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Uppsala Branch

1998/1999

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Research Report The aim of the research at the Uppsala Branch is to elucidate the signaling pathways in cells that control growth, differentiation, motility and apoptosis. Cancer cells are characterized by their lack of growth control and accumulating evidence support the notion that this is due to constitutive activation of pathways that, in the normal cell, are controlled by growth stimulatory factors, or by loss of components in growth inhibitory pathways. We focus in particular on soluble growth regulatory factors and their mechanism of action and role *in vivo*.

In September 1999, the group leader of our TGF- β group, Peter ten Dijke left to take up a position at the Netherlands Cancer Institute in Amsterdam. We wish Peter good luck and are looking forward to future collaborations with him and his colleagues. The TGF- β program at our Branch will continue and will be co-headed by Aristidis Moustakas and Serhiy Souchelnytskyi. As before the focus will be on signal transduction of members in the TGF- β superfamily, which, in addition to TGF- β , consists of *e.g.* activin and bone morphogenetic proteins. These factors are pleiotropic molecules which regulate the cell cycle, extracellular matrix production, cell differentiation and apoptosis, and have important roles during the embryonal development. They act by binding to type I and type II serine/threonine kinase receptors. Much effort is devoted to the studies of downstream signaling molecules of the Smad family and the elucidation of mechanisms that regulate cell morphogenesis and growth, especially in the context of cancer development.

Another new group leader has recently been recruited; Dr. Johan Ericsson will head the Gene Expression Group. Johan has just returned from a five-year postdoctoral training with Dr. P. Edwards at the University of California in Los Angeles. At our Branch he will focus on mechanisms involved in transcriptional control.

In addition to the TGF- β and Gene Expression Groups, the Uppsala Branch consists of six groups. The aim of the Signal Transduction Group is to elucidate signaling pathways downstream of tyrosine kinase receptors which lead to cell growth and chemotaxis. The major interest is devoted to signaling by platelet-derived growth factor (PDGF), a mitogen primarily for connective tissue cells, and stem cell factor (SCF), a mitogen for mast cells and other cells. The functional roles of components in established signaling pathways are characterized, and novel signaling molecules are identified.

The Growth Regulation Group aims at finding ways to antagonize the effects of PDGF on cells. Such antagonists are clinically warranted since overactivity of PDGF

has been implicated in several diseases, including cancer. Another interest in the Group is protein tyrosine phosphatases, which counteract the effects of tyrosine kinases.

The Gene Targeting Group focuses on the functional roles of specific signal transduction molecules in intact animals using knock-out or knock-in of genes in the mouse.

The Cytoskeletal Regulation Group studies the roles of small GTPases of the Rho family in actin reorganization, and aim at elucidating signaling pathways leading to apoptosis of cells.

The Molecular Signaling Group focuses on intracellular signal transduction, and in particular on the role of cytoplasmic tyrosine kinases, like Pyk2, in mitogenic signaling via G-protein coupled receptors.

As before, the skills in micropreparative methods, protein sequencing, protein identification by mass spectrometry and peptide synthesis of the staff in the Protein Structure Group, have been very important for many aspects of our research program.

Below, we summarize some of our findings during 1998 and 1999.

C.-H. Heldin

Growth Regulation Group

The work of the Growth Regulation Group focuses on PDGF and DEP-1, a receptor-like protein tyrosine phosphatase (rPTP). PDGF, a major growth factor and chemoattractant for connective tissue cells, is implicated in a number of pathological situations (reviewed in ref. 89). The goal with our work on PDGF is to explore the potential of PDGF and its tyrosine kinase receptors as drug targets in the treatment of malignancies and other diseases. Our work includes studies on mechanisms of PDGF-induced receptor activation, development of novel PDGF antagonists and applications of PDGF antagonists in disease models. rPTPs are regulators of cell signaling and potential mediators of disease-associated dysregulated proliferation. Our studies on DEP-1 aims at identifying processes regulated by DEP-1, as well as characterization of substrate specificity and regulating mechanisms.

Recombinant forms of PDGF receptor extracellular domains

The extracellular domains of the PDGF α - and β -receptors are composed of five Ig-like domains. Formation of ligand:receptor complexes involves both receptor:receptor interactions, mediated by Ig-like domain 4, and ligand:receptor interactions. Using recombinant forms of the extracellular domain of the PDGF α -receptor, purified from transfected CHO-cells, PDGF-AA binding was mapped to Ig-like domains 2 and 3, and PDGF-BB binding epitopes to Ig-like domain 2 (31). Recombinant forms of the extracellular domains of the PDGF α - and β -receptors, purified as GST-fusion proteins from CHO cells, were also compared with their cleaved counterparts, lacking the GST domain, for PDGF antagonistic effects. A 100-1000 fold higher potency occurring as a consequence of GST domain-mediated dimerization of the extracellular domains, was observed for the GST fusion proteins (Leppänen et al., submitted for publication). We conclude that antagonists to bivalent ligands like PDGF will, in general, act at much lower concentrations if presented as dimers.

The dermatofibrosarcoma protuberans associated COL1A/PDGFB fusion gene encodes a transforming protein that is processed to functional PDGF-BB

Dermatofibrosarcoma protuberans (DFSP), an infiltrative skin tumor of intermediate malignancy, displays a t(17:22) translocation that fuses the N-terminal parts of the collagen type 1 alpha I gene to exon 2 of the PDGF B-chain gene. In a study performed together with Dr. J. Dumanski, Stockholm, Sweden, expression of the fusion gene in NIH3T3 cells was found to lead to morphological transformation, increased growth rate and *in vivo* tumorigenesis (89). These phenotypic changes,

accompanied by the presence of activated PDGF receptors, were sensitive to a PDGF receptor tyrosine kinase inhibitor, STI-571, suggesting that transformation involved a PDGF autocrine loop. The fusion gene was demonstrated to encode a dimeric collagen type 1 alpha I/PDGF-B precursor that was processed to mature PDGF-BB. The chromosome 17:22 rearrangement thus contribute to DFSP by ectopic production of PDGF-BB and the formation of a PDGF autocrine loop. A panel of primary cultures derived from DFSP tumors are presently being investigated with regard to presence of PDGF autocrine loop and sensitivity to STI-571.

Characterization of the chronic myelomonocytic leukemia associated TEL-PDGF β R fusion protein

The t(5;12) translocation, associated with chronic myelomonocytic leukemia, generates a constitutively activated form of the PDGF β -receptor. The fusion protein, TEL-PDGF β R, is composed of the 154 amino-terminal amino acids of the transcription factor TEL and the transmembrane and intracellular part of the PDGF β -receptor. Together with Dr. J. Ghysdael, Orsay, France, we found that tyrosine phosphorylation of the fusion protein can be attenuated either by treatment with AG1296, a PDGF receptor kinase inhibitor, or through overexpression of the TEL-domain of the TEL-PDGF β R (90). Comparison of BaF/3 cell lines expressing TEL-PDGF β R and ligand-stimulated PDGF β -receptor revealed that only TEL-PDGF β R expression conferred IL-3-independent growth, suggesting differences in signaling capacity of the two proteins. Indications for signaling differences between TEL-PDGF β R and ligand-stimulated PDGF β -receptor was provided with the identification of tyrosine residues 17 and 27 as unique autophosphorylation sites in TEL-PDGF β R.

