

ANNUAL SCIENTIFIC REPORT

Uppsala Branch

2001

Staff

Heldin, Carl-Henrik, Director

Growth Regulation Group

Östman, Arne, Associate Member, Group Head

Furuhashi, Masao, Postdoctoral Fellow, from October

Hellberg, Carina, Postdoctoral Fellow, from January

Bäckström, Gudrun, Senior Technical Assistant

Sandström Leppänen, Jill, Technical Assistant

Hägerstrand, Daniel, Ph.D. Student, from July

Leppänen, Olli-Pekka, MD, Ph.D. Student, to February

Persson, Camilla, Ph.D. Student

Pietras, Kristian, Ph.D. Student

Sjöblom, Tobias, Ph.D. Student

Sörby, Maria, Ph.D. Student, to June

Signal Transduction Group

Rönstrand, Lars, Associate Member, Group Head

Bracco, Enrico, Postdoctoral Fellow, to August

Demoulin, Jean-Baptiste, Postdoctoral Fellow

Palumbo, Roberta, Postdoctoral Fellow, from November

Voytyuk, Olexandr, Postdoctoral Fellow

Rorsman, Charlotte, Senior Technical Assistant

Gottfridsson, Eva, Technical Assistant, to November

Maria Hägg, Ph.D. Student, from August

Kallin, Anders, Ph.D. Student

Lennartsson, Johan, Ph.D. Student

Cytoskeletal Regulation Group

Aspenström, Pontus, Assistant Member, Group Head

Hájková, Lucie, Postdoctoral Fellow, (joint with Molecular Signaling Group)

Saras, Jan, Postdoctoral Fellow, from March

Wollberg, Patrik, Postdoctoral Fellow, to January

Ruusala, Aino, Senior Technical Assistant

Edlund, Sofia, Ph.D. Student

Johansson, Ann-Sofi, Ph.D. Student

Richnau, Ninna, Ph.D. Student

Gizatullina, Zemfira, Visiting Investigator, from November

Gene Targeting Group

Heuchel, Rainer, Assistant Member, Group Head

Åhgren, Aive, Senior Technical Assistant

Brodin, Greger, Ph.D. Student

Molecular Signaling Group

Dikic, Ivan, Assistant Member, Group Head

Hájková, Lucie, Postdoctoral Fellow, (joint with Cytoskeletal Regulation Group)

Shimokawa, Noriaki, Postdoctoral Fellow, to August and from November

Soubeyran, Philippe, Postdoctoral Fellow

Ivankovic-Dikic, Inga, Ph.D. Student

Haglund, Kaisa, Ph.D. Student, from March

Kowanetz, Katarzyna, Ph.D. Student, from September

Szymkiewics, Iwona, Ph.D. Student

TGF- β Signaling Group

Moustakas, Aristidis, Assistant Member, Group Head

Gal, Annamaria, Postdoctoral Fellow

Kurisaki, Akira, Postdoctoral Fellow

Morén, Anita, Senior Technical Assistant

Kowanetz, Marcin, Ph.D. Student, from September

Pardali, Katerina, Ph.D. Student

Shiraishi, Keiko, Ph.D. Student

Tentes, Yiannis, Visiting Investigator, to February and August to October

Integrated Signaling Group

Souchelnytskyi, Serhiy, Assistant Member, Group Head

Eichner, Annegret, Postdoctoral Fellow, from April

Kanamoto, Takashi, Postdoctoral Fellow

Preobrazhenska, Olena, Postdoctoral Fellow, to May

Stasyk, Taras, Postdoctoral Fellow, from April

Yakymovych, Ihor, Postdoctoral Fellow

Grimsby, Susanne, Senior Technical Assistant, to November

Apoptotic Signaling Group

Landström, Maréne, Assistant Member, Group Head

Bu, Shizhong, Postdoctoral Fellow, to June

Kozakai, Takaharu, Postdoctoral Fellow, from November

Schuster, Norbert, Postdoctoral Fellow, from November
Tagami, Seiichi, Visiting Investigator, to January

Gene Expression Group

Ericsson, Johan, Assistant Member, Group Head
Giandomenico, Valeria, Postdoctoral Fellow
Grönroos, Eva, Postdoctoral Fellow
Terentiev, Alexei, Postdoctoral Fellow, to July
Wu, Zhengyang, Postdoctoral Fellow, to September
Simonsson, Maria, Ph.D. Student, from May
Terentieva, Svetlana, Ph.D. Student, to March

Matrix Biology Group

Heldin, Paraskevi, Associate Investigator, Group Head, from September

Protein Structure Group

Hellman, Ulf, Associate Member, Group Head
Stasyk, Taras, Postdoctoral Fellow, to February 2001
Engström, Ulla, Senior Technical Assistant
Wernstedt, Christer, Senior Technical Assistant

Technical Support

Ejdesjö, Bengt, Purchasing Officer
Hedberg, Ulf, IT-support
Hermansson, Lars-Erik, Service Engineer
Pettersson, Gullbritt, Laboratory Assistant
Schönquist, Inger, Laboratory Assistant

Administration

Hallin, Eva, Finance and Administration Manager

Secretariat

Schiller, Ingegärd, Secretary

Introduction

The Ludwig Institute for Cancer Research was established in 1972, following a donation by Mr. Daniel K. Ludwig. The research within the Institute is carried out at ten different Branches located in Brussels, Lausanne, London (two), Melbourne, New York, San Diego, São Paulo, Stockholm and Uppsala. In addition, the Institute has administrative offices in New York, London and Zürich.

The Branch in Uppsala was established in 1986. The aim of our research is to elucidate the signaling pathways in cells that control cell growth. We study factors which stimulate or inhibit cell growth and try to elucidate the molecular mechanism whereby such factors exert their effects on cells. The uncontrolled growth of cancer cells is to some extent due to constitutive activation of pathways that in the normal cell are controlled by growth stimulatory factors or the loss of components of growth inhibitory pathways. A goal is to develop clinically useful antagonists of growth factor action.

The Branch consist of eleven groups which cover different aspects of the research on growth factors and signal transduction. 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, are important research topics at the Branch but other growth regulatory factors are also studied.

Since overactivity of PDGF is implicated in certain malignancies as well as in other disorders, clinically useful antagonists are warranted. One of the aims of the Growth Regulation Group is to explore the usefulness of such antagonists in animal models, as well as in clinical studies. This group also studies the importance of tyrosine phosphatases in control of intracellular signaling.

