

ANNUAL SCIENTIFIC REPORT

Uppsala Branch

2008

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Sorrentino, Alessandro, Postdoctoral Fellow, to October
von Bülow, Verena, Postdoctoral Fellow
Ekman, Maria, Ph.D. Student
Hamidi, Anahita, Ph.D. Student
Marcusson, Anders, Ph.D. Student, to January

Gene Expression Group

Ericsson, Johan, Associate Member, Group Head, to December
Bengoechea Alonso, Maria Teresa, Postdoctoral Fellow, to December
Punga, Tanel, Postdoctoral Fellow, to June
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Pettersson, Gullbritt, Laboratory Assistant (part time)
Sandström Leppänen, Jill, Technical Assistant (on leave of absence), to May
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Administration

Hallin, Eva, Finance and Administration Manager

Secretariat

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Introduction

Cancer cells are characterized by perturbations in signaling pathways that regulate cell growth, survival, differentiation and migration. The aim of the work at the Uppsala Branch is to elucidate the molecular mechanisms that regulate these events. The expectation is that such knowledge will make it possible to develop means for better diagnosis, prognosis and treatment of cancer patients.

We study in particular two growth regulatory factors, *i.e.* platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β). PDGF is a family of dimeric isoforms of A-, B-, C- and D-polypeptide chains, which exert their cellular effects by binding to α - and β -tyrosine kinase receptors. PDGF isoforms have important roles during the embryonal development in the formation of different kinds of mesenchymal cell types. Overactivity of PDGF is implicated in autocrine as well as paracrine stimulation of tumors. Our aim is to elucidate the molecular mechanisms of signal transduction via PDGF receptors, and to explore the clinical utility of PDGF antagonists.

TGF- β family members act via heteromeric complexes of type I and type II serine/threonine kinase receptors, and have important roles during the embryonal development. Most often, the members of this family inhibit cell growth, but they also stimulate matrix production and induce apoptosis. In cancer, TGF- β is therefore initially a tumor suppressor. However, at later stages of tumor progression TGF- β becomes a tumor promoter through its ability to induce epithelial-mesenchymal transition which links to increased migration and metastasis. Additional pro-tumorigenic effects of TGF- β include its suppressive effect on the immune system and its ability to stimulate angiogenesis. Our aim is to explore the molecular mechanisms whereby TGF- β acts, and to investigate the possibility that selective TGF- β antagonists can be made that inhibit the protumorigenic effects of TGF- β while leaving its tumor suppressor effects unperturbed. The ultimate goal is to explore the clinical utility of such antagonists.

During 2008, the Group Leader for the Cytoskeletal Regulation Group, Pontus Aspenström, left for a professorship at the Karolinska Institute in Stockholm. Moreover, the Group Leader for the Gene Targeting Group, Johan Ericsson, left for a professorship at the University of Dublin. We thank Pontus and Johan for all their important work at our Branch, and wish them and their group members the best success at their new universities.

Our Institute is located at the Biomedical Center in Uppsala, using laboratory space provided by the University of Uppsala. Some of our progress during 2008 is described on the following pages.

C.-H. Heldin

PDGF Signaling Group

Introduction

Tyrosine phosphorylation of proteins is an essential component of signal transduction pathways that regulate cell growth, survival and death, as well as adhesion, migration and differentiation. Both protein tyrosine kinases and protein tyrosine phosphatases control cellular phosphotyrosine levels. Growth factors synthesized by activated stromal cells participate in the growth and survival of tumor cells, often by signaling through receptor tyrosine kinases. In addition, tyrosine kinase receptors are involved in the signals inducing tumor vascularization, a process that is necessary for both growth and metastasis of tumors.

The appreciation of the role of tyrosine kinases in the generation and progression of cancer has led to development of a number of anti-tumor drugs that specifically target tyrosine kinases. For example Gleevec, which targets the Bcr-Abl kinase, the PDGF receptors and c-Kit, has been successfully used in treatment of CML and gastrointestinal stromal tumors. Further understanding of the molecular mechanisms underlying tumor formation should generate a new wave of target-specific drugs. This, in combination with improved screening of patient material to determine the pathogenic alterations in the cancer cell signaling network for each tumor, should allow individualized treatments with improved efficiency.

The aim of the work in the PDGF Signaling Group is to elucidate the mechanism for signaling via PDGF receptors, and to explore the utility of PDGF antagonists in the treatment of tumors. The work is performed in two sections.

Section for Translational Research

The research in the Translational Research section is focused on the following two areas:

- PDGF receptors as cancer drug targets
- Termination of PDGF β -receptor signal transduction

PDGF receptors as cancer drug targets

In experimental models, inhibition of PDGF receptors on stromal cells increases tumor uptake of low molecular weight chemotherapy drugs. VEGF receptor inhibitors also

lowers tumor IFP, and we are currently investigating the effects of the combination of PDGF and VEGF receptor inhibitors on Kat4 tumor IFP in collaboration with K. Rubin, Uppsala University and R. Reed, Bergen University (Fig. 1). Both agents lowered the tumor IFP when given as monotherapies, with increasing effects over a four day treatment period. Surprisingly, combination therapy given for four days resulted in tumors displaying the same IFP as vehicle treated tumors. However, when PDGF receptor inhibitor was given for four days and the VEGF receptor inhibitor for the last two days of therapy, the tumor IFP was further reduced compared to either monotherapy.

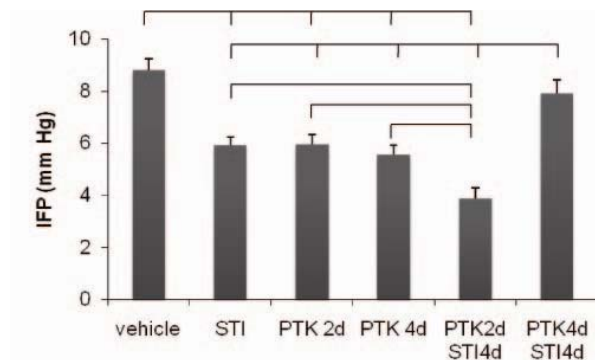


Figure 1. Effects of combination therapy targeting VEGFR and PDGFR on tumor IFP. Treatment of Kat4 tumors with a combination of PDGF receptor inhibitor (STI) for four days and VEGF receptor inhibitor (PTK) for two days was most efficient in lowering tumor IFP. The bars indicate statistically significant differences ($p < 0.05$).

