ANNUAL SCIENTIFIC REPORT

Uppsala Branch

2003

Staff

Heldin, Carl-Henrik, Director

Growth Regulation Group

Östman, Arne, Associate Member, Group Head to December
Hellberg, Carina, Assistant Investigator
Furuhashi, Masao, Postdoctoral Fellow
Kappert, Kai, Postdoctoral Fellow, from Augsut to December
Micke, Patrick, Postdoctoral Fellow, to December
Pietras, Kristian, Postdoctoral Fellow, to February
Sjöblom, Tobias, Postdoctoral Fellow, to August
Bäckström, Gudrun, Senior Technical Assistant
Sandström Leppänen, Jill, Technical Assistant
Hägerstrand, Daniel, Ph.D. Student, to December
Persson, Camilla, Ph.D. Student, to June
Sandin, Åsa, Ph.D. Student, from May to December
Sundquist, Christina, Ph.D. Student, from September to December

Signal Transduction Group

Demoulin, Jean-Baptiste, Postdoctoral Fellow, Section Head, to October
Chiara, Federica, Postdoctoral Fellow
Johannesen, Lene, Postdoctoral Fellow, from January to July
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Kallin, Anders, Ph.D. Student

Cytoskeletal Regulation Group

Aspenström, Pontus, Assistant Member, Group Head
Saras, Jan, Postdoctoral Fellow, to March
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Fransson, Åsa, Ph.D. Student
Johansson, Ann-Sofi, Ph.D. Student
Richnau, Ninna, Ph.D. Student, to December

Gene Targeting Group

Heuchel, Rainer, Assistant Member, Group Head
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Molecular Signaling Group

Dikic, Ivan, Assistant Member, Group Head, to January
Hájková, Lucie, Postdoctoral Fellow (joint with Cytoskeletal Regulation Group), to June
Soubeyran, Philippe, Postdoctoral Fellow, to September
Ivankovic-Dikic, Inga, Postdoctoral Fellow, to June
Haglund, Kaisa, Ph.D. Student, to June
Husnjak, Koraljka, Visiting Ph.D. Student, to February
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Szymkiewicz, Iwona, Ph.D. Student, to June
Zapart, Grzegorz, Ph.D. Student, from January to March

TGF-β Signaling Group

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Niimi, Hideki, Postdoctoral Fellow, from January
Valcourt, Ulrich, Postdoctoral Fellow
Morén, Anita, Senior Technical Assistant
Kowanetz, Marcin, Ph.D. Student
Pardali, Katerina, Ph.D. Student
Ritvos, Olli, Visiting Investigator, from March to August

Integrated Signaling Group

Souchelnytskyi, Serhiy, Assistant Member, Group Head
Dubrovska, Anna, Postdoctoral Fellow, from April
Iwahana, Hiroyuki, Postdoctoral Fellow
Lomnytska, Marta, Postdoctoral Fellow, from September to December
Stasyk, Taras, Postdoctoral Fellow, to July
Yakymovych, Ihor, Postdoctoral Fellow

Apoptotic Signaling Group

Landström, Maréne, Assistant Member, Group Head
Edlund, Sofia, Postdoctoral Fellow, from July
Kozakai, Takaharu, Postdoctoral Fellow, to March
Lee, So Young, Postdoctoral Fellow, from April

Zhang, Shouting , Postdoctoral Fellow, from August
Grimsby, Susanne, Senior Technical Assistant

Gene Expression Group

Ericsson, Johan, Assistant Member, Group Head
Grönroos, Eva, Assistant Investigator
Bengoechea Alonso, Maria Teresa, Postdoctoral Fellow
Sundqvist, Anders, Postdoctoral Fellow
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Simonsson, Maria, Ph.D. Student

Matrix Biology Group

Heldin, Paraskevi, Associate Investigator, Group Head (joint appointment with Department of Medical Biochemistry and Microbiology, Uppsala University)
Asteriou, Trias, Postdoctoral Fellow
Li, Yuejuan, Postdoctoral Fellow, from October
Takahashi, Yoshinori, Postdoctoral Fellow, to December
Kamiryo, Masaru, Ph.D. student, from July
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Protein Structure Group

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Introduction

Malignant cells are characterized by perturbations in the signaling pathways that regulate cell growth, chemotaxis and migration. The aim of the work at the Uppsala Branch is to elucidate the molecular mechanisms involved in the regulation of these events, and to use such knowledge for the development of useful treatment regimens for cancer patients.

During 2003, the Branch has consisted of 11 groups working on different aspects of signal transduction mechanisms. Important themes of the work are, as before, studies on platelet-derived growth factor (PDGF), a major mitogen for connective tissue cells, and transforming growth factor-β (TGF-β), which inhibits the growth of most cell types.

During the year, the Head of the Molecular Signaling Group, Ivan Dikic, has left to take up a position as professor at Frankfurt University, the Head of the Signal Transduction Group, Jean-Baptiste Demoulin, has left for a Group Leader position at the Catholic University of Louvain in Brussels, and the Head of the Growth Regulation Group, Arne Östman, has left for a professorship at the Karolin's Institute in Stockholm. We wish Ivan, Jean-Baptiste and Arne good luck in their future work and are looking forward to future interactions with them. Aspects of the program of these groups that are related to PDGF signaling, will be continued at our Branch in a novel group with Carl-Henrik Heldin as Group Leader, and with Johan Lennartsson as Head for a section on Signal Transduction, and Carina Hellberg as Head for a section of Translational Research. The aim of this group will be to elucidate the molecular mechanism for PDGF-induced cell growth and chemotaxis, and to explore the utility of PDGF antagonists in cancer treatment using preclinical models and patient studies.

The Cytoskeletal Regulation Group studies the roles of small GTP binding proteins in the regulation of cell growth, migration and actin reorganization, with a special emphasis on members of the Rho family.

The Gene Targeting Group uses mouse models to study the *in vivo* function of signaling components. On one hand, knock-in mutants of PDGF receptors with altered functional properties are being studied, on the other hand the Smad7 gene is being knocked out.

The TGF-β Signaling Group investigates signaling pathways that regulate cell growth and differentiation in response to TGF-β. Special attention is given to the role of Smad

molecules in transcriptional regulation, and to the dual role of TGF-β as a tumor suppressor and a tumor promoter.

The Integrated Signaling Group also studies the role of TGF-β signaling in normal and tumerous tissues. This group takes a proteomics approach and studies the global proteome, phosphoproteome and glycoproteome of TGF-β stimulated cells, using 2D gel electrophoresis followed by mass spectrometry.

The Apoptotic Signaling Group aims at elucidating the molecular mechanisms whereby TGF-β induces apoptosis in prostate cancer cells. A mechanism whereby Smad7 promotes the activation of a kinase cascade consisting of TAK1, MKK3 and p38, has been demonstrated.