PDGF receptor kinase inhibitor reduces tumor interstitial hypertension and increases transcapillary transport

Many types of solid tumors display increased interstitial fluid pressure (IFP), and reduced transcapillary transport. PDGF and PDGF receptors are expressed in many tumors, either by tumor cells, stromal cells or in vessels. Signaling through PDGF β -receptors has recently been shown to increase IFP in dermis after anaphylaxis-induced lowering of IFP. The possible involvement of PDGF receptor signaling in the increased IFP of syngenic subcutaneous tumors of rat PROb colon cancer cells was investigated in a study performed with Dr. K. Rubin, Uppsala, Sweden (Pietras et al., submitted for publication). Four-day treatment with the PDGF receptor kinase inhibitor STI-571, reduced tumor IFP and increased tumor uptake of a systemically

administered radioactive tracer, ^{51}Cr -EDTA. In the tumors, PDGF receptor expression was limited to the stroma compartment. Our study thus suggests that PDGF β -receptors in the tumor stroma are positive regulators of tumor IFP and that interference with PDGF receptor signaling might increase drug uptake in tumors.

PDGF SELEX aptamers block neointimal hyperplasia in a rat model of restenosis

In a collaboration with NeXstar Pharmaceuticals Inc., Co., USA, we have developed and characterized a set of DNA ligands (SELEX aptamers) that act as PDGF antagonists by interfering with PDGF-AB and -BB receptor binding. To probe the role of PDGF in post-angioplasty restenosis, a study with PDGF SELEX aptamers was performed in a rat model of restenosis. After balloon-catheter injury, rats were treated two weeks with aptamer or placebo, or for one week with aptamer followed by one week with placebo. The effect on restenosis was determined morphometrically two or eight weeks after injury. In the two-week endpoint study, animals treated with aptamer for two weeks displayed a reduction with 52% and 49% in intimal area and intima/media ratio, respectively, as compared to control animals (Leppänen et al., submitted for publication). However, neither in the case of a one-week treatment and a two-week endpoint, nor in the case of a two-week treatment and an eight-week endpoint, were statistically significant effects observed. The lack of sustained effect by two-week treatment with aptamer suggest that extended administration, or a combination therapy with other agents, may be required to obtain optimal results.

Site-selective dephosphorylation of the PDGF β -receptor by DEP-1

Dephosphorylation of PDGF β -receptor by tyrosine phosphatases could either lead to complete turning off of the signal, by general dephosphorylation or dephosphorylation of the regulatory Tyr857, or modulation of signaling output through site-selective dephosphorylation. In a collaboration with Dr. F. Böhmer, Jena, Germany, DEP-1-mediated dephosphorylation of PDGF β -receptor was analyzed in detail; these studies revealed that both *in vivo* and *in vitro* dephosphorylation of the PDGF β -receptor by DEP-1 led to site-selective dephosphorylation (Kovalenko et al., submitted for publication). Under conditions where 50% total dephosphorylation occurred *in vitro*, the extent of dephosphorylation of different sites varied between 5% and 70%. The regulatory Tyr857 was not a preferred site of dephosphorylation, suggesting that DEP-1 mediated dephosphorylation of PDGF β -receptors leads to modulation, rather than

general down-regulation of PDGF β -receptor signaling. The site-selectivity seen in dephosphorylation of intact receptor was reproduced when phospho-peptides derived from the receptor was used as substrate. Thus, the primary sequence surrounding auto-phosphorylation sites appear to be a major determinant of DEP-1 site-selectivity.

Signal Transduction Group

The receptors for platelet-derived growth factor (PDGF) and stem cell factor (SCF) belong to the class III tyrosine kinase receptors. Activation of the receptors is brought about by ligand-induced dimerization of the receptors. In the case of the α and β -receptors for PDGF, this can result in either formation of homodimers α - α or β - β , or heterodimerization leading to formation of α - β heterodimers. The activated receptors are capable of phosphorylating tyrosine residues on itself as well as on other molecules. These phosphorylation sites constitute docking sites for SH2 domain containing molecules. Binding, and in some cases also phosphorylation, of such molecules leads to their activation and initiation of signaling.

The overall aim of the Signal Transduction Group is to study and characterize the mechanisms by which the two receptors for PDGF, as well as the receptor for stem cell factor (c-kit), initiate signals leading to growth, chemotaxis and differentiation. This involves among other things the identification of phosphorylation sites in the receptors and in the signaling molecules, studies on their function, as well as identification of novel signaling molecules.

Identification of a novel autophosphorylation site in the PDGF β -receptor that mediates interaction with SHP-2

We were able to demonstrate that Tyr763 is an autophosphorylation site and by peptide affinity chromatography we could identify SHP-2 as the docking partner to Tyr763 (85). SHP-2 is protein tyrosine phosphatase containing two SH2-domains, that previously has been shown to bind to autophosphorylated Tyr1009 in the PDGF β -receptor. By expressing mutants of the PDGF β -receptor with Tyr763 and Tyr1009 altered to phenylalanine residues, either individually or in combination, we could show that mutation of either Tyr763 or Tyr1009 leads to a significant decrease in binding of SHP-2, whereas mutation of both tyrosines led to a complete loss of SHP-2 binding. We could demonstrate a dramatic decrease in both PDGF-BB induced Ras-GTP loading and MAP kinase activation. However, when assaying for PDGF-BB induced mitogenesis, no difference was seen compared to wild-type PDGF β -receptor expressing cells. Ras has previously been implicated as a mediator of chemotaxis in PDGF-stimulated cells. When assaying for PDGF-BB induced

chemotaxis we could see a decrease in chemotaxis in the Tyr763F/Tyr1009F mutant cells, compared to wild-type receptor expressing cells, thus demonstrating an importance of SHP-2 in PDGF-mediated chemotaxis.

Increased mitogenic signaling of a α - β heterodimeric complex correlates with decreased RasGAP binding

Upon ligand-binding PDGF α - and β -receptors form homodimers as well as heterodimers, depending on the ligand used. PDGF-AA binds only to PDGF α -receptor, while PDGF-AB induces both PDGF α - β heterodimers as well as α - α homodimers. PDGF-BB binding leads to formation of all three dimeric complexes. It has been shown that the α - β heterodimeric receptors transmit a stronger mitogenic response than either α - α or β - β homodimers. We have shown that the increased mitogenic signaling in heterodimers correlates with lowered RasGAP binding to the PDGF β -receptor in the heterodimer compared to homodimers (61). In the heterodimeric complex, the PDGF β -receptor does not become phosphorylated on Tyr771, the docking site for RasGAP. PAE cells expressing the PDGF α -receptor together with a PDGF β -receptor, with Tyr771 mutated to a phenylalanine residue, showed an increased mitogenic response to PDGF-AB compared to cells expressing wild-type receptors. Thus, it is likely that the decrease in binding of RasGAP in the heterodimeric configuration at least in part is responsible for the stronger mitogenic response of PDGF-AB, compared to either PDGF-AA or PDGF-BB.

Overactivation of phospholipase C- γ 1 renders PDGF β -receptor expressing cells independent of the PI3'-kinase pathway for chemotaxis

We have previously shown that PAE cells expressing the Y934F PDGF β -receptor mutant respond to PDGF-BB in a chemotaxis assay at about one hundred times lower concentration than do wild-type PDGF β -receptor expressing cells (Hansen et al. (1996) EMBO J. 15, 5299-5313). We now show that the increased chemotaxis correlates with increased activation of phospholipase-C γ 1 (PLC- γ 1) measured as inositol-3,4,5-trisphosphate release (86). By two-dimensional phosphopeptide mapping, the increase in phosphorylation of PLC- γ 1 was shown not to be selective for any site, rather a general increase in phosphorylation of PLC- γ 1 was seen. To assess whether increased activation of PLC- γ 1 is the cause of the hyperchemotactic behaviour of the Y934F mutant cell line, we constructed cell lines expressing either wild-type or a catalytically compromised version of PLC- γ 1 under a tetracycline-inducible promoter. Overexpression and concomitant increased activation of wild-type PLC- γ 1 in response to PDGF-BB led to a hyperchemotactic behaviour of the

cells, while the catalytically compromised PLC- γ 1 mutant had no effect on PDGF-BB induced chemotaxis. Furthermore, in cells expressing normal levels of PLC- γ 1, chemotaxis was inhibited by LY294002. In contrast, the increase in chemotactic response seen upon overexpression of PLC- γ 1 was not inhibited by the PI3'-kinase inhibitor LY294002. These observations suggest the existence of two different pathways which mediate PDGF-induced chemotaxis; depending on the cellular context the PI3'-kinase pathway or the PLC- γ 1 pathway may dominate.

Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and *c-fos* induction

The juxtamembrane region of the stem cell factor receptor (Kit/SCFR) shows striking similarity to the PDGF receptors, in that it contains two tyrosine residues, that in the PDGF receptors have been shown to bind to Src family kinases. To study the functional role of Src kinases in Kit/SCFR signaling, Tyr568 and Tyr570 were mutated to phenylalanine residues, and the mutated Kit/SCFR expressed in PAE cells. We could demonstrate that Tyr568 and Tyr570 are responsible for activation of Src family kinases by the Kit/SCFR (79). However, no stable association was seen. An almost complete loss of *c-fos* induction was seen, as compared to wild-type Kit/SCFR expressing cells. It is well established that the Ras/MAP kinase pathway is essential for *c-fos* induction by a number of growth factors. We were able to show that both Ras GTP-loading as well as Erk2 activation was severely abrogated in mutants of the Kit/SCFR unable to activate Src family kinases. Tyrosine phosphorylation of Shc has previously been demonstrated to lead to binding of Grb2-Sos to Shc, and activation of the Ras/MAP kinase pathway. We could demonstrate that in the Y568F/570F mutant, phosphorylation of Shc was dramatically lowered, suggesting an involvement of Src family kinases in Kit/SCFR mediated phosphorylation of Shc. This provides a probable explanation to the lowered Ras-GTP loading and Erk2 activation seen in Y568F/Y570F mutant cell lines, as well as the loss of *c-fos* induction.

In contrast to the Src activation data, the mitogenic potential of the Y568F mutant and Y570F mutant were almost as high as that of the wild-type Kit/SCFR. However, the Y568F/Y570F mutant cell line had a dramatically lowered mitogenic response to SCF. This suggests that additional molecules bind to Tyr568 and Tyr570, that might influence the mitogenicity of cells expressing Kit/SCFR.

Identification of Tyr703 and Tyr936 as autophosphorylation sites and the primary association sites for Grb2 and Grb7 in the Kit/stem cell factor receptor

We have demonstrated the presence of two novel *in vivo* autophosphorylation sites in the Kit/stem cell factor receptor (Kit/SCFR), Tyr703 in the kinase insert and Tyr936 in the carboxyterminal tail of the Kit/SCFR (92). The adapter protein Grb2 was shown to be a specific binding partner for both phosphorylated Tyr703 and Tyr936, whereas the adapter protein Grb7 selectively binds to phosphorylated Tyr936. Grb2 has been shown to link receptor tyrosine kinases to the Ras/MAP kinase pathway. Grb7 is an adapter protein, but its precise function remains to be elucidated. Its high similarity to Mig-10, a protein found in *Caenorhabditis elegans* which is involved in the migration of neuronal cells during embryonic development, points to an important function for Grb7 in cellular signaling. It was shown that the association to the Kit/SCFR occurs through the SH2 domains of Grb2 and Grb7.

Gene Targeting Group

Information obtained from cell culture studies regarding interactions and cross talks between signal transduction molecules enable us to speculate about specific gene functions. However, it is important to verify such hypotheses in the intact animal. One of the best characterized responses of the PDGF β -receptor is actin cytoskeleton rearrangements and chemotaxis mediated by PI3-kinase binding to the phosphorylated tyrosine residues 740 and 751 in the human β -receptor. In collaboration with P. Soriano, Fred Hutchinson Cancer Institute, Seattle, we have generated mice with the wild-type β -receptor replaced with one in which the tyrosines of the PI3-kinase binding sites have been mutated to phenylalanine residues. Surprisingly, mice homozygous for this mutation showed no overt phenotype. However, embryonic fibroblasts derived from such mutant mice exhibited several cytoskeletal defects, such as the lack of circular ruffles and a strongly reduced chemotactic response after stimulation with PDGF-BB. In addition, cells with mutant receptors were greatly impaired in their ability to contract collagen gels after stimulation with PDGF-BB compared to wild-type fibroblasts. In an *in vivo* model for the control of interstitial fluid pressure in dermis, a drop in interstitial fluid pressure provoked by edema induction has been shown to be counteracted by local injection of PDGF-BB. In a collaboration with K. Rubin, Uppsala University and R. Reed, University of Bergen, Norway, we found that this occurs only in wild-type mice, but not in mice carrying a homozygous mutation for the PI3-kinase binding sites in their PDGF β -receptor (67). These observations indicate a role for PDGF-BB/PDGF β -receptor/PI3-kinase in the homeostasis of interstitial fluid pressure, by modulating the tension between cells and extracellular matrix structures.

Phospholipase-C γ (PLC- γ) is another well studied molecule activated by binding to the PDGF β -receptor, thereby increasing mitogenic response and cytoskeletal rearrangements/chemotactic response after PDGF stimulation. Since the single (PI3-kinase-binding) mutant mice were seemingly unaffected, we generated mice that were mutant for the binding of PI3-kinase in combination with PLC- γ . The phenotype of these mice is currently under investigation.

TGF- β signaling in prostatic cancer

TGF- β 1 and its type II receptor (T β R-II) and type I receptor (T β R-I) are expressed in the prostate and has important regulatory effects on normal epithelial cells differentiation. After castration, the mRNA levels for TGF- β 1 rises in parallel with the onset of apoptosis in the normal epithelial cells, which makes TGF- β 1 a candidate for initiating the apoptotic program. However, in advanced prostatic tumors, expression levels of TGF- β 1 are higher than in the normal prostate, while losses of function of T β R-I and decreased expression of the T β R-II have been demonstrated. The loss of TGF- β receptors could explain the loss of sensitivity for the ligand, while the increased expression of TGF- β 1 instead could actually promote tumor growth through action of TGF- β 1 on angiogenesis and the immune system.

We have earlier found an increased expression of TGF- β 1 and its receptors in a highly differentiated rat prostatic tumor model, Dunning R3327 PAP, which was more pronounced when estradiol was given in combination with castration. The increased expression of TGF- β 1 showed a high correlation to apoptosis in the tumor cells and there was also a strong correlation between a rapid infiltration of immunocompetent cells, which preceded apoptosis. Recently, by immunohistochemical investigations of samples from the same tumor model and from normal ventral prostate before and after castration, we observed an increased expression of Smad2, Smad3 and Smad4 in tumors as well as in normal prostate after castration (59). Smad2 was also found to be activated, as analyzed by antibodies specifically recognizing phosphorylated Smad2. There was no increased expression of Smad1, and the Smad5 expression was only slightly higher, compared to before castration. Interestingly, apoptotic cells, showing morphological hallmarks like shrinkage of cell volume, nuclear condensation and fragmentation, often had Smad proteins located in the nucleus, revealing a link between Smad signaling and apoptosis in the normal prostate induced by castration. In the prostatic tumor model, fewer cells showed increased expressions of the Smad proteins and also the number of tumor cells undergoing apoptosis after castration were lower when compared to the normal prostate. Furthermore, we found an increased expression of the inhibitory

Smad6 and Smad7 after castration. In particular, Smad7 was present in some of the epithelial cells with morphological signs of apoptosis (59).

