universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Charakterisierung von p14“

Verfasserin

Sonja Hinterndorfer

angestrebter akademischer Grad

Magistra der Naturwissenschaft (Mag. rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 419
Studienrichtung lt. Studienblatt:	Diplomstudium Chemie
Betreuerin / Betreuer:	Univ.-Prof.Dr.med. Lukas A. Huber

Tu es oder tu es nicht,
aber hör auf es zu versuchen
Zen-Weisheit

Je öfter du fragst,
wie weit du zu gehen hast,
desto länger scheint die Reise
aus Australien

Danksagung

An erster Stelle bedanke ich mich bei meinem Diplomarbeitsbetreuer Lukas Huber sowie David Teis für die Anleitung, Betreuung und Unterstützung im Rahmen meiner Diplomarbeit und danach.

Bei meinen Laborkollegen Eva, Bibu, Bernhard und Robert sowie auch David für die Auflockerung des Laboralltags während wir die Pipetten strapazierten und im Anschluss nach der Arbeit.

Ganz besonderer Dank gilt Bibu für die wertvollen fachlichen Gespräche während und nach meiner Diplomarbeit und wesentliche Unterstützung in der Zeit der Verfassung dieser Arbeit wofür ich mich in der ETH einbunkern durfte. Und vor allem für Deine Freundschaft im Labor und seitdem, wodurch so manches viel leicher zu bewältigen wurde.

Mama und Papa, Euch danke ich für Euren Glauben an mich und alles was ihr mir auf meinen Weg mitgegeben habt; an Ausbildungsmöglichkeit genauso wie die sportliche Ausdauer bzw. prinzipiell Durchhaltevermögen, womit ihr mir diesen Weg ermöglicht habt.

Und letzendlich bin jenen dankbar, die mich unterstützt haben, damit ich zu guter Letzt diesen finalen Schritt zum Abschluss machen konnte.

Danke an alle, die an mich glauben und mir dadurch Kraft geben.

Table of contents

1 SUMMARY 5

1.1 ZUSAMMENFASSUNG 5
1.2 SUMMARY 6

2 INTRODUCTION 7

2.1 SIGNALING: MEASURES TO OBTAIN TEMPORAL AND SPATIAL SPECIFICITY 7
2.1.1 SIGNALING VIA CELL SURFACE RECEPTORS 7
2.1.2 SPECIFICITY VIA COMPARTMENTALIZATION 9
2.1.3 SCAFFOLD AND ADAPTOR PROTEINS - INTRODUCTION OF MP1 AND P14 10
2.2 POSTTRANSLATIONAL MODIFICATION (PTM) 15
2.2.1 GENERAL 15
2.2.2 PHOSPHORYLATION 17
2.3 AIM OF THIS STUDY 18

3 MATERIALS AND METHODS 19

3.1 HELA CELL CULTURE 19
3.2 HELA CELL TRANSFECTION 19
3.3 HELA CELL EXTRACT PREPARATION 20
3.4 IMMUNOPRECIPITATION 20
3.5 PROTEIN EXPRESSION IN SF9 AND HI5 INSECT CELLS 20
3.6 PROTEIN PURIFICATION FROM HI5 INSECT CELLS 21
3.6.1 AFFINITY CHROMATOGRAPHY: 21
3.6.2 IMMOBILE METAL AFFINITY CHROMATOGRAPHY (IMAC): 22
3.7 INTERACTION STUDIES 22
3.8 COOMASSIE STAINING 23
3.9 SILVER STAINING 23
3.10 IN VITRO DEPHOSPHORYLATION ASSAY 23
3.11 IN VITRO KINASE ASSAYS 24
3.12 IMMUNOFLUORESCENCE MICROSCOPY 24
3.13 REAGENTS 24

4 RESULTS 27

4.1 PURIFICATION OF P14 FROM INSECT CELLS 27
4.2 PURIFIED P14 IS PHOSPHORYLATED 29
4.3 PHOSPHORYLATION OF P14 IS NOT REQUIRED FOR ITS INTERACTION WITH MP1 30
4.4 CHARACTERIZATION OF POTENTIAL P14 PHOSPHORYLATION SITES 31
4.5 P14 AND MP1 FORM HOMO-OLIGOMERES IN VITRO 37

5 DISCUSSION 39

6 REFERENCES 43

1 SUMMARY

1.1 Zusammenfassung

Die extrazellulare signal-regulierte Kinase Kaskade reguliert Zellprolilferation, Differenzierung und Überleben in multizellularen Organismen. Gerüstproteine regulieren intrazellulare Signale, indem sie der kritischen räumlichen und zeitlichen Spezifizierung dienen. Das Gerüstprotein MEK1- (mitogen-activated protein kinase and ERK kinase 1) Partner (MP1) lokalisiert an späten Endosomen mit Hilfe des Adaptorproteins p14.

