

ANNUAL SCIENTIFIC REPORT

Uppsala Branch

2002

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Growth Regulation Group

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Signal Transduction Group

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Chiara, Federica, Postdoctoral Fellow, from February

Demoulin, Jean-Baptiste, Postdoctoral Fellow

Palumbo, Roberta, Postdoctoral Fellow, to February

Voytyuk, Olexandr, Postdoctoral Fellow, to August

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Kallin, Anders, Ph.D. Student

Lennartsson, Johan, Ph.D. Student, to August

Cytoskeletal Regulation Group

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Gene Targeting Group

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Brodin, Greger, Ph.D. Student, to June

Molecular Signaling Group

Dikic, Ivan, Assistant Member, Group Head

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

Shimokawa, Noriaki, Postdoctoral Fellow, to August

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Ivankovic-Dikic, Inga, Ph.D. Student

Haglund, Kaisa, Ph.D. Student

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Kowanetz, Katarzyna, Ph.D. Student

Szymkiewicz, Iwona, Ph.D. Student

TGF- β Signaling Group

Moustakas, Aristidis, Assistant Member, Group Head

Gaal, Annamaria, Postdoctoral Fellow, to February

Kurisaki, Akira, Postdoctoral Fellow, to May

Valcourt, Ulrich, Postdoctoral Fellow, from February

Morén, Anita, Senior Technical Assistant

Kowanetz, Marcin, Ph.D. Student

Kurisaki (nee Shiraishi), Keiko, Ph.D. Student, to May

Pardali, Katerina, Ph.D. Student

Integrated Signaling Group

Souchelnytskyi, Serhiy, Assistant Member, Group Head

Eichner, Annegret, Postdoctoral Fellow, to April

Filyak, Yevhen, Postdoctoral Fellow, from August to November

Iwahana, Hiroyuki, Postdoctoral Fellow, from April

Kanamoto, Takashi, Postdoctoral Fellow, to March

Lomnytska, Marta, Postdoctoral Fellow, from January to December

Stasyk, Taras, Postdoctoral Fellow

Yakymovych, Ihor, Postdoctoral Fellow

Apoptotic Signaling Group

Landström, Maréne, Assistant Member, Group Head

Kozakai, Takaharu, Postdoctoral Fellow

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Bender, Herdis, Ph.D. Student, from August to December

Gross, Juliane, Ph.D. Student, to January

Gene Expression Group

Ericsson, Johan, Assistant Member, Group Head

Bengoechea Alonso, Maria Teresa, Postdoctoral Fellow, from May

Giandomenico, Valeria, Postdoctoral Fellow, to October

Grönroos, Eva, Postdoctoral Fellow

Sundqvist, Anders, Postdoctoral Fellow, from February

Simonsson, Maria, Ph.D. Student

Matrix Biology Group

Heldin, Paraskevi, Associate Investigator, Group Head (joint appointment with
Department of Immunology, Microbiology and Medical Biochemistry, Uppsala
University)

Asteriou, Trias, Postdoctoral Fellow, from February

Takahashi, Yoshinori, Postdoctoral Fellow, from April

Li, Lingli, Ph.D. Student, from February

Protein Structure Group

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Engström, Ulla, Senior Technical Assistant

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Secretariat

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Introduction

Malignant cells are characterized by perturbations in signaling pathways which regulate cell growth, survival and migration. The aim of the work at the Uppsala Branch is to elucidate signaling mechanisms induced by factors that stimulate or inhibit cell growth.

The Branch consists of eleven groups which cover different aspects of the research on growth regulatory factors and signal transduction. Special efforts are put on studies of platelet-derived growth factor (PDGF), an important mitogen for connective tissue cells, and transforming growth factor- β (TGF- β), which inhibits the growth of most cell types, but other growth regulatory factors are also studied.

During 2002, the Group Leader for the Signal Transduction Group, Lars Rönnstrand, left to take up a position as Professor in Molecular Medicine in Malmö. We wish Lars and his associates the best success in their future work, and are looking forward to future interactions with them. The work on stem cell factor receptor in the Signal Transduction Group will now be continued by Lars in Malmö; however, some aspects of the work on PDGF signaling will be continued at our Branch.

The Growth Regulation Group continues their work on PDGF antagonists, using animal models for autocrine and paracrine effects of PDGF in tumorigenesis. The aim is to explore the usefulness of PDGF antagonists for the treatment of cancer patients. This group also studies the importance of tyrosine phosphatases in control of intracellular signaling.

The Cytoskeletal Regulation Group focuses on the molecular mechanisms that control cell migration and growth. In particular, the role of members of the Rho family of small GTPases in the control of the organization of the actin cytoskeleton is studied.