We investigated how TGF- β 1 induces apoptosis in a prostatic carcinoma cell line *in vitro*. By using anti-sense techniques together with inducible ectopic expression of Smad7, we found in collaboration with Dr. N.-E Heldin, Uppsala University, that Smad7 plays a pivotal role for activation of a caspase-dependent apoptotic pathway (Landström et al., submitted for publication). In Smad7 overexpressing cells, Smad7 is primarily located in the nucleus of prostate cancer cells, while upon ligand stimulation it is rapidly exported to the cytoplasm, as earlier reported in other cells (21). Whether the nuclear localization of Smad7 is important as well for its newly identified function as a mediator for TGF- β 1 induced apoptosis, is currently being investigated.

Cytoskeletal Regulation Group

The work within the Cytoskeletal Regulation Group is aimed at elucidating signaling pathways that control cell growth and cell migration during normal physiological conditions as well as during disease.

Cell signaling by Rho GTPases

The Rho GTPases is a group of small enzymes with homology to Ras. Up to this moment, 17 distinct Rho GTPases have been found in mammalian cells, but, by far, the best studied family members are Rho, Rac and Cdc42 (131). These proteins have been found to regulate the morphological and migratory properties of mammalian cells by regulating specific aspects of the actin cytoskeleton. Furthermore, the Rho GTPases have important roles in signaling pathways that control gene transcription, cell cycle regulation and cell survival (130). The Rho GTPases were originally shown to exhibit only weak transforming activity, yet several of their activating proteins, the GEFs, have been characterised as proto-oncogenes. Recently, it has become apparent that the Rho GTPases have important roles in tumor progression, for instance in the maintenance of the transformed phenotype of Ras transformed cells. It has, furthermore, been reported that the expression of Rho GTPases are upregulated in certain human tumors.

Analysis of proteins that bind to Cdc42

During the recent years, a lot of effort has been put into the characterization of target proteins, or effectors, for the Rho GTPases in order to understand the mechanisms by which they control the diverse signaling pathways (130). We have focused the studies

on the Cdc42-regulated pathways employing the yeast two-hybrid system to isolate cDNAs encoding proteins that bind specifically to the activated, GTP-bound, Cdc42. This way, seven different proteins, designated CIP1-7 (for Cdc42 interacting protein), were isolated including the Wiskott-Aldrich Syndrome protein (WASP), 14-3-3 β and MLK3, whereas CIP4-CIP7 represented novel proteins.

CIP4 is a protein of 545 amino acid residues containing a C-terminal SH3 domain. The N-terminus of the protein bears resemblance to the non-kinase domain of the Fer/Fes family of tyrosine kinases and this domain is, in addition, present in a number of CIP4-like proteins. Overexpression of CIP4 in Swiss 3T3 fibroblasts reduced the amount of stress fibres in these cells, which suggested a role for CIP4 in the regulation of the actin cytoskeleton. The development of a functional antiserum raised against CIP4 will facilitate the studies of the endogenous CIP4. In order to find out more about the function of CIP4, work has been initiated to find additional binding partners for CIP4. In a yeast two-hybrid system screen using the SH3 domain as a bait a number of potential CIP4 interacting proteins were isolated including a novel RhoGAP domain containing protein.

CIP5 was shown to contain a PDZ domain and its Cdc42-binding domain was different from such domains present in other known Cdc42-binding proteins. In the yeast two-hybrid system, the protein binds both Rac and Cdc42 but immunoprecipitation experiments as well as *in vitro* binding studies indicate that Cdc42 is the physiological partner for CIP5. This protein was recently identified in *C. elegans* as a gene-product, PAR-6, necessary for the asymmetric cleavage and polarisation of the early embryo. Work has been initiated in order to elucidate if CIP5/PAR-6 can have similar roles also in mammalian cells.

Wiskott-Aldrich Syndrome Protein

The Wiskott-Aldrich syndrome is a severe, but rare, immunodeficiency disorder. Earlier studies demonstrated that the Wiskott-Aldrich Syndrome protein (WASP) was a target for Cdc42. Recently, it has been reported that WASP is a key component in the regulation of actin polymerization. The expression of the protein has been shown to be restricted to hematopoietic cells and the rat basophilic leukemia, cell-line RBL-2H3, is currently used as model system for the studies of WASP function *in vivo*. It was demonstrated that WASP became tyrosine phosphorylated in response to ligation of the high affinity receptor for IgE, Fc ϵ RI (14). *In vivo* labeling of the RBL-2H3 cells with ^{32}P -orthophosphate, followed by phospho-peptide analysis showed that only a minor fraction of WASP is tyrosine phosphorylated. WASP was

phosphorylated on serine and threonine residues already in unstimulated cells. Stimulation of WASP with IgE or stem cell factor gave rise to a robust increase in WASP phosphorylation, mainly on serine residues but to a minor extent also on tyrosine. The precise nature of the receptor-induced phosphorylations of WASP and their implications for the biological function of the protein is currently being studied.

Activation of Stat molecules by the PDGF receptors

Stat molecules are important signal transducers downstream of cytokine receptors, but are also activated by PDGF receptors. The phosphorylated tyrosines in the β -receptor that are involved in binding and activation of Stat5 have been identified (51). The phosphorylation of Stat5b was found to be increased after PDGF stimulation, on tyrosine as well as serine/threonine residues (94). Since PDGF activates the MAP kinase cascade, the possibility was investigated that kinases in this cascade phosphorylated Stat5b on serine/threonine residues. Whereas no effects of phosphorylation on nuclear translocation were observed upon incubation with MEK inhibitor PD98059, the DNA binding activity and transcriptional activity of Stat5b were markedly increased. These observations indicate that MEK is a negative modulator of PDGF-induced activation of Stat5; the mechanism involved is currently under investigation.

Induction of reorganisation of vimentin filaments by PDGF

PDGF-stimulation of cells leads to a dramatic reorganisation of the actin filament system. Moreover, we observed that the vimentin filaments in PAE cells expressing the PDGF β -receptor also reorganised in response to PDGF stimulation; within 20 min the well-spread fine fibrillar vimentin aggregated into a dense coil around the nucleus (50). The redistribution of vimentin filaments was found to be dependent on PI3-kinase and Rac, similar to previous observations regarding PDGF-induced actin reorganisation.

Involvement of different PI3-kinase isoforms in PDGF- and insulin-induced actin reorganisation

The family of PI3-kinases consists of several variant forms; both the regulatory subunit of 85 kDa and the catalytic subunit of 110 kDa occur as different structurally related isoforms. Using microinjection of neutralizing antibodies specifically recognising p110 α and p110 β , respectively, we observed that p110 α antibodies inhibited PDGF-induced, but not insulin-induced, actin reorganisation; in contrast, p110 β antibodies inhibited insulin-induced but not PDGF-induced, actin

reorganisation (Hooshmand-Rad et al., submitted for publication). These data indicate that the two PI3-kinase isoforms have different roles in signaling via two major receptor tyrosine kinase receptors.

Molecular Signaling Group

Molecular characterization of signaling pathways that regulate cell growth and differentiation and their involvement in processes of malignant transformation represents a scientific focus of our group. Signaling by mitogenic G protein-coupled receptors (GPCRs) and functional characterization of cytoplasmic tyrosine kinase Pyk2 and CIS/SOCS proteins are investigated.