Unter Verwendung von sowohl *in vitro* als auch *in vivo* Methoden, wurde die Auswirkung der Phosphorilierung von p14 auf dessen Interaktion mit MP1 und den Einfluss auf dessen Lokalisierung an späten Endosomen analysiert. Zwei Phosphorilierungs-Orte wurden auf p14 mittels Massenspektrometrie detektiert. Ein dritter wurde durch Analyse mit einem Programm zur Beurteilung möglicher Proteinmodifikationen anhand der Proteinsequenz vermutet. p14 und MP1 wurden in Insektenzellen exprimiert und aufgereinigt und die Interaktion zwischen diesen beiden Bindungspartnern wurde untersucht. Dabei zeigte sich, dass p14 in Insektenzellen phosphoriliert wird. Diese Phosphorilierung wirkt sich jedoch nicht auf dessen Interaktion mit MP1 *in vitro* aus.

Punktmutanten von p14, die gemeinsam mit MP1 in HeLa Zellen transient exprimiert wurden, zeigten keine Veränderung des Lokaliserungsmusters. Immunpräzipitation mit den enstsprechenden Zelllysaten wies jedoch eine verringernde sowie eine verstärkende Wirkung auf die Bindung mit MP1 und den Phosphomutanten, die Phosphorilierung vorgeben, T41E und S31E, auf.

Die in vivo-Funktion dieser Phosphorilierung bleibt vorerst ungeklärt, wenn auch diese Ergebnisse unterstützt werden dadurch, dass auch in HeLa Zellextrakten eine schwache Doppelbande beobachtet wurde, die möglicherweise diese Modifikation von p14 in vivo darstellt.

Weiters konnte gezeigt werden, dass sowohl p14 als auch MP1 aus Insektenzelllysaten in vitro homo-oligomerisieren, was vermutlich auf die sehr ähnliche Topologie zurückzuführen ist.

1.2 Summary

The extracellular signal-regulated kinase (ERK) cascade regulates proliferation, differentiation, and survival in multicellular organisms. Scaffold proteins regulate intracellular signaling by providing critical spatial and temporal specificity. The scaffold protein MEK1 (mitogen-activated protein kinase and ERK kinase 1) partner (MP1) is localized to late endosomes by the adaptor protein p14.

Using both an *in vitro* and an *in vivo* approach, we analyzed the importance of phosphorylation of p14 on its interaction with MP1 and the impact on its proper localization to endosomes. Two phosphorylation sites on p14 were detected by MS, and a third phosphorylation site was predicted by informatic means. Using p14 and MP1 purified from insect cells, the interaction between the two binding partners was investigated showing that phosphorylation on p14 occurs in insect cells, however has no impact on its interaction with MP1 in vitro.

Expressing respective point mutants of p14 in Hela cells did not impact the localization pattern of transiently transfected p14 and MP1, nor could a difference in their interaction be visualized. However, pulldown assays from these cells showed that two phospho-mimicking mutants, T411E and S31E, had an enhancing and inhibitory effect on MP1 binding, respectively.

The function of this phosphorylation remains rather unclear, however, supporting our findings, also a faint double band, potentially representing the p14 protein modification, was observed in Hela cell extracts.

Furthermore, p14 as well as MP1 purified from insect cells were shown to be able to homo-oligomerize in vitro which may be due to their highly similar structure.

2 INTRODUCTION

2.1 Signaling: Measures to obtain temporal and spatial specificity

2.1.1 Signaling via cell surface receptors

All cells need to interprete their environment. In order to fulfill this task, extracellular information is received and translated accordingly into a specific biological response. The extracellular signal needs to be transduced to the cell's interior to trigger appropriate responses. Cell surface receptors are often used to transduce extracellular information to the cell. A great variety of ligands, representing the extracellular information, bind to and regulate the activity of cell surface receptors.

Upon ligand binding, receptor signaling is activated. Most activated receptors are efficiently cleared from the cell surface by endocytosis and sorted to lysosomes for degradation. In contrast, inactive cell surface receptors are constitutively internalized and recycled back to the cell surface. Thus, control mechanisms exist that survey the internalization and endosomal traffic of cell surface receptors.