The Gene Targeting Group uses gene targeting in the mouse to explore the *in vivo* importance of specific signaling pathways initiated by growth factors. Special attention is given to knock-in of PDGF receptor mutants with altered functional properties, and to the knock-out of the Smad7 gene in the TGF- β signaling pathway.

The Molecular Signaling Group has during the last year focused on the elucidation of mechanisms regulating endocytosis and degradation of receptor tyrosine kinases through actions of scaffold proteins and ubiquitin ligases. An important role of Cbl-mediated

monoubiquitination on multiple sites of EGF and PDGF receptors for their endosomal sorting and lysosomal degradation has been demonstrated.

The TGF- β Signaling Group investigates signaling pathways that regulate cell growth, differentiation and tumorigenesis in response to TGF- β . In particular, the role of Smad molecules in transcriptional regulation is studied. An important method in these studies is the use of cDNA microarrays.

Also the Integrated Signaling Group explores TGF- β signaling in normal and malignant cells. Special attention is given to the regulation of signaling by phosphorylation events. Two-dimensional gel electrophoresis and other proteomic techniques are often used in these studies.

The Apoptotic Signaling Group aims at identifying the precise mechanisms whereby TGF- β causes apoptosis in prostate cancer cells. Important roles for Smad7 and the TAK1-MKK3/6-p38 MAP kinase cascade have been elucidated.

The Gene Expression Group studies mechanisms of transcriptional regulation by the transcription factors YY1, SREBP and Smads. Crucial regulatory roles of acetylation of these components are being unraveled.

The Matrix Biology Group studies the importance of matrix molecules, such as hyaluronan, for growth and migration of normal and malignant cells. Effects of hyaluronan on tumor growth and on endothelial cell differentiation have been demonstrated.

The Protein Structure Group uses mass spectrometry to identify proteins and posttranslational modifications, and Edman degradation of proteins to determine phosphorylation sites. This group also has expertise in the synthesis of different types of modified peptides.

Some of the highlights from 2002 are summarized below.

C.-H. Heldin

Growth Regulation Group

Work in the Growth Regulation Group continues to explore the therapeutic opportunity constituted by dysregulated tyrosine kinase signaling in cancer. More specifically, our work concerns 1) platelet-derived growth factor (PDGF) receptors as cancer drug targets, 2) function and regulation of protein tyrosine phosphatases (PTPs) and 3) tumor stroma as a therapeutic target.

PDGF receptors as cancer drug targets

The PDGF family consists of five isoforms, PDGF-AA, -AB, -BB, -CC and -DD, which exert their cellular effects by binding with different affinities to α - and β -tyrosine kinase receptors. PDGF receptors are involved in different tumor associated processes, *e.g.* autocrine stimulation of tumor cells, modulation of tumor stroma and stimulation of tumor angiogenesis (reviewed in 85).

Autocrine stimulation of malignant cells occurs *e.g.* in sarcomas and glioblastomas and recent clinical studies, inspired by animal studies in our group, indicate that the skin tumor dermatofibrosarcoma protuberans responds to treatment with PDGF antagonists. For identification of PDGF-dependent subsets of glioblastomas, a panel of primary glioblastoma cultures is presently being characterized with regard to PDGF dependency and, in parallel, subjected to cDNA microarray gene expression profiling.

Recent animal studies, performed in collaboration with Prof. Kristofer Rubin, have suggested a highly interesting therapeutic relevance of PDGF receptors expressed in tumor stroma. Inhibition of PDGF receptors in tumor stroma, in two different tumor models, enhanced tumor uptake of cytotoxic drugs and increased anti-tumor effects (Pietras et al., submitted for publication, 26) (Fig. 1). The effects occurred without increasing toxicity. The mechanism(s) underlying the increased tumor drug uptake might include a decrease in tumor interstitial fluid pressure, induced by PDGF antagonists. The clinical relevance of these findings will be tested during 2003 in a pilot study on twenty breast cancer patients.

