled-coils have a characteristic heptad repeat with hydrophobic residues at positions a (one), d (four), the register of the coiled-coil a-helices is imposed on the downstream TM α-helix and intracellular domain.

When each of the seven possible dimeric orientations were imposed by the coiled-coil on the fused TM and intracellular domain of the EpoR, only two fusion proteins stimulated the proliferation of cytokine-dependent cell lines and erythroid differentiation of primary fetal liver cells (7). Since the predicted dimeric interfaces of the two active fusion proteins are very close, a unique dimeric EpoR conformation appears to be required for activation of signaling. In this active conformation TM residues L241 and L244 and JM residue W258 are predicted to be in the interface.

Similar studies are undertaken for the related TpoR and G-CSFR. Like the EpoR, the TpoR is thought to signal by activation of JAK2, of several STATs (STAT1, 3 and 5) as well as of MAP-kinase, PI-3-kinase and AktB. However, TpoR and EpoR signal quite differently since only TpoR can induce hematopoietic differentiation of embryonic stem cells or stimulate the earliest stages of hematopoiesis in immature hematopoietic cells.

STRUCTURE AND FUNCTION OF JUXTA-MEMBRANE SEQUENCES IN TRANS-MEMBRANE PROTEINS

A. Dusa, J. Van Hees, C. Mouton, R.-I. Albu

To define the interfaces of the active and inactive EpoR dimers we performed cysteine scanning mutagenesis of the extracellular juxtamembrane and TM domains (10). We isolated three constitutively active novel mutants of the EpoR where residues L223, L226 or I227 were mutated to cysteine (10). These three mutants as well as cysteine mutants of residues 220-230 formed disulfide-bonded dimers. Cysteine-mediated maleimidyl crosslinking indicated that the first five TM residues are not helical and that the interface of the active EpoR dimer contains residues L241 and L244. Replacement of the first 4-5 predicted transmembrane residues of the EpoR, which form a helix-cap, with a stretch of leucine residues-which form an α-helix- leads to constitutive receptor activation (8).

These studies led to the notion that sequences flanking the transmembrane domain might play important roles in receptor function as «switch» regions and also may regulate transmembrane protein oligomerization. In a collaborative study with Jean-Noel Octave and Pascal Kienlen-Campard, we noted that the juxtamembrane and transmembrane domains of the Alzheimer's Precursor Protein (APP) contains three adjacent Gly-x-x-x-Gly motifs, that are predicted to promote tight dimerization of APP transmembrane domains. Replacement of the middle Gly-x-x-x-Gly motif by a Leu-x-x-x-Leu motif changed the dimerization interface of the APP transmembrane domain and abolished production of amyloidogenic peptides Abeta40 and Abeta42 (9). These results may be relevant for efforts to identify small molecules able to block the APP dimer into a dimer conformation that is unfavorable for the production of amyloidogenic peptides Aβ40 and Aβ42.

TRAFFIC OF CYTOKINE RECEPTORS TO THE CELL-SURFACE

J. Kota, C. Pecquet, R.-I. Albu

We have observed that, in hematopoietic cells, over-expression of JAK proteins leads to enhanced cell-surface localization of cytokine receptors (i.e. EpoR TpoR, IL9R, IL2R, γc). For some receptors, the effect of the cognate JAK is to promote traffic from the endoplasmic reticulum (ER) to the Golgi apparatus (12). For others, such as the TpoR, JAK2 and Tyk2 also protect the mature form of the receptor from degradation by the proteasome, and thus JAKs enhance the total amount of cellular receptor (2). In collaboration with Pierre Courtoy, we are employing confocal microscopy of epitope tagged receptors in order to define the precise intracellular compartments where receptors and JAKs interact. Our working hypothesis is that the N-terminus FERM domain of JAK proteins exerts a generic pro-folding effect on cytosolic domains of cytokine receptors. We are testing this hypothesis on several different cytokine receptors and are investigating the link between proper folding in the ER and transport to the cell-surface. Using a fetal liver retroviral cDNA library cloned in pMX-IRES-CD2, we are attempting to clone novel proteins that can regulate traffic and stability of TpoR.