The Gene Expression Group focuses on the roles of posttranslational modifications, such as acetylation, ubiquitination and phosphorylation, for the functional properties of certain transcription factors, including SREBP, Smads and YY1.

The Matrix Biology Group elucidates the importance of matrix molecules, in particular the polysaccharide hyaluronan, in the growth, differentiation and migration of normal and malignant cells.

Finally, the Protein Structure Group provides skills and know-how in protein identification and characterization by mass spectrometry and Adman degradation, as well as in the synthesis of different types of modified peptides.

Brief descriptions of our progress during 2003 follow below.

C.-H. Heldin

Growth Regulation Group

In the Growth Regulation Group, work has continued along two lines, *i.e.* "PDGF receptors as cancer drug targets" and "Function and regulation of protein tyrosine phosphatases". In addition, we have initiated a project aiming at characterization of tumor stroma with the ultimate goal to identify novel drug targets in this tumor compartment.

PDGF receptors as cancer drug targets

PDGF receptor signaling contribute to many tumor-associated processes, *e.g.* autocrine growth of malignant cells, regulation of tumor fibroblasts and stimulation of tumor angiogenesis (reviewed in 96, 104, 105, 106).

PDGF-dependent gliomas. 23 primary human glioblastoma multiforme (GBM) cultures were used to study the role of PDGF in GBM growth. One third of the cultures were growth inhibited by treatment with the PDGF receptor kinase inhibitor Glivec (Hägerstrand *et al*, in preparation). Sensitivity correlated with the levels of PDGF receptor expression. Supervised analysis of gene-expression showed that expression of less than 5 genes could predict response. The findings identify a PDGF-dependent GBM subset, characterized by high PDGF receptor expression and a specific gene expression pattern.

PDGF-induced pericyte recruitment. Most solid tumors show perivascular PDGF β-receptor expression (Sjöblom *et al.*, submitted for publication). The role of PDGF in tumor pericyte recruitment was analyzed by investigating the effects of PDGF overproduction in tumors with PDGF receptor expression restricted to pericytes (63). Increased pericyte coverage, in the absence of increased vessel density, and increased tumor growth was found in the PDGF producing tumors. Thus, PDGF can contribute to tumor growth by enhancing tumor vessel function. This suggests targeting of PDGF-dependent pericyte recruitment as a novel way to enhance anti-angiogenic effects of *e.g.* VEGF antagonists.

PDGF antagonists enhance tumor drug uptake. In experimental tumors, inhibition of PDGF receptors in tumor stroma enhance tumor uptake of chemotherapy drugs (reviewed in 38, 96). This effect is specific for the tumors and requires a minimum of three days of treatment with PDGF antagonists (38). A clinical study investigating if

these interesting findings can be reproduced in a clinical setting have been initiated and is expected to be completed during the autumn of 2004.

Function and regulation of PTPs

TC-PTP is a negative regulator of PDGF receptors. In our studies of PTPs acting as negative regulators of PDGF receptor signaling, we have identified TC-PTP as an antagonist to PDGF-induced signaling (78). PDGF receptor phosphorylation was enhanced in TC-PTP -/- embryos and in cultured fibroblasts from these mice. TC-PTP preferentially dephosphorylates pY1009 of the PDGF β-receptor. Consistent with these findings, TC-PTP -/- cells displayed an increased PLC-γ activation and an enhanced migratory response to PDGF stimulation.

Regulation of PTPs by oxidation. Oxidation of the active site is a general, yet poorly characterized, mechanism for regulation of PTPs. We have developed a novel antibody-based method for monitoring of PTP oxidation (77). With this antibody, UV-induced oxidation of rPTP-α was demonstrated. Interestingly, the oxidation occurred preferentially in the second catalytically inactive PTP domain. This suggests a previously unrecognized function as redox sensor of this domain.

Gene-expression based characterization of tumor fibroblasts

Cancer-associated fibroblasts (CAFs) constitute a significant part of most solid tumors, and contribute through paracrine effects to tumor growth (102). To characterize this poorly defined cell type, a strategy have been developed which combines laser-capture microdissection, RNA amplification and array analyses (75). RNA from normal fibroblasts and CAFs of basal cell cancer have been isolated, amplified and analyzed with cDNA microarrays. Differential expression of selected genes was confirmed by real-time PCR of un-amplified RNA. This procedure will be applied to a larger set of samples to find genes consistently up-regulated in CAFs.

Signal Transduction Group

The Signal Transduction Group focuses on the mechanism of action of PDGF. A first line of research deals with the mechanism of activation of the PDGF receptor tyrosine kinase, using crystallography and mutagenesis. In a second type of projects, members of the group are analyzing the role of signaling proteins associated with the PDGF

receptor, mainly Gab1 and NHERF. Finally, we investigated the regulation of gene expression by PDGF in fibroblasts.

Role of the C-terminal tail in the regulation of the PDGF β-receptor kinase

In an effort to better understand the activation of the PDGF receptor tyrosine kinase, we investigated the role of the C-terminal tail of the PDGF β-receptor in the control of the receptor kinase activity. Using a panel of PDGF β-receptor mutants with progressive C-terminal truncations, we observed that deletion of the last 46 residues, which contain a proline- and glutamic acid-rich motif, increased the autoactivation velocity *in vitro* and the V_{max} of the phosphotransfer reaction, in the absence of ligand, compared to wild-type receptors. 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 presented an active conformation even in the absence of PDGF. A soluble peptide containing the Pro/Glu-rich motif specifically inhibited the PDGF β-receptor kinase activity. Whereas deletion of this motif was not enough to confer ligand-independent transforming ability to the receptor, it dramatically enhanced the effect of the weakly activating D850N mutation in a focus formation assay. These findings indicate that allosteric inhibition of the PDGF β-receptor by its C-terminal tail is one of the mechanisms involved in keeping the receptor inactive in the absence of ligand.

Role of Gab1 in PDGF signaling

Gab1 is a scaffolding/docking protein that has been suggested to play a role in signal transduction downstream of certain plasma membrane receptors including platelet-derived growth factor (PDGF) receptors. We found that PDGF induced a rapid Gab1 phosphorylation, which depended on the recruitment of Grb2, indicating that Grb2 acts as a bridge between Gab1 and the PDGF β-receptor. PDGF also enhanced the binding of Gab1 to the phosphatase SHP-2, but not to p85. To further study the role of Gab1 in PDGF signaling, we transfected porcine aortic endothelial cells with a doxycyclin-inducible Gab1 construct. Increased Gab1 expression enhanced the recruitment and activation of SHP-2, as well as the phosphorylation of the mitogen-activated protein kinases Erk and p38 by PDGF. Gab1 expression also enhanced the formation of lamellipodia and cellular protrusions. In Gab1-deficient mouse embryonic fibroblasts, the same phenotype was induced by restoring the expression of wild-type Gab1, but not a mutant Gab1 that was unable to associate with SHP-2. These effects of PDGF on the actin cytoskeleton were not altered by inhibition of p38 or Erk, but could be blocked by

a dominant negative form of Rac (N17). Finally, Gab1-deficient fibroblasts showed a decreased chemotactic response towards gradients of PDGF as compared to wild-type cells. In conclusion, Gab1 plays a selective role in regulation of the mitogen-activated protein kinases Erk and p38 downstream of the PDGF β-receptor, and contributes to cytoskeletal reorganization and chemotaxis in response to PDGF.