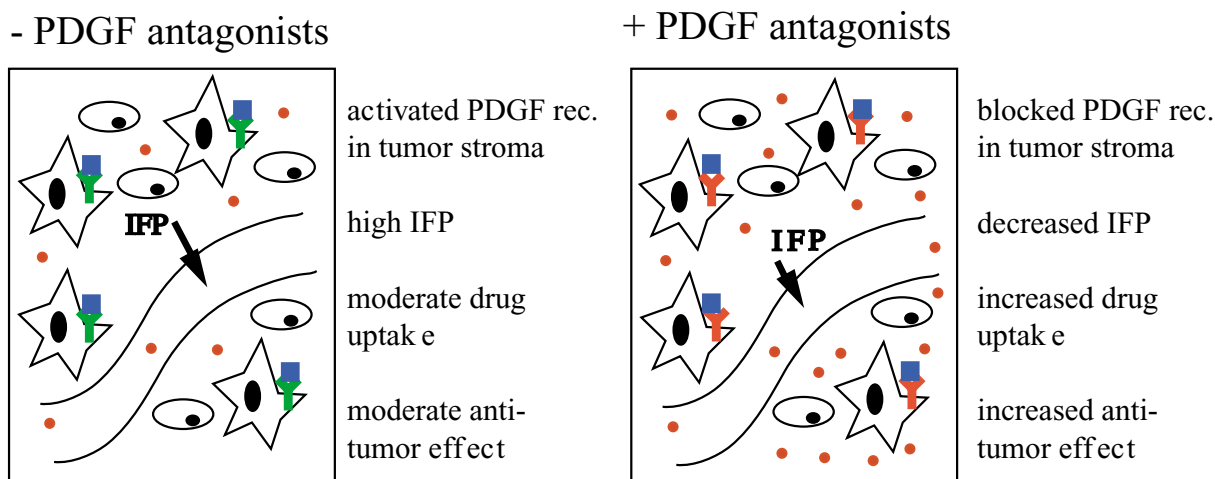


Fig. 1. Effect of PDGF antagonists on IFP, drug uptake treatment efficiency of tumors.

A role for PDGF receptor signaling in tumor angiogenesis is suggested by the perivascular expression of PDGF-receptors in more than 90% of tumors (Sjöblom *et al.*, unpublished observation). If PDGF receptors on pericytes regulate tumor angiogenesis is therefore currently investigated.

Function and regulation of PTPs

As naturally occurring tyrosine kinase antagonists, PTPs represent a class of enzymes of potential importance in tumor biology. A receptor-like PTP, DEP-1, was recently identified as a candidate tumor suppressor displaying loss of heterozygosity in 50% of colon, lung and breast cancer. Mechanism(s) of DEP-1 regulation and the specific activity of allelic variants of DEP-1 are investigated.

TC-PTP has been identified as a novel physiological negative regulator of PDGF receptors, by comparing PDGF signaling in wild-type and TC-PTP knock-out cells (Persson *et al.*, unpublished observation). TC-PTP knock-out cells displayed an enhanced chemotactic response to PDGF and a site-selective increase in receptor phosphorylation. This work will be extended by analyses of PDGF receptor signaling also in other PTP knock-out cells.

A novel antibody-based method for the detection of PTPs inactivated by oxidation has been established. With this method, oxidation of PTP α was shown after UV irradiation (Persson *et al.*, submitted for publication). The assay will be used to investigate the physiological relevance of PTP oxidation, and to compare oxidation of PTPs in normal and cancer cells.

Tumor stroma as a compartment of therapeutic potential

Accumulating evidence suggests that tumor growth involves interdependent interactions between tumor cells and the tumor stroma. Our findings of therapeutic effects after targeting of PDGF receptors in tumor stroma, highlights the therapeutic potential of this tumor compartment. To provide a platform for further studies aiming at targeting of the tumor stroma, we will perform gene-expression profiling of tumor stroma. Procedures for laser-capture micro-dissection, RNA extraction and RNA amplification, have been optimized (Micke *et al.*, unpublished observation).

Signal Transduction Group

The work in the Signal Transduction Group is focused on the mechanisms of signal transduction via the tyrosine kinase receptors for PDGF and stem cell factor (c-Kit).

Role of Gab1 in PDGF signaling

Signal transduction via tyrosine kinase receptors involves the docking of SH2 domain containing molecules to specific phosphorylated regions of the receptors. In addition, certain adaptor proteins associate with the receptors and mediate interactions with additional signaling molecules. We have investigated the role of one such adaptor molecule, Gab1. We found that Gab1 interacts indirectly with the PDGF β -receptor, via Grb2, and that Gab1 is phosphorylated by the receptor kinase, as well as by Src. Overexpression of Gab1 in porcine aortic endothelial (PAE) cells led to an increase in the activation of the Erk MAP kinase in response to PDGF (Kallin *et al.*, unpublished observations). Moreover, mouse embryo fibroblasts (MEFs) from Gab1 knock-out mice showed a more transient Erk activation compared to wild-type MEFs. Although no difference was observed in PDGF-induced proliferation of MEFs from wild-type and mutant mice, MEFs from mutant mice showed a decreased chemotactic response.

The PDZ domain protein NHERF controls cytoskeletal reorganization by PDGF

Using the C-terminal tail of the PDGF β -receptor as a bait in affinity chromatography, we isolated two PDZ domain proteins, NHERF/EBP50 and NHERF2 (Demoulin *et al.*, submitted for publication). The two proteins were shown to bind to the extreme C-terminus of the receptor; the interaction was dependent on ligand-induced receptor dimerization, but not on the kinase activity of the receptor. Receptor mutants unable to bind NHERF and NHERF2, showed an enhanced PDGF-induced actin reorganization,

however, no difference in terms of PDGF-induced mitogenicity, chemotaxis or pH regulation were observed compared to the wild-type receptor.