Mitogenic signaling by bradykinin B2 G protein-coupled receptor

Activation of bradykinin B2 GPCRs has been shown to elicit growth-promoting effects including cell proliferation and stimulation of DNA synthesis in several cell types. We have previously shown that G α q-dependent pathways stimulated by bradykinin receptors activate protein tyrosine kinases Pyk2 and Src leading to stimulation of MAP kinases in PC12 cells (reviewed in 121). In the last year, we have shown that bradykinin-induced MAP kinase activation depends significantly on the cellular milieu in which the B2 receptors are expressed. For example, in PC12 and COS7 cells bradykinin activates MAP kinase predominantly via calcium and tyrosine kinase-dependent pathways, whereas in human fibroblasts and 293T cells bradykinin employs the serine/threonine kinase PKC and Ptx-sensitive pathways to mediate MAP kinase activation. The functional characterization of these signaling pathways in intracellular transmission of mitogenic signals by B2 receptors are under investigation.

Recent evidence suggests that serine/threonine phosphorylation of GPCR may play critical roles in signaling to the MAP kinase cascade. All *in vivo* serine phosphorylation sites of B2 receptors which are phosphorylated upon ligand stimulation have been identified and shown to link B2 receptors with internalization. However, mutant B2 receptors impaired in receptor phosphorylation and internalization are fully capable to activate MAP kinase. Bradykinin-induced long-term effects on mitogenic signaling monitored by measuring transcriptional activity of Elk1 were identical in cells expressing the wild-type or mutant B2 receptors. We have concluded that G protein-coupled bradykinin receptors activate the MAP kinase pathway independently of receptor phosphorylation and internalization (58).

Functional characterization of Pyk2 and Pyk2-H

Pyk2 together with FAK constitute a distinct family of non-receptor protein tyrosine kinases that are regulated by a variety of extracellular stimuli. Pyk2 is predominantly expressed in the central nervous system and in cells and tissues derived from hematopoietic lineages. A new isoform of Pyk2 (Pyk2-H) that is generated by alternative RNA splicing and is specifically expressed in hematopoietic cells has been cloned (9). Engagement of T-cell or B-cell antigen receptors leads to rapid tyrosine phosphorylation of Pyk2-H. Pyk2-H is also activated in response to HIV envelope proteins and chemokines RANTES and MIP-1 β in T cells. Pyk2 and Pyk2-H are implicated in signaling by numerous transmembrane receptors, including GPCRs, integrins and growth factors and have been shown to activate the MAP kinase and JNK pathways (132).

The precise molecular mechanisms by which Pyk2 activates distinct MAP kinase pathways are not yet fully understood. We have recently shown that the protein tyrosine kinase Src and adaptor proteins Grb2, Crk and p130Cas act as downstream mediators of Pyk2 leading to the activation of Erk and JNK. Expression of dominant interfering forms of Grb2 and Sos reduced Pyk2-induced Erk activation, while expression of dominant interfering mutants of p130Cas or Crk specifically inhibited JNK but not Erk activation. Our data revealed specific pathways which couple Pyk2 with MAP kinases: the Grb2/Sos complex connects Pyk2 to the activation of Erk, while adaptor proteins p130Cas and Crk link Pyk2 with the JNK pathway (57).

More recently, we have provided evidence that Pyk2 acts as an integrating point in signaling by growth factors receptors and integrins in neuronal cells. A cellular model whereby activation of both growth factor receptors and integrins is necessary for neuronal differentiation of PC12 cells has been established. EGF and PDGF induce Pyk2 activation and neurite outgrowth in PC12 cells plated on laminin or collagen, but not on poly-L-lysine or plastic. Moreover, expression of dominant interfering forms of Pyk2 in PC12 cells plated on extracellular matrix leads to strong inhibition of EGF- and PDGF-induced neurite outgrowth. These results indicate that activation of Pyk2 is necessary for signal transmission downstream of the integrin-growth factor receptor complex leading to induction of neurite outgrowth (Ivankovic-Dikic et al., submitted for publication).

In addition to tyrosine residues, Pyk2 is strongly phosphorylated on serine/threonine residues in living cells. We have undertaken a laborious and detailed approach to carefully identify these phosphorylation sites *in vivo* by using ortho-phosphate

labeling of endogenous Pyk2 in PC12 cells. To date, we have identified two serines located in the kinase domain of Pyk2 which are phosphorylated *in vivo* and we are currently studying their function in Pyk2 activation and signaling.

The role of CIS/SOCS proteins in down-regulation of receptor tyrosine kinase signaling

Recently, a group of proteins, alternatively called CIS, SOCS, SSI or JAB, were identified and shown to act as negative regulators of cytokine-induced STAT signaling. These proteins contain an N-terminal region, followed by a SH2 domain which potentially could interact with phosphorylated tyrosines, and a novel C-terminal motif called the CIS homology (CH) domain or SOCS-box.

In collaboration with Dr. Akihiko Yoshimura (Kurume University, Japan) we are studying the mechanisms of receptor type tyrosine kinase modulation by CIS/SOCS proteins, initially using the PDGF β -receptor as a model system. Previous work from our institute has demonstrated that PDGF receptors activate several STAT molecules. Our studies indicate that several members of CIS/SOCS family associate with activated PDGF receptors and inhibit PDGF-induced STAT signaling. However, the exact mechanism of this inhibition remains elusive. We plan to expand our studies to receptors for epidermal growth factor, fibroblast growth factor, stem cell factor and insulin to test if different CIS/SOCS proteins have a specific role in controlling signaling by receptor tyrosine kinases.

TGF- β Group

Transforming growth factor- β (TGF- β) family members are important multifunctional signaling proteins, which include TGF- β s, activins and bone morphogenetic proteins (BMPs). Our goal is to elucidate the molecular mechanisms by which TGF- β family members elicit their effects on growth arrest, extracellular matrix formation, differentiation, chemotaxis and apoptosis, and apply this knowledge in developing novel treatments for diseases that are caused by perturbation in TGF- β signaling. In particular, our group is focused on the activation and regulation of Smads and their function in transcriptional regulation.

The TGF- β /Smad pathway

Members of the TGF- β family exert their effects through distinct combinations of two types of serine/threonine kinase receptors, *i.e.* type I receptors and type II receptors. Receptor activation involves ligand-induced hetero-oligomerization of two sequentially acting kinases with the type I receptor acting as a substrate for the type II

receptor kinase. The activated type I receptor propagates the signal downstream through transient interaction with and phosphorylation of Smad proteins, which assemble in heteromeric complexes that translocate into the nucleus, where they regulate the transcription of target genes.

Consistent with the current model for TGF- β family receptor activation, type I receptors were found to determine the specificity of the intracellular signals induced by different TGF- β family members. An exposed region in the kinase domain of type I receptors, termed the L45 loop, was found to be important in determining signaling specificity. In collaboration with Dr. K. Funahashi, Gothenburg, Sweden, we examined the responses of a mutant TGF- β type I receptor and a mutant BMP type IB receptor, in which the L45 regions of these two receptors were exchanged. Swapping the four amino acid residues that are different in BMP type IB receptor for those in TGF- β type I receptor, and *vice versa*, switched their type I receptor-restricted Smad activation and specificity in transcriptional responses (39).

Pathway-restricted and common-mediator Smads

Pathway-restricted and common-mediator Smads share two regions of homology at their N- and C-termini, termed Mad homology domain 1 (MH1) and MH2, respectively. Pathway-restricted Smads, but not common-mediator Smads, couple directly to different type I receptors. Whereas Smad2 and Smad3 act in the TGF- β /activin pathway, Smad1, Smad5 and Smad8 mediate BMP intracellular signals.