When the anti-angiogenic drugs were given in combination with the chemotherapeutic agent taxol, anti-PDGF receptor inhibition and continuous combination therapy potentiated the effect of taxol to the same extent (Fig. 2). Metronomic anti-angiogenic therapy, with PDGF receptor inhibitor given four days before taxol injection in combination with VEGF receptor inhibitor present during the last two days, also improved the effect of taxol, but to a lesser extent than the continuous treatments.

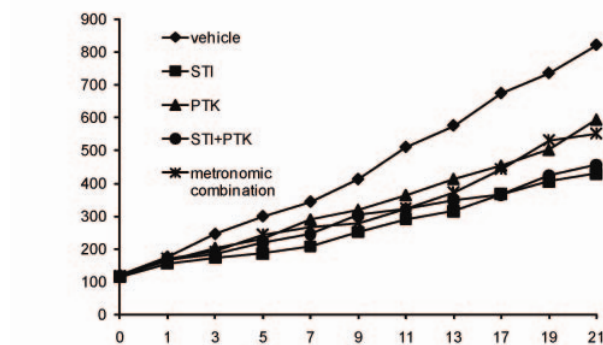


Figure 2. Effects of combination therapy targeting VEGF and PDGF receptors on tumor taxol sensitivity. Daily administration of PDGF receptor inhibitor (STI) induced the greatest potentiation of the ability of taxol to inhibit Kat4 tumor growth. No further effect of VEGF receptor inhibitor (PTK) was observed.

To further investigate the discrepancy observed between the effects of anti-angiogenic drugs on IFP and taxol treatment, we investigated the effects on the tumor water content. The therapies that provided the greatest effect on tumor growth in combination with taxol increased the extracellular water content in the tumor, indicating that

increased convection correlated better with the therapeutic outcome than the decrease in IFP did.

Termination of PDGF β -receptor signal transduction

Following ligand stimulation, the PDGF β -receptor signaling is terminated by dephosphorylation of the receptor autophosphorylation sites in parallel with receptor internalization and subsequent degradation. We have identified the T-cell protein tyrosine phosphatase as a site-selective regulator of PDGF β -receptor phosphorylation. The screening for tyrosine phosphatases that are regulating PDGF β -receptor phosphorylation and signal transduction is being continued, with a current focus on the LAR tyrosine phosphatase. Initial experiments showed that LAR, in contradiction to what was expected, was needed for maximum PDGF β -receptor phosphorylation to occur. It is possible that this phosphatase is required for activation of other kinases, such as Src family kinases, that participate in PDGF β -receptor phosphorylation.

We previously found that the increased PDGF β -receptor phosphorylation observed in T-cell phosphatase $-/-$ fibroblasts is paralleled by an induction of PDGF β -receptor recycling, which occurs through Rab 4 positive recycling endosomes (Karlsson *et al.*, Mol. Biol. Cell 17:4846-4855, 2006). Recycling of the PDGF β -receptor is also induced by stably knocking down the T-cell phosphatase in HeLa cells, providing a model system for studying which domains of the receptor that is required for sorting towards recycling. We have further utilized these two systems to identify pathways involved in receptor trafficking. By screening the trafficking of PDGF β -receptors carrying point mutations in HeLa cells, we identified the Ras pathway as a potential regulator of receptor trafficking. We have also identified activation of protein kinase C (PKC) α as a critical component in the sorting of the PDGF β -receptor into Rab 4 positive recycling endosomes (Hellberg *et al.*, submitted for publication). PKC α activation by the G-protein coupled lysophosphatidic acid (LPA) receptor also induced PDGF β -receptor recycling. Furthermore, activation of PKC by LPA potently increased the chemotactic response of wt fibroblasts to PDGF-BB. These findings suggest that the sorting of PDGF β -receptors on early endosomes could be a point of crosstalk between receptor subclasses.

Section for PDGF Signal Transduction

The research in the group is focused on the mechanisms by which PDGF induces cell proliferation and migration. Deregulation of these processes is involved in various

pathological conditions, including cancer. Through our studies of the molecular details of signal transduction the short term goal is to generate basic cell biological knowledge and in extension to use this information to identify and exploit novel drug targets for cancer treatment.

Negative and positive regulation of MAP kinase phosphatase 3 controls PDGF-induced Erk activation

The Erk MAP kinase has a well established role in regulating cell proliferation in response to growth factors and a large portion of human tumors has deregulated Erk activity, underscoring the importance of this signaling pathway in tumor formation. The activity of Erk is tightly regulated by various feed-back loops and one important regulator is MAP kinase phosphatase 3 (MKP3). We found that PDGF regulates MKP3, which is considered to be a phosphatase highly selective for Erk (39). Data obtained indicate that PDGF stimulation leads to a rapid phosphorylation and proteasomal degradation of MKP3, which was sensitive to Mek1/2 inhibition. By performing 2-dimensional tryptic phospho-peptide mapping on [³²P]orthophosphate labelled material from cells stimulated with PDGF in the absence or presence of a Mek1/2 inhibitor (UO126), we were able to identify two phosphorylated serine residues, Ser174 and Ser300. Mutation of Ser174 to alanine resulted in stabilization of MKP3, suggesting that phosphorylation of this site is important for MKP3 to enter the ubiquitin-proteasome degradation pathway. Furthermore, we found that after prolonged PDGF stimulation, the *mkp3* gene was activated and this resulted in re-synthesis of MKP3 protein which then dephosphorylated Erk. Downstream of the PDGF β -receptor the re-emerging MKP3 was again degraded allowing for a second wave of Erk phosphorylation. Reducing the MKP3 level by siRNA led to an increased Erk activation and mitogenic response to PDGF. In summary, Erk exploits MKP3 to regulate its own activity in two signal transduction phases: first in the initial amplification of PDGF-BB-induced Erk activation through phosphorylation-induced MKP3 degradation and later, after prolonged PDGF-BB stimulation, in the reduction in Erk activity via re-synthesis of MKP3.

Activation of Erk5 by PDGF is important for the kinetics of Akt phosphorylation

Erk5 is a less well characterized member of the MAP kinase family which has been implicated in regulation of cell proliferation, migration and survival in a cell type-dependent manner. Our studies have shown that PDGF stimulation transiently activates Erk5 in a manner requiring Src kinases. Furthermore, siRNA-mediated downregulation

of Erk5 results in a prolonged activation of the pro-survival kinase Akt. Preliminary data indicate that the effect of Erk5 downregulation on Akt phosphorylation may be mediated by a reduction in the phosphorylation status of PTEN. Current work aims to verify the preliminary data obtained so far and subsequently to investigate the importance of Erk5 in regulation of PDGF-mediated survival.

