“TRANSLOCATION PROTEOMICS: A NOVEL PROTEOMIC APPROACH FOR THE IDENTIFICATION OF SIGNALLING INTERMEDIATES”

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IV LIST OF ABBREVIATIONS

2-DE	**Two dimensional gel electrophoresis**
ACN	**Acetonitirile**
ACVRII	**Activin type II receptor**
ACVRII-B	**Activin type II-B receptor**
ALK1	**Activin receptor like kinase 1**
ALK2	**Activin receptor like kinase 2**
ALK3	**Activin receptor like kinase 3**
ALK4	**Activin receptor like kinase 4**
ALK5	**Activin receptor like kinase 5**
ALK6	**Activin receptor like kinase 6**
ALK7	**Activin receptor like kinase 7**
ALK8	**Activin receptor like kinase 8**
AMH	**Anti-Müllerian hormone**
AMHRII	**Anti-Müllerian hormone type II receptor**
APS	**Ammonium persulfate**
BAMBI	**BMP and activin membrane-bound inhibitor**
BMP	**Bone morphogenetic protein**
BMPRII	**Bone morphogenetic receptor 2**
CA	**Carrier ampholitcs**
CE	**Capillary electrophoresis**
CF	**Cytosolic fraction**
CE-MS	**Capillary electrophoresis-Mass spectrometry**
CHAPS	**3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate**
cDNA	**Complementary deoxyribonucleic acid**
Co-Smad	**Common Smad**
CRAC	**Cultured rat articular chondrocytes**
DAPI	**4´, 6´-diamidino-2-phenylindole**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
DTT	**Dithiothreitol**
ECD	**Extracellular domain**

EDTA	**Eythelene diamino tetra acetic acid**
EGF	**Epidermal growth factor**
EGFR	**Epidermal growth factor receptor**
EGTA	**Ethylene glycol bis (2-aminoethyl ether)-*N,N,N'N'* tetraaceticacid acid**
ERK	**Extracellular signal regulated kinase**
ESI	**Electrospray ionisation**
ESI-TOF-MS/MS	**Electrospray ionisation-time of flight-mass spectrometry/mass spectrometry**
FBP	**FUSE-binding protein**
FCS	**Fetal calf serum**
FITC	**Fluorescein isothiocyanate**
FPAH	**Familial pulmonary arterial hypertension**
FRET	**Fluorescence resonance energy transfer**
FUSE	**Far upstream element-binding protein 1**
GC-MS	**Gas chromatography-mass spectrometry**
GDNF	**Glial cell-line-derived neurotrophic factor**
GFP	**Green fluorescent protein**
HGF	**Hepatocyte growth factor**
HHT	**Hereditary hemorrhagic telangiectasia**
hnRNA	**Heterogeneous nuclear RNA**
HPLC	**High performance liquid chromatography**
HPLC-MS	**High performance liquid chromatography-mass spectrometry**
HRP	**Horseradish peroxidase**
HSP	**Heat shock protein**
IEF	**Isoelectric focusing**
IDA	**Iminodiacetic acid**
IL-1	**Interleukin-1**
IMAC	**Immobilized metal-ion affinity chromatography**
I-Smad	**Inhibitory Smad**
IPG	**Immobilized *pH* gradient**
JNK	**Jun N-terminal kinase**
KHSRP	**K homology type splicing regulatory protein**

LC-MS/MS	**Liquid chromatography**
MALDI	**Matrix assistant laser desorption ionisation**
MALDI MS	**Matrix assistant laser desorption ionisation mas spectrometry/mass spectrometry**
MALDI-TOF-MS	**Matrix assistant laser desorption ionisation-time of flight-mass spectrometry**
MAPK	**Mitogen-activated protein kinase**
MH1	**Mad homology domain 1**
MH2	**Mad homology domain 2**
MS	**Mass spectrometry**
MS/MS	**Mass spectrometry-mass spectrometry**
MW	**Molecular weight**
NF	**Nuclear fraction**
NLS	**Nuclear localization signal**
NTA	**Nitriloacetic acid**
PBS	**Phosphate-buffered saline**
PCR	**Polymerase chain reaction**
pI	**Isoelectric point**
PMDS	**Persistent müllerian duct syndrome**
PMAC	**Phosphate Metal Affinity Chromatography**
PKA	**Protein kinase A**
PKC	**Protein kinase C**
PMF	**Peptide mass fingerprint**
pSmad2	**phospho Smad2**
PTM	**Posttranslational modifications**
RNA	**Ribonucleic acid**
R-Smad	**Receptor Smad**
RT	**Reverse transcriptase**
SARA	**Smad anchor for receptor activation**
SDS-PAGE	**Sodium dodecyl sulfate polyacrylamide gel electrophoresis**
α-SMA	**Alpha smooth muscle actin**
Smurf	**Smad mediated ubiquitin regulatory factor**
TFA	**Trifluoro acetic acid**
TED	**Tris-(carboxymethyl)-ethylendiamine**

TEMED	***N,N,N',N* - tetramethyl ethylenediamine**
TGF-β	**Transforming growth factor-beta**
TOF	**Time of flight**
TβRII	**Transforming growth factor-beta type II receptor**
TβRII-B	**Transforming growth factor-beta type II-B receptor**
TβRIII	**Transforming growth factor-beta type III receptor**
UIP	**Usual interstitial pneumonia**
UTR	**Untranslated region**

SUMMARY

Members of the transforming growth factor (TGF)-β superfamily are key regulators of lung development and homeostasis, as well as alveolar epithelial cell function. TGF-β signalling involves ligand-dependent phosphorylation of receptor serine/threonine kinases, phosphorylation of pathway-specific transcription factors (Smads), nuclear translocation of Smads, and ultimately, modulation of gene expression. Although Smad-dependent responses represent the primary signalling system activated by TGF-β receptors, alternative signalling systems have recently been described, which also mediated TGF-β-induced effects. In this study, a proteomic approach was employed to identify: a) candidate proteins that are subjected to nuclear-cytoplasmic shuttling in response to TGF-β stimulation and b) novel proteins phosphorylated within TGF-β signaling pathway. In order to reduce complexity of the total proteome subcellular fractionation and phosphoproteome enrichment of A549 cells had been performed. Cytoplasmic, nuclear, and phosphoprotein enriched fractions were subjected to two dimensional polyacrylamide gel electrophoresis, tryptic digestion, and mass spectrometry in order to identify novel candidates/mediators of the TGF-β signaling pathway. In the first part of this study, a rapid increase of KHRSP, FUBP1, hnRNP-L, and hnRNP-H1 localization in the cytosol of A549 cells was observed, concomitant with a decrease in their nuclear localization after TGF-β stimulation. Proteomic data were confirmed by immunofluorescence and immunoblotting analyses. In the second part of this study, 25 phosphoproteins were identified after TGF-β stimulation, 20 of them with different phosphorylation pattern in 2-DE and 5 of them without changes after TGF-β stimulation. In conclusion, we showed that proteomic approach can be used for

detecting novel intermediate proteins in signalling pathways and that regulatory functions of TGF-β signalling are broader than previously thought.

ZUSAMMENFASSUNG

Mitglieder der TGF-β Familie stellen Schlüsselfaktoren für die Entwicklung und Homöostase der Lunge dar, sowie für die Funktion des alveolären Epithels. Die TGF-β Signalkaskade erfolgt durch ligandenabhängige Phosphorylierung von Rezeptor Serin/Threoninkinasen, anschließende Phosporylierung von spezifischen Transkriptionsfaktoren (Smads), die Translokation der Smads in den Nukleus und schließlich die Regulierung der Genexpression. Obwohl die smadabhängige Signaltransduktion das primäre Signalsystem darstellt, das durch TGF-β Rezeptoren aktiviert wird, wurden kürzlich auch andere Signalsysteme beschrieben, die ebenfalls durch TGF-β induzierte Effekte vermitteln. In der vorliegenden Studie nutzten wir einen proteomischen Ansatz um a) mögliche Proteine zu identifizieren, die als Reaktion auf TGF-β-Stimulation zwischen dem Nukleus und dem Zytoplasma pendeln und b) neue Proteine zu finden, die durch den TGF-β Signalweg phosphoryliert werden. Um die Komplexität des Proteoms zu reduzieren wurde eine subzelluläre Fraktionierung und eine Anreicherung der Phosphoproteine von A549 Zellen durchgeführt. Mit den zytoplasmatischen, den nukleären und den Phosphoproteinfraktionen wurden jeweils zweidimensionale Polyacrylamid Gelelektrophoresen durchgeführt, anschließend eine Verdauung durch Trypsin und schließlich wurden mit Hilfe von Massenspektrometrie neue Kandidaten bzw. Mediatoren des TGF-β Signalweges identifiziert. Im ersten Teil der Studie wurde nach der TGF-β Stimulation ein rapider Anstieg von KHRSP, FUBP1, hnRNP-L und hnRNP-H1 im Zytosol der A549 Zellen beobachtet, einhergehend mit einer Abnahme dieser Proteine im Zellkern. Diese Daten wurden mit Hilfe von Immunfluoreszenz und Immunblots bestätigt. Im zweiten Teil der Studie wurden nach TGF-β

Stimulation 25 Phosphoproteine identifiziert, von denen 20 ein verändertes Phosphorylierungsmuster in der 2 dimensionalen Gelelektrophorese aufwiesen und 5 Proteine keine Veränderung zeigten. Insgesamt zeigten wir, dass ein proteomischer Ansatz geeignet ist, um neue vermittelnde Proteine in Signaltransduktionswegen zu identifizieren und dass die regulatorischen Funktionen des TGF-β Signalweges umfassender sind als bisher angenommen.

1 INTRODUCTION

General

Transforming Growth Factor-β (TGF-β) signalling controls various cellular processes, including cell proliferation, recognition, differentiation, apoptosis, and specification of developmental fate during embryogesis as well as in mature tissues, in species ranging from flies, worms to mammals (Massague, 2000; Ten Dijke et al., 2002). A TGF-β ligand initiates signalling by binding to and directing the dimerization of type I and type II receptor serine/threonine kinases on the cell surface. This allows the type II receptor to phosphorylate and activate the type I receptor kinase domain, which in turn transmit signals through phosphorylation of the intracellular Smad second messenger proteins.

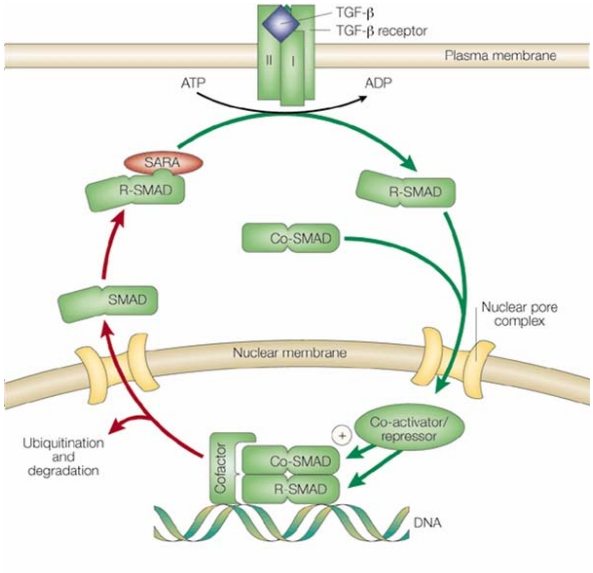

Figure 1.1. The canonical TGF-β Smad pathway. Transcription factors (Smads) require transforming growth factor-β to induce phosphorylation of receptor Smads (R-Smads) and direct their complex formation with the common Smad (co-Smad). To be accessible to membrane receptors, R-Smads bind in the cytoplasm to proteins such as SARA (Smad anchor for receptor activation). Phosphorylation decreases the affinity of R-Smads for SARA, and increases binding affinity for the co-Smad. Smad complex is free to move into the nucleus and associate with transcriptional coactivators or corepressors. The R-Smads that translocate into the nucleus can return to the cytoplasm, although their ubiquitination- and proteasome-dependent degradation in the nucleus is one of the ways to finish TGF-β response (after Massague, 2000).

After activation, the Smad2 and Smad3 (R-Smads) form complexes with the common mediator Smad, Smad4. The complex then accumulate in the nucleus where they associate with cofactors and are directly involved in the transcriptional regulation of target genes. Via direct contact with the nuclear pore complex, Smad2 and Smad3 undergo constant shuttling, providing a dynamic pool, which involves many cytoplasmic and nuclear signal transduction partners (Figure 1.1). Understanding covalent modifications of Smads and their regulation is essential for the in depth knowledge of their function. (Shu et al., 2004).

The two major events in TGF-β signalling pathway are translocation and phosphorylation. In this study proteomic approach was used to elucidate novel intermediates in TGF-β signalling pathway.

1.1 TGF-β SIGNAL TRANSDUCTION

1.1.1 The TGF-β superfamily of ligands

Members of the TGF-β superfamily of ligands (Table 1) are synthesised as large precursor polypeptides that are later cleaved to generate mature, active ligands. Ligands are synthesised as 100 kDa pro-proteins which consist of an amino-terminal pro-region and a carboxy-terminal mature region (Gentry et al., 1988). The amino-terminal facilitates dimerisation of the pro-proteins. Following dimerisation, the amino-terminal region are cleaved by endo-proteases at a conserved RXXR sequence (R, arginine; X, any amino acid residue) located upstream of the mature active domain of the TGF-β ligand (Kingsley, 1994). Another typical characteristic of most TGF-β family ligand members is the seven conserved cysteine residues in their mature region.

Table 1. A network controlling a transforming growth factor-β signalling pathway

TGF-β superfamily ligand	Type II Receptor	Type I Receptor	R-Smads	Co-Smad	Ligand inhibitors
Activin A	Activin type II receptor	Activin receptor like kinase 4	Smad2 Smad3	Smad4	Follistatin
Growth differentiation factor 1	Activin type II receptor	Activin receptor like kinase 4	Smad2 Smad3	SmadS4	
Growth differentiation factor 11	Activin type II-B receptor	Activin receptor like kinase 4	Smad2 Smad3	Smad4	
Bone morphogenetic proteins	Bone morphogenetic protein receptor II	Activin receptor like kinase 3, Activin receptor like kinase 6	Smad1 Smad5 Smad8	Smad4	Noggin Chordin, DAN
Nodal	Activin type II-B receptor	Activin receptor like kinase 4, Activin receptor like kinase 7	Smad2 Smad3	Smad4	Activin, Lefty
Transforming growth factor betas	Transforming growth factor beta type II receptor	Activin receptor like kinase 5	Smad2 Smad3	Smad4	Latent transforming growth factor beta binding protein1, thrombospondin decorin

Six cysteine residue form a knot-like structure, whereas the residual cysteine, the fourth in the primary sequence, mediates binding of the two ligand monomers to one another, forming a dimer via a disulfide bond (Daopin et al., 1992) (Schlunegger and Grutter, 1992). The TGF-β superfamily of ligands consists of structurally-related proteins with molecular masses of approximately 25 kDa. Three mammalian genes encoding TGF-β ligand isoforms have been identified, TGF-β1 (Derynck et al., 1985), TGF-β2 (de Martin et al., 1987) and TGF-β3 (Derynck et al., 1988). Different isoforms share 70-80% amino acid sequence identity and have similar activities. The difference in biological activity of the ligands are associated with the differential binding affinity to TGF-β receptors (Wrana et al., 1992) and to additional binding proteins including endoglin and betaglycan (Table 1).

1.1.2 TGF-β signalling via serine/threonine kinase receptors

Using a mutagenized lung epithelial cell line Mv1Lu resistant to TGF-β-induced growth inhibition it was demonstrated that only the type I and type II receptors were required for TGF-β signalling (Laiho et al., 1990). To date, seven type I and five type II receptors have been identified in mammals. The type I and type II receptors are structurally related to serine/threonine kinases, with highly conserved properties between the two subfamilies. These receptors are responsible for the binding of the TGF-β superfamily ligand members. All receptors contain non-homologous N-glycosylated cysteine-rich extracellular domains, which participate in ligand binding. The ligand-binding extracellular domain is separated from the conserved serine/threonine kinase domain and carboxy-terminal tails with a single transmembrane domain (Derynck and Feng, 1997). The first type I receptors identified were activin receptor like kinase 1 and activin receptor like kinase 4

(ALK1-ALK4) (ten Dijke et al., 1993) and subsequently other type I receptors were identified, and were named according to specificity of their ligand-binding properties. All type I receptors (ALKs) have a molecular mass of 55-65 kDa and share several general structural motifs with the type II receptors. Similar to the type II receptors, the ALKs contain cysteine-rich extracellular domain with putative glycosylation sites, a short transmembrane region, and an intracellular serine/threonine kinase domain. In contrast to the type II receptors, the type I receptors contain a functionally important, highly conserved, ~30 amino acid long glycine/serine (G/S) rich region with a consensus SGSGSG sequence, called as GS-box. The GS-box is important for the activation of type I receptors. The C-terminal intracellular regions of the ALKs are also substantially shorter than those of the type II receptors (Massague, 1998). The five type II receptors consist of approximately 500 amino acids, the molecular masses of which vary between 75-85kDa. The type II receptor, represent by TβRII, is constitutively phosphorylated at several serine residues and ligand-binding does not affect its phosphorylation status (Luo and Lodish, 1997).

However, upon ligand binding, a tetrameric complex containing two type I and two type II receptors is formed, and the constitutively active type II receptor then transphophorylates the type I receptor at several serine and threonine residues in its GS-box (Chen et al., 1997; Wieser et al., 1995).