Microarray analysis of PDGF-induced gene expression

We analyzed the transcriptional program elicited by stimulation of normal human fibroblasts with PDGF-BB using cDNA microarrays produced by the Sanger/Ludwig/CRUK consortium. We identified 103 significantly regulated transcripts that had not been previously 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, and their expression correlated with increased membrane lipid production. 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. 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 β–receptor tyrosines that bind p85. Fibroblast growth factors (FGF-2 and FGF-4) mimicked the effects of PDGF on NIH3T3 and human fibroblasts. In conclusion, our results suggest that growth factors induce membrane lipid synthesis via the activation SREBP and PI3K.

Cytoskeletal Regulation Group

The work in the Cytoskeletal Regulation Group is focused on studies of the molecular mechanisms that control cell migration and cell growth under normal physiological conditions as well as during disease. Signaling pathways involving the Rho family of small GTPases have been found to be of particular importance for the organization of the actin cytoskeleton and thereby the control of cell morphogenesis and cell migration. Our studies aim to identify the factors and mechanisms that determine the specific outcome of signal transduction that employ Rho GTPases with the object to understand complex biological processes such as cell migration, cell proliferation and cell survival.

TGF-β-mediated activation of Rho GTPases

TGF-β signaling results in rapid responses seen as a release of Ca^{2+} from intracellular stores as well as a mobilization of the actin cytoskeleton (15, 62). The selective activation of Cdc42 and RhoA is important during TGF-β-induced actin reorganization. Interestingly, we found that the inhibitory Smad7 is required for the TGF-β-induced activation of Cdc42 (62). This observation implicates a novel role for Smad7 in TGF-β-dependent actin regulation. In addition, we could show that the PI3K signaling pathway is required for the TGF-β-induced mobilization of the actin cytoskeleton (62).

The family of Rho GTPases

We have recently shown that the family of Rho GTPases consists of 22 members (59). Several of the newly identified Rho GTPases function in a manner distinct from the classical Rho GTPases. For instance, Miro-1 and -2 (for mitochondrial Rho) have roles in mitochondrial homeostasis and apoptosis (12). Moreover, the Cdc42-related protein Wrch1 has an N-terminal proline-rich extension, which mediates binding to the adapter protein Nck (Saras *et al.,* submitted for publication). The kinetic behavior of Wrch1 is different from the classical Rho GTPases since the GTPase has an unusually high intrinsic nucleotide exchange activity. As a result, the protein is likely to be constantly active in the cell (Saras *et al*., submitted for publication).

Signaling via Wiskott-Aldrich syndrome protein

The Wiskott-Aldrich syndrome protein (WASP) was originally identified as the gene defective in the severe immunodeficiency disorder Wiskott-Aldrich syndrome. WASP is an adapter protein, with a phosphoinositide-binding domain, a Cdc42 binding domain, and an extended proline-rich domain, which binds SH3 domain-containing proteins. In addition, the WASP family of proteins, which also includes N-WASP and Scar/WAVE 1-3, binds directly to actin and to the so-called Arp2/3 complex. The WASP family of proteins is now recognized as important regulators of actin polymerization in vertebrate cells.

The activity of the WASP proteins is governed by a number of associated proteins, such as the recently identified mammalian verprolin WIRE (for WIP-related). WIRE was shown to participate in the mobilization of the actin cytoskeleton and internalization of the PDGF β-receptor by employing distinct domains and affecting distinct signaling

pathways (Aspenström, submitted for publication). Thus, WIRE constitutes a possible link between the actin cytoskeleton and receptor endocytosis machinery.

Signaling mediating cell motility

In addition to the projects described above, the current studies involve a number of Rho GTPases, as well as effectors for the Rho GTPases, such as CIP4, PAR-6, diaphanous and CNK1 (67). The aim is to understand how these components convey signals to control cell migration under normal conditions, as well as during disease.

Gene Targeting Group

This group uses gene targeting in the mouse in an effort to explore the *in vivo* importance of specific signaling pathways initiated by different growth factors.

Analysis of PDGFR-β function by the use of knock-in mice

The receptors for PDGF dimerize upon ligand binding resulting in phosphorylation of specific tyrosine residues within the intracellular part of the receptor. These phospho-tyrosines provide docking sites for SH2-domain containing proteins initiating signals for mitogenesis and actin cytoskeletal rearrangements. In collaboration with Dr. Phil Soriano's group in Seattle, we generated mice bearing point mutated PDGF β-receptors, that are unable to bind and therefore activate phosphatidylinositol-3´-kinase (PI3K) upon ligand stimulation, a prerequisite to signal actin cytoskeletal rearrangements, proliferation and inhibition of apoptosis in cell culture experiments. Surprisingly, these mice had no obvious phenotype, but showed a defect in the regulation of the interstitial fluid homeostasis after a challenge leading to edema formation. In order to further restrict signaling from the β-receptor, we introduced an additional point mutation, such that neither PI3K nor phospholipase-Cγ (PLCγ) were able to bind to the activated PDGFR-β. Although double mutant mouse embryonic fibroblasts exhibited reduced proliferation and migration in response to PDGF, mutant mice showed no overt phenotype. However, in a model of experimental glomerulonephritis, mesangial cell defects were observed. Furthermore, in chimeric analysis, *i.e.* an *in vivo* competition situation between mutant and wild type cells, it was observed that the double mutant cells were defective in colonizing the vascular smooth muscle cell compartment.

In order to investigate the possible involvement of the PDGF β-receptor in disease we generated a mouse with a point mutation in the activation loop of the kinase domain.

Analogous mutations in the hepatocyte growth factor receptor and the stem cell factor receptor have been found in patients with hereditary papillary renal carcinoma and mastocytosis, respectively. In both cases the mutations turned out to be of the gain of function type. At present we are investigating the phenotype of PDGF β-receptor mutant mice, as well as the biochemical properties of the mutant PDGF β-receptor in mouse embryonic fibroblasts. Preliminary experiments, performed in mouse embryonic fibroblasts, point to significant differences in the tyrosine phosphorylation kinetics and pattern between the wt and the mutant PDGFR-β. These differences result for example in ligand-independent PI3K activation.

Very recently an activation loop mutation in the PDGFR-α was reported to be responsible for a certain percentage of gastrointestinal stromal tumors in human. We have generated ES-cells with the identical mutation in the PDGFR-β in order to produce mutant mice.