Gene regulation by PDGF using cDNA microarrays

The effect of PDGF-BB on the induction/repression of specific genes in human foreskin fibroblasts, have been investigated using microarray technique. Several interesting observations are currently followed up.

The adapter protein APS associates with the multifunctional docking sites Tyr568 and Tyr936 in c-Kit - potential role in tumorigenesis

The receptor for stem cell factor, c-Kit, is structurally related to the PDGF receptors and is expressed in cell types of diverse origins. It regulates different functions in different cell types. The aim of our work is to understand the mechanisms by which c-Kit signals under normal and pathological conditions, such as in neoplasia.

The adapter protein APS has previously been shown to be involved in recruiting the ubiquitin E3 ligase c-Cbl to several receptor tyrosine kinases, including the PDGF β -receptor, leading to increased degradation of the receptors and inhibition of mitogenesis. We have shown (63) that APS preferentially associates with phosphorylated Tyr568 and Tyr936 in c-Kit. We could demonstrate that the critical determinant for binding of APS is the presence of either a leucine or an isoleucine residue in position +3 relative to the phosphorylated tyrosine. This allowed us to design mutants that selectively failed to associate with APS while still associating with the Src family members, SHP-2 and Grb2. These mutants are currently being analyzed.

In this context, it is interesting that in the viral form of Kit, v-Kit, the sequence surrounding Tyr568 is altered so that the leucine residue is no longer in position +3. Furthermore, there is a carboxyterminal deletion involving Tyr936, which has been shown to be important for transformation. It is tempting to speculate that part of the transforming ability of v-Kit might arise from reduced ability to recruit c-Cbl to the receptor, thus leading to decreased degradation of the receptor and sustained signaling.

Splice-form specific signaling by c-Kit

Two splice forms of c-Kit exist that differ by the insertion of four amino acid residues (GNNK) in the extracellular domain, close to the transmembrane region. 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+ is activated with slower kinetics and remains active over a long period of time (64). Furthermore, the GNNK- form has a stronger transforming potential than the GNNK+ form. We have shown that several differences in downstream signaling exist. While the phosphatidylinositol-3'-kinase (PI3-kinase) pathway is about equally activated by the two receptor splice forms, the Ras/Erk pathway is activated more efficiently by the GNNK-form, which can be explained by differential activation of Src family kinases by the two splice forms. Using a selective inhibitor of Src family kinases, SU6656, we demonstrated that inhibition of Src family kinases led to a behavior of the GNNK- form resembling that of the GNNK+ form. Furthermore, ligand-induced degradation was higher in the GNNK- form compared to the GNNK+ form and shown to be partly dependent on the activity of Src family kinases.

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.

Future work is aimed at understanding the differences in downstream signaling by the two splice forms of c-Kit, using DNA microarray methods as well as by employing the technique of two-dimensional protein gel analysis.

Cytoskeletal Regulation Group

The work in the Cytoskeletal Regulation Group is focused on studies on 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 organisation of the actin cytoskeleton and thereby in the control of cell morphogenesis and cell migration. Our studies aim to identify signaling 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 signaling by the TGF- β receptor resulted in a robust mobilization of the actin cytoskeleton, seen as a rapid formation of membrane ruffles, as well as a long-term appearance of stress-fibers (7). In addition, stimulation of the TGF- β receptor resulted in the activation of Cdc42 and Rho. Cdc42 was needed for both the rapid and the long-term response, whereas Rho was needed only for the long-term response. We are currently studying the molecular mechanism underlying the activation of Rho GTPases by TGF- β , as well as the cross talk with other TGF- β regulated pathways.

The Rho family of GTPases

We have recently identified a number of novel Rho GTPases and we believe that this family consists of 22 family members. Currently, we are comparing the functions of these novel GTPases with the functions of the classical Rho GTPases. Recently, we found that Miro-1 and -2 (for mitochondrial Rho) have roles quite distinct from the classical Rho GTPases (47). These proteins have two putative GTP-binding domains separated by a linker region containing EF hand motives. Genes encoding Miro-like proteins were found in several eukaryotic organisms from *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* to mammals, indicating that these genes evolved early during evolution. Immunolocalization experiments showed that Miro was present in mitochondria. Overexpression of a constitutively active mutant of Miro-1 (Miro-1/V13) induced an aggregation of the mitochondrial network and resulted in an increased apoptotic rate (47). These data indicate a role for Rho-like GTPases in mitochondrial homeostasis and apoptosis.