1.1.3 Intracellular signalling molecules

1.1.3.1 Smads

Smads are the only by far known direct substrates of type I TGF-β receptors with a signalling function. They were first identified in *Drosophila* as *Mad* and *C. elegans* as *Sma* genes, which lie downstream of the BMP ligand-receptor systems in

these organisms. The human genome encodes eight Smad family members [Mad-homologues (*MADH*)] and homologue of Smads are also known to be present in rat, mouse, *Xenopus*, zebrafish, the helminth *Schistosoma mansoni* beside*Drosophila* and *C. elegans* (Moustakas et al., 2001). Smads are expressed throughout development: embryogenesis and in all adult tissues. Many of them (Smad2, Smad4, Smad5, Smad6 and Smad8) are produced from alternatively spliced mRNAs (Gene encyclopaedia, GeneCards) (Figure 1.2).

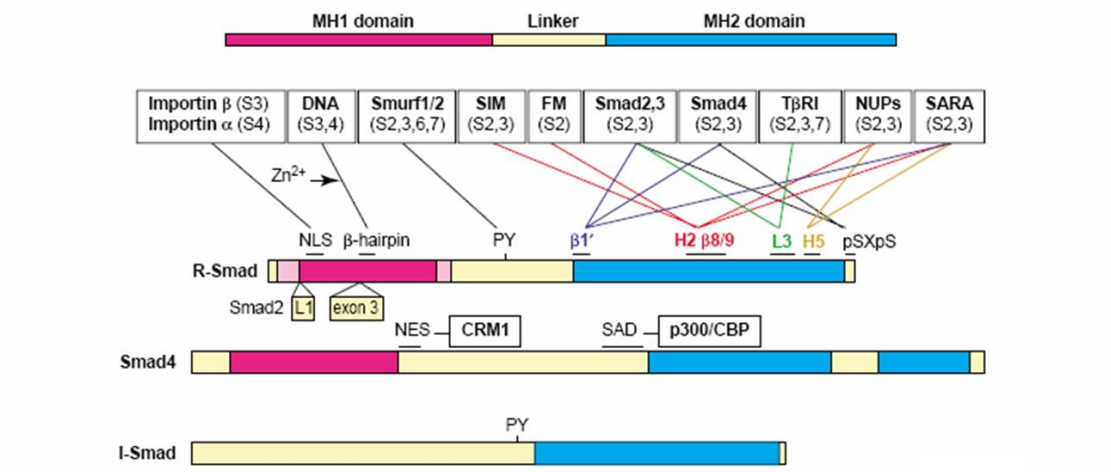

Figure 1.2. Structure of R-Smads, Smad4 and I-Smads (Smad6 und Smad7) The MH1 (dark pink) and MH2 (cyan) domains are conserved between Smads. The motifs shown are the β10-strand (dark blue), α-helix 2 (red), β-strands 8 and 9 (β8/9, red), L3 loop (L3, green), α-helix 5 (H5, brown), and pSXpS, the phosphorylated C-terminal SxS motif of the R-Smads (black). The MH1 domain of Smad3 and Smad4 binds DNA. Both the MH1 and the MH2 domain interact with other transcription factors. FM, Fast or FoxH1 motif; MH, Mad homology; NES, nuclear export signal; NLS, nuclear localization signal; NUPs, nucleoporins; PY, the PPxY motif that mediates binding to Smurf1 and Smurf2; SAD, Smad activation domain; SIM, Smad interaction motif (after P. ten Dijke and CS. Hill, 2004).

Functionally, Smads can be divided into three subfamilies:

1. Receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, Smad8), which are phosphorylated by the type I receptors;

2. Common mediator Smads (Co-Smad: Smad4), which oligomerise with activated R-Smads.

3. Inhibitory Smads (I-Smads: Smad6 and Smad7), which are induced by TGF-β family members. Both Smad6 and Smad7 exert a negative feedback effect by

competing with R-Smads for receptor interaction and by marking the receptors for degradation (Moustakas et al., 2001).

Smads have two conserved domains, the N-terminal Mad homology 1 (MH1) and C-terminal Mad homology 2 (MH2) domains (Figure 1.2). The MH1 domain is highly conserved among R-Smads and Co-Smads; the N-terminal regions of I-Smads have only weak sequence similarity to MH1 domains. Sequence and structural analyses indicated that the MH1 domain is homologous to the diverse histidine-metal-ion (His-Me) finger family of endonucleases. The MH1 domain regulates nuclear import and transcription by binding to DNA and interacting with nuclear proteins (Reguly and Wrana, 2003). The MH2 domain is also highly conserved between all Smads, containing several α-helices and loops which surround a β-sandwich structure, resembling the forkhead associated (FHA) domain, a phosphopeptide-binding domain common in transcription and signalling factors (Shi, 2001). The MH2 domain regulates Smad oligomerisation, recognition by type I receptors, and interacts with cytoplasmic adaptors and several transcription factors (Moustakas et al., 2001).

1.1.3.2 Smad phosphorylation and dephosphorylation

The TGF-β receptor-induced phosphorylation of the C-terminal SXS motif of R-Smads is the signal for interaction with Smad4. The complex accumulates in the nucleus and binds to transcription factors and DNA to modulate gene transcription. The identities of Smad phosphatases however remain under investigation. Smad phosphatases can act either in the cytoplasm to limit the amount of phosphorylated R-Smads that enter the nucleus or they can act inside the nucleus to terminate the transcriptional regulation by R-Smads because dephosphorylation of R-Smads is required for nuclear export of R-Smads and Smad4 (Inman et al., 2002; Xu et al.,

2002). Recently, an RNAi screen of Ser/Thr phosphatases in cultured *Drosphilia* S2 cells identified PDP (*P*yruvate *D*ehydrogenase *P*hosphatase) as a Smad phosphatase (Chen et al., 2006). In has been demonstrated that PPM1A (protein phosphatase 1A magnesium-dependent, alpha isoform) can dephosphorylate Smad2 and Smad3. This phosphatase is predominantly localized in the nucleus and its activity facilitates nuclear export of Smad2/3 (Lin et al., 2006). It has been reported that the *S*mall *C*-terminal Domain *P*hosphatases (SCP) dephosphorylate Smad1 and subsequently increase transcriptional activation of target genes in response to BMP (Knockaert et al., 2006). Dephosphorylation studies on Smads may yield a new insight into downregulation of TGF-β signalling, which is an important mechanism of regulation of this pathway.

1.1.3.2.1 Smad phosphorylation by other pathways

In addition to phosphorylation of R-Smads by TGF-β, phosphorylation of endogenous R-Smads can be undertaken by other pathways as well. These phosphorylations are induced by different extracellular signals and the targeted sites are primarily localized in the N-terminal linker region of Smads. The mitogen activated protein kinase (MAPKs) can phosphorylate Smad1 upon epidermal growth factor (EGF) stimulation (Kretzschmar et al., 1997). Phosphorylation of Smad2, Smad3 and Smad4 by MAPKs has been also reported (Javelaud and Mauviel, 2005). The linker region of Smad2 and Smad3 is subjected to phosphorylation by calmodulin dependent protein kinase II (CaMKII); the N-terminal and linker regions of Smad3 have been shown to be phosphorylated by cyclin dependent kinases (CDKs), which are regulated in a cell-cycle-dependent manner (Matsuura et al., 2004). The G protein-coupled receptor kinase 2 (GRK-2) mediated phosphorylation of Ser197 in Smad2, inhibited the carboxy-terminal phosphorylation in response to TGF-β, thereby

preventing nuclear accumulation of Smad2 (Ho et al., 2005). Both MAPK- and CaMKII-mediated phosphorylation does not interfere with the carboxy-terminal phosphorylation of R-Smads by TGF-β or BMP receptor kinases, but in turn it was shown to inhibit nuclear accumulation of Smad1, Smad2, and Smad3 (Kretzschmar et al., 1997). The N-terminal and linker phosphorylation of Smads by CDKs resulted in a reduced transcriptional activation potency of Smad3. Whether this was due to a decreased interaction of Smad3 with transcription co-factors remains to be tested, but there is no apparent effect on the carboxy-terminal phosphorylation of Smad3.

1.1.3.3 Nucleocytoplasmic Smad Shuttling

Smads constantly shuttle between the cytoplasm and the nucleus. They can shuttled both in an uninduced state and during stimulation with TGF-β superfamily ligands (Inman et al., 2002; Xu et al., 2002). In uninduced cells, Smad2 and Smad3 are primarily cytoplasmic, and Smad4 is dispersed throughout the nucleus and cytoplasm (Figure 1.3) (Pierreux et al., 2000; Watanabe et al., 2000; Xu et al., 2002). Receptor-regulated Smads (R-Smads) require TGF-β induced phosphorylation to form transcription regulatory complexes with partner Smads. In order to be accessible for membrane receptors, R-Smads are found in a complex with accession, proteins including SARA (Smad anchor for receptor activation). The R-Smad phosphorylation decreases the affinity of R-Smads for SARA, and increases their affinity for the co-Smad. The Smad complex is free to move into the nucleus and can form complex with transcriptional coactivators or corepressors. The R-Smads that move into the nucleus may return to the cytoplasm. Their ubiquitylation and proteasome-dependent degradation in the nucleus provide means of termination TGF-β responses (Massague, 2000). However, the nucleocytoplasmic shuttling of Smads and its regulation has not been completely resolved. Studies by Xu and Massague (Xu et al., 2003; Xu et al.,

2000; Xu et al., 2002) have suggested that Smad nucleocytoplasmic shuttling is independent of the transport of receptors, and is mediated only through direct interactions with nucleoporins, primarily Nup214 and Nup153. Smad interactions with the nuclear pore appear to be independent of their phosphorylation state and whether they are monomeric or oligomeric (ten Dijke and Hill, 2004).

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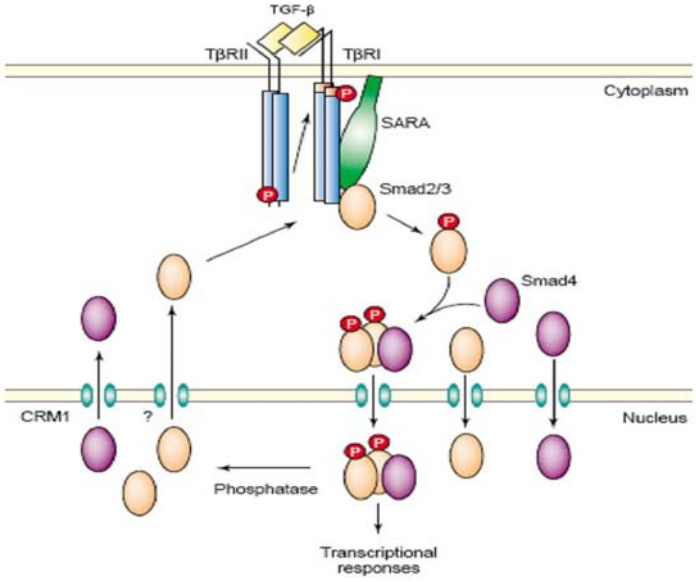

Figure 1.3. Model of nucleocytoplasmic shuttling of Smads. Smad proteins shuttle between the cytoplasm and nucleus both in uninduced cells and in cells stimulated with TGF-β superfamily ligands. In active signalling, continuous Smad shuttling provides a mechanism whereby intracellular transducers can monitor receptor activity. Export of Smad4 (purple) is mediated by CRM1 (ten Dijke and Hill, 2004).

The monomeric Smads (R-Smads) have a higher affinity for cytoplasmic retention factors like SARA. Complexed Smads have a higher affinity for nuclear retention factors such as transcription factors and DNA (Xu et al., 2003; Xu et al., 2002). This model might explain the cytoplasmic localization of Smads in unstimulated cells and their accumulation in the nucleus after TGF-β treatment. An alternative model proposes the localization of Smads in given situation as a function of relative rates of import and export: monomeric Smads are predominantly exported from the nucleus, while complexed Smads are rather imported. Complex formation might either

stimulate import or inhibit export (Kurisaki et al., 2001; Watanabe et al., 2000). Mechanisms of nucleocytoplasmic shuttling of Smads in the TGF-β pathway deserve further examination. In addition, since the TGF-β pathway provides an excess of interacting signalling factors, proteomic screens could help to revel the complete repertoire of proteins that undergo nucleocytoplasmic shuttling.

1.2 NEGATIVE REGULATION OF TGF-β SIGNALLING

1.2.1 Inhibitor Smads

The I-Smads (Smad6 and Smad7) are the inhibitors of TGF-β induced Smad mediated signalling. The MH2 domain of the I-Smads is structurally related to the R-Smads and co-Smads but their N-terminal region is highly different from other Smads (Figure 1.2). The I-Smads do not have the SSXS domain and according to Miyazono *et. al*. (Miyazono et al., 2003) this allows them to stably bind to the activated type I receptor. The expression of I-Smads is upregulated after activation of R-Smads, most likely in order to prevent excessive stimulation of the cell, therefore I-Smads form a negative feedback loop (Miyazono, 2002).

Smad6 appears to be an antagonist of BMP signalling, while Smad7 can shown to antagonise signalling by TGF-β and activin (Liu et al., 2002). The I-Smad interference with the Smad signalling pathways exist on two levels: binding and formation of R-Smad-Smad4 complex. Overexpression of I-Smads blocks Smad signalling by binding to the R-Smad-type I receptor interaction site or through their MH2 domains, (Imamura et al., 1997). At lower expression levels, Smad6 competes with Smad4 for binding to R-Smads, which blocks the formation of R-Smad-Smad4 complexes.

1.2.2 Smad ubiquitination

Negative regulation of TGF-β signalling can also occur through degradation of R-Smads by the ubiquitin-proteosome system. Proteosomal degradation of Smads is assisted by Smad-mediated ubiquitin regulatory factors (Smurfs, Smurf1 and Smurf2). Smurf1 induces ubiquitination and later degradation of the BMP-specific Smad1 and Smad5 by binding to the PPXY motif in their linker region (Zhu et al., 1999). Smurf2 associates with Smad1 and Smad2, and targets them for degradation (Lin et al., 2000; Zhang et al., 2001). Smad4 lacks a PPXY motif to engage the Smurfs. Recently Dupont *et al*. (Dupont et al., 2005) reported the suppression of Smad4 functions by ectodermin/TIF1-γ, a protein harboring a RING domain in addition to a number of other functional domains, and thus acting as Smurfs for R-Smads .

1.3 SMAD-INDEPENDENT SIGNALING CASCADES ACTIVATED BY TGF-β FAMILY MEMBERS

Prior to the discovery of the canonical TGF-β signalling pathway, the small GTPase Ras and the mitogen-activated protein kinases (MAPKs; ERK, p38 and c-Jun N-terminal kinase (JNK)) were implicated in TGF-β signalling (Yue and Mulder, 2000). The TGF-β type II receptor interacts with the proapoptotic adaptor protein Daxx, which leads to activation of JNK and induction the of apoptosis in epithelial cells and hepatocytes (Perlman et al., 2001). Furthermore, TGF-β signalling in cultured rat articular chondrocytes (CRAC) is regulated by protein kinase A (PKA) and protein kinase C (PKC) (Hirota et al., 2000). Genetic evidence in normal mammary epithelial cells stably-transfected with a mutant type I TGF-β receptor that cannot bind and thus activate R-Smads, implicated the p38 and JNK pathways in mammary cell apoptosis (Itoh et al., 2003; Yu et al., 2002). The type I receptor kinase activates the p38 and JNK pathways indepently of other factors (including Smads)

that bind to the receptor. Biological responses to TGF-β depend on a balance between multiple signalling pathways, each activated in a cell-type and context-dependent manner, involving both Smad-dependent and Smad-independent pathways (Derynck and Zhang, 2003).

1.4 TGF- β SIGNALLING IN HUMAN DISORDERS

1.4.1 Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive and lethal fibrotic lung disease of unknown etiology. Histologically, the lung is characterised by temporally heterogeneous lesions where normal lung is interspersed with areas of fibrosis, aggregates of fibroblasts called fibroblast foci and enlarged distorted airspaces called honeycomb cysts (Gross and Hunninghake, 2001; Khalil et al., 2001). It has been demonstrated that epithelial cells express a number of fibrogenic cytokines, such as TGF-β, platelet-derived growth factor (PDGF) (Antoniades et al., 1990), interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) (Nash et al., 1993; Pan et al., 1996). Data from patients with IPF and animal models of this disease support the idea that TGF-β is a key player involved in the development of IPF. It has been shown that macrophages and lavage fluid from patients with IPF contain large amounts of TGF-β. The presence of TGF-β in fibroblastic foci was also demonstrated in biopsies from patients with IPF. Gene expression studies in human tissue show obvious up-regulation of TGF-β-responsive genes in IPF (Kaminski et al., 2000). Furthermore, transgenic mice overexpressing TGF-β and overexpression models of TGF-β have demonstrated that the presence of this cytokine alone is sufficient to induce a progressive fibrotic response independent of inflammation (Zhang et al., 2004), (Lee et al., 2004), thereby demonstrating important role of TGF-β in IPF.