TGF- β and activin signal through two closely related type I receptors, T β R-I and ActR-IB, respectively, which both have the potential to activate Smad2 and Smad3. However, in certain biological assay systems, *e.g.* human keratinocytes (HaCat), TGF- β and activin elicit different responses. In collaboration with Dr. K. Miyazono, Tokyo, Japan, we showed that activin induced activation of Smad3 and, to lesser extent, Smad2, in HaCat cells. On the other hand, TGF- β induced efficiently the phosphorylation and nuclear translocation of both Smad2 and Smad3. In transfected COS cells, constitutively active ActR-IB efficiently stimulated heteromeric complex formation of Smad3 and Smad4. The observed differential Smad activation by activated T β R-I and ActR-IB may provide an explanation for different effects of TGF- β and activin on these cells (45).

We showed that osteogenic protein-1 (OP-1), also termed BMP-7, binds predominantly to BMP type II receptor and BMP type IB receptor in the ROB-C26

osteoprogenitor-like cell line. Smad1, Smad5 and Smad8 were shown to have potential to interact with activated BMPR-IB. However, only Smad5 was found to be activated in the ROB-C26 cells upon OP-1 challenge. Moreover, transfection studies with dominant negative forms of Smad5 or Smad4 revealed their critical importance in OP-1 intracellular signaling (49).

In collaboration with Dr. Hill (ICRF, London, UK), we have identified two Smad4s in *Xenopus*: XSmad4a, which is the *Xenopus* orthologue of human Smad4, and a distinct family member XSmad4b. Both XSmad4s were found to act as co-Smads forming ligand-dependent complexes with receptor-regulated Smads and synergizing with them to activate transcription in *Xenopus* and cultured mammalian cells. Interestingly, the two XSmad4 genes have reciprocal temporal expression patterns in *Xenopus* embryos and are expressed in varying ratios in adult tissues, suggesting distinct functional roles *in vivo* (68).

Transcriptional regulation by Smads

Smads can function as transcriptional regulators of target genes. In collaboration with Dr. J.-M. Gauthier, Paris, France, we identified a TGF- β /Smad-responsive element, termed CAGA box, in the plasminogen activator-inhibitor-1 (PAI-1) promoter; PAI-1 is directly and potently induced by TGF- β . CAGA boxes confer responsiveness to stimulation by TGF- β and activin, but not BMP, of a heterologous promoter reporter construct. Mutation of the CAGA boxes present in the PAI-1 promoter was found to abolish TGF- β responsiveness. TGF- β was found to induce binding of Smad3/Smad4 containing nuclear complexes to CAGA boxes. Bacterially expressed Smad3 and Smad4, but not Smad1 or Smad2, bind directly to this sequence *in vitro* (8). In addition, in collaboration with Dr. W. Kruijer, Groningen, Netherlands, we mapped a Smad responsive element, termed SBE, in the TGF- β target gene *JunB*. Multimerization of SBE created a powerful TGF- β inducible enhancer that is also responsive to activins and BMPs (26).

TGF- β 1 is a positive regulator of IgA production. The TGF- β 1 responsive region in Ia promoters upstream of IgA switch regions contains binding sites for Smad and AML. In collaboration with Dr. P. Sideras (University of Umeå, Sweden) we found that Smad3, Smad4 and AML bind to Ia1 promoter and have a critical role in the activation of Ia1 promoter by TGF- β 1 (110).

The transcriptional activity of Smad protein complexes in the nucleus can be modulated by their interaction with co-repressors, among which the co-activator

CBP/p300 is the best characterized. The histone acetyl transferase (HAT) activity of these co-activators is known to contribute to the acetylation of histones and the transcriptional activation of the modified chromatin. Thus, we have investigated the signaling pathway and identified P/CAF as a novel co-activator which specifically associates with Smad3 and positively cooperates with Smads to mediate transcriptional responses to TGF- β .

TGF- β 1 potently upregulates the expression of cell cycle inhibitors such as p15 and p21 in a cell type-specific manner and this provides one mechanism to explain the growth inhibitory action of this family of ligands. We have previously analyzed the role of Smad proteins in p21 promoter regulation and provided evidence for functional cooperation between Smads and the ubiquitous transcription factor Sp1 (33). In collaboration with Dr. D. Kardassis, Heraklion, Greece, we have extended the analysis of the mechanisms of regulation of the p21 promoter and showed that Jun family proteins physically interact and functionally cooperate with Sp1 in regulation of the p21 promoter (75). We further demonstrated that Smad2, Smad3 and Smad4 physically interact with Sp1 and this association results in enhanced DNA binding of Sp1 to its cognate sites on the p21 promoter and cooperativity in transcriptional activation which is also modulated by the co-activator p300 (Pardali et al., in preparation).

Inhibitory Smads

Inhibitory Smads, *i.e.* Smad6 and Smad7, act oppositely from the pathway-restricted Smads by competing with them for type I receptor binding. In collaboration with Dr. N.-E. Heldin, Uppsala, Sweden, we showed that the transcription of inhibitory Smads is upregulated by TGF- β family members, suggesting that they may function in an auto-regulatory feedback loop in TGF- β family signaling (1).

In collaboration with Dr. J. Christian, Portland, Oregon, we found that the C-terminal domain of Smad7 is sufficient for its antagonistic activity. In addition, we could show that Smad7 interacts with, in addition to TGF- β and activin receptors, BMP receptors. Smad7 interfered with BMPRI-mediated Smad activation, and phenocopied the effect of known BMP antagonists, when overexpressed in ventral cells of *Xenopus* embryos (48).

In collaboration with Dr. T. Nishihara, Tokyo, Japan, Smad7 was found to block activin and BMP-induced growth arrest and apoptosis in mouse B cells (20). Consistent with previous findings in other cell types, Smad7 was found to abrogate

activin-induced Smad2 phosphorylation and BMP-induced Smad1 phosphorylation. Smad6, however, blocked specifically BMP-induced responses, including Smad1 phosphorylation in these cells. Thus, Smad6 inhibits preferentially BMP signaling, whereas Smad7 appears to be a general inhibitor of TGF- β family signaling (20,, Ishisaki et al., submitted for publication).

Interestingly, in the absence of ligand, Smad7 was found to localize to the nucleus, whereas Smad7 accumulated in the cytoplasm upon TGF- β receptor activation. The differential localization of different Smad7 deletion mutants indicate that an intact MH2 domain is important for nuclear localization and TGF- β -induced nuclear export (21). The nuclear localization of Smad7 suggests a function distinct from its antagonistic effect in the cytoplasm.

Together with Dr. G. Stenman, Gothenburg, Sweden, we mapped the chromosomal localization of the human *Smad7* gene to 18q21.1, close to the locations of the *Smad2* and *Smad4* genes (42). The colocalization indicates that these Smads may have originated from a common ancestral gene which after duplication diverged in sequence and function.

TGF- β /Smad signaling in cancer

In collaboration with Dr. N.-E. Heldin, Uppsala, Sweden, we investigated TGF- β signaling in various thyroid carcinoma lines. One cell line, out of six, was not growth inhibited by TGF- β . Analysis of the intracellular signaling pathway in this cell line revealed an intact TGF- β /Smad pathway, indicating a novel mechanism for TGF- β insensitivity (66). In addition, we analysed the expression of TGF- β receptors and Smad proteins in glioblastoma cell lines with distinct responses to TGF- β . Our results suggest a complex balance between several components mediating the TGF- β responsiveness in glioblastoma (84).