A major aim in the Signal Transduction Group is to elucidate signaling pathways downstream of the tyrosine kinase receptors for PDGF and stem cell factor, which leads to cell growth and motility. Autophosphorylation sites in homo- and heterodimeric receptor complexes are identified, and their importance for the activation of the receptors and as docking sites for SH2-domain-containing signaling molecules, are determined.

The Cytoskeletal Regulation Group focuses on the roles of small GTP binding proteins in signal transduction. Special interest is devoted to the involvement of members of the Rho family in rearrangements of the actin filament system.

The Gene Targeting Group uses gene knock-out and knock-in techniques in the mouse to elucidate the function of signaling components. Special attention is devoted to the knock-in of PDGF receptor mutants with altered functional properties, and the knock-out of the Smad7 gene in the TGF- β pathway.

The Molecular Signaling Group studies oncogenic signaling networks. The role of the related tyrosine kinases Pyk2 and FAK in transformation is being elucidated. Components that bind to the E3 ubiquitin ligase Cbl have been identified, and their roles in the regulation of tyrosine kinase receptors are being investigated.

The TGF- β Signaling Group investigates signaling pathways which regulate cell growth differentiation and tumorigenesis in response to TGF- β . Special attention is given to the mechanism of nuclear import of Smads and the role of Smad molecules in transcriptional regulation. An important method in the studies is cDNA microarray analyses of mRNA profiles in TGF- β stimulated cells.

The Integrated Signaling Group also studies the molecular mechanism of TGF- β signaling. In particular, the phosphorylation events involved in the modulation of different signaling pathways are characterized. Proteomic techniques, including analysis by 2D gel electrophoresis, are important methods in these studies.

The aim of the Apoptotic Signaling Group is to elucidate the molecular mechanisms for TGF- β -induced apoptosis of prostate cancer cells. Crucial roles for Smad7 and the MAP kinase p38 in the apoptotic pathway have been demonstrated.

The Gene Expression Group has recently found that Smad7, as well as the transcription factors YY1 and SREBP undergo acetylation, and is currently investigating the importance of this posttranscriptional modification for the functional properties of these proteins.

The aim of the work in the Matrix Biology Group is to elucidate the importance of matrix molecules, in particular the polysaccharide hyaluronan, in the growth, differentiation and migration of normal and malignant cells.

The Protein Structure Group focuses on identification and structural characterization of proteins using mass spectrometry and Edman degradation. In addition, this group has expertise in the synthesis of different types of modified peptides.

The accomplishments during 2001 are described in more detail below.

C.-H. Heldin

Growth Regulation Group

The focus of the Growth Regulation group is on the role of platelet-derived growth factor (PDGF) in disease, which is analyzed with a range of approaches spanning from molecular cell biology to phase-1 clinical studies. Experimental therapy studies with PDGF antagonists in cancer and restenosis models have a central place. We also engage in basic studies of protein tyrosine phosphatases (PTPs), a class of yet not therapeutically exploited endogenous PDGF receptor antagonists.

Autocrine effects of PDGF in tumor growth

Glioblastomas and fibrosarcomas are examples of malignancies with well-documented co-expression of PDGF and PDGF receptors (53). Dermato-fibrosarcoma protuberans (DFSP) is a special case where constitutive production of PDGF-BB occurs because of a translocation involving the collagen IA1 and PDGF-B genes. Growth of primary DFSP cells, and DFSP tumor xenografts, is reduced by the PDGF receptor inhibitor STI571 through induction of apoptosis (22). The mechanism(s) whereby inhibition of PDGF receptor signaling leads to apoptosis will be investigated by selective blocking of different receptor activated pathways.

Ongoing and planned clinical trials with PDGF receptor antagonists will benefit from possibilities to monitor PDGF receptor activation before and after treatment. To this end, a panel of antibodies specific for activated PDGF receptors has been generated. Protocols for use of these antibodies in immunohistochemistry is under development.

PDGF receptor signaling in tumor stroma

Most solid tumors express PDGF receptors in the fibroblasts of the tumor stroma. Inhibition of PDGF receptor signaling in tumor stroma of experimental tumors reduces tumor interstitial fluid pressure (16). PDGF antagonist-triggered reduction in tumor interstitial fluid pressure leads to an increased tumor uptake of chemotherapeutic drugs and enhances the anti-tumor effect of chemotherapy (Pietras *et al.*, submitted for publication). Together these findings suggest the highly interesting possibility that PDGF receptor inhibition can be used as a general strategy to increase the efficiency of chemotherapy. A phase-1 clinical trial testing this concept is under planning.

Novel and classical PDGF isoforms in development of restenosis

Surgical treatment of atherosclerosis is frequently unsuccessful because of restenosis, which involves PDGF stimulation of smooth muscle cells. PDGF antagonists show beneficial effects in restenosis models. However, effects are transient, possibly because of absence of endothelialization. A study in a rabbit restenosis model was performed to explore if combination of stimulation of endothelialization, by VEGF-C gene transfer, and inhibition of smooth muscle cell activation, by STI571, represented a viable treatment strategy. The combined treatment, in contrast to the single treatments, led to persistent reduction in restenosis (Leppänen *et al.*, submitted for publication). In agreement with the expected effects of VEGF-C gene transfer and STI571, lesions from combination-treated animals displayed increased endothelialization and reduced smooth muscle cell number.

Antibodies against PDGF-CC and -DD will be used to analyze expression of these novel PDGF isoforms in various stages of atherosclerosis and restenosis, and to study the effects of neutralization of PDGF-CC and -DD in animal models of restenosis.

PTPs as endogenous antagonists to PDGF receptor signaling

PDGF receptors, like other receptor tyrosine kinases, are negatively regulated by PTPs (52). A novel role for PTPs in control of PDGF receptors was identified with the finding that PDGF receptor dimerization is associated with reduced susceptibility to receptor dephosphorylation (21). This finding adds to the accumulating evidence indicating that ligand-induced tyrosine kinase receptor phosphorylation is a consequence of both increased kinase activity and reduced receptor dephosphorylation. Activation of receptor-targeting PTPs thus appears as an interesting possibility for interference with pathological receptor activation.

PDGF β -receptor dephosphorylation by the receptor-like PTP DEP-1 involves site-selective dephosphorylation of specific SH2 domain binding phospho-tyrosines rather than general receptor dephosphorylation. DEP-1 selectivity is primarily determined by the primary sequences surrounding PDGF receptor phosphorylation sites (C. Persson *et al.*, submitted for publication). Basic amino acids at position -4 and +3, relative to the phospho-tyrosine, act as major negative determinants. A set of PTP knock-out fibroblasts, together with a panel of site-specific PDGF β -receptor antibodies, are used to further study the occurrence and consequences of site-selective PDGF receptor dephosphorylation.