One large family of cell surface receptors is represented by receptor tyrosine kinases (RTKs). All of the RTKs are monomers in the plasma membrane, with the exception of the insulin receptor. Ligand-induced activation of RTKs stabilizes a receptor dimer and results in autophosphorylation of tyrosine residues within the cytoplasmic domain of the RTK. The phosphorylated tyrosine residues serve as binding sites for a variety of signaling proteins. The recruitment of signaling proteins allows the assembly of signaling complexes on the cytoplasmic tail of the activated receptor, connecting activated receptors to the respective signal transduction cascades. Stimulation of all RTKs triggers the activation of the small G protein Ras. Ras activation is controlled by the guanidine exchange factor (GEF).

A GEF complex is recruited from the cytoplasm to specific phosphotyrosine

(pTyr) residues on the activated epidermal growth factor receptor (EGFR). This recruitment stimulates Ras activation at the site of the activated EGFR. Activated Ras is known to interact with more then 10 different effectors, thereby modulating a multitude of downstream signaling cascades. One of them is the highly conserved mitogen-activated protein kinase (MAPK) signaling or extracellular signal regulated protein kinase (ERK) cascade, which is involved in the regulation of cell survival, proliferation and differentiation.

These cascades are highly conserved among eukaryotes and consist of a three-kinase module that includes a MAPK, which is activated by MAPK/ERK kinase (MEK), which itself is activated by a MEK kinase (MEKK) [1]. One of the best characterized MAPK cascades consists of MEK1/2, which are Raf isoformes, and ERK1/2, and is regulated by Ras.

By activating Raf, Ras stimulates signaling in the MAPK cascade. Sequential phosphorylation events within the cascade transduce signals downstream from Ras to effector proteins. Activated Ras binds to and activates Raf (the MAPKKK). Raf phosphorylates MEK (the MAPKK) on a critical serine in the activation loop. Next, MEK activates ERK (the MAPK) by phosphorylating threonine and tyrosine residues on ERK. Activated ERK, in turn, phosphorylates a great variety of targets in the cytoplasm and on membranes. In addition, activated ERK rapidly dissociates from MEK and translocates to the nucleus to activate transcription factors.

This pathway is found in non-proliferating cells, but mitogenic signals stimulate it and proliferation can be blocked when it is inhibited. Several MAPKs have been indentified, demonstrating the existence of several MAPK pathways in metazoans (as reviewed in Robinson et al., Curr.Op.Cell Bio 1997 [2]).

Interestingly, the great number of ligands and their respective cell surface receptors use only a limited repertoire of signaling molecules to transduce their information, and yet signal transduction mediates a specific biological response. Therefore, signaling requires precise spatial and temporal regulation to evoke a unique biological response.

Biochemical and genetic methods have identified the basic setup of signaling cascades, it is however yet not clear how the variety of extracellular cues employs only a limited number of signaling cascades to transduce and execute a unique biological response. Composition and compartmentalization of signaling complexes, by the use of scaffold and adaptor proteins, as well as the duration and amplitude of signaling might be crucial to define a specific biological signal.

2.1.2 Specificity via compartmentalization

Signal transduction through the MAPK pathway requires spatial and temporal specification. The signaling specificity is regulated by scaffold proteins, which coordinate the assembly of MAPK-signaling units [3]. Spatial information is provided by adaptor proteins, which define the localization of scaffold complexes [4].