1.4.2 Other disorders

The anti-mitogenic tumor suppressive effect of TGF-β is lost in several tumor-derived cell lines, and it has been proposed that mutations in the TGF-β signalling cascade are involved in a large fraction of pancreatic and colorectal cancers (de Caestecker et al., 2000). Inactivating mutations in genes encoding the TGF-β type II receptors have been detected in colorectal and gastric cancers (Luo and Lodish, 1997). Furthermore, gene encoding serine/threonine kinase receptors are also found to be mutated in some hereditary pre-malignant syndromes. Mutations in ALK3 have been shown to be responsible for variants of juvenile polypotic syndromes, where an affected individual are at a high risk for the development of malignant tumors from pre-existing benign polyps. Mutations in the gene encoding Smad2 or deletions have been reported in several cancers, including colorectal, lung and helatocellular. The chromosomal region where Smad4 is located is deleted in more than 50% of human pancreatic carcinomas (Blobe et al., 2000). In addition, mutations in the gene encoding Smad4 have been studied in ovarian, prostate, pancreatic and colorectal cancers. Mutations in the gene encoding ALK1 cause hereditary haemorrhagic telangiectasia (HHT) type 2, and mutations in endoglin, an accessory TGF-β receptor cause hereditary haemorrhagic telangiectasia type 1 (a TGF-β type III receptor) (Blobe et al., 2000). Similary mutations in the gene encoding BMPRII may cause the rare autosomal dominant genetic disorder familial pulmonary arterial hypertension (FPAH).

1.5 PROTEOMICS

General

The term proteomics encompass studies on proteins, particularly their structure and function, as well as identification and localization studies of proteins and interaction studies using different methods like two dimensional gel electrophoresis and mass spectrometry, immunochistochemistry, immunoprecipitation or the yeast two-hybrid system (Pandey and Mann, 2000).

The Figure 1.4 presents a one of the proteomic approaches, which includes the separation of the proteome by two-dimensional gel electrophoresis and identification of proteins or polypeptides from a gel using mass spectrometry. Different subcellular compartments contain different amount of proteins in order to provide suitable biochemical environments for their particular function. The identification of proteins and their subcellular localization is, an important initial step for the understanding of cellular function (Dreger, 2003b).

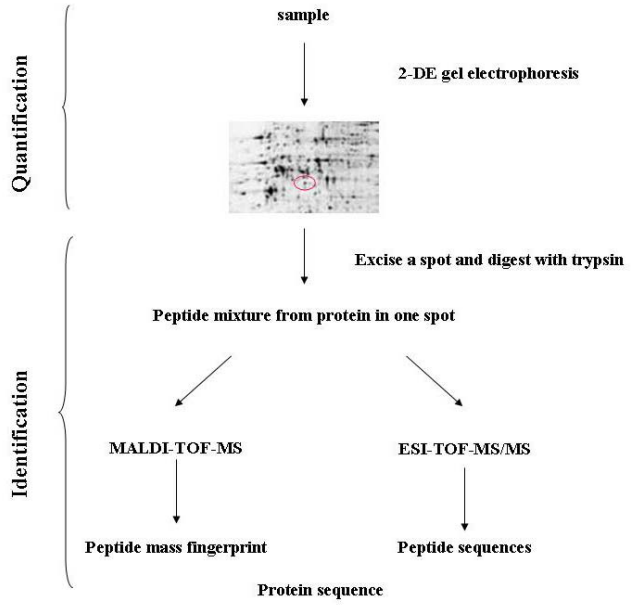

Figure 1.4. General proteome analysis using two-dimensional gel electrophoresis and mass spectrometry.

1.5.1 Subcellular proteomics

Proteins within the cell are able to shuttle between membrane receptors, the cytoplasm and nucleus (Dreger, 2003b). Two dimensional electrophoresis (2-DE) has exceptional analytical power to separate large number of proteins. There are certain classes of proteins like phosphoproteins or proteins which are major constituent of specific cellular compartments and in complete crude homogenate they might not be detected on 2-DE gel (Mann et al., 2002). The classic approach may fail in the discovery of novel proteins, which undergo cytoplasmic/nuclear shuttling. Performing proteomic analysis at a subcellular level is therefore an appropriate strategy for this kind of study (Dreger, 2003a).

1.5.2 Phosphoproteomics

The primary aim of signal transduction is the spread and amplification of signals from membrane receptors to the nucleus. One major stream of information is mediated through reversible phosphorylation of proteins (Pawson and Scott, 1997) (Hunter et al., 1985). In eukaryotes, phosphorylation occurs mainly on serine, threonine, and tyrosine residues. Phosphorylation on serine and threonine residues is more abundant than on tyrosine residues. The phosphoserine: phosphothreonine: phosphotyrosine ratio is 1800:200:1 in vertebrates (Hunter and Cooper, 1985). The degree of phosphorylation is regulated by the interplay between the activities of protein kinases and protein phosphatases.

The TGF-β ligand signal via receptor serine/threonine kinases and intracellular Smad molecules. Several intracellular proteins that mediate signalling by receptor tyrosine kinases, G protein-coupled receptor or cytokine receptors also participate in TGF-β signalling (Javelaud and Mauviel, 2005). Some previous studies combined immunoprecipitation (IP) with anti-phosphorylation antibody and SDS-PAGE or two

dimensional electrophoresis (2-DE) and mass spectrometry (MS) to reveal epidermal growth factor receptor, interferon receptor-α, interleukin-2, MAPK and fibroblast growth factor signalling pathways (Steen et al., 2002), (Pandey et al., 2000), (Hinsby et al., 2003). Immunoprecipitation of phosphotyrosine containing proteins (Mann et al., 2002) is still much more frequent than immunoprecipitation using mixtures of phosphoserine or threonine antibodies (Gronborg et al., 2002). Up to date, specific antibodies for serine/threonine phosphorylation are not available. Enrichment of phosphorylated proteins and/or peptides followed by mass spectrometry is possibly the most widely-used approach in elucidating signaling by receptor serine/threonine kinases (Kalume et al., 2003).

1.5.2.1 Immobilized metal-ion affinity chromatography: enrichment of phosphoproteins

Immobilized metal-ion affinity chromatography (IMAC) was first introduced by Porath *et al.* (Porath et al., 1975) for the purification of His-tagged proteins. At present, it is used primarily for the enrichment and purification of phosphopeptide (McLachlin and Chait, 2001). Phosphorylated peptides and proteins are bound to the stationary phase by electrostatic interactions with positively charged metal-ions, such as Fe^{3+}, Ga^{3+}, Al^{3+}, Zr^{4+}, Ti^{4+} that are bound to the column material *via* iminodiacetic acid (IDA), nitriloacetic acid (NTA) or tris-(carboxymethyl)-ethylendiamine (TED) linkers (Liu et al., 2003; Nuhse et al., 2003). Non-phosphorylated species are removed in the flowthrough, and the phosphopeptides and/or proteins may be eluted by salt- and/or pH-gradients. In this way, multiple phosphorylated peptides or proteins are enriched, as a very acidic peptides that are bound to the column (Stensballe et al., 2001). Nevertheless, reaction conditions have to be chosen with care to avoid incomplete esterification and side reactions, for instance on asparagines, in order to

avoid increased sample complexity which would interfere with mass spectrometry analysis. The IMAC technology is described to be compatible with following separation and detection techniques like capillary electrophoresis (CE), (Cao and Stults, 1999), LC-MS/MS (Heintz et al., 2004) and direct MALDI-MS of phosphopeptides bound to IMAC beads on the target (Reinders and Sickmann, 2005).

1.5.3 Two dimensional gel electrophoresis

Two dimensional gel electrophoresis (2-DE) combines isoelectric focusing (IEF) in the first dimension, which facilitates the separation of very complex mixtures of proteins or polypeptides according to their isoelectric point (pI) and by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, where separation of complex mixtures is performed according to molecular weight (MW). 2-DE can be used to separate more than 5000 proteins simultaneously, and can detect less than 1 ng of protein per spot and later used for identification by mass spectrometry (Gorg et al., 2004). To date, this is only method which facilitate the study of intact proteins, isoforms or post-translational modifications (PTM) at a global basis.

1.5.4 Protein detection and quantitation

After 2-DE, the separated proteins have to be visualized, either by universal (coomassie blue or silver staining) or by specific staining methods. The right selection of visualization method is one of most important steps in the assays, which will be followed by quantification. Therefore, the method should be of high sensitivity, high linear dynamic range (for quantitative accuracy), reproducibility, and compatibility with identification procedures, for example fluorescent stainer SyproRuby. Already, mentioned universal staining methods are coomassie blue (anionic dyes staining), but only in case of having enough sample (detection limits 100ng); silver staining method

is a very sensitive one, although this procedure does not have high linear dynamic range. Fluorescence staining or labelling is employed mostly because of its very high sensitivity, quantification possibilities and subsequently sample can be applied to mass spectrometry (Corthals et al., 2000). The dynamic range of quatitation obtained with fluorescent stains matches the dynamic range of protein expression levels measured in yeast (Velculescu et al., 1997).

A widely used fluorescence based visualization method is SyproRuby, a ruthenium complex bind robustly to proteins like a Coomassie Brilliant Blue-type mechanism, involving lysine, arginine and histidine residues (Berggren et al., 2000). This fluorescence stain can be visualized by commonly-used image analysis systems, and the dye maximally emits signall at about 610 nm (Berggren et al., 1999; Patton, 2000).

After enrichment of phosphoproteins and separating by different analytical methods, they can be visualized either directly in 2-DE gels using phosphospecific stains (Cutting and Roth, 1973; Patton, 2002) or using one dimensional electrophoresis followed by western blotting techniques (Kaufmann et al., 2001). The radioactive labelling is still the most sensitive detection method by ^{32}P or ^{33}P, followed by autoradiography (Bendt et al., 2003; Hathaway and Haeberle, 1985). Commercially available phosphospecific-stains, for example Pro-Q Diamond, are less sensitive than radioactive methods but the use of these “inactive” reagents is far more safe (Steinberg et al., 2003; Valles et al., 2003). The good compatibility of these staining methods and mass spectrometry has been reported in many studies for identification of phosphoproteins without additional assays like immunoblotting.

1.5.5 Protein identification and characterization

1.5.5.1 Mass spectrometry and protein analysis

One of the major advancement in proteomics to date so far has been the mass spectrometric identification of proteins separated by SDS-PAGE. Since eightie´s mass spectrometry has replaced the more classical Edman degradation, since it is much more sensitive, can be used with protein mixtures, has a much higher throughput applicability (Mann et al., 2002). Mass spectrometry use a peptide or several peptides to identify a protein, where peptides have been derived from digestion of a protein with one or more proteases. Only a few peptides are required in order to identify protein (Rappsilber and Mann, 2002).

Prior to detection and analysis, peptides must be ionised and later fragmentated to single amino acids. Fragmentation fingerprints can be used for identification by comparison with fragment databases, which are available online. Complex mixtures can be analysed with coupling of mass spectrometers with different separation methods like gas chromatography-mass spectrometry (GC-MS) and high preassured liquid chromatography-mass spectormetry (HPLC-MS) or capillary electrophoresis (CE-MS) (Pandey and Mann, 2000).

1.5.5.2 Matrix assisted laser desorption/ionization

Many different mass spectrometers are available on the market, each consist of an ion source, a mass analyser that measures the mass-to-charge ratio (m/z) of the ionised analytes and a detector that registers the number of ions at each m/z value. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two techniques most commonly used to ionise proteins or peptides for mass spectrometric analysis (Karas and Hillenkamp, 1988). First reports about use of MALDI come form group of Hillenkamp in 1988 (Karas and Hillenkamp, 1988),

where sample was applied on a crystalline matrix and MALDI sublimed and ionises the samples with laser pulses . One of the frequently used analysers in MALDI mass spectrometers is the time of flight mass analyser (TOF). The MALDI-TOF methodology is used to identify many compounds including sugers, lipids, nucleic acids and proteins. Identification of proteins is known as peptide mass fingerprinting. Proteins are identified by matching a list of experimental peptide masses with the calculated list of all peptide masses of each entry in a database. Peptide mass mapping by MALDI-TOF has become very efficient at the identification of gel-separated proteins (Aebersold and Mann, 2003).

2 AIM OF THE STUDY

There are two major events in TGF-β signal transduction: a) constant shuttling between the cytoplasm and nucleus of the downstream Smad signalling molecules and b) phosphorylation of TGF-β receptors, which is a trigger for the initiation and transmission of signals from transmembrane receptors to the nucleus. In eukaryotes, phosphorylation occurs on serine, threonine, and tyrosine residues, where phosphorylation on serine and threonine residue is far more abundant than phosphorylation on tyrosine residues (Hunter, 1998). The aims of this study, therefore, were as follows:

1) to identified proteins that are subjected to nuclear-cytoplasmic translocation in response to TGF-β stimulation.

2) to identified proteins that are subjected to phosphorylation in response to TGF-β stimulation.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Equipments

Table 2. Laboratory equipments

Equipment	Company
Cell Culture Incubator; Cytoperm2	Heraeus, Germany
Developing machine; X Omat 2000	Kodak; USA
Electrophoresis chambers	Bio-Rad, USA
Fluorescence microscope; LEICA AS MDW	Leica, Germany
Freezer -20 °C	Bosch, Germany
Freezer -40 °C	Kryotec, Germany
Freezer -80 °C	Heraeus, Germany
MALDI-TOF-TOF	Bruker Daltonics, Germany
Protean IEF cell	Bio-Rad, USA
Fridge +4 °C	Bosch, Germany
Mini spin centrifuge	Eppendorf, Germany
Multifuge centrifuge, 3 s-R	Heraeus, Germany
Light microscope; LEICA DMIL	Leica, Germany
Pipetboy	Eppendorf, Germany
Pipetmans: P10, P20, P100, P200, P1000	Gilson, France
Power Supply; Power PAC 300	Bio-Rad, USA
Western Blot Chambers:	Bio-Rad, USA
Mini-Protean 3 Cell	Bio-Rad, USA
Vortex machine	Eppendorf, Germany
Film cassette	Sigma-Aldrich, Germany
Filter Tip FT: 10, 20, 100, 200, 1000	Greiner Bio-One, Germany
Filter units 0.22 µm syringe-driven	Millipore, USA
Glass bottles: 250, 500, 1000 ml	Fisher, Germany
Pipette tip 10, 200, 1000 µl	Gilson, USA
Radiographic film X-Omat LS	Sigma-Aldrich, Germany
FX scanner	Bio-Rad, USA
Serological pipette: 50 ml	Falcon, USA
Test tubes: 15, 50 ml	Greiner Bio-One, Germany
Tissue culture chamber slides	BD Falcon, USA

Tissue culture dish 100, 150 mm	Greiner Bio-One, Germany
Tissue culture flask 75 ml	Greiner Bio-One, Germany

3.1.2 General chemicals

Table 3. General laboratory chemicals

Compounds	**Company**
β-Mercaptoethanol	Sigma-Aldrich, Germany
3 mm Whatman paper	Bioscience, Germany
12% Ready Gel Precast gels	Bio-Rad, USA
Acetic acid	Merck, Germany
Agarose	Promega, USA
Ammonium Persulfate (APS)	Promega, USA
Bradford reagent	Bio-Rad, USA
Bromophenol blue	Sigma-Aldrich, Germany
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Sigma-Aldrich, Germany
Protease inhibitor cocktail tablets	Roche, Germany
Dithiothreitol (DTT)	Promega, USA
HEPES	Sigma-Aldrich, Germany
Detection reagents ECL western blotting	Pierce, USA
Ethanol	Fluka, Germany
Eythelene diamino tetra acetic acid	Promega, USA
Glycerol	Promega, USA
Iodoacetamide	Sigma-Aldrich, Germany
IPG strips pH 3-10	Bio-Rad, USA
Isopropanol	Merck, Germany
Methanol	Fluka, Germany
Mineral oil	Bio-Rad, USA
Potassium chloride	Meck, Germany
Protein standards dual colour precision	Bio-Rad, USA
Rotiphorese® gel 30	Roth, Germany

Silver stain kit	Bio-Rad, USA
Sodium acetate	Sigma-Aldrich, Germany
Sodium chloride	Merck, Germany
Sodium dodecyl sulfate (SDS)	Promega, USA
Sodium ortho vanadate	Sigma-Aldrich, Germany
Nitro-cellulose membrane	Bio-Rad, USA
Igepal (Nonidet P-40)	Sigma-Aldrich, Germany
Phosphate-buffered saline	Laboratories, Austria
Phosphoprotein enrichment kit	Bioscience, Germany
Pro-Q-Diamond phosphoprotein stain	Invitrogen, UK
SyproRuby	Bio-Rad, USA
Urea	Sigma-Aldrich, Germany
Temed	Bio-Rad, USA
Trifluoroacetic acid	Merck, Germany
Tris	Roth, Germany
Triton X-100	Sigma-Aldrich, Germany
Tween-20	Sigma-Aldrich, Germany

3.1.3 Cell culture reagents and other laboratory chemicals

Table 4. Cell culture reagents

Compounds	**Company**
Trypsin-EDTA	Laboratories, Austria
Dulbecco's modified eagle medium	Gibco BRL, Germany
TGF-β1	R&D Systems, USA
Fetal calf serum (FCS)	Laboratories, Austria

3.1.4 Antibodies

Table 5. Primary antibodies

Catalogue No.	Description	Company
SC5286	tubulin	Santa Cruz
SC20681	lamin	Santa Cruz
#07-392	phospho Smad2	Upstate
SC28726	hnRNP L	Santa Cruz
H00008570-A01	KHSRP	Abnova
Ab10374	hnRNP H	Abcam
ARP 35703-T200	FUBP1	Aviva System Biology

Table 6. Secondary Antibodies

Name	Description	Company
Mouse horse-radish peroxidase	Anti-mouse IgG(H+L)	Pierce, USA
Goat horse-radish peroxidase	Anti-goat IgG (H+L)	Pierce, USA
Rabbit horse-radish peroxidase	Anti-rabbit IgG (H+L)	Pierce, USA

3.1.5 Cell lines and media

The A549 epithelial cell line (human lung adenocarcinoma) was cultured in DMEM medium with 10% (v/v) FCS. The primary human fibroblasts from idiopathic pulmonary fibrotic patients and donors were cultured in DMEM medium with 10% (v/v) FCS under sub-confluent conditions. The cells were cultivated on 150 mm tissue culture plastic dishes (Sarstedt, Newton, NC, USA) at 37 ºC in 5% CO_2 environment and 98% humidity.