Regulation of PTPs

Given the importance of PTPs in control of tyrosine kinase receptor signaling the issue of how PTPs are regulated constitutes an important area of research. The variability in structure of the extracellular domain of the receptor-like PTPs has for long time suggested that these act as ligand-binding domains. By a combination of cell-based and *in vitro* studies, Matrigel, a preparation of extracellular matrix proteins, was shown to contain an activating DEP-1 ligand (56). This provides the first evidence for the existence of extracellular agonists of receptor-like PTPs. Additional mechanisms for regulation of PTPs that are investigated include reversible oxidation and dimerization.

Signal Transduction Group

Research at the Signal Transduction group is aiming at elucidating the mechanisms by which the receptors for stem cell factor (SCF; c-Kit) and PDGF elicit their responses. This involves identification of proteins that associate with and/or become phosphorylated by the respective receptors, and identification of phosphorylation sites in the receptors. The ultimate goal is to identify the downstream signal transduction components activated by the receptors and link their activation to the different biological responses elicited upon stimulation of the respective receptors.

Two splice forms of c-Kit show major differences in their ability to induce tumorigenesis and in the way downstream signal transduction components are activated

Several splice forms of c-Kit have been described. Two splice forms exist that differ by the presence or absence of a four amino acid insert in the extracellular part of the juxtamembrane region of the receptor. The presence or absence of the four amino acids GNNK leads to dramatic differences in signaling capabilities of the receptors. Despite the fact that they both bind SCF with similar affinity, the GNNK⁻ form is rapidly and strongly activated, followed by rapid degradation in lysosomes. On the other hand, the GNNK⁺ form is activated with slower kinetics and remains active over a long period of time (Voytyuk *et al.*, submitted for publication). Furthermore, the GNNK⁻ form is more efficient than the GNNK⁺ form in inducing anchorage independent growth of NIH3T3 cells, as well as tumor growth in nude mice. We have shown that several differences in downstream signaling exists. While the PI3-kinase pathway is about equally activated by the two receptor splice forms, activation of the Ras/Erk pathway could be explained

by differential activation of Src family kinases by the two splice forms. We have previously shown (Lennartsson *et al.* (1999) *Oncogene 18*, 5546-5553) that Src-mediated phosphorylation of Shc is essential for c-Kit mediated activation of the Ras/Erk pathway. Using a selective inhibitor of Src family kinases, SU6656, we could demonstrate that inhibition of Src family kinases led to a behaviour of the GNNK⁻ form resembling that of the GNNK⁺ form.

Furthermore, ligand-induced degradation was more rapid for the GNNK⁻ form compared to the GNNK⁺ form, and was in part dependent on the activity of Src family kinases. Others have previously shown that Src-mediated phosphorylation of c-Cbl leads to increased ubiquitination of the PDGF α -receptor.

Future work is aimed at understanding the differences in downstream signaling by the two splice forms of c-Kit, using DNA microarray technique, as well as by employing the technique of two-dimensional protein gel analysis. We have demonstrated, by use of phosphospecific antibodies against individual phosphorylation sites in c-Kit, that the two splice forms induce phosphorylation of individual sites at different rates and magnitude; the GNNK⁻ form shows a strong phosphorylation of Tyr568, the Src binding sites, and Tyr823, in the activation loop, while the GNNK⁺ form shows strong phosphorylation of Tyr936, the Grb2 association sites. On the other hand, Tyr721 being the association site for PI3-kinase, is phosphorylated to the same extent by both isoforms.

Signaling through heterodimeric PDGF receptor complexes

Binding of PDGF to its receptor leads to dimerization and activation of their intrinsic tyrosine kinase activity. This in turn leads to binding of and, in many cases, phosphorylation of downstream signal transduction molecules. In total five isoforms of PDGF exist: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. Depending on the isoform used, PDGF stimulation leads to formation of either homo- or heterodimers of the structurally related α - and β -receptors. Stimulation with PDGF-AB leads to preferential formation of heterodimers of PDGF α - and β -receptors. Formation of heterodimers have been shown to lead to a stronger mitogenic response than homodimeric receptor complexes. We have investigated the mechanisms behind this difference in signaling and found that Tyr771 in the PDGF β -receptor was phosphorylated to a much lower stoichiometry in the heterodimeric β -receptor than in the homodimeric β -receptor. This correlated with reduced binding of RasGAP to the heterodimer and decreased activation of the Ras/Erk pathway. The mechanisms behind

this difference was investigated, and was found to at least in part involve the action of the SH2-domain containing protein tyrosine phosphatase SHP-2. Mutation of the tyrosine phosphorylation sites that mediate interaction with SHP-2 in either the PDGF α - or β -receptor, was shown to lead to increased phosphorylation of Tyr771 (35).

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 phosphotyrosines provide docking sites for SH2-domain-containing proteins initiating signals for *e.g.* mitogenesis and actin cytoskeletal rearrangements. In collaboration with Dr. Phil Soriano's group in Seattle, we generated mice bearing point mutant PDGF β -receptors, that are unable to bind and therefore activate phosphatidylinositol-3'-kinase (PI3'-kinase) 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 (Heuchel *et al.* (1999) Proc. Natl. Acad. Sci. USA 96, 11410-11415). In order to further restrict signaling from the β -receptor, we introduced an additional point mutation, such that neither PI3'-kinase nor phospholipase-C γ (PLC γ) were able to bind to the activated PDGF β -receptor. 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 wildtype cells, it was observed that the double mutant cells were defective in colonizing the vascular smooth muscle cell compartment (Tallquist *et al.* (2000) Genes Dev. 14, 3179-3190).

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.

Regulation and *in vivo* function of Smad7

Transforming growth factor- β (TGF- β) family members, which include TGF- β s, 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 lead 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 (Brodin *et al.* (2000) J. Biol. Chem. 275, 29023-29030).

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.

Cytoskeletal Regulation Group

The work in the Cytoskeletal Regulation Group is focused to studies on the molecular mechanisms that control cell migration and cell growth under normal physiological conditions, as well as during disease. Signalling pathways involving the Rho family of small GTPases have been found to be of particular importance for the organisation of the actin cytoskeleton and thereby the control of cell morphogenesis and cell migration. Our studies aim to identify signalling pathways that lead to the activation of the Rho GTPases. In addition, we aim to identify effectors downstream of the Rho GTPases,

which influence biological processes such as cell migration, cell proliferation and cell survival.