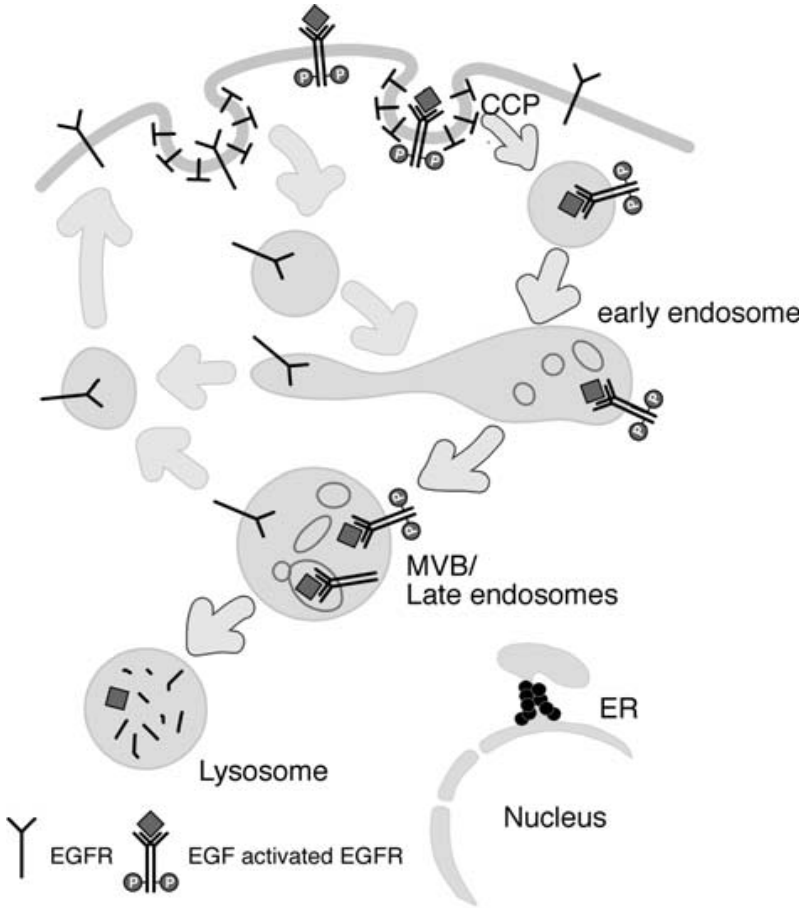
Upon EGF stimulation, Ras becomes activated at the plasma membrane. However, after endocytosis of the activated EGFR, Ras activity persists on endosomes. Furthermore, all components of the Ras-MAPK signaling cascade, namely, Raf, MEK, and ERK have been found to reside on endosomes. Internalized EGFRs show all the characteristics of receptors that are activated at the plasma membrane. They are bound to EGF, exist as dimers, are tyrosine-phosphorylated and interact with the known domains of their adaptor proteins to connect receptor activation to downstream effectors.

All components of downstream signaling cascades have been found to reside not only at the plasma membrane but, notably, also on endosomes. It appears likely that signaling from the EGFR is not terminated upon internalization. Internalized receptors continue to signal until they are sorted to internal vesicles of MVBs. This sorting step would disconnect the receptor from its ‘cytoplasmic’ downstream effectors, and signaling would be shut off. Cell surface receptors are active at the plasma membrane and in the endosomal system. Therefore, activated cell surface receptors could engage a variety of different signaling complexes in a sequential manner, first at the plasma membrane, then on early endosomes and, finally, on the limiting membrane of MVBs.

The activation of diverse signaling complexes by activated receptors along the endocytic pathway would increase the signaling potential. Receptor signaling from endosomes has been suggested by Teis et al to provide a window of opportunity that could contribute to the spatial and temporal regulation of signal transduction. Thus, signal transduction could be compartmentalized at the plasma membrane and on endosomes. Compartmentalization would provide high flexibility to an otherwise limited number of signaling cascades to transduce specific signals from a multitude of signaling cues.

2.1.3 Scaffold and adaptor proteins - introduction of MP1 and p14

Internalized receptors continue to signal until they are sorted to internal vesicles of MVBs. This sorting step would disconnect the receptor from its 'cytoplasmic' downstream effectors, and signaling would be shut off. Activated cell surface receptors could engage a variety of different signaling complexes in a sequential manner, first at the plasma membrane, then on early endosomes and, finally, on the limiting membrane of MVBs (Picture 1) [5].