3.1.6 Samples from Patients

Lung tissue biopsies were obtained from six patients with IPF [usual interstitial pneumonia (UIP) pattern; mean age 51.3 ± 11.4 years], and six control subjects (organ donors, mean age 47.5 ± 13.9 years). The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol.

3.2 METHODS

3.2.1 Western blotting

A) Protein isolation from cells/tissues

Medium was aspirated from the cells, and the cells were washed twice with ice-cold 1× PBS phosphate-buffered saline (PBS). In the case of tissues, tissue was ground to powder under liquid nitrogen and lysed in lysis buffer [20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1% (v/v) Nonidet P-40] containing a mixture of protease and phosphatase inhibitors. The lysates were vortexed every 5 min for 40 min and centrifuged at 13,000 rpm for 15 min. Protein concentration in the supernatant was measured and samples were stored at -80 °C.

B) Preparation of cytoplasmic and nuclear protein extracts

The human lung epithelial cell line A549 was obtained from the German Microbe and Cell Culture Collection (DSMZ, Braunschweig, Germany). Maintained at 37 °C in D-MEM/F-12 A549 cells were rinsed twice with ice-cold PBS, scraped in PBS and finally collected by centrifugation at 5000 rpm for 4 min at 4°C. Cell lysis was performed in ice-cold hypotonic lysis buffer [20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 0.2 % (v/v) NP-40, 10 % (v/v) glycerol] supplemented with protease inhibitors (Roche, Mannheim, Germany) for 10 min.

Nuclear and cytoplasmatic fractions were separated by centrifugation at 5000 rpm for 5 min at 4 °C. The resulting supernatant (cytoplasmc fraction) was stored at –80°C until further analysis. The supernatant was then resuspended in ice-cold hypertonic lysis buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4, 20% (v/v) glycerol] supplemented with protease inhibitors and incubated for 30 min at 4°C. Soluble compounds were then isolated by centrifugation at 13,000 rpm for 5 min at 4°C. The resulting supernatant (nuclear fraction) was stored at -80°C until further analysis.

C) Protein seperation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The separating gel mixture was poured between two glass plates and allowed to polymerise. The stacking gel mixture was poured on the top of separating gel and a comb was inserted in to the gel to form the wells (Table 13 and Table 14). The gel was run in 1× Laemmli-running buffer [25 mM Tris-Cl pH 8.3, 0.2M glycine and 0.1% (v/v) SDS] until the desired distance had been reached.

D) Immunoblot analysis

After gel electrophoresis, proteins were transferred to a nitrocellulose membrane using a Bio-Rad transfer chamber containing transfer buffer [24 mM Tris base, 193mM glycine, 10% (v/v) methanol]. Transfer was performed at 4°C overnight at 12V.

Blots were incubated for 1 h in blocking buffer [5% (m/v) non-fat dry milk powder in PBS with 0.1% (v/v) Tween-20] at room temperature. Incubation with the respective primary antibodies (1 µg/ml) was performed in blocking buffer for 1 h at room temperature. After washing 3 times for 10 min with buffer [PBS 0.1% (v/v) Tween-20], blots were incubated with the respective horseradish peroxidase (HRP)-labelled secondary antibodies at room temperature for 1 h. After washing 3 times for 10 min

with wash buffer, blots were developed by enhanced chemi-luminescence (Pierce) and visualised with Hyperfilm ECL (Kodak).

To re-probe with another antibody, the blots were stripped with stripping buffer [63mM Tris-Cl pH 6.8, 2% (m/v) SDS, 0.8% (m/v) DTT] at 50 °C for 30 min and re-probed as described above.

Table 7. Composition of 10% resolving gels (40 ml)

Component	**Volume**
Distilled water	15.9 ml
30% acrylamide mix	13.3 ml
1.5 M Tris-Cl (*pH* 8.8)	10 ml
10% (m/v) SDS	400 µl
10% (m/v) APS	400 µl
TEMED	16 µl

Table 8. Composition of 4% stacking gels (20 ml)

Component	**Volume**
Distilled water	13.6 ml
30% acrylamide mix	3.4 ml
1.5 M Tris-Cl (*pH* 6.8)	2.5 ml
10% (m/v) SDS	200 µl
10% (m/v) APS	200 µl
TEMED	20 µl

3.2.2 Two-dimensional gel electrophoresis

3.2.2.1 Sample preparation and protein solubilisation

Proteins in the sample must be denatured, disaggregated, reduced and solubilised to achieve disruption of molecular interactions in order that each spot represents an individual polypeptide (Gorg et al., 2004). Sample solubilisation is

usually carried out in a buffer containing chaotropes (for example urea and/or thiourea), nonionic and/or zwitterionic detergents e.g. Triton X-100 or 3-[(3-Cholamidopropyl)dimethylammonio]- 1-propanesulfonate (CHAPS), reducing agents, carrier ampholytes (CA) and depending on the type of sample or protease inhibitors. Enriched cytosolic and nuclear protein fractions were purified with a ReadyPred™ 2-D Clean Kit (Bio-Rad Labaratories, CA, USA) as per manufacturer's protocol, in order to concentrate proteins from the sample which are too diluted, for higher protein loads, prior to solubilisation in rehydration buffer [50 mM DTT, 4 % (m/v) CHAPS, 9 M urea]. The protein concentration of both protein fractions was determined using Quick Start™ Bradford Dye Reagent on a SmartSpec™ 3000 spectrophotometer (both Bio-Rad Laboratories, CA, USA).

3.2.2.2 First dimension: Isoelectric focusing

Immobilised *pH* gradient IPG strips were induced by Görg in the eighties (Gorg et al., 1988). The IPG can be a linear or nonlinear wide (for example IPG 3-12), narrow (for example IPG 4-7) and/or ultra-narrow (for example IPG 4.9-5.3) *pH* range. The IPGs can be cast in different *pH* ranges between *pH* 2.5-12. Also in different lengths, usually from 7–24 cm. Prior to IEF the IPG dry strips must be rehydrated (usually overnight) to their original thickness of 0.5 mm with a rehydration buffer containing urea (or alternatively thiourea), nonioninc or zwitterionic detergents (for example CHAPS) and CA (Gorg et al., 2004). Sample can be added to rehydration buffer (in-gel rehydration) or can be added to the strips before starting isoelectric focusing (cup loading). The longer the IPG strip and the narrower the *pH* gradient, the more voltage required to achieve steady state separation for high reproducibility (Gorg et al., 2004). Isoelectric focusing was carried out using the PROTEAN IEF Cell system (Bio-Rad Laboratories, Munich, Germany). The IPG

strips (17 cm; *pH* 3-10 NL; Bio-Rad Laboratories, Munich, Germany) were rehydrated overnight at 50 V with 0.2 % ampholytes *pH* 3-10 (Bio-Rad, CA, USA). The IEF was performed under the following conditions: 250 V, 1h; 500 V, 1h; 750 V 1h; 1000 V 1h; 2500 V, 1h; 4000 V, 1h; 8000 V, until 60 kVh. The strips were maintained at -80°C until further analysis.

3.2.2.3 Second dimension: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to second dimension, the strips were equilibrated for 15 min in 10 ml equilibration buffer [6 M urea, 0.375 M Tris-HCl (*pH* 8.8), 2% (v/v) SDS, 20% (v/v) glycerol] containing 2% (w/v) DTT and subsequently for 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide. The SDS-PAGE was performed on a Protean® II XL system (Bio-Rad, CA, USA) with lab cast 0.75 mm SDS polyacrylamide gels (12.5%) for 3.5 h for 1100 Vh. The gels were individually stained with 200 mL SyproRuby™ fluorescent stain (Bio-Rad, CA, US) according to the manufacturer's instructions. Gel images were acquired with the Molecular Imager™ FX (Bio-Rad, CA, US) using an excitation wavelength of 532 nm and an emission wavelength filter of 610 nm. A software-based image analysis was carried out for spot detection, matching and quantification using the PDQuest 2D Analysis Software (Bio-Rad, CA, USA). Finally, the gels were stained with silver according to the method described by Shevchenko et al (Shevchenko et al., 1996).

Table 9. Composition of 12% gel (40 ml)

Component	**Volume**
Distilled water	15.4 ml
30% acrylamide mix	16 ml
1.5 M Tris-Cl (*pH* 6.8)	10 ml
10% (m/v) SDS	400 µl

10% (m/v) APS	400 µl
TEMED	40 µl

3.2.2.4 Gel analysis

The gels were individually stained with 200 mL Sypro®Ruby fluorescent stain (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. Each sample (cytosolic and nuclear) was analyzed three times. All gels were scanned at 100-µm resolutions using the Molecular Imager™ FX (Bio-Rad, Hercules, CA, USA) using an excitation wavelength of 532 nm and an emission wavelength filter of 610 nm. Gel images were converted into digital TIF files. Spot detection and pattern evaluation and normalization was performed using the PDQuest 2D Analysis Software (version 7.2, Biorad, Hercules, CA, USA). One non stimulated gel was chosen as a master gel. The protein spots were automatically detected and vizually checked for undetected or incorrectly detected spots and then matched to their corresponding spots in a digitized master gel. Intensity levels were normalized between gels by the total quantity in valid spots in gel images. Protein spots with significant changes (using student's t-test, $P \leq 0.05$) were selected and cut out for identification. Finally, the gels were stained with silver according to the method described by Shevchenko et al (Shevchenko et al., 1996).

3.2.3 Tryptic in-gel digest and MALDI TOF MS analysis

3.2.3.1 Protein identification by peptide mass fingerprinting

Selected protein spots were excised from the gel. The gel pieces were washed alternately three times with 15 µl 10 mM NH_4HCO_3 and 5 mM NH_4HCO_3/50% acetonitrile. After drying the gel pieces, 2 µl of trypsin solution (33 ng/µl in 10 mM NH_4HCO_3, *pH* 7.8) was added to digest the proteins for several hours at 37°C.The

tryptic peptides were extracted from the gel pieces with 5-10 µl 0.1% (v/v) trifluoro acetic acid (TFA) and spotted onto prespotted AnchorChip targets (Bruker Daltonics, Bremen, Germany) according to the manufactures instructions.

The MALDI-TOF mass spectra were recorded on an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), operated in the reflectron mode. For acquisition of peptide mass fingerprint spectra (PMF, MS), 200 single shot spectra were averaged and the peak finding was undertaken using the SNAP algorithm. Protein identification was achieved by searching the mass spectra against the NCBI human protein database (http://www.ncbi.nlm.nih.gov/), using the external search algorithms Mascot™ (version 1.9.03). For PMF spectra, the mass tolerance was set at 50 ppm.

3.2.3.2 Protein identification by peptide sequencing and peptide mass fingerprints

Proteins were identified using Pick ‘n Post Protein identification service (Alphalyse, Denmark) essentially as previously described (Mortz et al., 2001; Shevchenko et al., 1996). Briefly, gel-excised protein spots were reduced, alkylated with iodoacetamide and digested with trypsin. The resulting peptides were concentrated on a ZipTip C18 column (Millipore,USA) and eluted onto an anchorchip target for analysis on a Bruker Autoflex III MALDI TOF/TOF instrument (Bruker Daltonics, Bremen, Germany). The peptide mixture was analyzed in positive reflector mode for accurate peptide mass determination and some of the peptides analyzed by MS/MS fragmentation for partial peptide sequencing. For acquisition of peptide mass fingerprint spectra (PMF, MS), 3000 single shot spectra were averaged and the peak finding was undertaken using the SNAP algorithm. Peptide fragmentation spectra (PFF, MS/MS) were acquired when possible. The MS and MS/MS spectra were combined and used for a Mascot database search (Mascot version 2.1.03) in the NCBI

nrdb protein database protein database. For PMF spectra, the mass tolerance was set at 60 ppm allowing 1 missed cleavage site.

3.2.3.3 Phosphoprotein enrichment by phosphate metal affinity chromatography

Phosphoprotein enrichment was carried out as described in the phosphoprotein enrichment kit user manual (BD Biosciences, USA) Briefly, the cells were treated with 2 ng/ml of TGF-β1 for 10, 30, 60, 120 minutes and then washed 3 times with ice-cold PBS, suspended with 30 µl of solubilisation buffer (buffer A) for each mg of cells. The cell pellet was mixed and centrifuged at 13 000 rpm for 20 min at 4°C and the supernatant fraction stored at -80°C until use. Total protein extracts from every fraction were added to columns, which had been preequilibrated with buffer A for the phosphoprotein enrichment. The column was gently agitated with sample at 4°C for 20 min on a platform shaker to allow the phosphoproteins to bind to a matrix. The non-absorbed material (nonphosphorylated protein fraction) was allowed to flow through the column. The column was washed 4 times by adding 5 ml buffer A and allowing it to flow through the column, and at the same time the first and the last fractions were collected. Finally, 1 ml of elution buffer (buffer B) was added to elute the adsorbed material (phosphorylated protein fraction). The procedure was repeated 4 times.

3.2.3.4 Concentrating and desalting the phosphoprotein fraction

For 2-DE analysis, phosphorylated proteins (the eluant fraction) was concentrated and desalted. This was achieved by reducing the volume of the eluted fraction to 500 µl by centrifigation using the Amicon ultra-4-YM-10 ultrafiltration column (Millipore, USA) and adding 3 ml of LC-MS water. The procedure was repeated 2 times. The concentrated and desalted proteins were purified with a ReadyPred™ 2-D Clean Kit according to the user manual (Bio-Rad Labaratories, CA,

USA). Protein concentration was determined by the BCA protein assay (Pierce, USA) using a SmartSpec™ 3000 spectrophotometer (both Bio-Rad Laboratories, Munich, Germany).

3.2.3.5 Separation of enriched phosphoproteins by two-dimensional electrophoresis

The total phosphoprotein fractions enriched and purified from the cell extracts were subjected to IEF, which was carried out using the PROTEAN IEF Cell (Bio-Rad).The IPG strips (11 cm; pH 3-10 NL) (Bio-Rad) were rehydrated overnight at 50V with 0.2 % ampholytes *pH* 3-10 (Bio-Rad, CA, USA). The first dimension was performed under the following condition: 1000 V linear for 4 h; 1500 V rapid for 1 h; 2500 V rapid for 1 h; 4000 V rapid for 1 h; 6000 V rapid untill 35 kVh. The strips were maintained in -80°C until use. Prior to running the second dimension, strips were equilibrated as described previously (Chapter 3.2.2.3). Criterion™ precast Gels 12% (Bio-Rad, CA, USA) were used for the second dimension. The gels were fixed according to protocol for Pro-Q® Diamond Phosphoprotein Gel Stain in 50% methanol and 10% acetic acid, destained by washing 4 times in 50 mM sodium acetate, *pH* 4.0, 4% acetonitril (ACN) (400 ml *per* gel) for 1 h. Gel images were acquired with the Molecular Imager™ FX (Bio-Rad, CA, US). After scaning gels were incubated in SyproRuby overnight and scaned with the same software. Identification of proteins was carried as described in the section 3.2.2.4.

3.2.4 Immunofluorescence

Alveolar epithelial cells were seeded on eight-well chamber slides at 10 X 10^3 per well and treated as indicated. Cells were then washed with cold PBS and fixed with ice-cold methanol for 10 min at -20°C. After washing twice with PBS, slides were incubated in blocking buffer [5% (v/v) FCS in 1 × PBS] for 1 h at room temperature

followed by an overnight incubation with the primary antibodies at 4°C, as described. After washing, incubation with FITC-labelled secondary antibodies, cells were washed for 5 times with PBS, the plastic border of the slide was removed and slides were covered with mounting medium and a cover slide. Nuclei were visualised by 4,6-diamidino-2-phenylindole (DAPI) staining and individual cells analysed by deconvolution fluorescence microscopy using a Leica AS-MDW miscroscope.

4 RESULTS

4.1 SUBCELLULAR FRACTIONATION

The classic proteomics approach (analysis of total crude homogenate) may fail in the discovery of proteins that are constituent proteins of particular subcellular compartments, but minor proteins of the whole crude homogenate. A study by Dreger *et. al*. (Dreger, 2003a) suggested subcellular fractionation prior to MS analysis in order to detect and identify proteins of particular subcellular compartments. In this study, the classical proteomics approach has been modified. The new strategy based on: stimulation of cells at different time points with TGF-β1, performing subcellular fractionation, and separating protein fractions by two-dimensional electrophoresis was applied (Figure 4.1). Proteins of interest, potential translocoms, have been selected for identification. Since Smads, which are downstream molecules of TGF-β signalling pathway, translocate into the nucleus within 30 min after stimulation (Nicolas et al., 2004), cells were stimulated for 30, 60 and 120 minutes with TGF-β. In order to separate and enrich nuclear and cytosolic fractions, buffers with different tonicity were used. After extracting the cytoplasmic fraction using hypotonic buffers, samples were stored at -80°C, the nuclear fraction in the pellet was dissolved in hypertonic buffer and after 30 minutes of vortexing, samples were centrifuged and proteins from the nuclear fraction, now in supernatant fraction were stored at -80°C.