The role of Alix and HD-PTP in PDGF receptor downregulation

Appropriate PDGF signaling depends on receptor activation as well as receptor deactivation. Defects in either of these processes may contribute to malignant disease. We have previously studied the involvement of the adaptor protein Alix in the downregulation process and this work has during the last year been expanded by the studies on the related protein HD-PTP. Alix and HD-PTP are related proteins, but HD-PTP contains a putative phosphatase domain not present in Alix. We have found that when we overexpress HD-PTP the PDGF receptor's ability to induce Cbl phosphorylation is diminished. Phosphorylation of Cbl has been associated with an increase in its ability to induce ubiquitination of target proteins. In concurrence, preliminary data suggest that overexpression of HD-PTP cause defective PDGF receptor ubiquitination. Current work aims to elucidate how HD-PTP can influence Cbl tyrosine phosphorylation, for example can HD-PTP dephosphorylate Cbl or does it act as an adaptor protein.

The role of G3BP in PDGF-induced proliferation

G3BP is a nuclease that has been implicated in mRNA metabolism. Initially, G3BP was discovered as a RasGAP binding protein thus establishing a link between tyrosine kinase receptor signaling and post-transcriptional RNA turn-over. Using synthetic peptides corresponding to different phosphorylation sites in the PDGF receptor as baits in a proteomic screen, we detected an interaction between G3BP and a peptide corresponding to phosphorylated Tyr716, which is a well established Grb2 binding site. When we performed GST-Grb2 pulldown we found that G3BP bound to the Grb2 SH3 domains, but only in cells that had not been treated with PDGF. When we analyzed the gene expression of the AP-1 components *c-fos*, *c-jun*, *junB* and *junD* under conditions where G3BP expression was silenced using siRNA, we found a reduced expression of *c-fos* and *c-jun*, the pro-proliferative AP-1 components, and an increased expression of *junB*, an anti-proliferative AP-1 component. Preliminary data suggest that PDGF-induced proliferation is severely inhibited in the absence of G3BP. Our data suggest that G3BP interacts with Grb2 in resting cells and upon PDGF-BB stimulation

this complex is translocated to the active receptor due to the ability of the Grb2 SH2 domain to bind phosphorylated Tyr716, resulting in release of G3BP from Grb2. G3BP then modulate the expression of different AP-1 components, in a manner favoring cell proliferation.

Cytoskeletal Regulation Group

Our group studies various aspects of cell signalling mediated by Rho GTPases. This family of proteins has emerged as key regulators of cell morphogenesis, cell migration and cell polarity but they also participate in signalling pathways that control cell growth, cell cycle progression and cell survival. We have previously shown that the family of Rho GTPases consists of 20 members and that their effect on the organisation of the actin filament system is more intricate and complex than recognised before. Our goal is to identify how signalling involving Rho GTPases contribute to processes such as cell migration, cell growth and tumour progression.

The Rho GTPases

The Rho GTPases can be divided into two main categories, the classical Rho GTPases and the atypical Rho GTPases. We have put efforts into the identification of the atypical Rho GTPases (RhoBTB1-3, Rnd1-3, RhoH, Rac1B, Chp and Wrch-1). Previous studies have demonstrated that Wrch-1 is involved in the regulation of cell adhesion and cell morphology and cell transformation. We employed a yeast two hybrid approach to identify Wrch-1 targets and found the non-receptor tyrosine kinases Pyk2 and FAK. Interestingly, we found that the activity of Src was needed for the formation of a Wrch1-Pyk2 complex, as well as for the Wrch1-induced formation of filopodia. We propose a model in which Pyk2 and Src function to coordinate the Wrch1-dependent effects on cell adhesion and cytoskeletal dynamics (24). These observations demonstrate a novel functional link between Rho GTPases and signalling by non-receptor tyrosine kinases.

The RhoBTBs are involved in the regulation of protein stability and they have been suggested to function as tumour suppressors. Together with Cul3 and the ring-finger domain protein Roc1, RhoBTB forms an ubiquitin ligase complex, which ubiquitinylates specific substrate proteins and targets them for proteasomal degradation (2). Our preliminary results indicate that the RhoBTB/Cul3/Roc1 complex targets key components in cancer signalling pathways.

Miro GTPases

The Miro GTPases form a separate subfamily of the Ras-like GTPases. Miro harbours two putative GTP-binding domains separated by a linker region containing two calcium-binding EF hands. We have previously shown that Miro binds to mitochondria via a C-terminal transmembrane motif and that the Miro GTPases are essential components in the machinery responsible for mitochondrial motility along the microtubules. The present study demonstrates that Miro GTPases function as Ca^{2+} sensors by regulating mitochondrial motility in a Ca^{2+} -dependent manner. Resting Ca^{2+} concentrations allow mitochondria in neuronal cells to move in anterograde as well as retrograde direction. In contrast, Ca^{2+} pulses, triggered for instance by neurotransmitter release, result in a rapid arrest of mitochondrial movement. Interestingly, this Ca^{2+} -dependent mitochondrial arrest is dependent on Miro (26). We are currently evaluating the role of Miro GTPases in neuropathological conditions as well as diseases caused by deregulated mitochondrial function.

Regulators and effectors of Rho GTPases

We have studied signalling components responsible for linking receptor tyrosine kinases (RTKs) to Rho-dependent actin reorganisation. The Nck adapters constitute one important group of RTK-regulated proteins. The present study employed mouse embryonic fibroblasts (MEFs) derived from Nck knock out mice. The mutant cells had a reduced ability to form edge ruffles in response to PDGF and the presence of Nck was obligatory for the formation of dorsal ruffles (25). In addition, the mutant cells had a reduced chemotactic and migratory potential. Importantly, KO cells had reduced cell attachment properties and a reduced ability to form focal adhesions in response to signalling involving the Rho GTPases (64). F-BAR domain-containing proteins, such as the Cdc42-binding protein 4 (CIP4), also have critical roles in linking RTK signalling to actin reorganisation. The F-BAR domain binds lipid bilayers and induces membrane deformation, a process occurring in vesicle formation and endocytosis. We have found a role for CIP4-like proteins in the regulation of trafficking of tyrosine kinase receptors (43, 64). We are currently studying the involvement of CIP4 and other F-BAR proteins in receptor activation and trafficking in health and disease.