Regulation and *in vivo* function of Smad7

Transforming growth factor-β (TGF-β) family members, which include TGF-βs, activins and bone morphogenetic proteins (BMPs), are secreted molecules that regulate a plethora of cellular responses, such as proliferation, differentiation, migration and apoptosis. Deregulated TGF-β family signaling has been implicated in multiple disorders and in various human diseases, including cancer, fibrosis and autoimmune diseases. TGF-β family members signal through specific type I and type II serine/threonine kinase receptors which in turn activate a subset of Smad proteins. These molecules relay signals into the nucleus where they direct transcriptional responses in concert with other proteins. The mRNA expression of a particular member of this family, namely Smad7, had been shown to be induced by TGF-β itself. Overexpression of Smad7 leads to downregulation of TGF-β signaling, suggesting an auto-regulatory feedback mechanism. We investigated the mouse Smad7 promoter and found not only an essential DNA binding site for the TGF-β activated Smads 2, 3 and 4, but also the requirement for cooperation of these Smads with Sp1 and Ap1 transcription factors in order to guarantee an efficient TGF-β response of the Smad7 promoter.

In order to shed more light on the *in vivo* function of Smad7, we generated mice with a null mutation in the Smad7 gene in collaboration with Dr. Tony Pawson's lab in Toronto, Canada. The phenotype of the Smad7 knockout animals is currently under investigation. Preliminary data suggest that the lack of Smad7 leads to early postnatal lethality when kept on a C57Bl/6 mouse background in contrast to when the outbred

ICR strain is used as background. In line with the idea of increased TGF-β signaling in the absence of Smad7 and TGF-β's growth inhibitory effect on lymphocyte proliferation, we have first indications for an immunologic phenotype in these mice.

Molecular Signaling Group

Our laboratory is dedicated to studying the molecular mechanisms by which signaling pathways control cell growth and differentiation and the reasons why they are deregulated in diseases such as cancer. We are primarily interested in signal transduction mediated by growth factor activated receptor tyrosine kinases (RTKs). More recently, we became interested in mechanisms underlying RTKs endocytosis and protein degradation through actions of the scaffold protein CIN85 and the ubiquitin ligase Cbl. We currently utilize genetically altered mice and cells derived from such animals to understand how components of these modules regulate cell functions in vivo.

EGF receptor endocytosis and degradation

EGF receptor signal inactivation is accomplished by endocytosis and degradation of activated receptors and associated signaling proteins (84). These processes are essential to avoid constitutive signaling and tumorigenesis. Ligand-induced ubiquitination of EGF receptors has been linked to their negative regulation by internalization and endocytic targeting to destruction in the lysosome. The Cbl family of ubiquitin ligases plays pivotal roles in these processes (85). Cbl can directly bind to phosphorylated EGF receptors via its tyrosine kinase binding (TKB) domain, while the RING finger domain of Cbl recruits ubiquitin-conjugating enzymes (E2, Ubc) and mediates the transfer of ubiquitin to the receptor. Several lines of evidence have converged to show that RTK ubiquitination is important both for receptor internalization and for degradation in lysosomes (84, 85).

We have recently shown that Cbl directs monoubiquitination, rather than polyubiquitination, of activated EGF and PDGF receptors in mammalian cells (17). RTKs are monoubiquitinated on multiple sites, which ensures proper endosomal sorting and subsequent degradation of receptors in the lysosome (17). Furthermore, a single ubiquitin attached to EGF receptors was sufficient to mediate internalization as well as

degradation of receptors (17). These results are consistent with the hypothesis that a single ubiquitin carries intrinsic signals for internalization at the plasma membrane and sorting for lysosomal destruction.

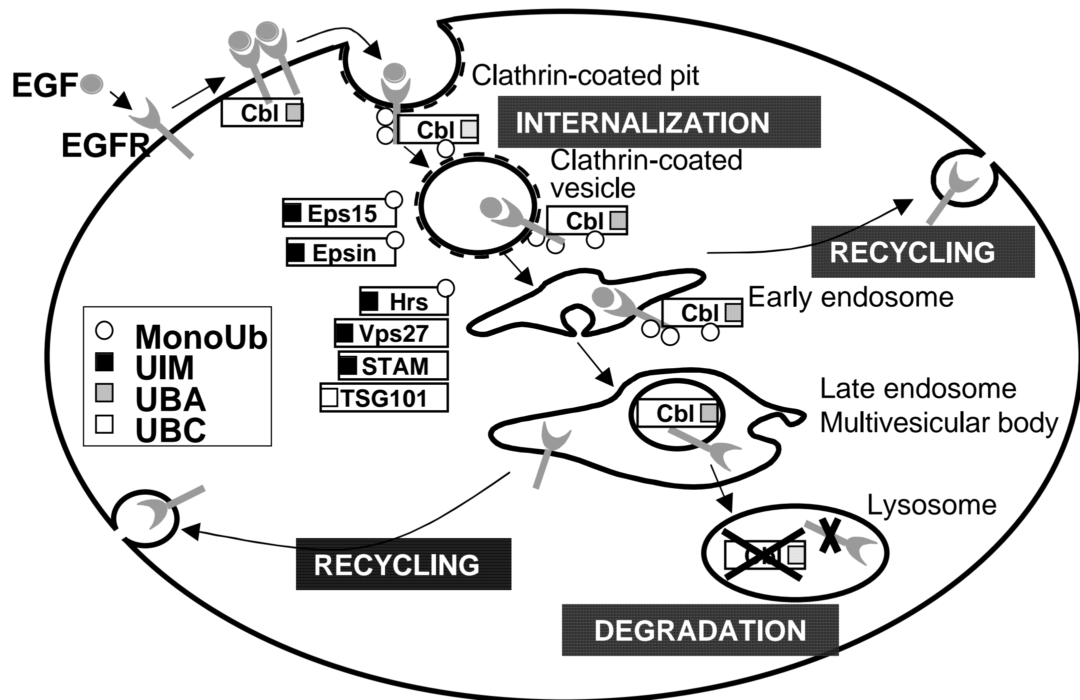

Figure 1. Intracellular trafficking of activated receptors.

The activation of EGF receptors by EGF induces its phosphorylation and the recruitment of Cbl that mediates its multi-monoubiquitination. This process is rapidly followed by internalization of activated receptors/Cbl complexes and their subsequent trafficking toward various intracellular compartments leading either to recycling of the receptors to the cell surface, or their concomitant degradation in the lysosome. This mechanisms are regulated by divers proteins able to interact with ubiquitin moieties via specific domains (UIM, UBiquitin Interacting Motif; UBA, UBiquitin Associated; UBC, UBiquitin Conjugating enzyme like) and that can also undergo mono-ubiquitination.

Major questions we are currently addressing deal with the role of monoubiquitin signals in endocytic sorting of cargo (*i.e.* EGF receptor and associated receptor complexes), as

well as functions of ubiquitin binding proteins that serve as ubiquitin receptors along the endosome (87). In addition, we are particularly interested to understand the mechanisms which define the specificity in determining mono-, multi- or poly-ubiquitination of distinct Cbl-substrates.