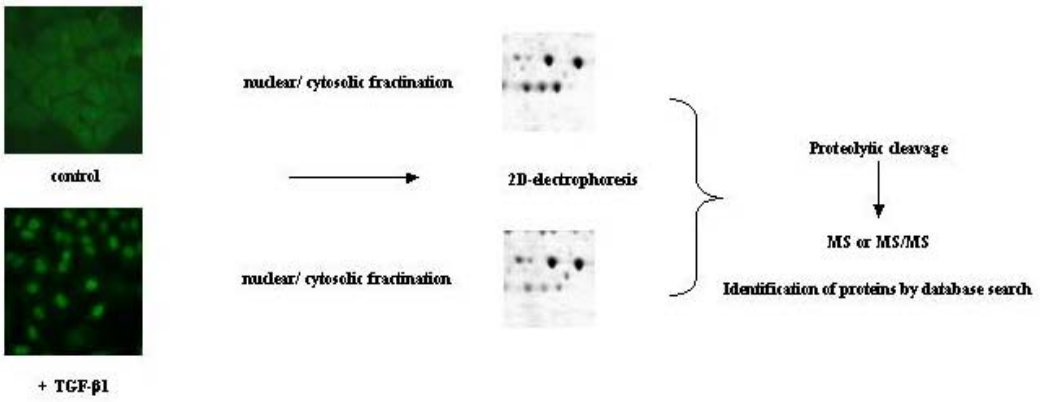

Figure 4.1. Schematic illustration of a strategy to study translocation events. A lung epithelial cell line was stimulated with TGF-β at different time points (0, 30, 60, 120 min), after stimulation subcellular fractionation was performed. Enriched fractions were separated by two-dimensional gel electrophoresis and candidates of interest were identified with mass spectrometry.

Prior to constructing a two-dimensional map of subcellular fractions, regular immunoblotting was performed to demonstrate enrichment of nuclear and cytosolic fractions after TGF-β1 stimulation in time-dependent manner. The protein α-tubulin was used as a cytosolic marker and lamin A/C and phosphoSmad2 were used as a nuclear marker in both control and TGF-β stimulated cells. Using hypertonic and hypotonic buffers it was possible to enrich cytosolic and nuclear fractions as illustrated in Figure 4.2. In addition, due to an increase in pSmad2 phosphorylation it can be concluded that stimulation with TGF-β1 was successful. Faint bands of lamin A/C were observed in the cytosolic fraction. Subcellular fractionation enriches certain cellular compartments but does not achieve complete separation which may be why some traces of lamin A/C present in cytoplasmic fraction. In fact, according to immunoblots against pSmad2, α-tubulin and lamin A/C cytosolic and nuclear fractions were enriched and further study could be carried on.

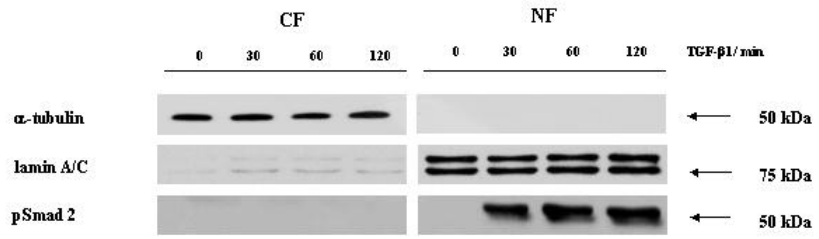

Figure 4.2. Subcellular fractionation of A549 cells. Subcellular fractions, cytosolic (CF) and nucler (NF), were subjected to immunoblot analysis with antibodies against α-tubulin (marker for cytosolic fraction), lamin A/C, and pSmad2 (markers for TGF-β stimulation and nuclear fraction respectivly) after TGF-β stimulation at different time points.

4.2 TWO-DIMENSIONAL SEPARATION OF CYTOPLASMIC AND NUCLEAR PROTEIN FRACTIONS FROM A549 CELL LINE

The TGF-β1 ligand starts signaling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface, which than trigger events inside the cell. Signalling is initiated by ligand-receptor binding at the cell membrane, which causes activation (phosphorylation) of the receptor and which in turn transduces the signal to the cytoplasmic proteins. In this study, cytosolic and nucear fractions were analysed with a focus on activated receptors and Smads interactions inside the cytoplasm. Two-dimensional electrophoretisis (2-DE) maps of nuclear and cytosolic fractions are illustrated in Figure 4.4. According to the result shown in Figure 4.2, maximal accumulation of pSmad2 in the nucleus occurred after 2 h, therefore 2-DE was carried out with enriched protein fractions, which had been stimulated with TGF-β1 for 2 h and for the control samples (those without stimulation). Proteins were visualized with SyproRuby followed by silver staining. Image analysis using PDQuest Image Analysis software. Among the resulting protein spots, only those with a fold change > 1.5 was selected for further analysis. A typical 2-DE analysis with the software, and quantification of proteins stained with SyproRuby dye (control samples and samples stimulated for 2 hours with TGF-β) is illustrated in Figure 4.5. Some of the protein spots did not show change in quantity

after analysis with the software. This spots were abundant and easily identified and were used as a control.

4.2.1 Two-dimensional gel electrophoresis analysis of cytosolic enriched fraction

As expected, large changes at the proteome level in cytosolic- and nuclear-enriched fractions, after cell stimulation with TGF-β (Figure 4.3), were not observed (Figure. 4.4). After analysis with PDQuest software 260 spots have been detected on both gels. A group of spots of basic *pI* and molecular mass between 60-80 kDa were detected only in the cytosolic fraction obtained from cells stimulated with TGF-β1 (spots 3, 4, 5, 6, 7 and 11; Figure 4.4, Table 10). In addition, some protein spots (spot 16, 17, 18, and 19) of acidic *pI* were more intense in control samples in comparison with stimulated samples. Several spots (spots 9, 10, 14, and 15) were excised and served as a control.

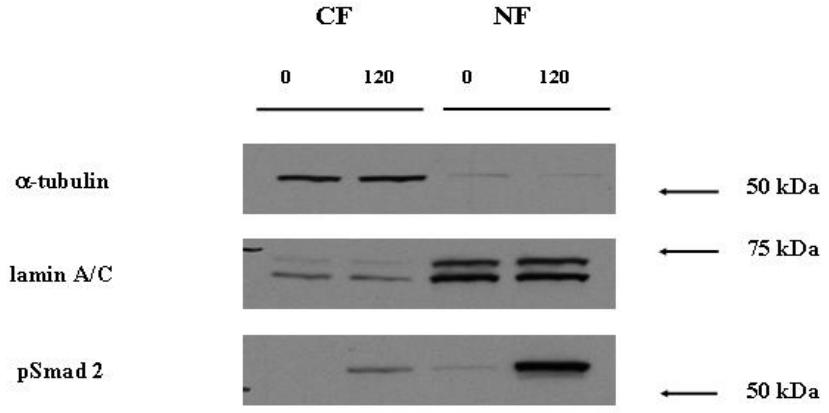

Figure 4.3. Control of subcellular fractionation of A549 cells after 120 minutes TGF-β stimulation. Prior to two dimensional separation of subcellular fractions immunoblotting-control experiments were performed against cytoplasmic (α-tubulin), nuclear (lamin A/C) and TGF-β1 stimulation (pSmad2) markers (CF-cytosolic fraction, NF-nuclear fraction).

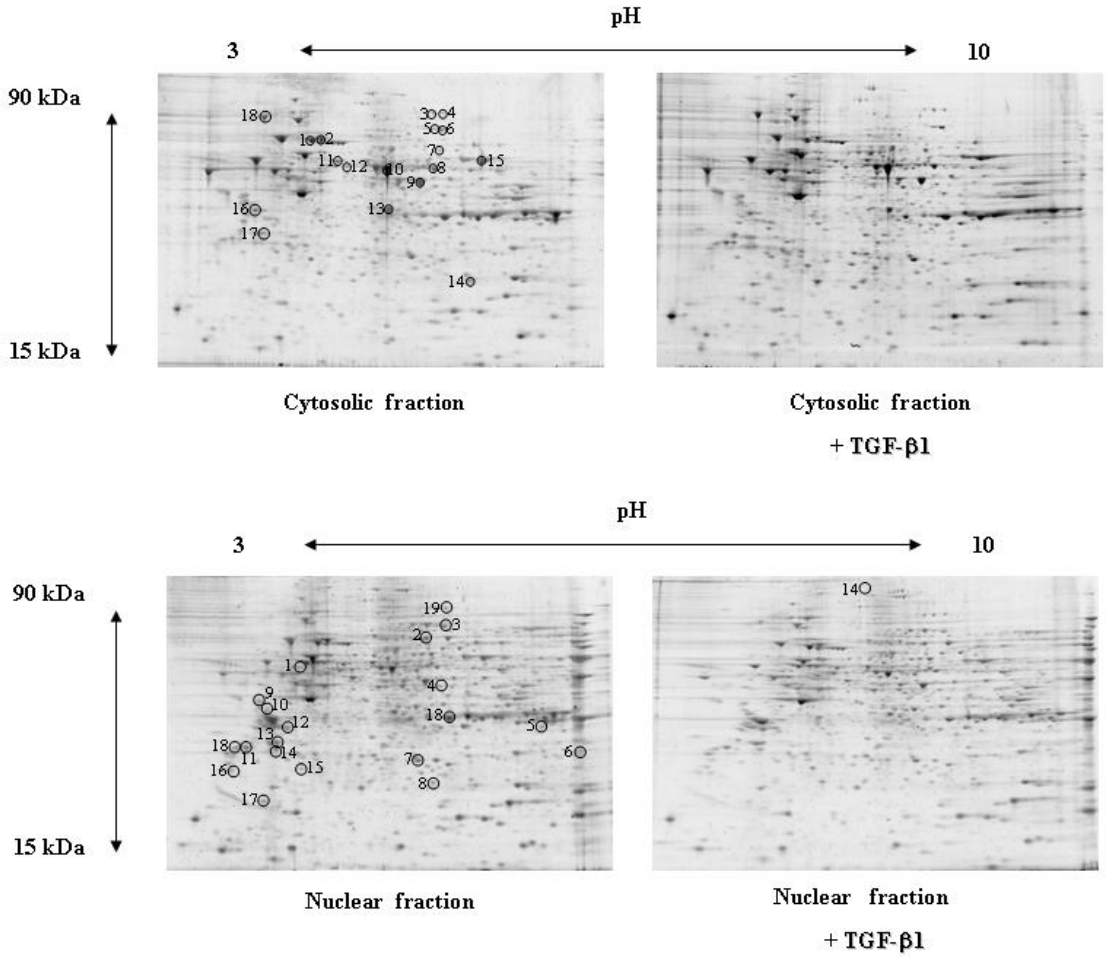

Figure 4.4. Two-dimensional separation of cytoplasmic and nuclear fractions. The Proteins were separated by 2-DE (first dimension, 11 cm 3-10 IPG strips, second dimension 12 % SDS PAGE) and visualised by Sypro®Ruby. The 18 annotated spots are labelled from the cytoplasmic fraction and 19 from the nuclear and corresponding identifications were made by MS and MS/MS as reported in Table 1 and 2.

The group of 19 protein spots was excised from the gels with enriched cytosolic fraction and a tryptic digest was preformed, proteins were identified by MS or MS/ MS. From 19 picked protein spots 18 could be identified (Table 10).

Table 10. Identified proteins from cytoplasmic fraction

Spot (#)	GI-number	Protein	Mascot Score	TGFβ/ control ± s.d	Sequence coverage	Peptides identified by MS/MS
1	5729877	Heat shock 70kDa protein 8 isoform 1	336	2.26±1.82	40%	^{138}TVTNAVVTVPAYFNDSQR^{155} ^{160}DAGTIAGLNVLR^{171} ^{300}ARFEELNADLFR^{311} ^{302}FEELNADLFR^{311}
2	12653415	Heat shock 70kDa protein 9B	367	1.12±1.01	30%	^{188}NAVITVPAYFNDSQR^{202} ^{349}AQFEGIVTDLIR^{360} ^{499}LLGQFTLIGIPPAPR^{513}
3	54648253	KHSRP	238	0.40±0.44	47%	^{123}LASQGDSISSQLGPIHPPPR^{142} ^{321}IGGGIDVPVPR^{331} ^{395}SGPPGPPGGPGMPPGGR^{411} ^{449}AINQQTGAFVEISR^{462}
4	4501867	Aconitase 2 precursor	362	0.29±0.45	36%	^{69}IVYGHLDDPASQEIER^{84} ^{412}SQFTITPGSEQIR^{424} ^{634}NAVTQEFGPVPDTAR^{648}
5	17402900	Far upstream element-binding protein	142	0.77±1.06	40%	^{272}IGGNEGIDVPIPR^{284} ^{309}IQFKPDDGTTPER^{321}
6	37078490	Far upstream element-binding protein	195	0.25±0.44	45%	^{271}IGGNEGIDVPIPR^{283} ^{308}IQFKPDDGTTPER^{320}
7	46812638	hnRNP-L	115	0.55±0.85	33%	^{67}TPASPVVHIR^{76} ^{404}AITHLNNNFMFGQK^{417}
8	189998	M2-type pyruvate kinase	366	1.24±0.656	65%	^{44}NTGIICTIGPASR^{56} ^{74}LNFSHGTHEYHAETIK^{89} ^{279}RFDEILEASDGIMVAR^{294} ^{384}EAEAAIYHLQLFEELRR^{400}
9	62896593	Enolase 1 variant	311	1.14±0.54	47%	^{33}AAVPSGASTGIYEALELR^{50} ^{240}VVIGMDVAASEFFR^{253}
10	21361176	Aldehyde dehydrogenase 1A1	377	0.53±0.19	33%	^{144}TIPIDGNFFTYTR^{156} ^{379}GYFVQPTVFSNVTDEMR^{395} ^{396}IAKEEIFGPVQQIMK^{410} ^{421}ANNTFYGLSAGVFTK^{435} ^{477}ELGEYGFHEYTEVK^{490}
11	5031753	hnRNP-H1	201	0.43±0.43	52%	^{99}HTGPNSPDTANDGFVR^{114} ^{263}DLNYCFSGMSDHR^{275} ^{317}VHIEIGPDGR^{326}
12	21361657	PDIA3	410	1.138±0.402	42%	^{336}FVMQEEFSR^{344} ^{352}FLQDYFDGNLKR^{363} ^{449}GFPTIYFSPANK^{460} ^{472}ELSDFISYLQR^{482}
13	38327502	CArG binding factor	159	0.32±0.32	43%	^{41}DLTEYLSR^{48} ^{49}FGEVVDCTIK^{58} ^{68}GFGFVLFK^{75}

(Continuation of Table 10. from page 42)

Spot (#)	GI-number	Protein	Mascot Score	TGFβ/ control ± s.d	Sequence coverage	Peptides identified by MS/MS
14	99893	Triosephosphate isomerase	233	1.10±0.37	73%	^{33}VPADTEVVCAPPTAYIDFAR^{52} ^{99}RHVFGESDELIGQK^{112} ^{100}HVFGESDELIGQK^{11}
15	30354488	PDI-related protein	93	0.63±0.37	33%	^{355}GSFSEQGINEFLR^{367} ^{374}GSTAPVGGGAFPTIVER^{390}
16	34234	Laminin-binding protein	317	0.62±0.80	50%	^{54}AIVAIENPADVSVISSR^{70} ^{93}FTPGTFTNQIQAAFR^{107}
17	57997573	Hypothetical protein	428	1.49±0.2	40%	^{13}KIQVLQQQADDAEER^{27} ^{55}RIQLVEEELDR^{65} ^{112}KLVIIEGDLER^{126}
18	4507651	Tropomyosin 4	275	1.1±0.49	46%	^{55}RIQLVEEELDR^{65} ^{56}IQLVEEELDR^{69}

Some of the identified proteins (spot 1, 2) are involved in binding to nascent polypeptides to facilitate correct folding. It is well know that TGF-β signalling regulates the rate of protein synthesis. Studies from Takenaka et. al.(Takenaka and Hightower, 1993) reported the rapid induction of HSP70 and HSP90 protein family members within first 5h of TGF-β1 treatment. It is possible that in preparation for increased rates of protein synthesis after stimulation with growth factors, the cell produces more heat shock chaperone proteins (HSPs) to modulate the newly synthesized proteins. The TGF-β ligand can activate p38 MAPK (Seay et al., 2005). Protein spot 3 identified as, K-homology type splicing regulatory protein (KHSRP), an important factor for AU-rich element (ARE)-directed mRNA decay, undergoes p38-dependent phosphorylation during muscle differentiation. Furthermore, KHSRP phosphorylated by p38 displays compromised binding to ARE-containing transcripts and fails to promote their rapid decay (Briata et al., 2005). Protein spots 5 and 6 were identified as far upstream element-binding protein (FUBP1); TGF-β induced p38

expression and promoted its translocation to nuceus for the regulation of FUBP1 and c-myc (Kim et al., 2003). The hnRNPs are RNA binding proteins (protein spot 7, 11) and they perform complex with heterogeneousnuclear RNA (hnRNA). These proteins are associated with pre-mRNA in the nucleus and appear to influence pre-mRNA processing and other aspects of mRNA metabolism and transport. While all of the hnRNPs are present in the nucleus, some seems to shuttle between the nucleus and the cytoplasm (Pinol-Roma and Dreyfuss, 1992). Signalling by TGF-β stimulates activity of glycolytic enzymes (Roy and Terada, 1999). Spots 4 and 8 represent aconitase 2 and M2-type pyruvate kinase, respectively. This suggests that TGF-β can also promote trafficking of glycolytic proteins, and such observations may provide insight into the molecular mechanism of TGF-β action in glycolytic processes. Three novel proteins without assigned function were also been identified (protein spots 13, 16, 18). Thus, we identified translocated proteins involved in diverse activities such as protein folding, transcriptional activation, mRNA metabolism and transport.