We examined the expression patterns of Smad proteins in colorectal cancer and found an increased expression of pathway-restricted Smads in a fraction of the tumor cells. Common-mediator Smad4 was detected in both tumor cells and normal tissues. Interestingly, we observed a distinct pattern of Smad4 immunostaining of epithelial cells along the colon crypts, with high expression in the zones of terminal differentiation (76).

TGF- β 1 has been shown to play an important role in the transition of epithelial cells into mesenchymal cells and in the malignant progression of benign tumors into

highly invasive and metastatic tumors. The TGF- β 1-mediated morphological epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells was found to be mediated through T β R-I/ALK-5 and Smad proteins (Piek et al., submitted for publication).

Gene Expression Group Cells respond to extracellular and/or intracellular signals by transmitting instructions to coordinate cellular responses. Most, if not all, signal transduction pathways ultimately affect gene transcription and alter the expression of specific genes. In a multicellular organism, all cell types contain the same genetic information. Yet each cell type expresses only a subset of the total number of available genes, usually in response to specific environmental or developmental signals. The development of a complex eukaryote requires the differential transcription of over 50 000 genes in a very precise manner. One of the key problems in cell biology is how an organism can achieve such diversity, while maintaining cell specificity and the ability to respond dynamically to its environment. The cell accomplishes this by cooperative and synergistic interactions between a limited repertoire of transcriptional activators/repressors (transcription factors). Most of these transcription factors are DNA-binding proteins that bind to regulatory DNA sequences in the promoters of their target genes. However, one of the major hurdles in activating transcription *in vivo* is the presence of nucleosomal structures that limit access of the transcriptional machinery to the DNA template. Acetylation of nucleosomal histones is thought to induce an open chromatin conformation, which allows the transcriptional machinery access to promoters. Our understanding of the relationship between histone acetylation and gene expression has been enhanced dramatically by the recent identification of proteins with intrinsic histone acetyltransferase and deacetylase activity. These histone acetyltransferases and deacetylases provide a critical link between chromatin structure and transcriptional output. This new signaling pathway is now accessible to experimental intervention.

The levels and activities of transcription factors are regulated in response to extracellular and intracellular cues, during development and under pathological conditions. The function of these factors may be affected in a variety of ways, including post-translational modification (*e.g.* phosphorylation and acetylation), ligand binding and interactions with other proteins. The aim of the Gene Expression Group is to elucidate the mechanisms involved in the transcriptional response to growth factors and metabolic signals and how this relates to the development of human disease.

Adipocyte differentiation and metabolic regulation of transcription

Atherosclerosis, including heart disease, is the most common cause of death in the Western world and is highly correlated with elevated levels of cholesterol in the blood. The level of cholesterol in plasma is tightly controlled by a complex feedback mechanism, *i.e.* the levels of cholesterol regulate the expression of genes involved in these processes. The factors that are involved in regulating the expression of these genes are central to our understanding of this control system and, therefore, central to our understanding of human disorders such as cardiovascular disease, obesity, certain cancers and diabetes.

Portions of our research efforts have been aimed at identifying the factors regulating genes in response to changes in cellular cholesterol levels. Key regulators of these processes belong to the Sterol Regulatory Element-Binding Protein (SREBP) family of transcription factors. Members of this family of transcription factors are activated and released from cellular membranes in sterol-deprived cells. Subsequently, they enter the nucleus and activate transcription of genes involved in cholesterol biosynthesis and metabolism. Interestingly, SREBPs are also activated during adipocyte differentiation. Our work has established a role for SREBPs in the transcriptional regulation of a number of cholesterol-regulated genes, as well as genes induced during adipocyte differentiation.

Our initial observations indicate that sterol- and SREBP-regulated transcription is dependent on the coactivator CBP/p300, a histone acetyltransferase (HAT). Furthermore, our results indicate that interactions between CBP/p300 and P/CAF, a separate protein with HAT-activity, is important for the function of CBP/p300 in these processes. We propose to continue and extend these studies and analyze the involvement of CBP/p300 and histone acetylation in sterol- and SREBP-regulated transcription of endogenous genes, both those involved in cholesterol metabolism and those induced during adipocyte differentiation. We will also determine if the HAT-activity of CBP/p300 and P/CAF is necessary for the function of these proteins during these processes. We would like to perform these studies on integrated DNA templates (as promoter-GFP constructs), rather than on transiently transfected promoter-reporter genes. These types of studies are very important since the chromatin structure associated with transiently transfected promoter-reporter genes is thought to differ significantly from that associated with endogenous genes. The use of promoter-GFP reporter genes will enable us to monitor gene expression on a cellular level *in vivo*, *e.g.* following microinjection of antibodies against transcription factors or coactivators.

It was recently reported that HATs could acetylate certain transcription factors, *e.g.* p53 and GATA-1, thereby affecting their DNA-binding and transcriptional activities. Our initial observations indicate that SREBP is acetylated by both CBP/p300 and P/CAF *in vitro*. We are currently trying to determine if SREBP is acetylated *in vivo* and the effect of this modification on its DNA-binding and/or transcriptional activity. The acetylated residues will be identified in collaboration with the Protein Structure Group. These residues will subsequently be mutated in order to analyze the functional consequences of defective acetylation. We will also generate acetylation-specific antibodies, which will enable us to monitor acetylation of SREBP in various situations (*e.g.* following changes in cholesterol levels or during adipocyte differentiation). The acetylation of SREBP will also be monitored in cells transfected with plasmids encoding this transcription factor and HAT-deficient versions of CBP/p300 and P/CAF. Similar studies will also be performed with other transcription factors that are associated with cholesterol biosynthesis and metabolism.

SREBPs are very potent transcriptional activators when overexpressed in cells. However, very little is known about the regulation of these transcription factors following their release from cellular membranes. This is especially true for the regulation of SREBPs during adipocyte differentiation. However, SREBPs are phosphorylated and this may regulate their function and/or activity in response to various signal transduction pathways. We propose to analyze the regulation of SREBPs by various signal transduction pathways, *e.g.* growth factor signaling and mitogen activated protein kinase (MAPK) signaling, with special emphasis on adipocyte differentiation. In addition, we intend to identify the phosphorylated residues in SREBPs, both in unstimulated cells and in response to the various stimuli (in collaboration with the Protein Structure Group). The functional consequences of SREBP phosphorylation will be studied in several systems (*e.g.* sterol-regulated transcription and adipocyte differentiation).

Characterization of the multifunctional transcription factor Yin Yang 1

Depending on the promoter context, the transcription factor Yin Yang 1 (YY1) has been shown to either stimulate or repress gene expression. The mechanistic basis for these two different activities has not been characterized. However, recent evidence indicates that the interaction of YY1 with the coactivator p300 may be relevant in determining whether YY1 functions as an activator or repressor. Furthermore, YY1 has been described as an initiator-binding protein that is able to stimulate basal

transcription *in vitro* in the absence of the TATA box-binding protein (TBP). YY1 interacts with a number of chromatin remodelling activities, *e.g.* histone acetyltransferases and deacetylases, indicating that YY1 may also be involved in aspects of chromatin organization. YY1 function and regulation have been linked to the proto-oncoprotein c-Myc, which directly interacts with and alters the function of YY1. In addition, YY1 can inhibit c-Myc function and is a potent inhibitor of c-Myc transforming activity. Furthermore, YY1 has been implicated in the expression of both the c-Myc and p53 gene. These findings point to a role for YY1 as a negative regulator of cell growth, with a possible involvement in tumor suppression. Almost all YY1-repressible genes identified to date are either associated with differentiation or are inducible genes that are targets of cytokines or stress responses. YY1 also regulates the expression of a large number of viral genes and functions as a repressor of human immunodeficiency virus type 1 (HIV-1) transcription and virion production when bound the HIV-1 LTR. Our initial studies have also indicated that histone deacetylation may be important for SREBP-regulated gene expression and that the transcription factor YY1 may be involved in these processes.