TGF- β -mediated activation of Rho GTPases

We found that treatment of PC-3U human prostate carcinoma cells with TGF- β 1 induced a robust rearrangement of the actin filament system visible as a rapid formation of lamellipodia (33). Interestingly, this short-term response was independent of the Smad signalling pathway; instead it required the activity of the Rho GTPases Cdc42 and RhoA. In contrast, long-term stimulation with TGF- β 1 resulted in an assembly of stress fibres; this response required both signalling via Cdc42 and RhoA, and Smad proteins. A known downstream effector of Cdc42 is the MAP kinase p38; treatment of the cells with the p38 inhibitor SB203580, as well as ectopic expression of a kinase inactive p38, abrogated the TGF- β -induced actin reorganisation. Moreover, treatment of cells with inhibitors of the RhoA target-protein Rho coiled-coil kinase (ROCK) as well as ectopic expression of kinase-inactive ROCK-1 abrogated the TGF- β 1-induced formation of stress fibres. Collectively, these data indicated that TGF- β -induced membrane ruffles occur via Rho GTPase-dependent pathways, whereas long-term effects require a co-operation between Smad proteins and Rho GTPases (33). We are currently investigating the possible involvement of signalling by the Rho GTPases on the Smad signalling pathway.

RICH, a Rho GTPases activating protein

A previously unidentified RhoGAP domain-containing protein was found in a yeast two-hybrid screen for cDNAs encoding proteins binding to the SH3 domain of Cdc42 Interacting Protein 4 (CIP4). The protein was named RICH-1 (RhoGAP Interacting with CIP4 Homologues) and in addition to the RhoGAP domain it contained an N-terminal domain with endophilin homology and a C-terminal proline-rich domain. Co-immunoprecipitation experiments, as well as colocalization assays of transiently transfected RICH-1, indicated that it bound to CIP4 *in vivo* (19). Moreover, *in vitro* assays demonstrated that the RhoGAP domain of RICH-1 catalysed GTP hydrolysis on Cdc42 and Rac1, but not on RhoA. Ectopic expression of the RhoGAP domain as well as the full-length protein interfered with PDGF-BB-induced membrane ruffling, but not with serum-induced stress-fibre formation, further emphasising the notion that, *in vivo*, RICH-1 is a GAP for Cdc42 and Rac1 (19). We are currently investigating the interesting possibility of a role for RICH-1 in receptor endocytosis.

Signalling 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 recognised as important regulators of actin polymerisation in vertebrate cells. This notion is illustrated by studies on B-cells from mice lacking WASP which suggested an important role for WASP in these cells (29).

The activity of the WASP proteins is governed by a number of associated proteins and we have identified a novel WASP interacting protein, WIRE (for WIP-related). WIRE was shown to bind WASP and N-WASP but not members of the Scar/WAVE family of proteins in co-immunoprecipitation experiments (Aspenström, submitted for publication). The C-terminus of WIRE was responsible for binding to the WH1 domain of WASP. WIRE was localised to actin filaments in transiently transfected porcine aortic endothelial cells and in cells simultaneously expressing WIRE and WASP, WIRE relocalized WASP to actin filaments, a relocalization that required the direct interaction between the two proteins. In addition, WIRE was able to bind the PDGF β -receptor substrate Nck β . In cells co-expressing WIRE and Nck β , both proteins were localised to sites of focal adhesions. Moreover, PDGF treatment of cells ectopically expressing WIRE resulted in formation of peripheral protrusions composed of filopodia and lamellipodia-like structures (4). Taken together, the data presented in this study indicated a role for WIRE in the regulation of the actin cytoskeleton and suggested that WIRE is a potential link between the activated PDGF receptor and the actin polymerisation machinery.

Signalling mediating cell motility

In addition to the above described projects, the current studies involve a number of Rho GTPases as well as Rho effectors, such as the Rac and Cdc42 binding protein PAR-6 and the Rho-binding protein Dia. The aim is to understand how these components convey signals to control cell migration under normal conditions as well as during disease.

Molecular Signaling Group

The aim of the work in the Molecular Signaling group is to characterize oncogenic signaling networks. Given the multigenic origin of cancer, we are focusing on various proteins that could participate in these networks including tyrosine kinase signaling module and protein degradation module. We use cDNA microarray screening and gene targeting to understand how components of these modules regulate cell transformation and metastasis.

Role of Pyk2/FAK tyrosine kinases in cell signaling and transformation

Much of our current work is focused on the characterization of the Pyk2/FAK family of non-receptor tyrosine kinases in normal and transformed cells. Pyk2 and FAK contribute to cell transformation by co-ordinating signaling networks induced by adhesion, mitogenic signals and oncogenes. They are highly overexpressed in several tumor cell lines and primary human tumors, and were shown to interact and co-operate with known oncogenes. We have recently shown that Pyk2 acts as a proximal effector of integrins and growth factor receptors leading to the regulation of neurite outgrowth (Ivankovic-Dikic *et al.* (2000) *Nature Cell Biol.* 2, 574-581). Pyk2 was also implicated in the control of glucose transporter translocation in response to hyperglycemia and sorbitol treatment (1, 41). Moreover, we have studied Pyk2-independent signaling pathways linking bradykinin B2 G protein-coupled receptors with activation of MAP kinase cascades as well as mechanisms underlying B2 receptor downregulation (3, 4, 17). In addition, we have identified a novel pathway that links Pyk2 with reorganization of the actin cytoskeleton (56 and Haglund *et al.*, manuscript in preparation). By using gene microarrays we are currently analyzing targets of Pyk2/FAK in transformed cell lines and primary tumors. This approach has a potential to identify specific genes controlled by Pyk2 and FAK during cell transformation.

Cdx1-regulated genes during adenocarcinoma development

The *Caudal*-like homeobox gene Cdx1 is an intestine-specific transcription factor involved in the control of proliferation and differentiation of epithelial cells. Deregulation of homeobox gene expression was also implicated in uncontrolled cell growth and oncogenesis. We have recently shown that expression of Cdx1 in intestinal epithelial cells (IEC-6) promotes anchorage-independent growth *in vitro* and adenocarcinoma development in nude mice (23). We are currently analyzing changes in gene expression during transformation of IEC-6/Cdx1 cells using cDNA microarrays.