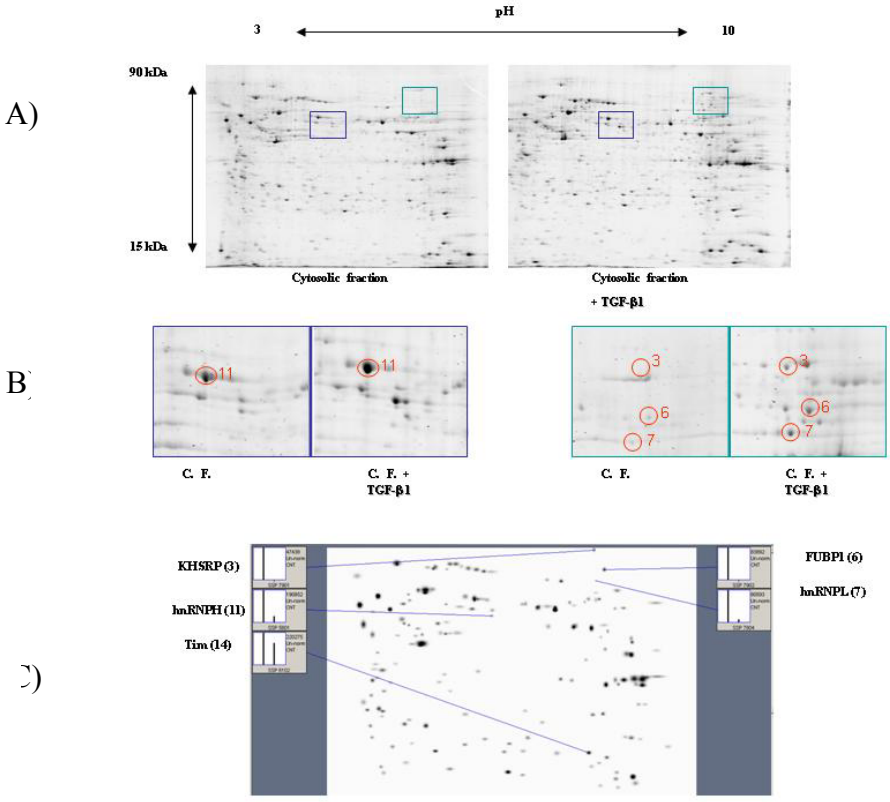

Figure 4.5. Alterations in protein profiles between cytosolic fractions from stimulated cells and unstimulated controls. Separation of cytosolic enriched proteome stimulated with TGF-β1 (2ng/mL) or unstimulated controls with two-dimensional gel electrophoresis (A). Magnification of a 2-DE zone stained with SyproRuby (B) (CF-cytosolic fraction). Master map at the bottom depicts spots, which were detected on both gels (stimulated and control gels) and the difference in spot quantity between conditions(C).

4.2.2 Validation of the translocation data

In order to validate our proteomic data (the enrichment of subcellular fractionation combined with proteomic analysis), we preformed immunoblotting and immunofluorescence with antibodies specific for selected identified proteins. Antibodies to some of the proteins were commercially available, including FUBP1, KHSRP, hnRNPL, hnRNPH. Activation of downstream TGF-β signaling molecules (Smads) and their accumulation in the nucleus occurs within 30 minutes (Nicolas et al., 2004). Therefore, A549 cells, were stimulated for 30 min, 1 h, and 2 h, subjected to immunobloting with antibodies against KHSRP (spot 3), FUBP1 (spot 6), hnRNP L (spot 7), hnRNP H (spot 11) (all identified using a proteomic approach).

Translocation of KHSRP from nucleus started after 30 min and was time-dependent (as illustrated in Figure 4.6). The increased accumulation of KHSRP was detected after 30 min and occured in a time-dependent manner after TGF-β1 stimulation.

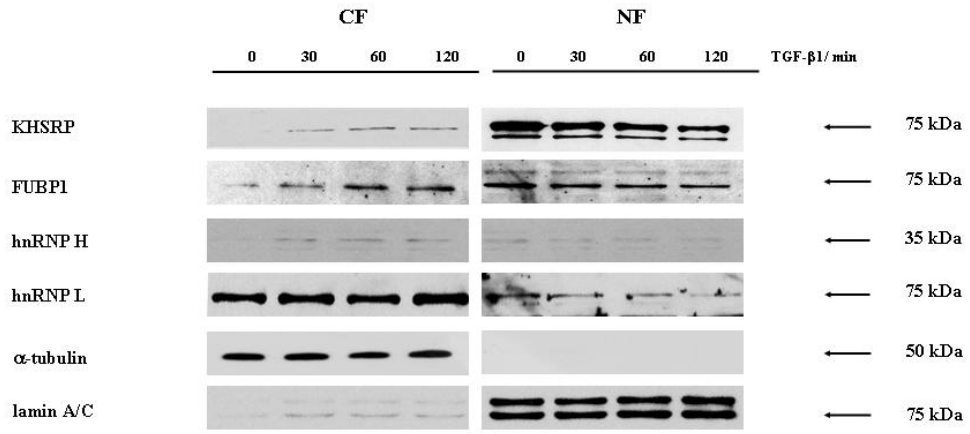

Figure 4.6. Translocation of KHSRP, FUBP1, hnRNP H, hnRNP L in A549 cells after TGF-β1 stimulation. The A549 cells were stimulated with **TGF**-β1 (2 ng/ml), after which subcellular fractionation (CF-cytosolic fraction, NF-nuclear fraction) and immunoblot analysis were preformed.

Using an immunofluorescence assay, KHSRP was detected to be localized in the nucleus and the cytoplasm. After stimulation with 2 ng/ml of TGF-β1, localisation of the KHSRP protein was evident predominantly in the cytoplasm (Figure 4.7).

The FUBP1 was translocated from nucleus after 2 h and increased signal was observed in enriched cytoplasmic fractions; according to the nuclear fraction, translocation was not complete (Figure 4.6); the hnRNP H translocation was confirmed by immunoblotting within 30 minutes stimulation with TGF-β1. The hnRNP L was depleted from nuclear-enriched fractions after 2 h stimulation with TGF-β1 and translocation was confirmed by immunoblotting and immunofluorescence assay (Figures 4.6 and 4.7). Thus, the identities of translocating proteins were established by mass spectrometry and were confirmed by immunoblotting and/or immunofluorescence assays.

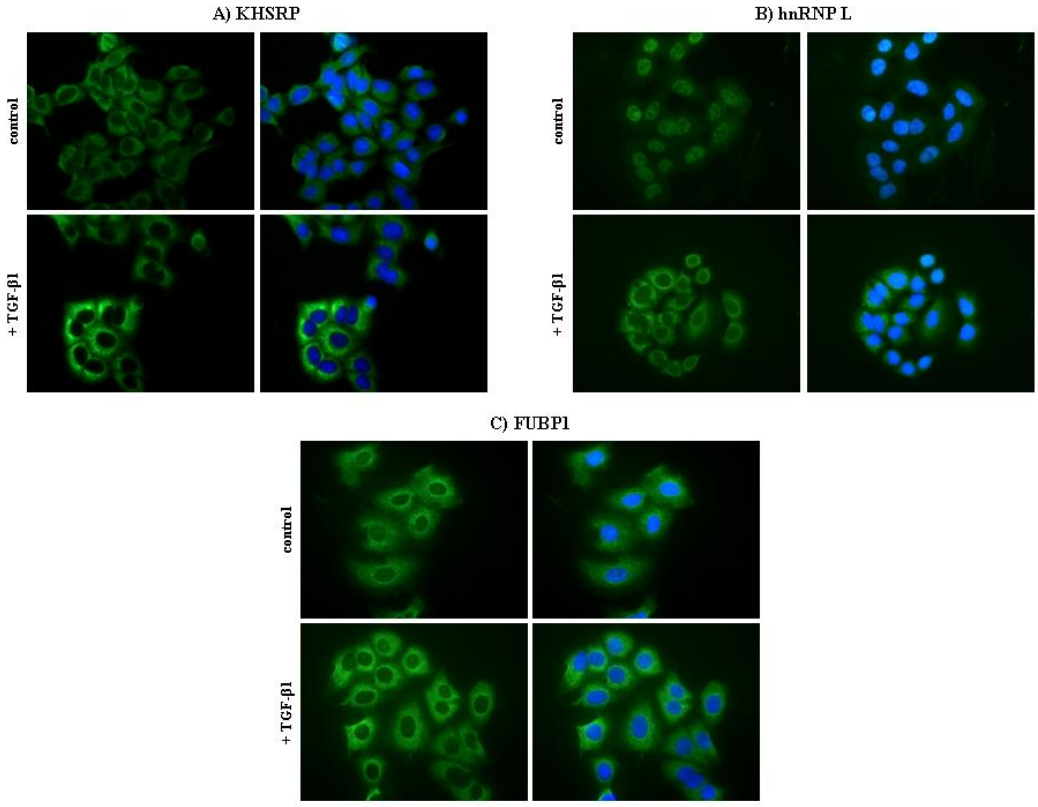

Figure 4.7. TGF-β1-dependent translocation of hnRNP L, FUBP1 and KHSRP. The A549 cells were plated on glass slides, treated as indicated and localisation of translocoms assessed by immunofluorescence analysis. All the translocoms were detected by fluorescein isothiocyanate (FITC)-labeled secondary antibody and nuclei were visualised by 4, 6-diamidino-2-phenylindole staining.

4.2.3 Two-dimensional gel electrophoresis analysis of nuclear enriched fraction

The enriched nuclear proteome is illustrated in Figure 4.3. After analysis with PDQuest software 321 spots have been detected on both gels. According to software analysis, 19 spots exhibited significant differences in quantity of signal and were selected for further analysis. After excision from the gel and in-gel tryptic digestion, proteins from the nuclear fractions were identified (Table 11). From 19 selected spots, 13 were identified. Protein spots 1, 3, 5, 15, 16, 17, 19 were not identified by the MASCOT program, perhaps because: the amount of sample was too low for identification or the peptide mass fingerprints with sound spectra did not match with the protein database. Some of the identified proteins in the nuclear fraction have reported roles in the nucleus (spots 2, 6, 11). Spots 10, 12, 13 and 14 belong to the

tropomyosin family of proteins. Isoforms of tropomyosin-1 were identified in spots 12 and 14. Protein spots 4, 8, 18 were identified like 3-hydroxyisobutyryl-Coenzyme A hydrolase, Chain A, hCG2017792 isoform CRA_b. These proteins have only been detected until now at the transcript level and this is the first time that were detected at the protein level. Protein spot 9 was identified as reticulocalbin, a member of CREC family of proteins primary localised in endoplasmatic reticulum. Protein spot 7 was identified as triosephosphate isomerase and was reported to be regulated at the protein level by TGF-β stimulation in human fibroblast (Berns et al., 2004; Bratt et al., 2001).

Table 11. Translocated proteins identified in nuclear fraction

Spot (#)	GI-number	NAME OF PROTEIN	Mascot Score	TGF-β/Control ± s.d.)	Sequence coverage	Peptides identified by MS/MS
2	16241370	myc far upstream elementbinding	336	0.62±0.2	38%	^{138}TVTNAVVTVPAYFNDSQR^{155} ^{160}DAGTIAGLNVLR^{171} ^{300}ARFEELNADLFR^{311} ^{302}FEELNADLFR^{311}
4	119631278	Coenzyme A hydrolase	367	0.49±0.11	24%	^{188}NAVITVPAYFNDSQR^{202} ^{349}AQFEGIVTDLIR^{360} ^{499}LLGQFTLIGIPPAPR^{513}
6	74735411	CPSF5	238	n.d	34%	^{123}LASQGDSISSQLGPIHPPPR^{142} ^{321}IGGGIDVPVPR^{331} ^{395}SGPPGPPGGPGMPPGGR^{411} ^{449}AINQQTGAFVEISR^{462}
7	39932641	triosephosphate isomerase	362	1.29±0.19	22%	^{69}IVYGHLDDPASQEIER^{84} ^{412}SQFTITPGSEQIR^{424} ^{634}NAVTQEFGPVPDTAR^{648}
8	14488599	Chain A	142	0.69±0.1	20%	^{272}IGGNEGIDVPIPR^{284} ^{309}IQFKPDDGTTPER^{321}
9	2493462	reticulocalbin 1	195	2.53±0.95	29%	^{271}IGGNEGIDVPIPR^{283} ^{308}IQFKPDDGTTPER^{320}
10	136090	beta tropomyosin	115	n.d	18%	^{67}TPASPVVHIR^{76} ^{404}AITHLNNNFMFGQK^{417}
11	4503477	EF-1-beta	366	1.28±0.22	24%	^{44}NTGIICTIGPASR^{56} ^{74}LNFSHGTHEYHAETIK^{89} ^{279}RFDEILEASDGIMVAR^{294} ^{384}EAEAAIYHLQLFEELRR^{400}
12	136092	skeletal muscle tropomyosin	201	0.43±0.21	22%	^{99}HTGPNSPDTANDGFVR^{114} ^{263}DLNYCFSGMSDHR^{275} ^{317}VHIEIGPDGR^{326}
13	24119203	tropomyosin 3 isoform 2	410	n.d	39%	^{336}FVMQEEFSR^{344} ^{352}FLQDYFDGNLKR^{363} ^{449}GFPTIYFSPANK^{460} ^{472}ELSDFISYLQR^{482}
14	89954539	tropomyosin 1	159	n.d	32%	^{41}DLTEYLSR^{48} ^{49}FGEVVDCTIK^{58} ^{68}GFGFVLFK^{75}

4.3 NEWLY IDENTIFIED PHOSPHOPROTEINS IN TGF-β SIGNALLING PATHWAY

4.3.1 Purification of phosphoproteins

Protein phosphorylation plays an important role in various cellular processes such as cell division, metabolism, survival and apoptosis. It is driven by a balanced interplay between phosphatases and tyrosine and serine-threonine protein kinases. In order to identify novel phosphorylated proteins after stimulation with TGF-β ligand, A549 cells were cultured in 1% FCS over 24 h and then stimulated for 1 h with TGF-β1 concentration 2ng/mL. After stimulation, cells were harvested, and proteins were extracted. As phosphorylated proteins are of a relatively low abundance in the cell in comparison to the total proteome, it was necessary to perfrom phosphoprotein enrichment prior to MS analysis (Pandey et al., 2000). For enrichment of phosphoproteins, a commercially available phosphor metal affinity chromatography (PMAC) was employed (Figure 4.8).

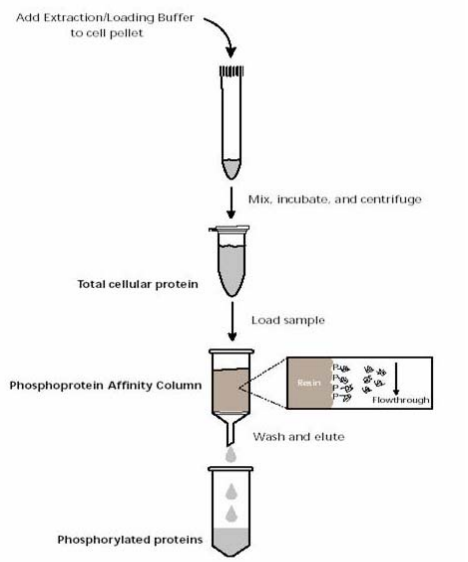

Figure 4.8. The phosphoprotein enrichment procedure. Extraction/Loading Buffer contains a mild, non-ionic detergent for efficient, non-denaturing extraction of cellular proteins.

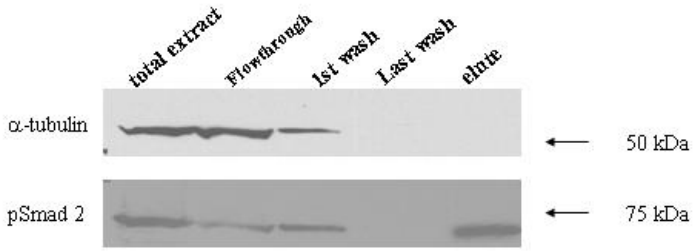

Figure 4. 9. Complete separation of non-phosphorylated and phosphorylated proteins.
Phosphoprotein affinity column was loaded with 8 mg of protein extracted from A549 cells. Cell extracts were divided into numerous fractions including total extract, flowthrough, first wash, last wash, and eluant. The α-tubulin, and pSmad2 were used as a control for immunodetection of enriched non-phosphorylated and phosphorylated proteins respectively.