TGF- β Signaling Group

The TGF- β Signaling Group studies new mechanisms by which the signaling pathways of TGF- β and of the related polypeptide factor bone morphogenetic protein (BMP) can

be regulated. The role of these growth factor pathways and of additional factors such as protein kinases and transcription factors on processes such as epithelial cell differentiation, proliferation, tumor cell invasiveness, metastasis and cancer stem cell self-renewal and pluripotency are major lines of research in the group.

Regulation of TGF- β /BMP/Smad signaling

TGF- β s, BMPs and other family members recognize receptor serine/threonine kinases on the cell surface (54). These receptors phosphorylate intracellular Smads called R-Smads and some additional signaling proteins. Phosphorylated R-Smads associate with the Co-Smad (Smad4), and rapidly accumulate in the nucleus. Nuclear Smad complexes regulate expression of a few hundred genes in a cell type-dependent manner by binding to DNA and to several transcription factors. One of the immediate-early TGF- β -responsive genes is *Smad7* that encodes for an inhibitory Smad (I-Smad). Smad7 inhibits signaling at the receptor and nuclear Smad level. Smad7 binds to the TGF- β /BMP receptors and inhibits R-Smad phosphorylation by the receptors, induces receptor de-phosphorylation by phosphatases and ubiquitination by ligases of the Smurf family that regulate TGF- β receptor internalization and turnover (60).

Continuing our activity in previous years, we attempt to identify novel regulators of Smad function in the nucleus that operate at the chromatin level. We also analyze Smad protein turnover and the role of ubiquitination in this process, by focusing on the Smurf family of ubiquitin ligases. Via genome-wide transcriptomic analysis, we previously found that the *salt-inducible kinase (SIK)* gene represents a new TGF- β -responsive gene, whose serine/threonine kinase product regulates turnover of the TGF- β receptor after ligand binding (14). SIK cooperates with Smad7 to regulate the TGF- β receptor (Fig. 3). We now extend this study by analyzing the role of the Smurf ubiquitin ligases as a co-factor of SIK. SIK is a member of the AMP-regulated kinases (AMPK), which are best known as substrates of the master kinase and tumor suppressor LKB1.

Peutz-Jeghers Syndrome (PJS) patients develop benign hamartomatous polyps in early age and are predisposed to intestinal or other forms of cancer in adult life. PJS is caused by loss-of-function mutations in the *LKB1* gene. PJS hamartomas resemble those in Juvenile Polyposis Syndrome (JPS) patients, who inherit inactivating mutations in the *Smad4* or the BMP type I receptor, *BMPRIA* genes. We try to uncover the molecular links between TGF- β , BMP and LKB1/AMPK signaling.

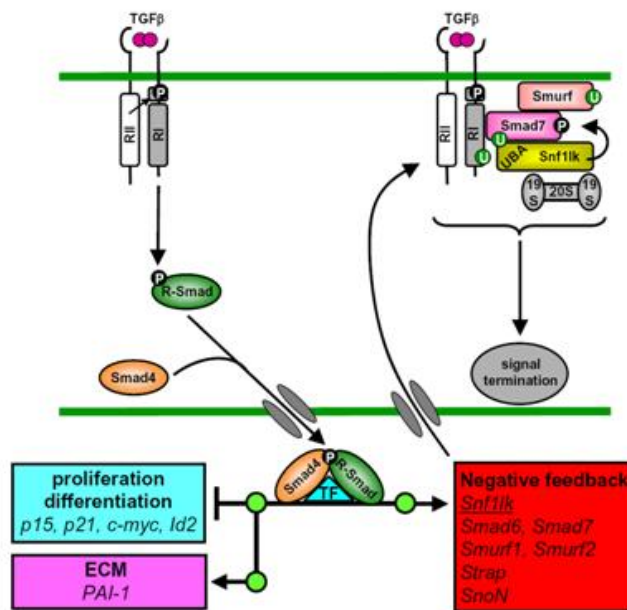


Figure 3. Diagrammatic picture of the negative feedback loop of TGF- β /Smad signaling emphasizing the role of SIK (SNF1LK). The incoming Smad complexes regulate various gene targets (green circles) leading to diverse physiological responses. SNF1LK/SIK binds to Smad7 and regulates the ubiquitin ligase Smurf, thus downregulating the TGF- β receptor complex.

Epithelial-mesenchymal transition and regulation of cancer stem cells

TGF- β regulates diverse aspects of adult tissue homeostasis (53). In addition, TGF- β plays important roles during cancer progression and one of its most intensely studied roles recently is its ability to promote the process of epithelial-mesenchymal transition (EMT), the terminal stages of tumor cell invasiveness and metastasis (58, 62). EMT and late processes of cancer progression, including metastasis, have recently been linked to the properties and actions of a rare cancer stem cell population.

In an effort to explore the relationship between EMT and cancer progression we found that long-term exposure of mammary epithelial cells to TGF- β promotes EMT and enhances tumor growth due to enhanced secretion of chemokines (9). Tumors obtained from cells that had undergone EMT were enriched in cells that could differentiate into mammary epithelia and thus might resemble mammary stem cells. Deeper molecular analysis of the EMT process established that the nuclear factor HMGA2, whose levels are induced by TGF- β , can bind directly to Smad proteins and together Smads and HMGA2 induce expression of major factors that promote EMT, such as Snail (29). This work has also revealed that HMGA2 and Smads may regulate the expression of many other effectors of EMT (Fig. 4), a direction that we currently follow. In addition, we analyze the regulation of *Snail*, and other transcription factors of the EMT program, by a network of signaling pathways including receptor tyrosine kinases and Notch. Influenced by our work on LKB1 and AMPKs we perform screens to identify members of the AMPKs that regulate the EMT process.

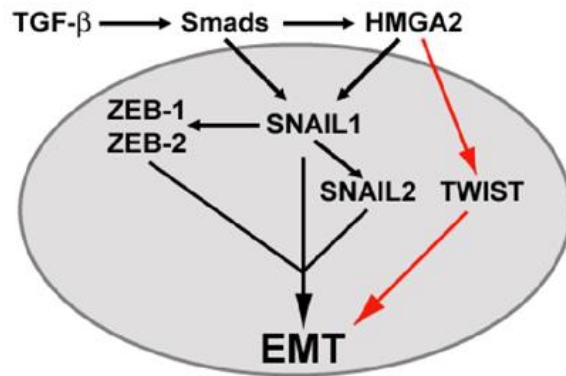


Figure 4. Diagrammatic model of the role of Smad and HMGA2 proteins in regulation of major transcription factors that control the process of EMT.