RANDOM MUTAGENESIS APPROACHES TO STUDY INTERACTIONS BETWEEN TRANSMEMBRANE DOMAINS AND STRUCTURE OF JAK2 V617F

A. Dusa

Two transmembrane viral envelope proteins (gp55-P and gp55-A) belonging to the polycythemic (P) and anemic (A) Spleen Focus Forming Virus (SFFV) strains, can activate the EpoR when co-expressed in the same cell. In collaboration with Yoav Henis, Tel-Aviv University, Israel, we have shown that both the gp55-A and gp55-P TM domains specifically interact with the TM domain of the EpoR (Figure 1C and D). gp55-A weakly activates the receptor leading to erythroleukemia with low number of red blood cells (anemia). gp55-P fully activates the EpoR inducing erythroleukemia with elevated levels of red cells (polycythemia). The basis for this difference between gp55-P and gp55-A is represented by differences in specific binding of the TM domains to the TM domain of the EpoR. Taking advantage of this specific interaction we are constructing a genetic system where the TM sequence of gp55-P is randomized and tested for the ability to bind and activate the EpoR. In this system activation of EpoR signaling will result in cell survival and proliferation, which represents a powerful selection.

Second, we employ a random mutagenesis approach coupled to retroviral gene transduction in order to examine the sequence requirements at position V617 of JAK2 for constitutive activation. Mutants of JAK2 V617 to each of the other 18 amino acid residues were tested for constitutive kinase activity, for induction of cell proliferation and activation of STAT5. We identifed several other mutations (V617FW, V617L, V617I and V617I) which also lead to activation of JAK2 (10). Among those, only V617FW induces strong activation comparable to V617F and is resistant to the down-modulation effect of the negative regulator SOCS3. Since the V617F mutation also activated JAK1 and Tyk2 (5), we will test whether any of the potentially activating mutations may also activate JAK1 and Tyk2. We expect these results to shed light on the uniqueness of the JAK2 V617F in patients and on the structural requirements at position V617 of the JH2 region for activation.

CONSTITUTIVE ACTIVATION OF JAK-STAT SIGNALING PATHWAYS AND GENES TARGETED BY STAT5 IN TRANSFORMED HEMATOPOIETIC AND PATIENT-DERIVED LEUKEMIA CELLS

M. Girardot

Cytokine stimulation of cytokine receptors, induces transient activation of the JAK-STAT pathway. In contrast, oncogenic forms of receptors or of JAKs (JAK2 V617F) transmit a continuous signal which results in constitutive activation of STAT proteins. In cultured cells this process is studied by expressing oncogenic forms of cytokine receptors or JAKs in cytokine-dependent cells and assaying for their transformation into cells that grow autonomously. In these transformed cells many of the transient signaling events induced by cytokines are detectable permanently, i.e. ligand-independent phosphorylation of JAK and STAT proteins or high levels of nuclear activated STATs especially STAT5 and STAT3. A similar picture has been noted in patient-derived leukemia cells. The critical questions we would like to answer concern the mechanisms by which the JAK-STAT remain permanently activated in transformed cells and which genes are regulated by constitutively active STAT proteins in leukemic cells. Using chromatin immunoprecipitation and sequencing of native promoters bound by STAT5 we noted that in transformed cells STAT5 can also bind to low affinity N4 sites (TTC-NNNN-GAA) not only to N3 sites, which are characteristic of ligand-activated STAT5. We are attempting to identify the promoters actually bound by STAT proteins in living cells in physiologic and pathologic situations. We use a modified version of the chromatin immunoprecipitation assay pioneered by Alex Varshavsky in conjunction with DNA microarray genomic profiling. The isolated genomic fragments are screened for the presence of STAT-binding sites and tested for the ability to regulate transcription of reporter genes. Newly identified genes regulated by such genomic sequences will be tested for function by cloning their cDNA expressed in bicistronic retroviral vectors that allow wide expression of candidate proteins at physiologic levels.

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