Protein extracts from A549 cells stimulated with TGF-β1 or control (non-stimulated cells) were separated into flowthrough, first wash, last wash, and eluant fractions by PMAC. Both pSmad2 and α-tubulin were used as a control for phosphoprotein enrichment. In a total cell extracts, pSmad2 and α-tubulin were deceted (Figure 4.9). Decreased amounts of pSmad2 were observed in flowthrough fractions, indicating some loss of phosphoproteins after phosphoproteome enrichment with a commercially available enrichment kit. The absence of non-phosphorylated α-tubulin in the eluant fraction (which is enriched with phosphorylated proteins) and presence of pSmad2 in the eluant demonstrated the effective separation of phosphorylated proteins from non-phosphorylated proteins using PMAC (Figure 4.9). Therefore, we could purify and enrich phosphorylated proteins from cellular extracts. Since Pro-Q Diamond binds exclusively to phosphoryl groups of phosphotyrosine, phosphoserine and phosphothreonine in phosphoproteins (Patton, 2000), it was used as a detection reagent. The gel was visualizated with Pro-Q Diamond to detect phosphoproteins and the same amount of sample was loaded to the gel that was stained with SyproRuby to detect total proteins (Figure 4.10). The amount of cellular phosphoproteins was increased in A549 cells stimulated with TGF-β1 compared with the unstimulated control samples after 10 minutes stimulation with TGF-β. Analysis of phospho

proteome induced by TGF-β after 2 and 4 hours of stimulation has been already reported (Stasyk et al., 2005) and in this study it was observed increaded phosphorylation level after 10 minutes of stimulation. For further analysis, 1 hour time point was chosen for stimulation in order to recognize interplay between kinases and phosphatases.

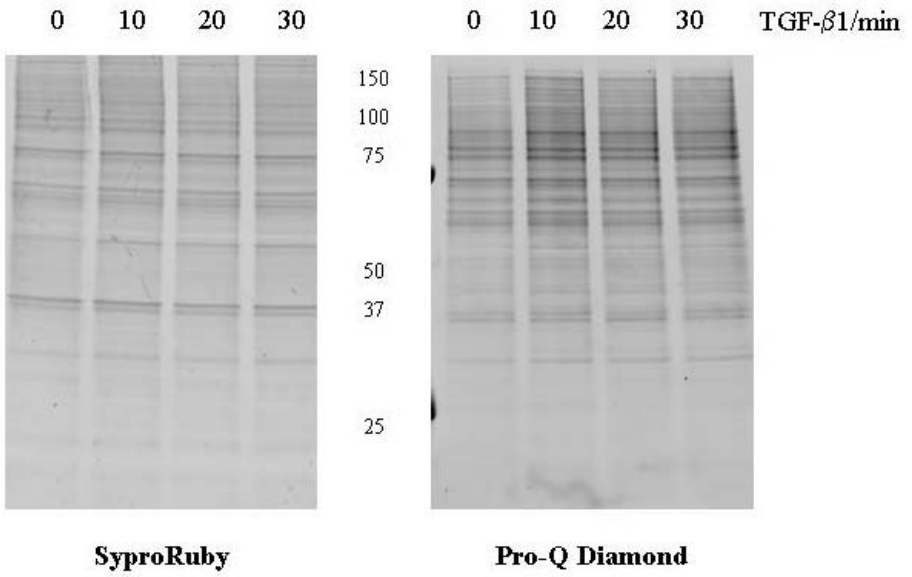

Figure 4.10. Phosphoproteome of A549 cells after elution of phosphoproteins from phosphate metal affinity chromatography (PMAC) separated by one dimensional gel electrophoresis. After stimulation with 2 ng/mL of TGF-β1 0, 10, 20, and 30 min proteins were extracted and the same amount of proteins (20 μg) was loaded to the gel. Phosphoproteins were visualized with Pro-Q Diamond and the total proteome was visualised with SyproRuby.

4.3.2 Analysis of total phosphoprotein using two-dimensional electrophoresis

It was previously demonstrated that TGF-β, can activate other signaling pathways like MAPKs (ERK1/2, p38 and c-Jun N-terminal kinase (JNK) pathways (Javelaud and Mauviel, 2005; Yue and Mulder, 2000). In addition to the Smads the number of phosphoproteins induced by TGF-β is still unclear. To address this question, PMAC-enriched phosphoprotein fractions isolated from cells that were either unstimulated or stimulated with TGF-β were separated on a 2-DE gel. The same gels were visualized with ProQ-Diamond (data not shown) and later with the SyproRuby (Figure 4.11) staining. The PDQuest 6.0. software was used to normalize

the total amount of protein. The protein spots detected were mainly located in the *pI* range of 4–7, which was consistent with the fact that phosphorylated proteins are usually acidic proteins, due to the addition of one or more phosphate groups. Spots differently induced by TGF-β1 were scored when they were greater than two-fold elevated on SyproRuby-stained gels or if protein spots showed up in a multiple pattern. That has been observed in three replicate gels from three independent experiments. The analysis revealed 35 reproducible protein spots and which were subjected to identification (Figure 4.12).

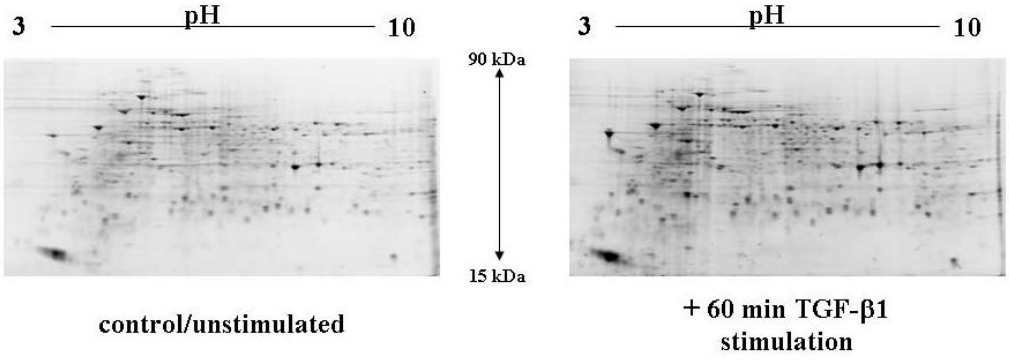

Figure 4.11. Two-dimensional electrophoretic analysis of A549 cell fractions after selective phosphoprotein enrichment. Proteins (40μg) were applied to a 11 cm *pH* 3-10 strip for the first-dimension and later proteins were separated by SDS-PAGE. The gels [control (unstimulated) and 60 min of stimulation with 2n/mL TGF-β1] were stained with SyproRuby.

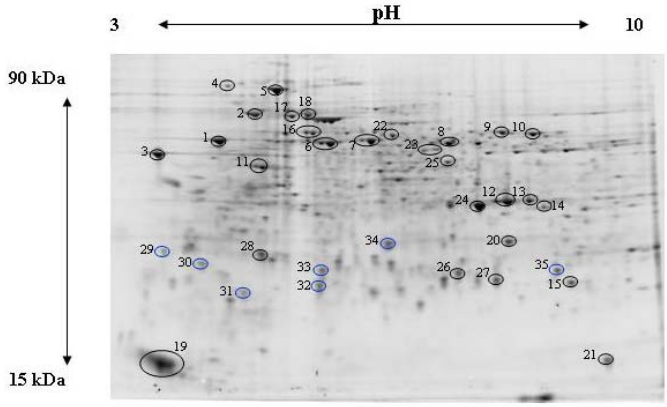

Figure 4.12. Two-dimensional electrophoretic analysis and alignment of phosphorylated proteins after stimulation with TGF-β for 60 minutes. A phosphoprotein affinity column was loaded with 8 mg of total protein. Total enriched phosphoproteins purified from 8 mg of total protein were separated by 2-DE with a *pI* 3–10 gradient after concentrating and desalting: 2-DE gel images of A459 cells stimulated with TGF-β1 for 60 min. Proteins spots which showed reproducible difference between two conditions, non-stimulated and stimulated with TGF-β (2ng/mL) are assigned in the figure.

4.3.3 Identification of differential phosphoproteins triggered by TGF-β1

Protein spots detected after Pro-Q Diamond and SyproRuby stained were subjected to in-gel tryptic digestion and protein identities were determined by PMF and by searching the NCBInr or Swiss-Prot or TrEMBL databases using the MASCOT and ProteinProspector programmes. Proteins with low-abundance were identified by pooling spots from more than two gels. Out of 35 protein spots detected on gels, that showed reproducible differences or remained the same (taken as a control), 25 proteins were identified in the enriched fraction from A549 cells and are listed in Table 12. Among them, 15 have been reported to be phosphorylated but not in response to TGF-β. Here, we report 10 proteins that have not previously been described to be phosphorylated. Protein spots identified as: PDI (spot 1), GRP 78 (spot 2), calreticulin (spot 3), endoplasmin (spot 4), TER ATPase (spot 5) did not show any change after stimulation with TGF-β. They were excised from the gel as a phosphoprotein controls. Some spots were identified as: PDIA3 (spots 6), keratin, type II (spots 17, 27), annexin A2 (spots 12, 13, 14), TCP-1-epsilon (spot 16), TCP-1-

beta (spot 7). These proteins with the same molecular mass but differing in their *pI* (two spots for the same protein), were previously reported as phosphoproteins but not directly involved in TGF-β signalling (Figure 4.12)

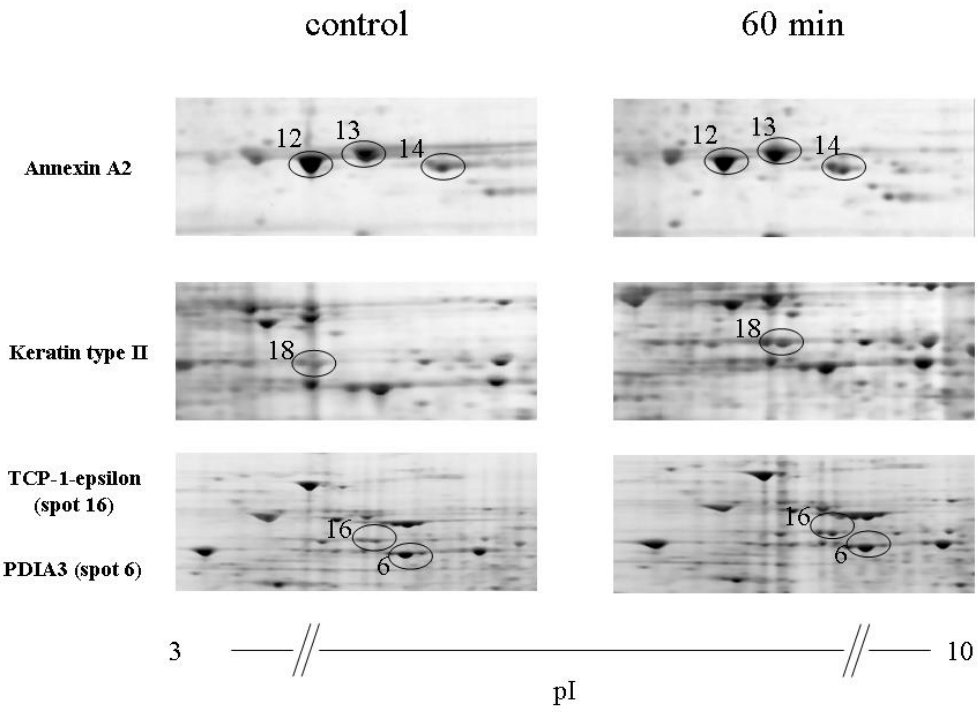

Figure 4.12. Two-dimensional electrophoretic analysis of proteins from TGF-β1 stimulated cells compared with the non-stimulated control. Magnification of a 2-DE zone stained with SyproRuby.

4.3.4 Prediction of phosphorylatrion sites

Eukaryotic proteins contain one or more motif domains for binding of kinases, phosphatases and phoshopeptides. They also contain characteristic sequence motifs for post-translational modifications such as phosphorylation, glycosylation or binding to specific modular domains (Obenauer and Yaffe, 2004). Scansite (http://scansite.mit.edu) is a computational tool developed on the basis of experimental results of binding and/or substrate information from oriented peptide library screening and phage display experiments to develop scoring algorithm that predicts protein–protein interactions and sites of phosphorylation (Obenauer et al., 2003). In this study, Scansite was used to analyse phosphorylated proteins; newly found to be triggered by TGF-β1 stimulation. From 25 identified proteins, 15 were

already reported to be phosphorylated (spots 1, 2, 4, 5, 6, 10, 11, 14, 17, 18, 19 20, 21, 23, 25). Six proteins identified as calreticulin (spot 3), glutamate dehydrogenase 1 (spot 8), TCP-1-eta (spot 9), TCP-1-zeta (spot 22), aldo-keto reductase family 1 member C1 (spot 24), cathepsin D (spot 28) were comfirmed by Scansite. Only for four protein spots: TCP-1-beta (spot 7), proteasome subunit beta type-1 (spot 15), TCP-1-epsilon (spot 16), proteasome subunit beta type-2 (spot 26) we could not find any current data about their phosphorylation up to now. In conclusion, from 25 identified proteins only for 4 does not exist in any kind of data as to be phosphorylated, in that manner PMAC enrichment colums showed high purification specificity of phosphorpoteins.

Table 12. Phosphorylated proteins identified by MALDI-TOF-MS

Spot number	Name of Protein	Matched Peptides	Accession Number	Coverage	Mascot Score
1	PDI	18	P07237	40%	197
2	GRP 78	33	P11021	45%	144
3	Calreticulin precursor	23	P27797	41%	122
4	Endoplasmin	34	P14625	35%	118
5	TER ATPase	24	P55072	45%	150
6	PDIA3	24	P30101	43%	148
7	TCP-1-beta	36	P78371	61%	165
8	Glutamate dehydrogenase 1	38	P00367	60%	300
9	TCP-1-eta	19	Q99832	37%	131
10	Pyruvate kinase isozymes M1/M2	25	P14618	46%	103
11	PDIA6	17	Q15084	38%	77
12,13,14	Annexin A2	24	P07355	57%	181
15	Proteasome subunit beta type-1	18	P20618	66%	99
16	TCP-1- epsilon	14	P48643	30%	93
17,27	Keratin, type I cytoskeletal 10	14	P13645	22%	69
18	Keratin, type II cytoskeletal 1	16	P04264	25%	79
19	Nucleolar phosphoprotein B23	4	P06748	46%	135
20	Proteasome subunit alpha type-4	10	P25789	35%	56
21	Single-stranded DNA-binding protein	10	Q04837	62%	60
22	TCP-1-zeta	14	P40227	30%	68
23	IMP dehydrogenase 2	14	P12268	28%	146
24	Aldo-keto reductase family 1 member C1	11	Q04828	30%	53
25	Alpha-enolase	18	P06733	50%	154
26	Proteasome subunit beta type-2	12	P49721	39%	116
28	Cathepsin D precursor	12	P07339	26%	50

Table 13. Classification of identified phosphoproteins divided into functional categories

Functional category	Protein
ATPs Family	transitional endoplasmic reticulum ATPase
heat shock protein 70 family	78 kDa glucose-regulated protein
heat shock protein 90 family	endoplasmin
TCP-1 chaperonin family	T-complex protein subunit 1 zeta
	T-compex protein 1 subunit epsilon
	T-complex protein 1 subunit eta
	T-complex protein 1 subunit beta
enolase family	α-enolase
nucleoplasmin family	nucleolar phosphoprotein B23
peptidase A1 family	cathepsin D
peptidase T1B family	proteasome subunit beta type-1
	proteasome subunit beta type-2
peptidase T1A family	proteasome subunit alpha type 4
IMPDH/GMPR family	IMP dehydrogenase 2
aldo/keto reductase family	aldo-keto reductase family 1 member C1
Glu/Leu/Phe/Val dehydrogenases family	glutamate dehydrogenase 1
annexin family	annexin A2
protein disulfide isomerase family	protein disulfide-isomerase precursor
	protein disulfide-isomerase A6
	protein disulfide-isomerase A3
intermediate filament family	keratin, type II cytoskeletal 1
	keratin, type I cytoskeletal 10
pyruvate kinase family	pyruvate kinase isozymes M1/M2
calreticulin family	calreticulin

5 DISCUSSION

5.1 TRANSLOCATION IN RESPONSE TO TGF-β STIMULATION

5.1.1 Analysis of cytoplasmic fraction

Understanding of TGF-β signalling is of great importance for the in depth knowledge of how this factor can regulate cell growth, cell differentiation, proliferation and some other cellular processes. In an unstimulated state, R-Smads are predominantly cytoplasmic and Smad4 is distributed through out the cytoplasm and nucleus. After ligand stimulation, both Smad4 and R-Smads rapidly accumulate in the nucleus. One of main features of Smad behaviour is the dynamic nucleo-cytoplasmic shuttling that is regulated by a variety of pathways (Reguly and Wrana, 2003). Till date, many studies have been performed in order to identify novel interaction partners of TGF-β receptors or downstream Smad molecules (Stasyk et al., 2005; Zakrzewicz et al., 2007). Here, we describe the combination of subcellular fractionation and a proteomic approach as a strategy for the identification of translocation events in the cell following TGFβ stimulation.

Cellular signalling starts at the plasma membrane and continue in the cytoplasm. After translocation of Smad molecules to the nucleus, expression of genes can be initiated. In order to investigate changes in translocation of intracellular proteins after TGF-β stimulation, proteome maps of subcellular fractions were determined. The proteome maps were complex, out of a total 260 spots detected under various conditions, 22 spots in the cytoplasmic fraction and out of 321 detected spots in nuclear fraction, 19 spots in the nuclear fraction were taken for further analysis (Figure 4.3).