Signaling via Wiskott-Aldrich syndrome protein

The Wiskott-Aldrich syndrome protein (WASP) was originally identified as the gene product 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 polymerisation in vertebrate cells.

The activity of the WASP proteins is governed by a number of associated proteins, such as the newly identified 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 (1). WIRE was localized to actin filaments in transiently transfected cells. Moreover, 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 . We are currently studying the collaboration between the WASP and the WIRE/WIP families of proteins in the regulation of processes such as cell migration and cell proliferation.

Signaling 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.

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 PDGF β -receptor 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 mitogenesis and actin cytoskeletal rearrangements. In collaboration with Dr. Philippe Soriano's group in Seattle, we generated mice bearing point mutant PDGF β -receptors, that are unable to bind and activate PI3'-kinase upon ligand stimulation, a prerequisite to induce 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 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 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 are 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 wild-type and the mutant PDGF β -receptor.

Very recently, an activation loop mutation in the PDGF α -receptor was reported to be responsible for a certain percentage of gastrointestinal stromal tumors in human. We are generating mice with the identical mutation in the PDGF β -receptor to test its oncogenic potential.

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, is 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 knock-out 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.

Molecular Signaling Group

Our laboratory studies the molecular mechanisms by which signaling pathways control cell growth and differentiation and 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 the endocytosis of RTKs and protein degradation through the actions of scaffold proteins and ubiquitin ligases. We currently utilize genetically altered mice and cells derived from such animals to understand how components of these modules regulate cell functions *in vivo*.

The role of Cbl and CIN85 in endocytosis of receptor tyrosine kinases

Upon engagement by their cognate ligands, RTKs become ubiquitinated through the action of Cbl ubiquitin ligases (reviewed in 81). Cbl is associated with activated RTKs throughout the endosomal compartment leading to sustained receptor ubiquitination, thus targeting receptors for degradation in the lysosome (80). In addition, Cbl regulates RTK endocytosis via pathways that are dependent on the ability of Cbl to interact with multiple proteins, including CIN85 and Grb2 (66).

The major focus of our work during the year was to understand the mechanisms involved in Cbl- and CIN85 (Cbl interacting protein of 85 kDa)-mediated negative receptor signaling. We have discovered a novel pathway by which Cbl and Cbl-b regulate internalization of the EGF receptor (36, 37). This pathway is functionally separable from the ubiquitin ligase activity of Cbl and depends on binding of the adaptor protein CIN85 to Cbl and recruitment of endophilins in complexes with activated EGF receptors (36, 37) (Fig. 2). CIN85 binds to the carboxyl-terminal part of Cbl,

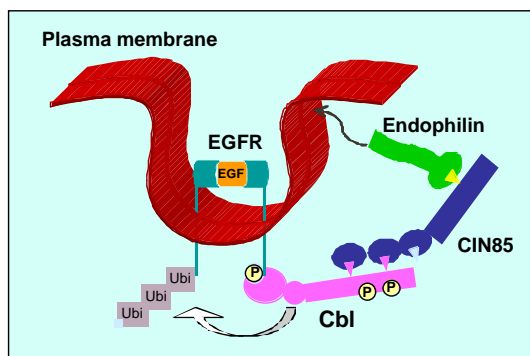


Fig. 2. CIN85 interactions with Cbl and endophilins are important for EGF receptor internalization.

recognizing a novel proline-arginine motif present in Cbl and Cbl-b, but not in Cbl-c (37). Furthermore, we have shown that CIN85 associates with Cbl/receptor complexes along the endocytic pathway, where Cbl directs monoubiquitination of CIN85 (13). These events seem to be critical for proper routing of EGF receptor complexes for degradation in the lysosomal compartment (13). Thus, we have proposed that CIN85 acts as a critical scaffold protein involved in endocytosis and degradation of activated RTKs.

Ubiquitination of receptor tyrosine kinases

Ubiquitination is a reversible modification of cellular proteins where a single ubiquitin (monoubiquitination) or a chain of ubiquitins (polyubiquitination) is attached to a target protein. Several lines of evidence have converged to show that RTK ubiquitination is important both for receptor internalization and degradation (80, 81). Despite a large number of studies on ubiquitination of RTKs, it remains to be determined whether receptor ubiquitination represents a critical signal for endocytosis or is only a modulator of this process. In particular, the nature of Cbl-directed ubiquitination of RTKs is still not resolved. It has been assumed that polyubiquitination of RTKs occurs *in vivo*. We have recently shown that Cbl directs monoubiquitination, rather than polyubiquitination, of activated EGF and PDGF receptors in mammalian cells (49). RTKs are monoubiquitinated on multiple sites, which ensures proper endosomal sorting and subsequent degradation of receptors in the lysosome (49). Furthermore, a single ubiquitin attached to EGF receptors is sufficient to mediate internalization as well as degradation of the receptors (49). These results are consistent with the hypothesis that a single ubiquitin moiety carries intrinsic signals for both internalization at the plasma membrane and sorting for lysosomal destruction.