HMGA2 has recently been shown to regulate the ability of normal brain stem cells to self renew. We therefore try to analyze the role of TGF- β and HMGA2 in the survival and tumor-initiating potential of cancer stem cells of the breast and brain (glioblastoma multiforme). We have therefore unraveled a complex regulatory network that defines the cytostatic and EMT programs of epithelial cells in response to TGF- β . Our findings have direct relevance to the mechanisms by which TGF- β acts as a tumor suppressor or a pro-metastatic factor and direct our research towards novel therapeutic approaches.

Evolutionary analysis of TGF- β signaling and bioinformatics

We have also analyzed the evolutionary origins of the TGF- β superfamily pathways based on new algorithms and sequence data of all available metazoan genomes and found that this pathway evolved together with the metazoan origins of life (37). Furthermore, we use mathematical modelling and bioinformatics to analyze novel behaviour of the signaling pathway and novel targets of TGF- β signaling in cancer.

Apoptotic Signaling Group

TGF- β plays an important role in the regulation of cell fate during embryogenesis. In the adult organism, it inhibits proliferation and induces apoptosis in most normal cell types, and therefore acts as a tumor suppressor, while at late stages of tumor progression TGF- β instead promotes tumorigenesis by facilitating tumor cell invasiveness, stimulating angiogenesis and suppressing the immuno-surveillance. High levels of TGF- β is closely related to initiation and progression of prostate cancer, leading to lethal metastases (63).

Smad7 is a target gene for TGF- β and we have demonstrated that Smad7 is required for TGF- β -induced apoptosis in prostate cancer cells. Furthermore, we have reported that

Smad7 is important for activation of the TGF- β activating kinase 1 (TAK1), mitogen activated protein kinase kinase 3 (MKK3) and p38 MAP kinase pathway, presumably by acting as an adaptor protein bringing the kinases close to each other.

The aim of our work is to in detail investigate the molecular mechanisms for the activation of the TAK1 – p38 MAPK pathway by cytokines like TGF- β , TNF- α and IL-1, as well as the potential cross-talks between these inflammatory cytokines. We also continue to investigate the targets for Smad7 and p38 in the TGF- β signaling pathway. We also use advanced molecular pathology techniques in prostate cancer tissues, to achieve a profound understanding of the role for TGF- β signal transduction in prostate cancer tumor biology, in order to identify novel potential drug targets and development of individual prognostic biomarkers.

The type I TGF- β receptor recruits the E3-ligase TRAF6 leading to apoptosis

We have reported that the TGF- β receptor interacts with TAK1, and that activation of TAK1 requires Lys63-dependent ubiquitination regulated by the E3-ligase TRAF6. This molecular modification of TAK1 determines biological responses downstream of the active receptor complex (27). This novel finding provides a molecular mechanism for a non-Smad signaling pathway from the TGF- β receptors. Interestingly, our results show that TGF- β -induced activation of the p38 MAPK pathway is not dependent on the kinase-activity in the type I TGF- β receptor. Intriguingly, we have identified a consensus binding site for TRAF6 in the type I TGF- β receptor (27) which explains how TRAF6 interacts with the type I TGF- β receptor. Ligand-induced oligomerization of the receptor-complex thus causes auto-ubiquitination and activation of TRAF6, followed by Lys63-dependent poly-ubiquitination and activation of the TAK1 – p38 MAPK pathway (Fig. 5).

Does Smad7 act in a cross-talk between TGF- β and Wnt signaling?

Signaling molecules downstream of TGF- β and Wnt receptors regulate both cell fate and proliferation during development and tissue homeostasis. We have recently reported that Smad7 interacts with β -catenin and lymphoid enhancer binding factor 1/T-cell-specific factor (LEF1/TCF), transcriptional regulators in Wnt signaling in a TGF- β -dependent manner. Furthermore, by the use of siRNA and anti-sense techniques, we have shown that Smad7 expression is required for TGF- β -induced stabilization of β -catenin, increase of c-Myc and subsequent apoptosis in human prostate cancer cells and keratinocytes. Interestingly, we have identified Smad7 together with p38, as novel

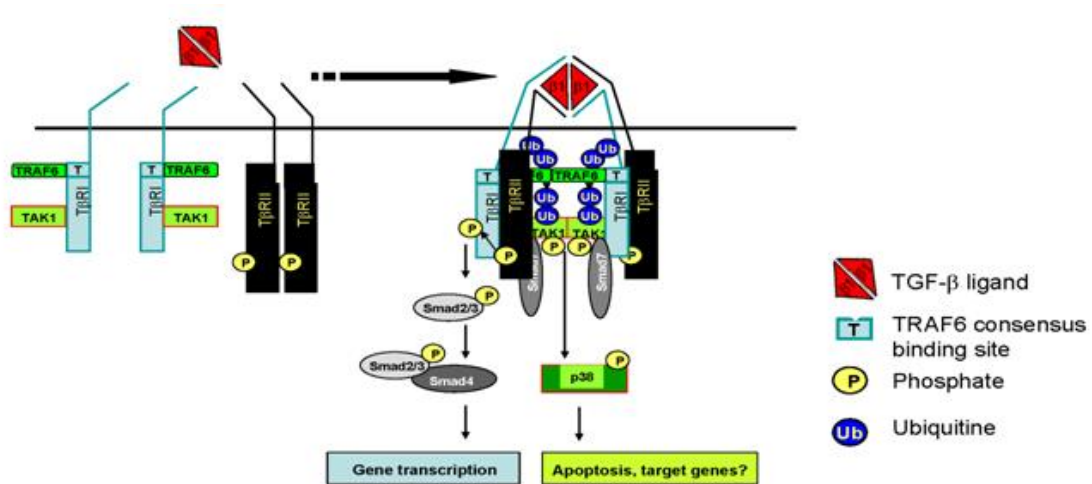


Figure 5. Dimerization of ligand induces receptor oligomerization, leading to kinase-independent Lys63-dependent autoubiquitination and activation of TRAF6 which next causes Lys63-dependent ubiquitination and activation of TAK1.

regulators of the activity of glycogen synthetase kinase-3 β (GSK-3 β) which in turn leads to stabilization of β -catenin (36). This event is crucial for TGF- β induced directed cell migration of prostate cancer cells. We will also continue to further examine the underlying molecular mechanisms by which Smad7 and p38 MAPK affect other key components in the Wnt-signaling pathway.

Smad7 target genes

Smad7 is a nuclear protein and TGF- β stimulation of cells leads to an export of Smad7 to the cytoplasm, where it by binding to the activated TGF- β receptor complex both acts as an inhibitor of Smad activation and as an adaptor protein for activation of the p38 MAPK pathway as mentioned above.