Functions of the adaptor protein CIN85 and its isoforms in vivo

Upon engagement by their cognate ligands, receptor tyrosine kinases (RTKs) become ubiquitinated through the action of Cbl ubiquitin ligases (reviewed in 85). In addition, Cbl regulates RTK endocytosis via pathways that are dependent on Cbl's ability to interact with multiple proteins, including CIN85 and Grb2 (83). We have previously shown that binding of the adaptor protein CIN85 to Cbl and recruitment of endophilins in complexes with activated EGF receptors is critical for EGF receptor internalization (84). More recently, we have shown that CIN85 binds to the distal carboxyl-terminus of Cbl, recognizing a novel proline-arginine motif (PxxxPR) present in several CIN85 effectors (24, 25). All three SH3 domains of CIN85 bind to PxxxPR motifs of Cbl/Cbl-b with high specificity and relatively low affinity, thus enabling full size CIN85 to simultaneously interact with multiple Cbl molecules, promoting clustering of Cbl/EGF receptor complexes in mammalian cells (24). In addition, CIN85 binds to a PKPAPR motif in Disabled-2 (Dab2), an endocytic adaptor molecule implicated in clathrin-coat assembly. CIN85 association with Dab2 is essential for its recruitment to clathrin coat and appears to be modulated by growth factor stimulation. Dab2 and clathrin dissociated from CIN85 following growth factor treatment, enabling other molecules, such as Cbl, to bind to CIN85. Taken together, our data indicate a dynamic interplay between CIN85 and its effectors during endocytosis of receptor tyrosine kinases. Our current interests are focused on the determination of the biological importance of CIN85 and its multiple isoforms by using gene targeting approaches in mice.

TGF-β Signaling Group

The TGF-β Signaling Group investigates signaling pathways that regulate cell growth and differentiation in response to transforming growth factor β (TGF-β), and pays special attention to processes that contribute to tumor cell invasiveness and metastasis.

TGF-β signaling and Smad regulation

The highly conserved TGF-β signaling engine consists of plasma membrane serine/threonine kinase receptors and Smad proteins, their cytoplasmic effectors (103). Smads, upon activation by the receptors, translocate to the nucleus and regulate gene expression (93, 94). TGF-β induces phosphorylation of Smad3, a receptor-activated (R-) Smad, which is rapidly imported to the nucleus, regulates gene transcription and eventually is exported back to the cytoplasm. We have analyzed the mechanism of Smad3 nuclear export and identified exportin-4 and the Ran GTPase as the major transporting factors for Smad3 (Kurisaki *et al.*, submitted for publication). In parallel, we study the regulation of Smad4 function. This Smad serves as the common effector for all TGF-β superfamily pathways. In carcinomas, specific amino acid substitutions in Smad4 lead to its enhanced poly-ubiquitination and proteolysis. Wild-type Smad4 can be mono- or oligo-ubiquitinated, which leads to efficient R-Smad/Smad4 oligomerization and enhanced transcriptional activity (34). We identified Smurf1 as an E3 ubiquitin ligase that induces poly-ubiquitination of wild-type Smad4 with the assistance of the adaptor protein Smad7. We now want to understand whether Smurf1 also catalyzes mono-ubiquitination of Smad4, and if so, whether the degree of ubiquitination is regulated by the expression level of Smurf1. Ultimately, we will examine Smurf1 expression levels in carcinomas that express mutant Smad4 to validate the relevance of our biochemical model in human cancer.

Transcriptional roles of Smads: Regulation of the cell cycle and cell differentiation

Smad proteins, upon signal-dependent translocation to the nucleus cooperate with several nuclear factors in order to regulate transcription (93). One such factor is the multifunctional nuclear protein YY1, whose specific association with Smads blocks their transcriptional activity in a gene-specific manner (27). As a result, YY1 cannot interfere with TGF-β/BMP-induced cell cycle arrest but rather represses genes involved in cell differentiation processes in response to either TGF-β or BMP. We now attempt to understand what specifies this selectivity of YY1. We have extended our previous studies on the transcriptional regulation of the cell cycle inhibitor *p21* gene by analyzing the expression of p21 in response to all signaling pathways of the superfamily. BMP-specific pathways and the corresponding R-Smad effectors show a stronger ability to induce p21 expression and we attempt to understand the molecular reason behind such specificity.

Dual role of TGF-β in tumor suppression and tumorigenesis

TGF-β plays a tumor suppressor role in early stages of carcinogenesis, yet it promotes carcinoma cell invasiveness and metastasis. Using mammary epithelial models that exhibit this dual response to TGF-β we focus on regulation of proliferation and epithelial-mesenchymal transition (EMT), a change in cell differentiation that precedes tumor cell migration and metastasis (27, 70). We found that chronic exposure of mammary epithelial cells to TGF-β leads to sensitization and relative resistance to growth inhibition, while cells remain in the fibroblastic phenotype, thus exhibiting sustained and long-term EMT (Gaal *et al*., submitted for publication). We showed that the EMT response is specific to TGF-β, and is not induced by BMP members of the superfamily and that Smads play critical roles during EMT. By analyzing immediate-early and late gene responses to TGF-β using cDNA microarrays, we identified several novel gene targets of the pathway with interesting functional characteristics that makes them candidate effectors of EMT and promoters of cell invasiveness (Valcourt *et al*., submitted for publication). We are in the process of functionally analyzing a set of these target genes for their contribution to EMT and invasiveness. We have also initiated a line of research whereby we try to understand crosstalk between TGF-β and Notch pathways. Specifically, we ask whether TGF-β signaling is required for Notch to exhibit its tumor suppressor functions and we analyze the role of Notch in EMT induced by TGF-β.

Using human mammary carcinoma cells, which are null for Smad4, and comparative cDNA microarray analysis of the TGF-β and BMP pathways, we found that gene regulation downstream of these pathways primarily depends on the presence of Smad4 (70). Two prominent targets of this screen were the transcriptional regulators Id2 and Id3. We showed that TGF-β represses these *Id* genes while BMP induces them and this is physiologically relevant as Id2 and Id3 block both cell cycle arrest and EMT responses to TGF-β. Our findings explain why TGF-β is a potent inducer of growth inhibition and EMT in epithelial cells, while BMP is not. Id2 and Id3 define the specificity of these signaling pathways. We currently analyze additional gene targets from the screen and attempt to understand the logic behind their regulation by TGF-β members.

Integrated Signaling Group

Activities in the Integrated Signaling group are concentrated on proteome profiling of breast epithelial cell transformation, and on studies of TGF-β signaling in cancer. We

have developed a proteomics platform for TGFβ studies, and develop and implement tools for incorporation of proteomics data into a systems biology approach.