TGF- β Signaling Group

The TGF- β Signaling Group investigates signaling pathways that regulate cell growth, differentiation and tumorigenesis in response to transforming growth factor β (TGF- β).

TGF- β signaling and Smad regulation

TGF- β signals via plasma membrane serine/threonine kinase receptors (79) and cytoplasmic effectors, the Smad proteins (74). The receptors activate the Smads, which move rapidly into the nucleus to regulate gene expression by associating with DNA and cooperating transcription factors (73). Smad3 is a receptor-activated (R-) Smad that after TGF- β stimulation is imported to the nucleus where it affects the transcription of specific genes; thereafter, it is exported back to the cytoplasm. We have studied the nuclear export pathway of Smad3 and attempt to define a novel exportin and corresponding nuclear export signal in Smad3 (Kurisaki, A. *et al.*, submitted for publication). In parallel, we focus on the fate of Smad4, Smad protein common for all TGF- β superfamily pathways. In human cancers, specific amino acid substitutions in Smad4 lead to its enhanced proteolysis. In normal cells, Smad4 is mono- or oligo-ubiquitinated, which leads to efficient R-Smad/Smad4 oligomerization (Morén *et al.*, submitted for publication). We also screen for E3 ubiquitin ligases that mediate Smad4 ubiquitination in normal and cancer cells.

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

The activated, nuclear Smads specifically interact with several transcription factors (73). We have analyzed the association of Smads with the transcriptional regulator YY1 (Kurisaki, K. *et al.*, submitted for publication). YY1 does not interfere with TGF- β /BMP-induced growth inhibition but it can specifically repress important target genes and cell differentiation processes in response to either TGF- β or BMP. After having defined a mechanism by which TGF- β , via Smads and the transcription factor Sp1, induces the *p21* gene, we studied the expression of this gene in response to other signaling pathways of the superfamily, and the importance of p21 induction for the negative regulation of growth of epithelial cells. Both YY1 and Sp1 are zinc-finger proteins that interact specifically with the conserved N-terminal Mad homology 1 (MH1) domain of Smads. We attempt to finely map the interaction interfaces and look for short peptide motifs common to transcription factors that may selectively interact with the Smad MH1 domain.

Dual role of TGF- β in 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. We study mammary epithelial models that exhibit this dual response to TGF- β . Murine NMuMG cells undergo epithelial to mesenchymal transition (EMT) *in vitro* in response to TGF- β , a change in cell differentiation important *in vivo* during tumor cell migration and metastasis (75). 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 (Gaal *et al.*, submitted for publication). We also analyze immediate-early and late gene responses to TGF- β using cDNA microarrays and attempt to identify novel regulators of the transition between regulation of cell growth and differentiation.

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 have examined their relevance to the tumor suppressor pathway. We have combined microarray screening with knock-down technologies based on RNAi to functionally establish the critical nature of specific gene targets in cell growth, differentiation and tumorigenesis.

Integrated Signaling Group

The work in the Integrated Signaling Group aims at exploring TGF- β signaling in normal and malignant cells. We direct our efforts at the unraveling of mechanisms of receptor activation, as well as Smad-dependent and Smad-independent signaling downstream of the receptors. An important aspect of our work is to elucidate the role of TGF- β in carcinogenesis. Proteomic techniques are among the tools we use to understand the complexity of integrated intracellular signaling.

Proteomics in studies of TGF- β signaling

We continue our global proteome profiling of TGF- β stimulated cells using two-dimensional gel electrophoresis combined with protein identification by mass spectrometry. We have unveiled a novel role of TGF- β in the regulation of genome stability; TGF- β 1-dependent down-regulation of Rad51 resulted in inefficient repair of

DNA double-strand breaks (18). This may have profound impact on our understanding of the dual role of TGF- β in tumorigenesis, suggesting a mechanism for the tumor-promoting activity of TGF- β .

We also study the phosphoproteome and glycoproteome of human breast epithelial cells (MCF7), and have identified a number of proteins, which change their phosphorylation or glycosylation upon TGF- β 1 treatment. An evaluation of the functional importance of these two post-translational modifications is on-going.

We have also identified a number of proteins whose expression and phosphorylation are affected by TGF- β in human endothelial cells (HMVEC). Functional proteome profiling of human epithelial and endothelial cells will be completed in 2003.