We are interested in the possibility that Smad7 has direct effects in the nucleus on gene regulation and have therefore performed microarray analyses on cells over-expressing Smad7. Candidate genes are presently being validated by RT-PCR and chromatic immunoprecipitation analysis (Thakur *et al.*, manuscript in preparation).

Inflammation in relation to prostate cancer biology

The role of TAK1 in chronic inflammatory reactions in the prostate is an interesting topic for our current investigations. We are therefore further exploring the role of active TAK1 for biological responses evoked by inflammatory cytokines like TNF- α and IL-1 β (manuscript in preparation). To further investigate the role for TGF- β 1 and

inflammation for tumor initiation and progression in prostate cancer, we are in the process of establishing novel model systems *in vitro* and *in vivo*.

Gene Expression Group

Our group is interested in how post-translational modifications regulate the activity of transcription factors. As model proteins, we have selected certain transcription factors involved in the regulation of cell growth and proliferation.

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 (Bengoechea *et al.*, *Curr. Opin. Cell Biol.* 19:215-222, 2007). We hypothesize that compounds that enhance the stability of SREBPs should increase the levels of transcriptionally active SREBP in cells. Such compounds could potentially be used to enhance the cholesterol-lowering activities of statins.

Transcriptionally active SREBP molecules are rapidly degraded by the ubiquitin-proteasome pathway. The ubiquitin ligase Fbw7 has been shown to control several proteins critical for cell cycle control, and is inactivated in cancers of the breast, endometrium, ovary and colon. We previously demonstrated that Fbw7 enhances the ubiquitination and degradation of SREBP following GSK-3-mediated phosphorylation of two specific Ser and Thr residues in SREBP, Thr426 and Ser430. We have also found that the GSK-3 β -dependent phosphorylation of these residues in SREBP1 is enhanced in response to DNA binding. DNA binding enhanced the direct interaction between the C-terminal domain of SREBP1 and GSK-3 β . Accordingly, we could demonstrate that GSK-3 β was recruited to the promoters of SREBP target genes *in vivo*. As a result of the phosphorylation of Thr426 and Ser430 in response to DNA binding, Fbw7 was recruited to SREBP molecules associated with target promoters. Using a reconstituted ubiquitination system, we could demonstrate that Fbw7-mediated ubiquitination of SREBP1 was dependent on its DNA binding activity. Thus, DNA binding could provide a link between the phosphorylation, ubiquitination and degradation of active transcription factors. Fbw7 and its interaction with SREBPs may be attractive targets for developing new cholesterol-lowering therapies for the fight against cardiovascular disease. During the last year, we have identified a number of new phosphorylated residues in SREBP1 that are involved in its Fbw7-dependent

degradation and started to identify the signals and factors controlling these modifications.

The SREBP family of transcription factors regulates genes involved in the synthesis of lipids, which are required for the formation of cell membranes. We recently found that SREBP-mediated transcription is regulated during the cell cycle as a result of specific modifications of the SREBP proteins. We found that the mature forms of SREBP1a and SREBP1c are hyperphosphorylated in mitotic cells, giving rise to a phosphoepitope recognized by the mitotic protein monoclonal-2 (MPM-2) antibody. We also found that mature SREBP1 was stabilized in a phosphorylation-dependent manner during mitosis. We have now mapped the major MPM-2 epitope to Ser439, in the C terminus of mature SREBP1. Using phosphorylation-specific antibodies, we could demonstrate that endogenous SREBP1 is phosphorylated on Ser439 during mitosis. Our results suggest that Cdk1-mediated phosphorylation of Ser439 is required for the hyperphosphorylation and stabilization of mature SREBP1 during mitosis, thereby preserving a critical pool of active transcription factors to support lipid synthesis. This possibility was supported by our observation that siRNA-mediated inactivation of SREBP1 arrested cells in the G1 phase of the cell cycle, thereby attenuating cell growth. It may be important to stabilize nuclear SREBP1 during mitosis, since the membrane systems that regulate the cleavage of the precursor form of the protein is dispersed during this phase of the cell cycle. We hypothesize that the hyperphosphorylated, stable, form of SREBP1 is associated with specific target genes during mitosis, ready to transactivate these genes as cells leave mitosis and an active chromatin structure is reformed. Thus, we speculate that SREBP1 is involved in an epigenetic mechanism that retains the expression of specific genes after cell division. Such a mechanism could be of utmost importance for cell cycle progression and cell growth. We now propose to test this hypothesis and identify the factors and mechanisms involved in this process. Altogether, our data support the hypothesis that deregulation of lipid synthesis could facilitate the growth and proliferation of cancer cells. During the last year, we have identified a number of new phosphorylated residues and factors involved in the hyperphosphorylation and stabilization of SREBP1 during mitosis. We are currently analysing the roles of these modifications and factors in SREBP-dependent processes.

Matrix Biology Group

Our research is focused on understanding how the stromal microenvironment influences the proliferative and invasive behavior of malignant cells. We aim to dissect signaling pathways downstream of the hyaluronan receptor CD44, and to elucidate mechanisms

whereby CD44 modulates the activation and signaling specificity of growth factor receptors, including the receptors for PDGF and TGF- β . We also investigate the growth factor regulation of hyaluronan biosynthesis and the biological significance of the interaction between hyaluronan and its receptor CD44 in promoting breast tumor progression, using 3D cell culture models in order to recapitulate the *in vivo* 3D milieu.

Hyaluronan promotes the malignant phenotype of tumor cells

Hyaluronan is an extracellular and cell-associated polysaccharide, which has a key role in tissue homeostasis. During cancer progression, hyaluronan acts as a microenvironmental stimulus that promotes both cell proliferation and migration, and may serve as a niche component for tumor cancer stem cell populations. The levels of hyaluronan are balanced through its biosynthesis by hyaluronan synthases (HASs) and catabolism by hyaluronidases (HYALs); these enzymes are differentially regulated by growth factors, including PDGF-BB and TGF- β that under pathological conditions are produced in excess. The general concept emerging from our studies is that HAS2 overexpression leads to a faster development of transplantable tumors in syngeneic rats, compared to mock transfectants. In contrast, HYAL1 overexpression suppressed the growth rate of tumor cells both *in vitro* and *in vivo*. The importance of HAS2 in the maintenance of the malignant and invasive phenotype of tumor cells was further studied by investigating the consequences of *HAS2* gene suppression, by specific silencing using siRNAs. Interestingly, suppression of *HAS2* slows down the growth as well as the migratory and invasive capacities of aggressive breast cancer cells.