Proteomics

We have developed a proteomics platform based on use of two-dimensional gel electrophoresis and proteome image analysis. We analyze protein expression, turnover, phosphorylation and glycosylation. We also use our proteomics platform to study protein-protein complexes, and for discovery of prognostic markers of human breast and ovarian cancers. Protein identification by mass spectrometry is performed in collaboration with Dr. Ulf Hellman at our Branch.

In various proteomics-based projects, we have identified more than 200 proteins affected by TGFβ. Fifty-four proteins affected by TGFβ1 in human endothelial cells have been identified; the novel targets of TGFβ1 provided an insight into regulation of endothelial cell proliferation and cytoskeleton rearrangement (73). Thirty-three proteins associated with type II BMP receptor were identified using two-dimensional gel electrophoresis and mass spectrometry (65).

Study of protein post-translational modifications is the main challenge of modern proteomics. We have developed approaches to monitor the phospho- and glyco-proteome of human epithelial breast cancer cells. Metabolic labeling of cells with radioactive isotopes (^{32}P, ^{14}C) and affinity purification of modified proteins with lectins have been found to be the most efficient techniques. Phosphoproteome profiling of TGFβ/Smad signaling in human breast cancer cells has led to identification of 35 proteins; TGFβ/Smad-dependent phosphorylation of one of the novel targets, Transcription Factor-II-I, at serine residues 371 and 743 was found to serve as a negative feed-back regulator of the TGFβ1/Smad3-transcriptional activity (Stasyk *et al.* submitted for publication).

O-Glycosylation has been shown to be a highly dynamic regulatory modification. Glycoproteome profiling has led to the identification of 27 proteins affected by TGFβ1 (Iwahana *et al.*, manuscript in preparation). Functional studies of one of the novel targets unveiled a mechanism of the TGFβ1-dependent regulation of apoptosis-induced fragmentation of genomic DNA.

We apply peptide chemistry approach to identify phosphorylation and O-glycosylation sites in proteins; one of them is the BEMAD (β-elimination followed by Michael

addition; Iwahana *et al.*, manuscript in preparation). Modifications of the BEMAD chemistry with introduction of various additive compounds are under investigation.

Our proteomics platform has been used in a number of collaboration projects studying TGFβ signaling and searching for markers of human cancers. In collaboration with clinicians, we have identified 23 proteins as potential markers of human breast and ovarian cancer in plasma obtained from 43 patients. Collection of plasma samples for a large cohort-validation, and follow-up of the primary cohort of patients whose plasma was used for the proteomics-based screen, are on-going (Lomnytska *et al.*, manuscript in preparation). Proteome profiling of the effect of expression of the non-coding RNA DD3, a marker of prostate cancer, has revealed changes in expression of 47 proteins (Oosterwijk *et al.*, manuscript in preparation). A number of proteins affected by TGFβ family receptors ALK1 and TβR-I have been identified in collaboration with Dr. Feige, Grenoble; some of the proteins are currently under validation in functional studies (Lamouille *et al.*, manuscript in preparation). The fact that we have been involved in several successful collaborative projects shows that we have established a technology platform and collected experience of proteome profiling, which can be of benefit for the scientific community, including scientists of the Ludwig Institute worldwide.

Generation of a significant amount of proteomics data requires their systematic analysis. The recent initiatives by the Human Proteome Organization (PSI, PEDRo/MIAPE) provide guidelines for acquisition of primary data. These initiatives allow also bridging of proteomics and mRNA/cDNA microarray results. We acquire and process our proteomics data using PEDRo, v1.4, and translate them using Systems Biology Workbench recommendation. Presentation in extensible markup language format allows further incorporation of our data into international efforts for modeling of human tumorigenesis.

TGFβ signaling in cancer

Our work has been concentrated on the elucidation of mechanisms of tumor suppressive and tumor promoting activities of TGFβ. This work should result in design of treatments or drugs affecting tumorigenesis.

We have shown that manipulation of Smad-dependent signaling in cells affects growth of tumor plugs in mice; increased expression of Smad2 resulted in inhibition of proliferation of tumor cells. Smad2-dependent effect on apoptosis and angiogenesis were also noted in tumors formed in mice (81). Further studies of TGFβ/Smad signaling in breast tumorigenesis are on-going.

We study cross-talk between TGFβ/Smad signaling and tumor suppressors and promoters which have been described in human breast cancer. We have found that Smad3 forms complexes with BRCA1 and BRCA2 (Kanamoto *et al.*, submitted for publication). Direct interaction of BRCA1 and Smad3 resulted in synergy in transcriptional regulation, but Smad3 inhibited BRCA1-dependent repair of DNA double-strand breaks. The opposing effects of TGFβ/Smad3 and BRCA1 on DNA repair may modulate sensitivity of tumors to chemotherapeutic drugs.

The observation by us that DNA damage repair can be regulated by TGFβ/Smad proteins, through interactions with BRCA1, Rad51, Rag1, and ORC5T, unveiled a novel pathway in TGFβ signaling which targets directly regulators of genomic DNA integrity. The discovery of this pathway is the result of combination of a direct hypothesis-driven approach and proteomics-based studies.

Apoptotic Signaling Group

TGF-β plays an important role for regulating of cell fate (*i.e.* proliferation, migration, differentiation and apoptosis) during embryogenesis. TGF-β inhibits proliferation and induces apoptosis in most normal cell types while during tumor progression, TGF-β instead promotes tumor growth due to its effects on transdifferentiation of cells, angiogenesis and immunosurveillance. Smad7 is a target gene for TGF-β and we have demonstrated that Smad7 is required for TGF-β-induced apoptosis in prostate cancer cells as well as in human keratinocytes. Furthermore, we have recently reported that Smad7 facilitates the TGF-β activating kinase 1 (TAK1), mitogen activated protein kinase kinase 3 (MKK3) and p38 mitogen activated protein (MAP) kinase pathway, presumably by acting as an adaptor protein bringing the kinases close to each other. The aim of our work is to more precisely investigate the role of Smad7 in the TGF-β-induced apoptotic signalling pathway.

Smad7 is required for apoptosis in prostate cancer cells

We have observed increased expression of the receptor-activated Smads (Smad2, Smad3 and Smad4), as well as the inhibitory Smad6 and Smad7, in normal and malignant prostate epithelial cells *in vivo,* preceding apoptosis induced by androgen withdrawal. The presence of Smad7 in apoptotic cells *in vivo,* together with our previous observation that Smad7 is required for induction of apoptosis, encourage us to continue our search for the detailed molecular mechanisms for how Smad7 can act as a mediator for TGF-β induced apoptosis in epithelial cells. As Smad7 is facilitating the

activation of p38, we are currently investigating possible novel target proteins downstream of the p38-Smad7 complex (Edlund S, Lee SY *et al.,* manuscript in preparation).

We have earlier described a previously unknown function for Smad7 as an adaptor protein in the TAK1-MKK3-p38 MAPK pathway. We are currently in more detail, investigating the molecular mechanism whereby TGF-β and Smad7 activate this pathway (Schuster N, Grimsby S *et al.,* manuscript in preparation).