Eighteen proteins in the cytoplasmic fraction and nine in the nuclear fraction were identified that are known to translocate after TGF-β1 stimulation (Tables, 10

and 11). In order to confirm the results obtained with this novel approach, two independent methods (immunoblotting and immunofluorescence) were emoloyed following translocoms: hnRNP H, hnRNP L, FUPB1 and KHSRP (Figure 4.6 and Figure.4.7). The identified translocated proteins fall into several groups with diverse activities such as protein folding, transcriptional activation, mRNA metabolism and transport.

The protein FUBP1 binds a pyrimidine-rich sequence in the 3′ UTR (untranslated region) of GAP-43 mRNA and has been postulated to modulate stability of the transcript (Kim et al., 2003). A FUSE-binding protein was previously identified as a sequence specific single- stranded DNA binding protein which targets a far upstream *cis* element (FUSE) of c-myc and stimulates its expression (Liu et al., 2006). In proliferating hematopoietic cells, FBP and c-myc mRNA expression have been shown to be co-ordinately regulated (Kim et al., 2003; Liu et al., 2006). It is known that TGF-β can induce p38 expression and promote its translocation in to nuclei for regulation of the FUSE-binding protein and c-myc (Yu et al., 2002), it is highly possible that this mechanism of translocation can be directed by p38 MAPK. Heat shock proteins (HSPs) can be classified according to their molecular weight into four major families, the small molecular weight HSP family, the HSP60 family, HSP70 family, and HSP90 family (Takenaka and Hightower, 1993). Upon exposure of cells to environmental stresses including heat shock, oxidative stress, heavy metals, or pathological conditions (such as ischaemia, inflammation, tissue damage, infection, and neoplastic transformation) cells respond in rapid increase in HSP expression. Recently Mattaj and Englmeier (Mattaj and Englmeier, 1998) reported that HSP70 promotes the formation and stability of a nuclear localisation signal (NLS) complex and regulates the import of proteins into the nucleus. Morimoto (Morimoto, 2002)

found that HSP could dynamically remodel transcription complexes. It is thus possible that HSP70 helps to facilitate molecular transport between the nucleus and cytoplasm in response to TGF-β stimultion.

hnRNPs are predominantly nuclear RNA-binding proteins that form complexes with RNA polymerase II transcripts. These proteins have a broad spectrum of cellular functions, from transcription and pre-mRNA processing in the nucleus to cytoplasmic mRNA translation (Pinol-Roma and Dreyfuss, 1992). Recent studies have suggested that several major characteristics of hnRNPs can explan their involvement in regulatory pathways. It has been reported that hnRNP A2/B1 is involved in mRNA splicing or mRNA localisation (Pinol-Roma and Dreyfuss, 1992). Yan (Yan et al., 2006) confirmed that hnRNP directly binds to Smad2 or hnRNP binds to an interacting partner of Smad2, so translocation of hnRNP proteins was expected and therefore we used it as a positive control in this study (Figure 4.6 and Figure 4.7).

Aconitase 2 and M2-type pyruvate kinase are predominantly mitochondrial proteins. Both of them have been identified to be increased in cytosolic fraction upon TGF-β1 stimulation. Study from Shyamal et.al (Roy and Terada, 1999) clearly showed that TGF-β modulated activities of pyruvate kinase which is involved in glycolysis and Krebs cycle. Mitochondrial translocation of aconitase 2 and pyruvate kinase in response to TGF-β1 stimulation can give us better insight into tricarboxylic acid cycle and glycolysis and the role of TGF-β signalling in their regulation.

5.1.2 Analysis of nuclear fraction

In the nuclear fraction 11 proteins were identified of which eight shuttled from the nucleus and three into the nucleus after stimulation with TGF-β. Among 11 proteins, members of tropomyosin family were identified and all of them according to proteomic data translocate from nucleus after TGF-β stimulation. The study by Piek

et. al. (de Caestecker et al., 2000) has shown how TGF-β induction of actin stress fibers depends on Smad signalling. Tropomyosins play an essential role in TGF-β induced stress fiber formation in mouse and human epithelial cell lines (Bakin et al., 2004)**.** Translocation study of tropomyosin family members can help in better understanding of TGF-β regulation of stress fibers and control of cell motility. In this study elongation factor 1-beta (EF-1) was identified to be translocated into nucleus after TGF-β stimulation. Iborra *et. al*. showed that 10–15% of cellular protein synthesis can occur in the nucleus (Iborra et al., 2001). Some elongation factors can obtain their function in the nucleus; EF-1 can be one of the candidates, although further analysis are required in support of this hypothesis. Cleavage and polyadenylation specificity factor subunit 5 (CPSF5) belongs to nudix hydrolase family and is involved in processing of pre-mRNA in the nucleus. In the study from Borsi L *et. al* (Borsi et al., 1990) it is presented that TGF-β may regulate the splicing pattern of pre-mRNA and translocation of CPSF5 together with other pre-mRNA regulatory proteins which we identified here to translocate in response to TGF-β stimulation. This might give us a better overview into implication of TGF-β signaling pathway into pre-mRNA processing.

Moreover, subcellular fractionation combined with proteomic approach gave good combination of analytical methods to identify translocated proteins in response to TGF-β1 stimulation. With this approach we can get better insight into uncovered regulatory processes of TGF-β signalling. The translocoms reported in this study could represent new directions to be followed to understand multiple functions of these TGF-β signalling. Further studies are required to clarify functional contribution of translocoms to TGF-β signalling.

5.2 IDENTIFICATION OF NOVEL PHOSPHORYLATED PROTEINS IN TGF-β SIGNALLING PATHWAY

Previously, after stimulation with TGF-β1 from 2 hours up to 4 hours, protein phosphorylation level was shown to have a relatively high amplitude of changes (Kanamoto et al., 2002), (Stasyk et al., 2005). In this study, A549 cells were incubated with TGF-β1 up to 1 hour. Phosphoproteome was enriched and proteins phosphorylated in respons to TGF-β stimulation were identified (Tables 12 and 13). Activation of TGF-β receptors leads to a large number of intracellular interactions, which include different effector molecules. The stimulus can lead to cytoskeletal changes, activation of transcription in the nucleus, and ultimately result in cell proliferation, differentiation or motility. The TGF-β ligands signal via membrane-bound heteromeric serine/threonine kinase receptor complexes. Upon ligand binding, receptor activation leads to the phosphorylation of cytoplasmic protein substrates of the Smad family (Derynck and Feng, 1997). Phosphorylation is one of the most important events in TGF-β signalling.

In this study, phosphoproteome profiling of TGF-β signaling in the lung alveolar epithelial cell line A549 was employed to reveal novel regulatory targets of TGF-β signalling. Two-dimensional gel electrophoresis and peptide mass fingerprinting using MALDI-TOF-MS in combination with phosphoprotein enrichment provided reproducible phosphoproteome maps and a high efficiency of protein identification (Table 12). It has been selected for identification of 35 phosphorylated proteins and 25 have been identified. To investigate this issue further, PMAC was employed for the enrichment of phosphorylated proteins from the cellular extracts (Figure 4.8). Phosphorylated and non-phosphorylated proteins were nearly completely separated (Figure 4.9). Phosphorylated proteins were enriched from total

proteome and were identified. These results indicated that PMAC can be used as a powerful tool to purify and enrich phosphorylated proteins from cellular extracts. Most of the protein spots (phosphoproteins) were detected on both gels, sometimes the same protein was detected in multiple spots (Figure 4.12). This can be due to different phosphorylated isoforms. Other post-translational modifications (including glycosylation, sulfation, acetylation and alkylation) are excluded to be present, since used fractions in this study phosphoproteins were exclusively purified from the cellular extract (Figure 4.9). As confirmed by MALDI-TOF-MS, they were indeed the same proteins with different phosphorylation status (Figure 4.12).

Yue et al.(Yue and Mulder, 2000) described the crosstalk between TGF-β and MAPK signalling pathways in *in vitro* cell models. Downstream targets of receptor tyrosine kinases, G-protein-coupled receptors or cytokine receptors also participate in the TGF-β signalling network (Derynck and Zhang, 2003). Experimental evidence for annexin A2 (spots 12, 13, 14) phosphorylation by pp60src was demonstrated in HEK 293 cells (Deora et al., 2004). Annexin A2 can also be phosphorylated after the activation of other membrane-associated kinases, for example insulin-like growth factor 1 and PDGF in CHO cells (Biener et al., 1996). Some studies have previously reported that TGF-β could activate PKC (Yakymovych et al., 2001). It can be hypothesised that phosphorylation of annexin A2 might be mediated by TGF-β via PKC.

The subunits of chaperonin containing TCP1 were identified from two-dimensional electrophoretic analysis in protein spots 7, 9, 16 and 22. It is known that TCP-1 chaperonin family of proteins play a role in the folding of some structural proteins like actin and tubulin. Previous studies have indicated that these proteins have a higher level of tyrosine phosphorylation in-stress induced conditions (Kim et al.,

2002). Berns et al. (Berns et al., 2004) used siRNA bar-code screens to identify TCP-1-beta as a downstream target of the p53 signaling pathway, indicating that members of thise protein family might be involved in the p53 pathways and indicating direct or indirect involvement of TGF-β in p53 signalling. Heat shock proteins [endoplasmin (spot 4) and GRP-78 (spot 2)] and protein-disulfide isomerase [PDI (spot 1)] were identified as a substrate for Akt signalling (Laugesen et al., 2006). In addition, HSPs change their phosphorylation in MCF-7 cells under stimulation with TGF-β1 within 24 hours (Stasyk et al., 2005). In this study both identified HSPs were detected not to change their phosphorylation up to one hour of stimulation with TGF-β; together with TER ATPase (spot 5), calreticulin (spot 3) and PDI (spot 1) they were cut out to be identified as a control for phosphorproteins. The biological significance of HSP phosphorylation induced by TGF-β after one hour of stimulation was not examined in the present study but phosphorylation of these proteins can regulate interaction with other chaperones and intracellular proteins. Protein-disulfide isomerases identified in this study [PDIA3 (spot 6), PDIA6 (spot 11)] are known to localize mainly in endoplasmatic reticulum, their role is to catalyze the -S-S- bonds in proteins. Phosphorylation sites on PDIA6 has been already identified (Beausoleil et al., 2006) and some members of PDI family has been described to play a role of a substrate in Akt signalling (Barati et al., 2006), what kind of role thise proteins play in TGF-β signalling up to now has not been explained.

The proteins that are components of the cytoskeleton and that regulate actin filament formation were also identified to alter their phosphorylation: keratin type II (spot 18) and keratin 10 type I (spots 17, 27). The TGF-β1 dependent regulation of expression of keratin type I and II and alpha-enolase (spot 25) was described in Mv1Lu cells (Kanamoto et al., 2002). The study from Stasky el. al (Stasyk et al., 2005) showed

keratin type I and keratn type II to change their phosphorylation rate upon TGF-β stimulation up to 24 hours in epithelial breast cancer cell line. Additional experiments need to be carried out in order to confirm involvement of those proteins in TGF-β signalling. Alpha-enolase (spot 25) plays a role in a number of different processes in the cell like carbohydrate degradation and glycolysis. Phosphorylation sites of it has been described in a human embryonic kidney 293T cells on TYR-57 and SER-63 (Kalume et al., 2003). These findings suggest the involvement of TGF-β signalling as a potential stimulus for induced tyrosine and serine phosphorylation.

As it has been expected in this study protein kinase was identified as phosphorylated protein: pyruvate kinase isozymes M1/M2 (spot 10). Protein spot 15 (proteasome subunit beta type-1), spot 20 (proteasome subunit alpha type-4) and spot 26 (proteasome subunit beta type-2) were identified as the members of peptidase T1B and T1A family. The proteasome contains at least 15 non identical subunits which form a highly ordered ring-shaped structure and role of phosphorylation of this subunits has not been very well understood. Induction of phosphorylation via TGF-β can give us new insight into TGF-β regulation of proteosomal degradation. Glutamate dehydrogenase (spot 8) and IMP dehydrogenase 2 (spot 23) are also identified in this study as a phosphoproteins. These are already known phosphoproteins; in this study their phosphorylation pattern changed after stimulated with TGF-β. Both of the proteins were detected on non-stimulated and stimulated phosphoprotein profiles as expected**.** Single-stranded DNA-binding protein (spot 21) located in mitochondrion, it binds ss-DNA and is involved in mitochondrial DNA replication and is also know to be phosphorylated. Direct or indirect implication of TGF-β signalling into mitochondrial replication still remain unclear.

Phosphorylation triggered by TGF-β (on direct or indirect way) combinaed with known functions of proteins in the cell can help us in a better understanding of the cellular signalling.

The detected phosporylated proteins on two-dimensional gels were identified based on the peptide mass fingerprint of the PMAC flow-through fraction with the high peptide coverage. Despite the limits of two-dimensional analysis to detect proteins of low abundance, this method in combination with enrichment procedures could be useful to characterise changes in the phosphoproteome under TGF-β stimulation.

It is necessary to further define the role of the proteins identified in the present study in the signalling pathways triggered by TGF-β1. Results from this study have revealed many previously unidentified signalling molecules or targets of signalling pathways triggered by TGF-β. In conclusion, regulatory functions of TGF-β signalling are broader than previously thought.

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7 DECLARATION

Ich erkläre: "Ich habe die vorgelegte Dissertation selbständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalte

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EDUCATION:

Year	Institution
2004-2008	PhD student, Laboratory of Dr. Oliver Eickelberg, Med. Klinik II, University of Gießen
2004-2006	Member of the graduate program „Molecular Biology and Medicine of the Lung (MBML)", http://www.uniklinikum-giessen.de/mbml/
2003-2004	Visiting scientist, University of Münster, Germany, Faculty of Natural Science and Mathematics
1996-2003	University of Zagreb, Croatia, Faculty of Natural Sciences and Mathematics, Master in Chemistry (Dipl.Ing. Chemistry)
1991-1995	High School, Slatina, Abitur
1983-1990	Elementary School, Nova Bukovica

RESEARCH EXPERIENCE:

2004-2008 Laboratory of Dr. Oliver Eickelberg, Med. Klinik II, University of Gießen, Germany

2003-2004 Institute for Medical Physics and Biophysics, Biomedical Analysis Group, University of Münster, Germany

2002 Diploma student guest at the Institute for Medical Physics and Biophysics, University of Münster, Prof. Jasna Peter-Katalinic

TEACHING EXPERIENCE:

2005-2006 Teaching assistant, Course: "Separation and Identification of Proteins from Cell Culture Extracts using 2D- Polyacrylamide Gel Electrophoresis and MALDI-TOF-MS" (Dr. Patrick Bulau), University of Giessen , Germany

2003 Teaching assistant, Course "Molecular Biotechnology" (Prof. Dr. J. Müthing), University of Münster, Germany

2001-2002 Teaching assistant, Course "Methods in Protein Analysis"(Prof. Dr. Marijana Krsnik-Rasol), Department of Molecular Biology, Faculty of Natural Sciences and Mathematics, University of Zagreb, Croatia

PARTICIPATION AT INTERNATIONAL MEETINGS/COURSES

2007 2nd Summer Course on Mass Spectrometry in Biotechnology and Medicine (Dubrovnik, Croatia)

2006 International Conference of the American Thoracic Society (San Diego, USA)

2005 30th Congress on Federation of European Biochemical Societies (Budapest, Hungary)

2004 36th Meeting of the German Society of Mass Spektrometry (Leipzig, Germany)

2003 14th Joint Meeting on Glycobiology (Lille, France)

2003 8th International Summer School on Biophysics (Rovinj, Croatia)

2002 1st Croatian Congress on Molecular Life Science (CCOMLIS) (Opatija, Croatia)

2000 3rd International Dubrovnik NMR Course and Conference (Dubrovnik, Croatia)

ORAL PRESENTATIONS

2006 Oral presentation entitled "Annexin 2 is a novel intermediate in the TGF-β pathway" at the ATS, San Diego

AWARD:

Traveling Award from American Thoracic Society, San Diego 2006

MEMBERSHIPS

DGMS-Deutsche Gesellschaft für Massenspektrometrie

ATS–American Thoracic Society

LANGUAGES

Croatian (nativ)

English (spoken and written)

German (spoken and written)

PUBLICATIONS:

B. Balen, **J. Milosevic**, M. Krsnik-Rasol: Protein and Glycoprotein Patterns Related to Morphogenesis in *Mammillaria Gracillis Pfeif* Tissue Culture. *Food Technol.Biotechnol.* (2002), **40,** 275-285

J. Muthing, I. Meisen, B. Kniep, J. Haier, N. Senninger, U. Neumann, M. Langer, K. Witthohn, **J. Milosevic**, J. Peter-Katalinic: Tumor-associated CD75s gangliosides and CD75s-bearing glycoproteins with Neu5Acalpha2-6Galbeta1-4GlcNAc-residues are receptors for the anticancer drug rViscumin. *FASEB J*. 2005; 19**(1)**: 103-5

B. Balen, M. Krsnik-Rasol , AD Zamfir **, J. Milosevic**, SY Vakhrushev , J. Peter-Katalinic: Glycoproteomic survey of Mammillaria gracillis tissues grown in vitro. *Journal of Proteome Research* (2006), 5**(7)**: 1658-66

J.Milosevic, P. Bulau, O.Eickelberg: Translocation Proteomics Reveals a Novel Signaling Intermediate in the TGF-β Pathway (manuscript in revision: Proteomics-2007-00604)

REREFENCES:

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