In order to elucidate the mechanism of the aberrant accumulation of hyaluronan during tumor progression and persistent inflammation, and unravel its essential roles in tissue homeostasis and cellular functions, we have undertaken to investigate the regulatory mechanisms of HAS activities. We could demonstrate that HAS2 forms a homodimer, as well as a heterodimer with HAS3; the functional importance of the dimerization remains to be determined. We have also demonstrated that HAS2 activity and expression are post-translationally regulated through both mono- and poly-ubiquitination (Karousou *et al.*, in preparation).

Additionally, we investigate the effects of peritumoral hyaluronan in breast cancer cell invasion and microvascular endothelium adherence. The analyses have revealed that breast tumor cell-retained hyaluronan is important for their adhesion to microvascular but not to lymphatic endothelium (Bernert *et al.*, in preparation).

Hyaluronan-CD44 complexes regulate growth factor receptor activity

CD44s is an adhesion receptor with an extracellular hyaluronan binding domain and a stalk region which varies as a result of differential splicing, as well as intracellular FERM-, ankyrin- and PDZ-binding domain motifs, all of which participate in intracellular signaling (Fig. 6). CD44 has been proposed to be an important marker for breast cancer-initiating cells, and its aberrant expression is associated with persistent inflammation and malignant transformation. We and others have demonstrated that there is a cross-talk between CD44 and growth factor receptors including the receptors for PDGF-BB, TGF- β , hepatocyte growth factor and epidermal growth factor. Recent studies in our laboratory revealed that the PDGF β -receptor and CD44 form a complex resulting in inhibition by hyaluronan stimulation of PDGF β -receptor phosphorylation and fibroblast migration. The effect most likely involves the CD44-mediated docking of a tyrosine phosphatase (PTP) to the PDGF β -receptor. In an attempt to identify the CD44-associated PTP, as well as other proteins that may interact with CD44 and explain the effect of CD44 on growth factor receptor signaling, we have used pull down approaches. As baits we have used a GST fusion protein of the intracellular domain of CD44, as well as synthetic peptides from the cytoplasmic domain of CD44 that were biotinylated and immobilized onto streptavidin-conjugated Dynabeads. Proteins from cell lysates that bound to the CD44 sequences were separated by SDS-PAGE and identified by Maldi-TOF-MS. The analysis revealed several interesting candidate proteins; their functional significance for CD44-mediated cellular functions are currently under investigation.

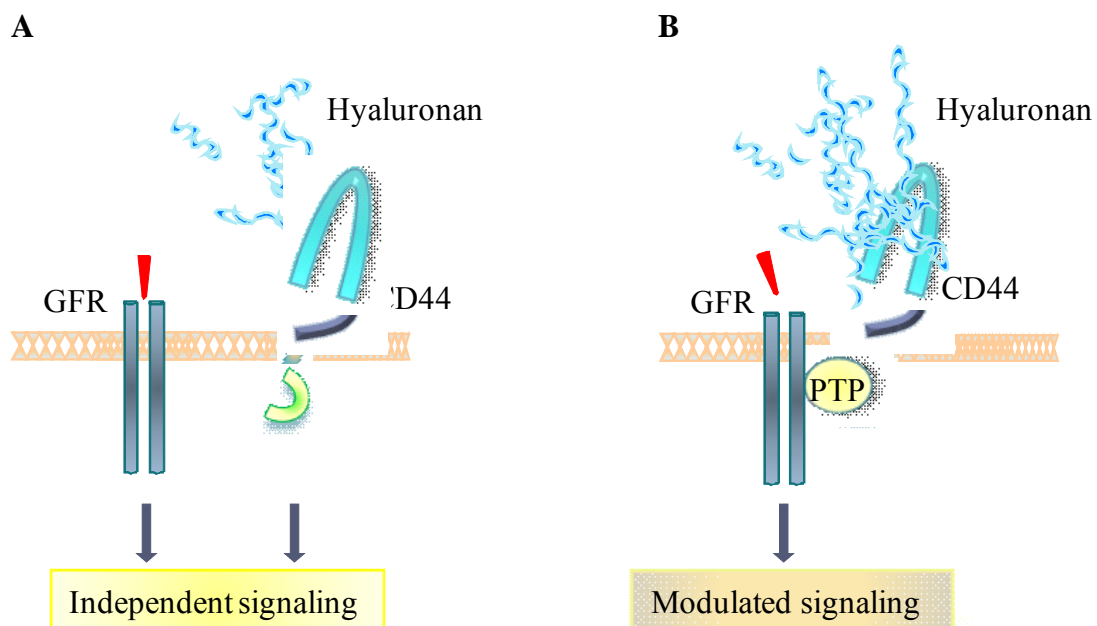


Figure 6. Mode of regulation of GFR signaling by CD44. (A), Receptors act independently. (B), Formation of a complex leads to modulation of signaling.

Cooperative activities between tumor epithelial cells and surrounding stroma are required for disease progression of various cancer types. The interplay of CD44 with its ligand hyaluronan and associating molecules modulates cancer cell behavior. Understanding of the underlying molecular mechanisms may help to design therapeutic interventions to prevent tumor invasiveness and metastasis.

Protein Structure Group

Our main mission is to support the activities within the Branch with our specialized techniques, and when possible, to interact with other Branches in Sweden and abroad. Also, when time allows, we gladly collaborate with other research groups. This has over the years provided a broad experience with applications onto various types of projects. The Protein Structure Group has a solid experience in peptide synthesis, radio-labeled amino acid sequencing and MALDI TOF mass spectrometry. For the latter activity, we use a top of the line matrix-assisted-laser-desorption-ionization-time-of-flight-mass-spectrometer (MALDI-TOF-MS). The present instrument (Bruker Ultraflex III TOF/TOF), installed in March 2007, offers a dramatic increase in sensitivity and accuracy for both MS and especially for MS/MS, user friendliness and peptide sequencing possibilities compared to the previous instrument. This latter property is a consequence of the extra “TOF” which allows the development full fragment spectra in few seconds. Our three main activities are described in more details below.