Furthermore, Smad7 is predominantly localized in the nucleus of resting cells, while stimulation of cells with TGF-β causes a rapid export of Smad7 to the cytoplasm, where it interacts with the TGF-β-activated receptor-complex. At longer timepoints after TGF-β-stimulation of cells, Smad7 accumulates in the nucleus again. We are currently investigating whether the apoptotic effect of Smad7 is dependent on its possibility to interact with other proapoptotic proteins which can shuttle between the nucleus and the cytoplasm (Zhang S *et al.,* manuscript in preparation).

In a collaboration with Pontus Aspenström, Group Leader for the Cytoskeletal Group, we have found that Smad7 expression is required for TGF-β-mediated cytoskeletal regulation which occurs mainly via the small GTP-ase Cdc42 (62). We will continue to investigate the role of Smad7 in TGF-β-dependent regulation of cytoskeletal processes such as migration of cells.

Smad7 target genes

Smad7 is a nuclear protein which upon TGF-β stimulation of cells is exported to the cytoplasm. We are very interested in the potential effects of Smad7 on gene regulation and have therefore performed microarray analyses on cells overexpressing Smad7. The possible candidate genes are currently validated.

The role of Smad7 in apoptosis induced by 2-methoxyestradiol

2-methoxyestradiol is an endogenous metabolite of estradiol-17β. We and others have previously shown that 2-ME have both anti-angiogenic and direct cytotoxic effects on several investigated tumor cells *in vitro* and *in vivo*. We are currently investigating the role of Smad7 in the apoptotic pathway induced by 2-ME in human prostate cancer cells. Preliminary data shows that Smad7 expression enhances 2-ME-induced apoptosis in prostate cancer cells, while in cells stably transfected with an anti-sense Smad7

construct, a reduced apoptotic response is observed and the apoptotic molecular pathway are under examination (Davoodpour *et al.,* manuscript in preparation).

Gene Expression Group

SMADs - mediators of transforming growth factor-β signaling

TGFβ belongs to a superfamily of cytokines that regulate diverse biological functions, ranging from differentiation, motility and apoptosis to the inhibition of cell growth. Inappropriate regulation of TGFβ signaling has been implicated in multiple human diseases, such as fibrosis, rheumatoid arthritis and carcinogenesis. Smad proteins regulate gene expression in response to TGFβ signaling. We have demonstrated that the inhibitory Smad7 interacts with the transcriptional coactivator p300, resulting in acetylation of Smad7 on two lysine residues in its N-terminus (Grönroos *et al.*, Molecular Cell *10*: 483-493, 2002). Acetylation or mutation of these lysine residues stabilizes Smad7 and protects it from TGFβ-induced degradation. Furthermore, we have demonstrated that the acetylated residues in Smad7 also are targeted by ubiquitination and that acetylation of these lysine residues prevents subsequent ubiquitination. In addition, acetylation of Smad7 protects it against degradation mediated by the ubiquitin ligase Smurf1, demonstrating that the acetylated lysines are important for Smurf-dependent degradation. Thus, our data suggest that competition between ubiquitination and acetylation of overlapping lysine residues constitute a novel mechanism to regulate protein stability. We propose that acetylation of specific lysines in Smad7 prevents subsequent ubiquitination of the same residues, thereby blocking proteasome-mediated degradation of Smad7. The ubiquitin-proteasome pathway regulates a large number of nuclear proteins and many of these are also acetylated. Therefore, it will be of utmost importance to determine if competition between acetylation and ubiquitination is a general mechanism to regulate protein stability.

SREBPs – key regulators of lipid metabolism

Members of the SREBP family of transcription factors control cholesterol and lipid homeostasis and play important roles during adipocyte differentiation. The transcriptional activities of SREBPs are dependent on the coactivators p300/CBP. We have demonstrated that SREBPs colocalize with p300 in nuclear speckles *in vivo*. In addition, we have found that SREBPs are acetylated by the intrinsic acetyltransferase activity of p300 and CBP (14). In SREBP1a, the acetylated lysine residue resides in the DNA-binding domain of the protein. Coexpression with p300 dramatically increases the

expression of both SREBP1a and SREBP2 and this effect is dependent on the acetyltransferase activity of p300, indicating that acetylation of SREBPs regulate their stability. Indeed, acetylation or mutation of the acetylated lysine residue in SREBP1a stabilized the protein. We have demonstrated that the acetylated residue in SREBP1a is also targeted by ubiquitination. Thus, our studies define acetylation-dependent stabilization of transcription factors as a novel mechanism for coactivators to regulate gene expression. We are currently using SREBP and Smads as model proteins to characterize the complex link between protein acetylation and the ubiquitin-proteasome pathway.

We have also demonstrated that the degradation of SREBP is dependent on its transcriptional activity, *i.e.* transcriptionally active molecules are degraded rapidly whereas transcriptionally inactive molecules are stable (47). We are currently investigating how the balance between coactivator-mediated acetylation and transcription-dependent degradation controls the stability of transcription factors, using SREBP as a model protein.

The most common treatment for elevated cholesterol levels in humans is a group of drugs called statins. These compounds block cholesterol synthesis and, therefore, activate SREBPs. Activation of SREBP leads to an enhanced expression of the LDL receptor gene and, thereby, increased clearance of LDL from the circulation. We hypothesize that compounds that enhance the stability of SREBPs should increase the levels of transcriptionally active SREBP in cells. Such compounds could potentially be used to enhance the cholesterol-lowering activities of statins.

Characterization of a new regulator of the p53 tumor suppressor

Mutations in the p53 gene or inactivation of the p53 protein are the most frequent alterations in cancer cells and are found in more than 50% of all human cancers. We have identified a nuclear protein that binds to p53 and inhibits its transcriptional activity (Terentiev *et al.*, manuscript in preparation). In addition, we have demonstrated that overexpression of this protein blocks the phosphorylation, stabilization and transcriptional activation of p53 in response to DNA damage. Furthermore, we have demonstrated that RNAi-mediated inactivation of this protein promotes p53-dependent apoptosis in response to DNA damage, indicating that the endogenous protein is involved in the control of p53-dependent processes. It will, therefore, be of interest to determine if this protein also regulates the tumor suppressor function of p53 *in vivo*.

Matrix Biology Group

The Matrix Biology Group studies the molecular mechanisms whereby the polysaccharide hyaluronan promotes tumor growth and invasion. Hyaluronan influence cell behavior, *e.g.* cell migration, proliferation and differentiation, by affecting the structural integrity of extracellular and pericellular matrices and initiation of cellular signals. We investigate how hyaluronan- and growth factor-induced signaling are interrelated, as well as whether hyaluronan influences tumor progression and invasion to the adjacent host tissue. Increased understanding of the regulation of hyaluronan biosynthesis and its implications in cell function may be of therapeutic value.