This approach has a potential to delineate Cdx1-induced factors that render normal epithelial cells into tumor cells. The information thus gained will be compared with information on human susceptibility genes.

Characterization of CLIPs – Cbl-interacting proteins

Recent evidence indicates that c-Cbl acts as a E3-RING dependent ubiquitin ligase that negatively regulates receptor tyrosine kinases by mediating their ubiquitination and degradation. Moreover, oncogenic forms of Cbl were shown to enhance ligand-independent signaling by growth factor receptors. By using the yeast two-hybrid system we cloned several Cbl interacting proteins (CLIPs). We are currently focused on molecular characterization of CLIPs in normal and transformed cells.

We have recently demonstrated that Cbl additionally regulates epidermal growth factor (EGF) receptor endocytosis. Cbl was shown to rapidly recruit CLIP5/CIN85, Cbl interacting protein of 85 kDa, and endophilins, regulatory components of clathrin-coated vesicles, in the complex with activated receptors thus controlling receptor internalization (43 and Fig. 1).

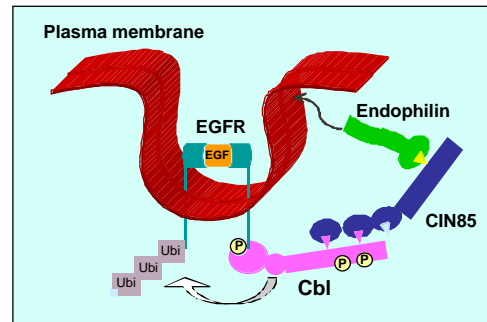


Fig. 1. Clip5/CIN85 interactions with Cbl and endophilins are important for EGF receptor internalization.

Inhibition of these interactions was sufficient to block receptor internalization, delay receptor degradation and enhance EGF-induced gene transcription, without perturbing Cbl-directed receptor ubiquitination. Thus, we have concluded that the evolutionary divergent carboxyl terminus of Cbl utilizes a novel mechanism, functionally separable from the ubiquitin ligase activity of Cbl, to mediate ligand-dependent downregulation of receptor tyrosine kinases. We are further interested to study the role of CIN85 in the regulation of receptor trafficking along the endocytic pathway. The ability of CIN85 to assemble larger Cbl-associated signaling networks places it in a position to co-ordinate multiple steps in endocytosis of tyrosine kinase receptors. Defining how this process is regulated in living cells is the challenge for future investigations.

TGF- β Signaling Group

We investigate signaling pathways that regulate cell growth, differentiation and tumorigenesis in response to TGF- β .

TGF- β signaling and Smad regulation

TGF- β signals via plasma membrane serine/threonine kinase receptors and cytoplasmic effectors, the Smad proteins. The receptors activate the Smads, which move rapidly into the nucleus to regulate gene expression by associating with DNA and co-operating transcription factors (51, 61). Eventually, Smads exit the nucleus to be degraded in the cytoplasm.

After having shown that the nuclear import of Smad3, a TGF- β pathway-specific Smad, is dependent on interactions with importins and the Ran GTPase (Figure 1; 12), we have focused on the nuclear export pathway of Smad3, which occurs late after the TGF- β signal has been received by the cell. We are in the process of characterizing a novel mechanism of Smad3 export. Thus, highly dynamic processes regulate the nucleo-cytoplasmic distribution of Smad3.

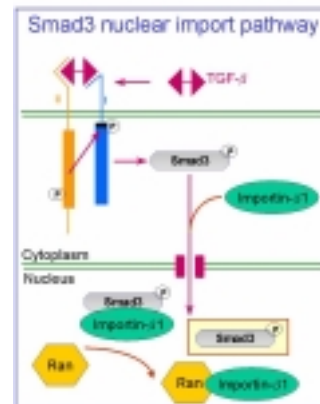


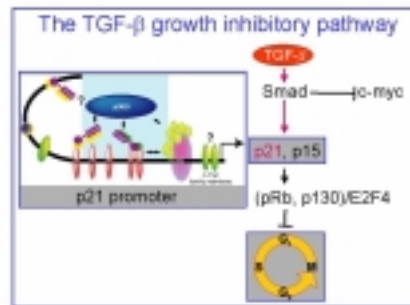
Figure 2

In parallel, we focus on the fate of Smad4, a Smad which is common to all TGF- β superfamily pathways. In human cancers, specific amino acid substitutions in Smad4 lead to its enhanced proteolysis. We analyze the mechanism and cellular signaling pathways that regulate Smad4 ubiquitination and proteasomal degradation.

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

The activated, nuclear Smads specifically interact with several transcription factors (51, 61). TGF- β inhibits epithelial cell proliferation by repressing *c-myc* expression and inducing gene expression of cell cycle inhibitors, p15 and p21, which lead to cell cycle arrest (Figure 3). Simultaneously, the Smad pathway modulates cell differentiation.

We have described the association of Smads with two general transcription factors, Sp1 and YY1, a finding relevant to many TGF- β - and BMP-responsive genes (51, 61). After having defined a mechanism by which TGF- β , via Smads, induces the *p21* gene (Figure 3), we dissect this gene response for other signaling pathways of the superfamily, and the importance of



F

figure 3

this response to the regulation of growth of different cell types. We have shown that YY1 does not interfere with growth regulation but it can specifically repress important target genes and the process of cell transdifferentiation in response to either TGF- β or BMP (Shiraishi *et al.*, submitted for publication).

Dual Role of TGF- β in tumor suppression and tumorigenesis

The TGF- β pathway acts as a tumor suppressor in early stages of epithelial cell carcinogenesis, yet it serves pro-tumorigenic actions in late, pre-metastatic stages of tumor development (Figure 4). It is important to define the signaling modules that lead to either tumor suppression or tumor promotion of specific epithelial cell types. We focus on mammary epithelial models. Murine

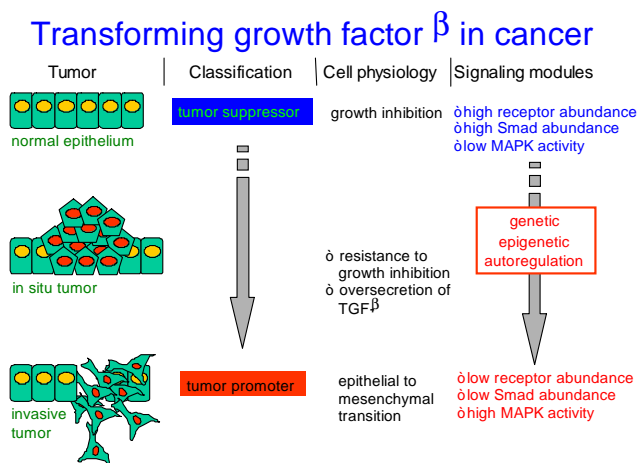


Figure 4

NMuMG cells lead to benign tumors *in vivo* but undergo epithelial to mesenchymal transition (EMT) *in vitro* in response to TGF- β , a morphogenetic and differentiative alteration important *in vivo* during tumor cell migration and metastasis (61). We have developed a two-cell model of CHO cells secreting latent TGF- β (the physiological form of this cytokine in the extracellular space) and responding NMuMG cells that leads to a significant increase in tumorigenesis in *scid* mice.