Peptide synthesis

Contrary to the trend of outsourcing this highly specialized skill, we prefer to take advantage of our long gained experience. Our synthesizer, a nine-year-old Applied Biosystems 433A instrument, is operated with Fmoc chemistry, and produces high quality peptides. The synthesis of peptides modified with phosphorylations, acetylations, oxidations or other chemical groups at a given amino acid residue, has been most useful for the different groups at our Branch. All peptides are worked up manually and often, depending on the intended use, purified to homogeneity by liquid chromatography. The products are quality controlled using MALDI-TOF/TOF-MS. The peptides produced are used to generate anti-peptide antibodies, as substrates or inhibitors, or as ligands in affinity chromatography experiments. An important extension has developed over the last years – we now also carry out affinity purification of anti-peptide antibodies in collaboration with other groups of our Branch. Another, now well established, development in our Group is that we, in collaboration with other Groups perform affinity based searches for interacting partners using immobilized

ligands (*i.e.* a modified synthetic peptide). The combination of the biotin-streptavidin system and magnet beads has showed to be very powerful.

Radiolabeled phosphopeptide mapping

The introduction in our group of MALDI-TOF-MS eleven years ago has made peptide sequencing (see below) faster, more sensitive and a dramatically lowered running cost, compared to classical chemical amino acid sequence analysis ("Edman degradation"). However, the designated peptide sequencer has been remodeled to perform position analysis of phosphorylated (^{32}P) Tyr, Ser and Thr residues in proteins.

Sample preparation for mass spectrometry

Over 95 % of the samples for analysis by MALDI-TOF/TOF-MS are delivered as bands or spots from one- or two-dimensional SDS-PAGE gels. With Coomassie-visible material only a few percent is needed for analysis; with silver-stained material, often the entire sample must be applied after concentration and desalting on micro RPC columns (*i.e.* $\mu\text{ZipTips}$). We have recently introduced a novel approach to enrich for acidic, especially phosphorylated peptides, highly important but notorious for low sensitivity by MALDI TOF MS. This is based on homemade micro columns comprising TiO_2 particles. This has proven to increase the sensitivity significantly for this group of highly relevant peptides.

Peptide mass fingerprinting (PMF)

Determining protein identity by PMF is a routine procedure for known proteins. After the generation of a proteolytic digest (most commonly performed *in-gel*) and determination of peptide masses by MALDI-TOF/TOF-MS, we employ a search engine (ProFound or MASCOT are preferred) to search a match with a protein in the sequence databases. If a significant mass spectrum is obtained, we are practically always able to identify the protein with high confidence. Should this not be the case (*i.e.* if the searched protein is not found in any database or if that particular protein has an unfavorable distribution of target amino acids), the identity may be established by obtaining the amino acid sequence of one or more tryptic peptides (see below). A sequence homology search by BLAST is, in contrast to PMF, tolerant to amino acid substitutions. We have taken up a technique of Lys-modification (an imidazol derivative), which renders lysine containing peptides more basic and therefore increases the sensitivity resulting in higher sequence coverage. As a bonus, such peptides are easy

to fragment for sequence analysis. Furthermore, the Lys-Tag reagent may be used to label a set of proteins with deuterium for relative quantifications. Over the last year, we have carried out interesting proteomics projects with groups the Karolinska Hospital (20, 21, 22) looking for proteins that are significantly changed in various tumors.

Post Source Decay (PSD) based peptide sequencing by MALDI-TOF/TOF-MS

Fragment analysis of peptides by MALDI-TOF/TOF-MS using PSD is a straightforward technique. The resulting fragment spectra are commonly used for protein identification, but are difficult to use for *de novo* sequencing. The TOF/TOF technology allows complete PSD spectra to be generated in seconds, so it is possible to scan through several peptides from one digest in a short time. This easy and quick PSD combines extremely well with the Chemically Assisted Fragmentation (CAF) derivatization protocol, leading to easily interpreted spectra, as they comprise a unique series of γ -ions. Hence, an amino acid sequence can unambiguously (with the single exception of the isobaric Leu/Ile) be determined faster, cheaper and more sensitive than was ever possible using chemical Edman degradation. We use CAF-PSD for identification of un-characterized species, as well as for analysis of modified peptides. An example where we identify a phospho-Ser by sequencing after sulfonation is given in Fig. 7. As the CAF reagent was discontinued early 2008, we have started to replace it by SPITC (4-sulfophenyl-isothiocyanate), which is a much cheaper compound with similar positive effects on peptide fragmentation by MALDI-TOF/TOF.

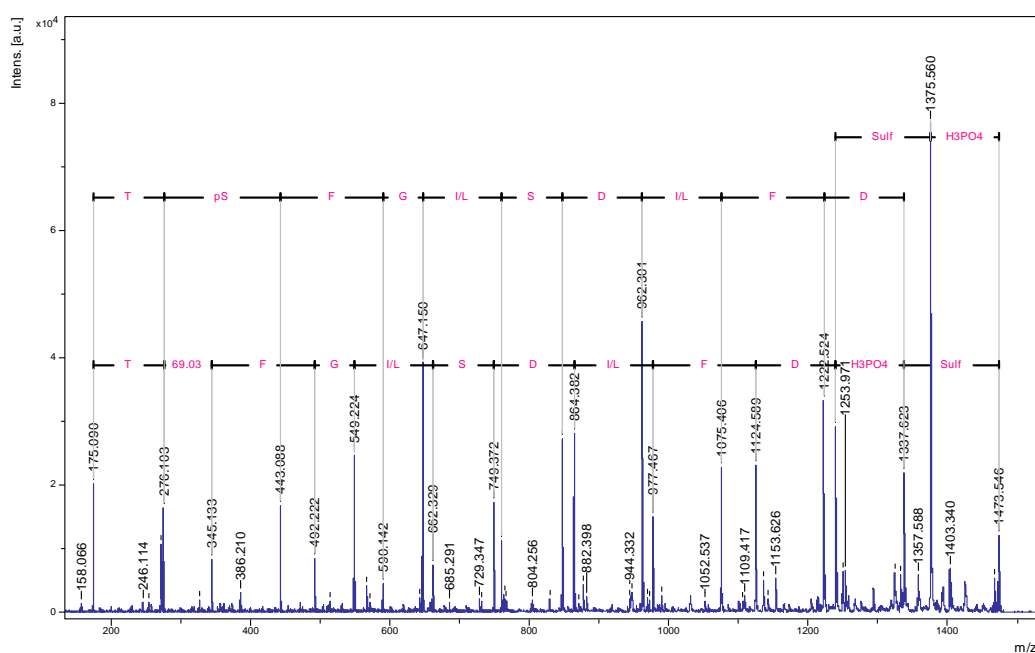


Figure 7. The peptide D F I/L D S I/L G F S TR is phosphorylated on a Ser residue, but as it has two Ser we cannot tell the exact position. By sequencing we could clearly determine that only the second Ser is modified.

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