Growth factor regulation of hyaluronan biosynthesis and degradation

Several studies have demonstrated a correlation between increased levels of hyaluronan and the malignant phenotype of tumors, in both animal models and human patients. Therefore, we have initiated studies to investigate the mechanisms underlying perturbation of hyaluronan synthesis and turnover during tumor progression. The regulation of hyaluronan syntheses (Has1, Has2 and Has3) and hyaluronidases (PH-20, Hyal1 and Hyal2) in response to growth factors released by tumor cells, have been studied. Platelet-derived growth factor-BB (PDGF-BB) was shown to have a potent stimulatory effect, which was partly mediated by activation of protein kinase C. Among the three Has isoforms studied, the Has2 isoform is more markedly upregulated or suppressed in response to external stimuli. Notably, transforming growth factor-ß1 (TGF-ß1) was found to stimulate Has activities but to strongly inhibit hyaluronidase activities. Additional studies revealed that newly synthesized hyaluronan is retained on the cell surface either by sustained attachment to the Has or through its interactions with hyaluronan receptors, such as CD44; hyaluronan is therefore crucial for the assembly of cell microenvironment or pericellular matrices. The composition of such matrices is differently regulated in response to growth factors and influence critical cell functions.

The importance of hyaluronan in tumor invasion

In another line of research we have investigated the effect of hyaluronan production on the malignant properties of tumor cells. Until now it has not been clear how hyaluronan produced by tumor cells or adjacent non-cancer stromal cells affect tumor-host microenvironment. In an effort to explore the relation between hyaluronan production by mesothelioma cells and their aggressiveness, we have compared the biological properties of a non-hyaluronan producing mesothelioma cell line with those of the same

cells made to produce hyaluronan after transfection of Has2 cDNA. Our data indicate that increased synthesis of hyaluronan leads to an increased malignant phenotype of mesotheliomas and facilitates their aggressive spread in a CD44-dependent manner. These results were further confirmed by additional studies in a colon carcinoma model both *in vitro* and *in vivo*. The interesting and novel finding in these studies was that Has2 overproduction promotes tumorigenicity, whereas Hyal1 overexpression suppressed tumor development. Given the opposite effects of hyaluronan synthesizing and hyaluronan degrading enzymes, studies on the molecular mechanism that regulate their activities are highly warranted.

Studies on the mechanisms through which hyaluronan fragments induce endothelial cell differentiation

We have also initiated studies to investigate how hyaluronan fragments affects neovascularization at the molecular level, We have studied the gene expression profile of endothelial cells in response to hyaluronan fragments using a microarray approach in the absence or presence of blocking CD44 antibodies. The effect of the fragments on the induction of tubuli structures was compared to the effect of the angiogenic factor, basic fibroblast growth factor (bFGF). Several distinct but also common classes of genes were up- or down-regulated after stimulation with hyaluronan fragments or bFGF during capillary endothelial cell assembly into tube-like structures. The role in endothelial cell differentiation of the genes regulated by hyaluronan fragments are currently under investigation.

Protein Structure Group

Close interactions with the other Groups within the Institute and at the Stockholm Branch, as well as with external research groups, have given us a solid experience in the relatively few activities we make use of. These are (1) peptide synthesis – contrary to the trend of outsourcing this labor-intensive technique we prefer to take advantage of our long gained know-how. (2) Radio-labeled amino acid sequencing. (3) Mass spectrometry using a top of the line matrix-assisted-laser-desorption-ionization-time-of-flight-mass-spectrometer (MALDI-ToF-MS).

The introduction of mass spectrometry into our lab has made chemical peptide sequencing obsolete, and has also reduced the quantity needed for a significant analysis (*e.g.* a protein identification) with a factor of about 100. In September 2003, we traded in the Autoflex MALDI and the Esquire Ion Trap for a new instrument, a Bruker

Ultraflex TOF/TOF and this has lead to a dramatic increase in accuracy, user friendliness and peptide sequencing possibilities.

Peptide synthesis

Our synthesizer, a four-year-old Applied Biosystems 433A instrument, is operated with Fmoc chemistry, and produces high quality peptides. These are worked up manually and often, depending on the intended use, purified to homogeneity by reversed-phase liquid chromatography. It has gradually become possible to prepare peptides carrying various modifications, *i.e.* phosphorylations, acetylations, oxidations etc., which has been most useful for the different Groups. All products are quality control analyzed by the MALDI-ToF-MS. The uses of the peptides produced vary from generation of anti-peptide antibodies, as substrates or inhibitors or as ligands in affinity chromatography experiments.

Radiolabeled phosphopeptide mapping

The introduction in our lab of MALDI-ToF-MS six years ago made us realize that classical amino acid sequence analysis was too slow, too expensive, too labor-intensive and too insensitive, all this is better done by MALDI-ToF-MS when working on well characterized species. However, the peptide sequencers from Applied Biosystems (the two ancient 477A instruments are fading out because of lack of spare parts; therefore we have slightly reconstructed the 494A instruments) are being used for the important positioning of phosphorylated Tyr, Ser and Thr residues. Thanks to the shielded phosphorimager from Fuji, these analyses have far higher sensitivities than we can achieve with mass spectrometry.

Sample preparation for mass spectrometry

Over 95% of the samples for analysis by MALDI-ToF-MS come as bands or spots from one- or two-dimensional gels followed by in-gel tryptic digestion. With Coomassie-visible material only a few percent is used for analysis; with silver stained material, often all the samples must be applied, and with weak silverstain, the sample must be concentrated and desalted on a hand-made reversed phase column in sub microlitre scale.

Peptide mass fingerprinting (PMF)

Determining protein identity by PMF is a routine procedure, provided the target protein's sequence is deposited in a sequence data bank. After generation of a proteolytic digest and analysis by MALDI-ToF-MS we utilize a search engine (ProFound or MASCOT are preferred). If a clear mass spectrum, with only few contaminating mass peaks, is obtained, we usually obtain the protein identity with high significance. When this is not the case, a protein's identity may be established by determining the amino acid sequence of one or more tryptic peptides (see below). A sequence homology search is, in contrast to PMF, tolerant to amino acid substitutions.

Post Source Decay (PSD) sequencing by MALDI-ToF-MS

Fragment analysis of peptides in MALDI-ToF-MS by PSD is an established procedure for MALDI instruments equipped with reflector. The process in standard MALDIs is, however, time-consuming, rather un-precise and very difficult to interprete, and therefore not often used. Our new Ultraflex, on the other hand, is a dedicated top-of-the-line MALDI instrument with a special feature for PSD, in addition to precision in low ppm range. The TOF/TOF technology allows PSD spectra to be generated in seconds, so it is possible to scan through several peptides from one digest in a short time. The easy and quick PSD combines extremely well with the two year old Chemically Assisted Fragmentation (CAF) derivatization protocol, leading to unsurpassed easily interpreted spectra, comprising a clean series of y-ions. We use CAF-PSD for identification of un-characterized species, as well as for analysis of modified peptides.

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