Human MDA-MB-468 cells are highly tumorigenic *in vivo* and lack critical tumor suppressors such as Smad4 and p53. Using cDNA microarray analysis we have identified specific gene targets whose regulation depends on the presence of Smad4. We examine their relevance to the tumor suppressor pathway. We have also screened for TGF- β gene targets in the NMuMG cell model. We combine microarray screening with knockdown technologies based on RNAi to functionally establish the critical nature of specific gene targets in tumorigenesis.

Integrated Signaling Group

The Integrated Signalling Group is concentrated on exploration of TGF- β family signalling in normal and malignant cells, and its role in integrated intracellular regulatory network. We direct our efforts on unraveling of mechanisms of receptor activation, as well as elucidation of Smad-dependent and Smad-independent signalling downstream of the receptors. These studies aim to elucidate the role of TGF- β signalling in carcinogenesis. Proteomics technique is one of the tools we use to understand the complexity of integration of the TGF- β intracellular signalling.

Proteome analysis of TGF- β stimulated cells

We have completed an analysis of the proteome of Mv1Lu lung carcinoma cells upon TGF- β 1 treatment (37). We have identified 28 new targets of TGF- β 1, suggesting new functions of TGF β . One of these proteins, Rad51, was shown to be the direct target of TGF- β in a regulation of genome stability. Our study provides the first description of the mechanism of regulation of genome stability by TGF- β . Our functional proteomics studies are aiming at creation of a database of proteins targeted by TGF- β in normal and human breast cancer cells.

We have participated in the development of new techniques for improving sample preparation for 2-dimensional electrophoresis, in collaboration with Amersham Biosciences (24).

We use our proteomics technology in searches for BMP receptor-interacting proteins. This work is performed in a collaboration with the group of P. Knaus. More than 80 proteins, specifically interacting with BMPR-II or BMPR-IB, have been selected. Identification of these proteins is performed in a close collaboration with Ulf Hellman in the Protein Structure Group at our Institute.

In collaboration with the group of J.-J. Feige and S. Bailly, we have performed an analysis of Smad activation by the ALK-1 receptor, which mediates TGF- β action on endothelial cells, affecting angiogenesis. We have found ALK-1 specific activation of Smad5 and Smad1; in contrast T β R-I (ALK-5) activates Smad 2 and Smad3. We have generated also a proteome arrays of endothelial cells for further analysis. By analyzing the phosphoproteome of endothelial cells, we have identified phosphoproteins affected by the ALK-1. These proteins currently are under identification.

Modulation of Smad function by phosphorylation

We have found that all Smad proteins are phosphoproteins. We showed that inhibitory Smad7 is phosphorylated by other kinase(s) than TGF- β receptors, and that this phosphorylation is important for a new function of Smad7 as a transcriptional regulator (18).

We have also studied the regulation of Smad functions by protein kinase C (30). We found the PKC directly phosphorylates receptor-regulated Smad proteins, and affects their ability to activate gene transcription. Interference with the PKC-Smad cross-talk results in an increased sensitivity of cells to tumor promoter PMA and to abrogation of pro-apoptotic action of TGF- β 1. Those findings provide an additional insight into relation of tumor growth promoting (PKC) and cell proliferation inhibiting (TGF- β /Smad) signalling pathways.

Search for specific inhibitors of TGF- β receptors

In our studies of receptor activation, we developed a system, which allows search for specific regulators of TGF- β receptor kinase activity. We have analyzed inhibitors of TGF- β type I receptor kinase, which competes at the ATP-binding pocket or at the substrate-recognition site of the kinase. This study provides a basis for the development of drugs affecting TGF- β functions (Yakymovych *et al.*, submitted for publication).

Effect of OP1 on osteoblast differentiation

We described also a functional interaction of osteogenic protein-1 (OP1 or BMP7) with vitamin D₃-regulated differentiation of human osteoblasts (34). We showed that OP1, unlike TGF- β 1, inhibits vitamin D₃-induced differentiation. These findings can have an impact on design of treatment of osteoporosis, as vitamin D₃ is often used in the clinic, and OP1 is also scheduled for clinical trials.

Smads in tumorigenesis

We have identified a cross-talk between TGF- β signalling and products of the human breast cancer susceptibility genes *BRCA1* and *BRCA2*. We showed that *BRCA2* interacts with Smad2 and Smad3, and potentiates transcriptional activity of Smad3 (Preobrazhenska *et al.*, submitted for publication).

Identified cross-talks of the TGF- β signalling between tumor promoters (30) and tumor suppressors (Preobrazhenska *et al.*, submitted for publication), together with the identification of receptor- and Smad-interacting proteins and the discovery of new function of TGF- β as the regulator of genome stability (37), provide new insights in the role of TGF- β in tumorigenesis.

Apoptotic Signaling Group

The aim of our work is to elucidate mechanisms for induction of apoptosis in epithelial tumor cells.

TGF- β signaling in the prostate

Prostate cancer is a common tumor form in elderly men, which continue to increase in frequency in the Western world. TGF- β is known to play an important regulatory role for proliferation, differentiation, migration and apoptosis of cells during embryogenesis as well as for maintenance of homeostasis in the adult organism. In the normal prostate, TGF- β has been shown to be essential for castration-induced apoptosis of epithelial cells. After removal of testosterone, 80-90% of the epithelial cells undergoes apoptosis within 1-3 days. Earlier reports have demonstrated a huge increase of the mRNA and protein levels of TGF- β early after castration, which has been functionally linked to apoptosis. During tumorigenesis, prostate cancer cells escape the growth inhibitory and apoptotic effects of TGF- β , and mutations in the type I and type II receptors have been found to correlate to a bad prognosis for patients with prostate cancer. We have reported that apoptotic cells showed increased expression levels of the receptors as well as the Smad proteins in normal and malignant prostate epithelial cells after castration.