Screen for plasma markers for breast and ovarian cancers

We have performed a screen for markers of breast and ovarian cancers by proteomics analysis of plasma samples from patients. Twenty-three samples of breast and twenty samples of ovarian cancers have been analyzed by two-dimensional gel electrophoresis, and compared to the plasma proteome of non-cancer individuals. Potential markers will be analyzed in a larger group of cancer patients, to explore their prognostic value.

Smad3 and BMP receptor interacting proteins

We have identified more than 20 novel Smad3- and BMP receptor-interacting proteins, which currently are being explored for functional importance in TGF- β and BMP signaling. These interacting proteins were identified by two-dimensional gel electrophoresis and mass spectrometry.

Involvement of BRCA2 and BRCA1 in TGF- β signaling

We have discovered a cross-talk between TGF- β signaling and the products of breast cancer susceptibility genes *BRCA2* and *BRCA1*. We have found that BRCA2 forms a complex and synergizes with Smad3 in transcriptional regulation (29).

TGF- β type I receptor kinase inhibitors

We have investigated six classes of low molecular weight compounds for inhibition of the kinase of the type I TGF- β receptor (41). We have found that compounds of the

imidazole and isoquinoline groups can be used as a backbone for the development of highly specific inhibitors acting via the ATP-binding site. We also described a novel class of inhibitors, *i.e.* peptidomimetics, which can inhibit the receptor kinase by interfering with the substrate-binding site of the kinase.

Effects of OP1 (BMP7) and TGF- β on vitamin D₃ regulation of osteoblast differentiation

We have described a functional interaction between osteogenic protein-1 (OP1/BMP7) and vitamin D₃ in the differentiation of human osteoblasts (8). We showed that OP1, unlike TGF- β 1, inhibits vitamin D₃-induced differentiation. Our findings can have an impact on the treatment of osteoporosis, as vitamin D₃ is often used in the clinic.

Apoptotic Signaling Group

TGF- β plays an important regulatory role in the proliferation, differentiation, migration and apoptosis of cells during embryogenesis, as well as for maintenance of homeostasis in organs and tissues in the adult organism. We have previously demonstrated that Smad7, a target gene for TGF- β , is required for TGF- β -induced apoptosis in prostate cancer cells, as well as in human keratinocytes. The aim of our work is to further elucidate the precise roles of TGF- β and Smad7 in the control of cell fate in prostate cancer cells.

Smad7 mediates TGF- β -induced apoptosis

We have observed an increased expression of the inhibitory Smad7 in normal and malignant prostate epithelial cells undergoing apoptosis *in vivo*, 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 have recently shown that Smad7 mediates TGF- β -induced apoptosis not only in prostate cancer cells, but also in human keratinocytes (HaCaT) cells. During the past year, we have focused our efforts on the molecular mechanisms by which Smad7 enhances TGF- β -induced apoptosis in epithelial cells. We have found that Smad7 is required for TGF- β 1-induced activation of a MAP kinase pathway consisting of TGF- β activating kinase 1 (TAK1), mitogen activated protein kinase kinase 3 (MKK3) and p38, resulting in apoptosis (46). It appears that Smad7, which upon TGF- β stimulation is exported from the nucleus to the activated receptor complex, acts as a scaffolding protein for TAK1, MKK3 and p38, thereby facilitating the activation of p38 (46). We are currently investigating the

molecular mechanisms by which TGF- β , and in particular Smad7, can activate the TAK1-MKK3-p38 MAP kinase pathway.

Smad7 target genes

Smad7 is localized in the nucleus. We have therefore explored the possibility that direct target genes for Smad7 exist, and have performed microarray analyses on cells overexpressing Smad7; a number of candidate genes have been identified which now are validated.

Induction of apoptosis in epithelial-derived 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. We have reported that 2-ME potently induces apoptosis in several prostate cancer cell lines *in vitro* and *in vivo*, via the activation of the MAP kinase c-Jun N terminal kinase (JNK), which was found to be crucial for the apoptotic response. Similar effects were observed in breast, colon and liver cancer cell lines. 2-ME does not bind to the known nuclear estrogen receptors. Moreover, our data suggest that the apoptotic effect of 2-ME is dependent on phosphorylation of Bcl-2, since the phosphorylation of Bcl-2 preceded the induction of cell death, and a prostate cancer line lacking Bcl-2 does not undergo apoptosis upon treatment with 2-ME (5). In collaboration with Prof. Mats Bergström and Padideh Davoodpour (Uppsala University) we investigate the effects of 2-ME on prostate cancer cells growing as aggregates *in vitro*, which is similar to the *in vivo* situation. Again we observed potent apoptotic effects of 2-ME. By the use of PET-tracers, we are currently investigating the effects of 2-ME on the metabolism and proliferation of prostate cancer cells (Davoodpour *et al.*, unpublished observation). We have also investigated the effects of 2-ME on other epithelial-derived tumor cell lines *in vitro* and *in vivo*, in collaboration with groups at the University Hospital in Uppsala (Li, L. *et al.*, submitted for publication).