One of the goals of the current project is to further elucidate the mechanisms involved in the different transcriptional activities of YY1 and the role of histone acetyltransferases and deacetylases in these processes. We recently demonstrated that YY1 is acetylated by both CBP/p300 and P/CAF. The DNA-binding activity and repressor activity of YY1 is greatly enhanced following acetylation. We are currently trying to determine if YY1 is acetylated *in vivo* and the effect of this modification on its transcriptional activity. The acetylated residues will be identified in collaboration with the Protein Structure Group. These residues will subsequently be mutated in order to analyze the functional consequences of defective acetylation for the different transcriptional activities (*i.e.* transcriptional activation and repression) of YY1. We will also generate acetylation-specific antibodies, which will enable us to monitor acetylation of YY1 in various situations.

The multifunctional transcription factor p53 is mutated in more than 50% of all human cancers. This tumor suppressor is a critical component of cellular mechanisms that respond to certain stresses to preserve genomic integrity by arresting cell-cycle progression or by inducing apoptosis. p53 is normally a short-lived protein that is maintained at low levels. However, in response to DNA-damaging agents, nucleotide depletion, or hypoxia, the p53 protein is transiently stabilized and accumulates in the nucleus in which it functions in part to induce or repress the expression of several target genes that regulate cell-cycle progression. DNA damage also activates the transcriptional activity of p53 through a phosphorylation and acetylation cascade. We

recently demonstrated that YY1 has the ability to inhibit the transcriptional activity of p53. We are currently trying to determine the mechanisms involved in this inhibition. We are currently mapping the domains of YY1 that are necessary for the inhibition of p53. In addition, we are analyzing if these proteins are involved in direct protein-protein interactions. Furthermore, we are trying to establish if YY1 also affects any of the physiological responses to activated p53 (*e.g.* cell-cycle arrest and apoptosis). The functional role of acetylation of p53 and YY1 in these processes is also studied.

Many of the transcription factors and genes regulated by YY1 are associated with major human diseases (*e.g.* atherosclerosis and cancer). The expression and/or transcriptional activity of YY1 may, therefore, be an attractive target when developing novel therapies for these disorders. We would, therefore, like to develop a screen that would enable us to monitor the expression and/or transcriptional activity of YY1. Such a screen would utilize cells transfected with either YY1 promoter-reporter genes or YY1-responsive promoter-reporter genes. Following treatment of cells with various compounds, the activity of the reporter genes will be used to monitor the expression or transcriptional activity of YY1. Once this screen is optimized, it could potentially be used for high-throughput screening for compounds that affect YY1 function. Once such compounds are identified, they will be tested for their effect on processes that are regulated by YY1.

Transcriptional regulation by Stat proteins

STAT (signal transducers and activators of transcription) is a family of transcription factors that mediate transcriptional regulation in response to a diverse group of cytokines and growth factors. Cytokine signaling is usually transient. Typically, STAT activation occurs rapidly after cytokine stimulation and declines within a few hours. Recently, a number of negative regulators of STATs have been identified, *e.g.* SOCS and PIAS proteins. As mentioned above, YY1 functions as a repressor for a number of cytokine-inducible genes. One example of this is the STAT5-dependent induction of the beta-casein gene in response to prolactin and PDGF. The transcriptional activity of STATs is also affected by MAPK signaling.

We propose to continue our work on the role of STATs in PDGF-signaling. We are especially interested in the functional role of YY1 and histone acetyltransferases and deacetylases in STAT-mediated transcription. In addition, we would like to extend our studies of the involvement of MAPK signaling in the control of STAT activation.

Protein Structure Group

The Protein Structure Group performs peptide synthesis and characterization of proteins via amino acid microsequencing and matrix assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF-MS). We continue to interact with, in particular, all other groups at our Branch and other Branches of the Institute, as well as with many other research groups in Sweden and abroad. This large contact area, plus frequent visits to scientific conferences give us an increasing experience of various research problems.

The introduction of the MALDI-TOF mass spectrometer in our laboratory was the single most important event during 1998. It has influenced the Group's activities greatly, for example the sensitivity at protein identification is about 50 times higher and results are obtained considerably quicker. Also the direct access to mass spectrometric analysis of synthetic peptides means easier and better control of their quality.

Peptide synthesis

The peptides produced in the PE-ABI 430A synthesizer are mainly used by research groups at the Branch. There are a number of important applications where synthetic peptides are required. Depending on the use, different degrees of purity are needed. When necessary, the crude peptides are purified by preparative reversed phase liquid chromatography; sometimes combined with complementary isolation techniques.

Since one strong line of research within the Branch is focused around signal transduction, one of the most important types of peptides are those that are phosphorylated, either done post-synthetically or by using phosphoamino acids as building blocks. Other applications include preparation of anti-peptide antibodies, substrates or inhibitors or the preparation of affinity matrices. In the latter case, peptides are immobilized onto a matrix, and a cell lysate is passed over the construct. Proteins interacting with the ligand are retained and can be eluted and further purified by SDS-PAGE for subsequent identification.

Micropurification and sequence analysis of peptides

Even if mass spectrometric analyses are replacing certain applications where Edman degradation normally is used, we still do a lot of traditional amino acid sequence analyses in the PE-ABI Procise 494A instrument. Preparation of samples for sequence analyses are almost exclusively done by polyacrylamide gel electrophoresis; a technique we have used extensively for the last six years. The

"in-gel" digestion procedure offers isolation of internal proteolytic fragments of practically any type of protein; of particular interest are of course those with hydrophobic character that are almost impossible to isolate by conventional techniques. Membrane bound receptors, which play a key role in signal transduction, are found in this group.

Although very efficient, protein isolation by SDS-PAGE is commonly preceded by another highly resolving purification step, such as affinity chromatography in order to get homogeneous bands. A typical example is the use of an immobilized synthetic peptide as affinity matrix, as described above.

Analysis of proteins isolated by electrophoresis can be carried out at the low pmol level, if Edman degradation is used to analyze the peptides obtained through "in-gel" digestion. When, on the other hand, MALDI-TOF-MS is used for the analysis of proteolytic peptides, considerably lower quantities are needed. Sensitivities in the order of about 50 fmoles are now routine.

The ^{32}P based mapping of phosphorylation sites in proteins, which is an important special procedure in our Group, requires a sequencer which can collect fractions. Since this activity tends to increase, we took advantage of an offer to obtain a second-hand PE-ABI 477A sequencer, which together with our other 477A is used exclusively to run this method.

Mass spectrometry

Our Bruker Biflex MALDI-TOF mass spectrometer continues to be an extremely useful analytical instrument. Many of the protein identification analyses, previously done by Edman degradation, are now done quicker and at a much lower cost by peptide mass fingerprinting after in-gel digestion. In addition, it is approximately 50 times more sensitive. Indeed, we can analyze a protein in a gel visualized by silverstaining. In most cases, since salts interfere with the ionization process, we are desalting and concentrating the peptide extracts on a "nanocolumn", *i.e.* a reversed phase column of about 1 μl , packed in a narrow pipette tip. This allows not only the needed desalting, but also, by stepwise elution, a simple but important separation of the peptides in order of hydrophobicity.

Since the databases with protein sequences are increasing rapidly, it is likely that this way of analyzing proteins will be of even greater value in the future.

**PRIMARY
RESEARCH
PAPERS**

Published 1998

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