Interestingly, we observed an increased expression of the inhibitory Smad7 in epithelial cells undergoing apoptosis, suggesting that Smad7, in addition to exerting a negative feedback on Smad signaling, might play an active role in regulation of apoptosis. By using antisense techniques, we found that Smad7 mediates TGF- β -induced apoptosis

not only in prostate cancer cells (PC-3U and DU145), but also in human keratinocytes (HaCaT) cells (Landström *et al.* (2000) *Curr. Biol.* 10, 535-538). During the past year, we have further investigated the molecular mechanisms by which Smad7 positively can regulate TGF- β -induced apoptosis in epithelial cells. We have now shown that Smad7 is required for TGF- β 1-induced activation of the TGF- β activating kinase 1 (TAK1) and p38 mitogen activated protein (MAP) kinase pathway, resulting in apoptosis (Bu *et al.*, submitted for publication). Our aim is now to elucidate the mechanisms for how Smad7 can activate the TAK1 - p38 MAP kinase pathway, as well as to identify the genes regulated by Smad7 and p38.

We have also identified several proteins which interacts with Smad7, in the nucleus as well as in the cytoplasm, and we will continue to investigate the functional importance of these interactions.

Induction of apoptosis in epithelial-derived common tumors by 2-Methoxyestradiol

In order to improve the therapeutic strategies for advanced prostate cancer, we have investigated the effects of 2-methoxyestradiol (2-ME), an endogenous estrogen metabolite, which previously has been found to exert potent growth inhibitory effects on endothelial cells. We have found that 2-ME potently induces apoptosis in several prostate cancer cell lines *in vitro* and *in vivo*. Similar effects were observed in breast, colon and liver cancer cell lines. 2-ME does not bind to the known nuclear estrogen receptors, instead the apoptotic mechanism involves an activation of the MAP kinase, c-Jun N terminal kinase (JNK). Our data suggests that the apoptotic effect of 2-ME is dependent on phosphorylation of Bcl-2, since a prostate cancer line lacking Bcl-2 do not undergo apoptosis upon treatment with 2-ME and since phosphorylation of Bcl-2 precedes the induction of cell death (Bu *et al.*, submitted for publication). We are currently investigating the effects of 2-ME on other epithelial derived tumors and cell lines *in vitro* and *in vivo*, in collaboration with groups at the University Hospital in Uppsala.

Gene Expression Group

Most, if not all, signal transduction pathways ultimately affect gene transcription and alter the expression of specific genes. A novel signal transduction pathway, protein acetylation, has been found to regulate the activity of many transcription factors. Our group recently identified a number of acetylated nuclear proteins that are direct targets

of this pathway. These proteins are involved in a very broad range of cellular events and our goal is to determine how protein acetylation affects these processes.

We have also identified a novel family of acetyltransferases that shuttle between the cytoplasm and the nucleus. Our goal is to further characterize these proteins and to identify their intracellular targets. We also plan to develop a proteomics strategy to identify novel targets and components of the protein acetylation pathway.

YY1 – 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 demonstrated that the nuclear transcription factor Yin Yang 1 (YY1) binds to p53 and inhibits its transcriptional activity (Terentiev *et al.*, manuscript in preparation). We have mapped the interaction domains in both proteins and identified the repressor domain in YY1. In addition, we have demonstrated that YY1 regulates the acetylation of p53. We are now trying to define the mechanisms of YY1-mediated repression of p53 and determine if YY1 affects any of the cellular responses to activated p53, *i.e.* cell cycle arrest and apoptosis.

SMADs - Mediators of transforming growth factor- β signaling

TGF- β 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 TGF- β signaling has been implicated in multiple human diseases, such as fibrosis, rheumatoid arthritis and carcinogenesis. Smad proteins regulate gene expression in response to TGF- β 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.*, submitted for publication). Acetylation or mutation of these lysine residues stabilizes Smad7 and protects it from TGF β -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 from 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 (Giandomenico *et al.*, manuscript in preparation). 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.

Our results suggest that acetylation regulates the stability and, thereby, the transcriptional activity of SREBPs. 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 either enhance the acetylation of SREBPs or inhibit their deacetylation should increase the levels of transcriptionally active SREBP in cells. Such compounds could potentially be used to enhance the cholesterol-lowering activities of statins.

Identification and characterization of a novel family of acetyltransferases

We have used a targeted screen to identify acetylated transcription factors. In this screen, we also identified a group of novel acetyltransferase proteins (NAPs). Some of these NAPs belong to the same family of transcription factors and contain potential

acetyltransferase domains. We have now established that these proteins have acetyltransferase activity *in vitro* and that they are autoacetylated *in vivo*. We have mapped the acetylated lysine residues to the nuclear localization signal (NLS) of these proteins, indicating that acetylation affects their subcellular localization. This is interesting since these proteins shuttle between the cytoplasm and the nucleus. We have now generated a large number of mutations in these proteins, including mutations in the putative enzymatic and acceptor sites.

Matrix Biology Group

Work in the Matrix Biology Group explores the biological functions of hyaluronan, *e.g.* its importance for growth, differentiation and migration of normal and malignant cells.

Regulation of hyaluronan biosynthesis and degradation

One of our major aims has been to investigate the molecular mechanisms that regulate hyaluronan biosynthesis. Increased knowledge about the regulation of hyaluronan synthesizing (HAS1, HAS2, HAS3) and hyaluronan degrading (PH-20, Hyal1, Hyal2, Hyal3) enzymes, will make it possible to regulate the content of hyaluronan in tissues, and may therefore be of therapeutic value. For example, since hyaluronan accumulation during organ transplantation often leads to interstitial oedema and graft rejection we have investigated possible beneficial effects of testicular hyaluronidase treatment on rat liver graft viability. We found that addition of hyaluronidase to the preservation solution limit the cell damage and considerably improve both the function and the viability of grafted livers (Papanikolaou *et al.*, submitted for publication).