Gene Expression Group

The work in the Gene Expression Group is focused on transcription factors. In particular, the regulation of Smad, SREBP and p53 by posttranslational modifications and interaction with other components, are studied.

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 (11). 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 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 (48). 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 target 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.

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.*, unpublished observations). 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 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 transduction of intracellular signals. We investigate how hyaluronan-induced and growth factor-induced signaling are interrelated, as well as whether hyaluronan influences tumor progression. 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, both in animal models and patients. Therefore, we have initiated studies to investigate the mechanisms underlying perturbation of hyaluronan synthesis and turnover during tumor progression. The regulation of hyaluronan synthases (Has1, Has2 and Has3) and hyaluronidases (PH-20, Hyal1 and Hyal2) in response to growth factors released by tumor cells have been studied. 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 regulated in response to external stimuli. Notably, TGF- β 1 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 pericellular matrices. The formation of such matrices is differently regulated in response to growth factors.

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 affects tumorigenesis. In an effort to explore the relationship 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 (17). These results were further confirmed by additional studies in a colon carcinoma model both *in vitro* and *in vivo*. These studies revealed that Has2 overproduction promotes tumorigenicity, whereas Hyal1 overexpression suppressed tumor development.

Effect of hyaluronan fragments on endothelial cell differentiation

We have also initiated studies to increase our knowledge of 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 3D collagen gel after stimulation with hyaluronan fragments and PH-20-like

hyaluronidase (30); the assembly of endothelial tubules was dependent on the hyaluronan receptor CD44. Thus, hyaluronan-CD44 interactions promote tumor progression.

Protein Structure Group

As in previous years, the Protein Structure Group has tight interactions with the other groups at the Uppsala and the Stockholm Branches, and also with other research groups within or outside Sweden. In the early days of our Branch, one of the main activities was to perform amino acid sequencing of novel proteins that were involved *e.g.* in signal transduction. However, now, when several genomes have been sequenced, it is no longer necessary to determine amino acid sequences. Instead, proteins can be identified by proteolytic fingerprints using mass spectrometry.

Much of our efforts are aimed at identifying proteins and describe their role. We also continue to prepare synthetic peptides; mainly for in-house collaborators.

Peptide synthesis

The Applied Biosystems 433A synthesizer, purchased three years ago, produces peptides of high quality. Still, a large part of the total time for preparation is manual work-up. This includes, for many applications, purification by preparative scale reversed phase chromatography, followed by quality check on our Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (MALDI-Tof-MS), and in particular, special modifications of selected residues. These can be phosphorylations, acetylations, oxidations etc. The uses of the synthetic peptides vary, many are used for production of anti-peptide antibodies, others as inhibitors or substrates and yet others are used in affinity chromatography.

Amino acid sequencing

Whereas classical Edman degradation is not used anymore after the introduction of mass spectrometry, analysis of phosphorylation sites in growth factor receptors and related proteins is still an important activity in the Group. We use two older instruments equipped with a fraction collector – a necessity since this strategy requires that the released amino acid is analyzed for ^{32}P by phosphorimaging. The two instruments used for this is getting difficult to service; therefore we plan to reconstruct the newer Procise 494 sequencer for sequencing of radiolabeled proteins.

Sample preparation for mass spectrometry

As analysis by mass spectrometry is extremely sensitive, also contaminants of low concentration are detected. Therefore, a large effort must be placed on sample preparation procedures. The most common technique is to utilize the high resolution of two-dimensional electrophoresis. However, since most samples are prepared from whole cells, containing tens of thousands of different proteins in different quantities, an efficient pre-fractionation step must precede the electrophoresis. After in-gel digestion of a spot or a band of interest (stained by Coomassie or silver) many samples need to be concentrated and desalted prior to mass spectrometry. This is done on hand-made reversed phase columns in the sub μl scale.

Peptide Mass Fingerprinting (PMF) on MALDI-ToF-MS

The identity of proteins is routinely determined by mass analysis of proteolytic peptides (usually after in-gel digestion using trypsin), followed by scanning a sequence database using one of many search engines, such as ProFound. The sensitivity is high, when *e.g.* the sample is from a weak silver stained spot (usually around 1-30 fmol, after digestion and extraction procedure) we can get a correct and significant identification.

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

The determination of amino acid sequences by MALDI using PSD has not been practical until a recent introduction of a sulfonating reagent (the CAF-chemistry). This improves PSD sequencing dramatically, and now we can perform de-novo sequencing of tryptic peptides in the low to mid fmol scale of peptides up to 20 amino acids. Also modified residues are identified and localized in the sequence, such as phosphorylated Tyr, Ser and Thr.

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