We have initiated studies of the regulation and the enzymatic properties of each HAS isoform. Our data suggest distinct functional roles of the three HAS isoforms in response to cell stimuli under normal and pathological conditions. Studies on the growth factor dependent regulation of hyaluronan synthesis in mesothelial cells, revealed that among the three HAS isoforms tested, HAS2 was most markedly upregulated in response to PDGF-BB and TGF- β 1; the HAS2 isoform was also most markedly down-regulated in response to pharmacological doses of hydrocortisone (Jacobson *et al.* (2000) *Biochem. J.* 348, 29-35). Furthermore, we investigated the molecular mechanisms involved in the perturbation of hyaluronan synthesis and catabolism during the early phase of lung injury, and how hyaluronan accumulation affects the pathogenesis of lung fibrosis. We found that hyaluronan accumulation during the early

phase of lung injury is due both to an increased hyaluronan synthesis, mediated through induction of the HAS2 gene, and to an impairment of the function of hyaluronan receptors (CD44) expressed on alveolar macrophages. In addition, HYAL2 contributes to the formation of hyaluronan fragments which induces the expression of types I and III collagen (Li *et al.* (2000) *Am. J. Respir. Cell Mol. Biol.* 23, 411-418).

Importance of hyaluronan production for the malignant properties of tumor cells

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 tumorigenesis. In an effort to explore the relation between hyaluronan production by mesothelioma cells and their clinical 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 (13). These results were further confirmed by additional studies on colon carcinoma cells. We found that HAS2 overproduction promoted tumorigenicity, whereas HYAL1 overexpression suppressed tumor development. Furthermore, the tumors formed in rats from HYAL1-transfectants had a significantly less pronounced viable zone compared to tumors formed of mock- and HAS2-transfectants (Jacobson *et al.*, submitted for publication). Our studies suggest that hyaluronan has an important role in tumor progression

We also have initiated studies to increase our knowledge about the effects of hyaluronan oligosaccharides and hyaluronidases on the differentiation and survival of endothelial cells. We have demonstrated that capillary endothelial cells form tube-like structures in a collagen gel after stimulation with hyaluronan oligosaccharides and certain populations of testicular hyaluronidase. The effect was dependent on the hyaluronan receptor CD44 (39). Our data suggest an important role of CD44 in the process of angiogenesis.

Protein Structure Group

The Protein Structure Group continues its close interaction with other groups at the Branch, as well as with other research groups in Sweden and abroad. Along with the release of the genomes from man and other species, the activity regarding protein

structure analysis has changed from de novo sequencing towards identification of known proteins and the analysis of modifications. Here we make very good use of our two mass spectrometers and the 2-D gel electrophoresis equipment. Our peptide synthesis work develops well, and we are able to make more and more sophisticatedly modified peptides.

Peptide synthesis

In the beginning of year 2000 we purchased a new peptide synthesizer, an Applied Biosystems 433A, which replaced the 430A that has been in use since 1986. The new instrument is controlled by an efficient computer, which allows a good flexibility in the design of, in particular, modified peptides. After synthesis, the products are analyzed by reversed phase liquid chromatography, and when necessary, purified by preparative chromatography. Every peptide is quality controlled by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) and sometimes by electrospray ionization mass spectrometry (ESI-MS) to generate complementary structural information.

Many of the peptide products are used as ligands in affinity chromatography experiments where the aim is to isolate interaction proteins, as inhibitors or substrates, or as antigens for production antibodies.

Of special interest are modified peptides; in particular we synthesize phosphorylated peptides, which are of great importance in signal transduction research. Now there is a growing interest in acetyl-lysine as a signalling compound (see report from the Gene Expression Group), and we have therefore started to gain experience in this modification.

Amino acid sequencing

Classical Edman degradation. Since the introduction of mass spectrometry at our Branch five years ago, automated peptide sequencing has gradually decreased and is now very seldomly performed. The major reason is the higher sensitivity of mass spectrometry, but there are also other advantages with MALDI-ToF-MS, as summarized below.

	Edman degradation	MALDI-TOF-MS
Sensitivity	Low pmol	Medium fmol

Running cost per peptide	Ca 150 USD	A few cents
Analysis time	Overnight	A few minutes
# cell culture flasks needed	100-150	1
Analysis of modifications	Only using standards	Possible

Radiolabeled amino acid sequencing. Identification of phosphorylation sites in growth factor receptors and related molecules continues to be an important activity in the Group. Since this is based on collection of cleavage product from Edman degradation, followed by high sensitivity analysis of ^{32}P on a phosphor imager, we are using two older sequencers equipped with fraction collectors for this purpose.

Sample preparation for analysis by mass spectrometry

The high sensitivity in analysis by mass spectrometry makes sample preparation of the minute amount of sample needed a rather sophisticated art. Since we are aiming at looking at samples in the low femtomole range (nanogram quantities of an average sized protein) it is difficult to avoid contaminating proteins. Most commonly samples are purified by polyacrylamide gel electrophoresis, either in one or two-dimensional gels. As the starting sample mixture normally is extremely complex (may comprise many tens of thousands of different proteins), every sample must pass several enrichment steps prior to the final electrophoresis. Once presented in a gel, visualized by *e.g.* silver staining, the gel piece containing the protein of interest is excised and subjected to in-gel digestion with a specific protease. Most samples have to be desalted and concentrated on a "nanocolumn". This is a reversed phase column with approximately 0.1 μl bed volume, packed in a gel-loader tip.

Peptide Mass Fingerprinting (PMF) Using Matrix Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry (MALDI-TOF-MS)

For the identification of proteins, peptide mass fingerprinting by MALDI-TOF-MS lends itself extremely well (see the table above). An unequivocal identification can often be made from proteins present in weak silver stained spots. The mass list from the spectrum is used to scan public sequence databases via search engines *e.g.* ProFound or Mascot. For heterogeneous samples, the interpretation is of course not as straightforward, but it is often possible to identify two proteins in one spot.

In February 2001, we were able to replace the Bruker Biflex III MALDI instrument with an Autoflex from the same manufacturer. This has significantly improved both quality

and speed of analyses, and more importantly, has made sequencing by Post Source Dissociation-MALDI much easier.

Post Source Dissociation (PSD) sequencing by MALDI-Tof-MS

It has for long been a desire to be able to generate even a small piece of peptide sequence from the same sample used for PMF. The new MALDI instrument has a higher degree of automation which makes PSD analyses easier. Another more important advancement was the introduction in December 2001, through a collaboration with Amersham Biosciences, of a chemistry called "Chemically Aided Fragmentation" which dramatically improves the possibilities of reading sequences by PSD.

Electrospray ionization mass spectrometry

A Bruker Esquire 3000 ESI-MS ion trap instrument was purchased in January 2001. It will complement the MALDI-MS in analysis of in particular post translational modifications, and hopefully also allow de novo sequencing. We are currently investigating whether the CAF-chemistry will be applicable at electrospray MS – preliminary results indicate that this is the case under certain conditions.

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