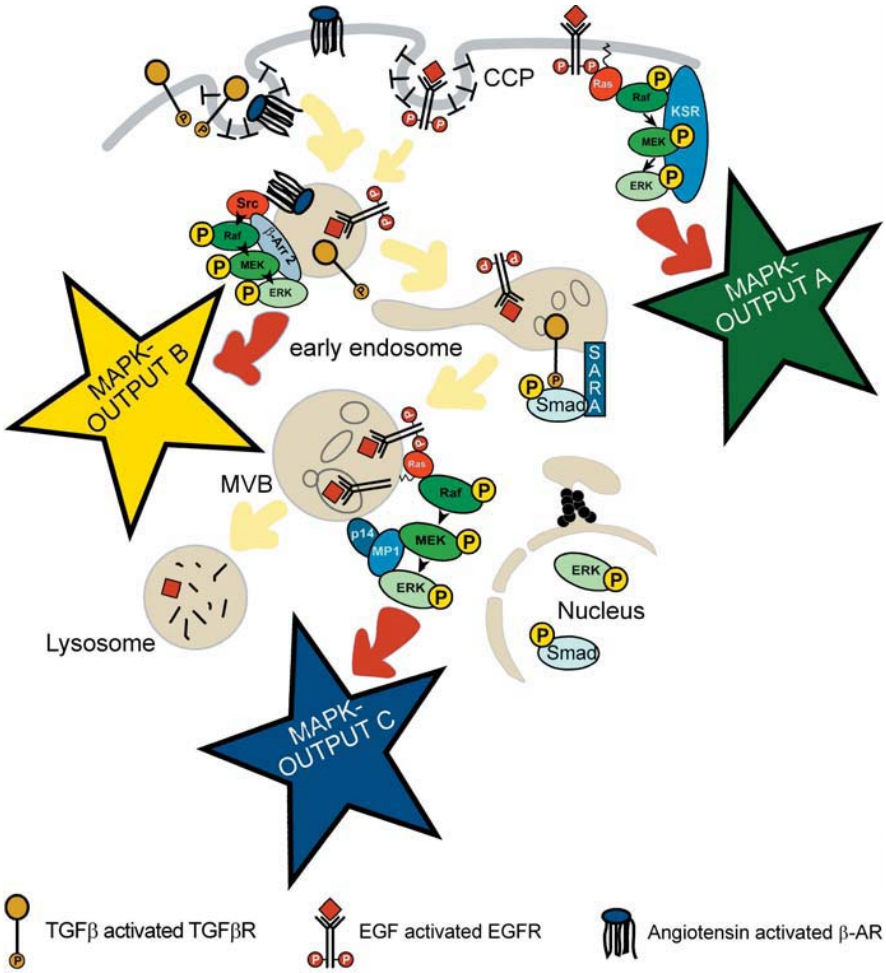


Picture 1 [5] Endosomal traffic of the activated EGFR. The inactive EGFR localizes in steady-state equilibrium to the plasma membrane. It is internalized at low rates and efficiently recycled back via early endosomes to the plasma membrane. In contrast, the activated EGFR (yellow arrows) is efficiently internalized via CCPs. In the early endosomes they are sorted to the late endosomal/MVB compartment. Sorting into the lumen of the MVB shunts the EGFR to the lysosomes, where they are finally degraded.

The specification of the signaling pathways can be achieved by organizing signaling components into multi protein complexes. Scaffold proteins are used to organize specific signaling units. Consistently, adaptor proteins, which interact with the activated EGFR, have been found to reside together with the cell surface receptor at the plasma membrane and also on endosomes.

Scaffold, anchor and adaptor proteins might bring the spatial distribution and activation of specific signaling complexes under the control of activated cell surface receptors. The subcellular distribution of scaffolded signaling complexes of one signal transduction pathway might finally contribute to the generation of different specific

signaling outputs. The formation of such membrane-based scaffold complexes would be ideal for the propagation of signals with high spatial as well as temporal resolution and specificity within cells [5] Picture 2.



Picture 2. Subcellular compartmentalization of signaling complexes. Different scaffold complexes reside at different subcellular compartments. The use of the KSR-scaffold module at the plasma membrane could result in the MAPK signaling output A. b-Arrestin 2 (b-Arr 2) assembles another MAPK signaling complex on early endosomes and might define MAPK signaling output B. The late endosomal p14/MP1 scaffold module generates yet another MAPK signaling complex, which might regulate a MAPK signaling output C. Thus, the MAPK pathway might be compartmentalized by a variety of scaffold proteins. This compartmentalization of signaling modules might be used to define a specific biological signal. In addition to the scaffold modules, other signaling adaptor modules (like the SARA/Smad module) have been found to localize on endosomes. [5]

In a yeast-two-hybrid screen for proteins which might be important in regulating this signaling cascade but act in a nonenzymatic way, a protein called MP1 (MEK

Partner 1) was identified that bound specifically to MEK1 and ERK1 and enhanced their activity [1], [6] and [13]. Overexpression of MP1 in cultured cells resulted in an enhacement of activation of ERK1, and an increased binding of ERK1 to MEK1, suggesting that MP1 functions as an adapter to enhance the efficiency of the MAP kinase cascade.

KSR (kinase suppressor of Ras) and MP1 (MEK1 partner) serve as MAPK-scaffold proteins to regulate signal transduction in higher eukaryotes. The MAPK-scaffold protein KSR mediates the interaction between Raf and MEK and is required for signal transduction in the Ras-MAPK pathway. MP1 functions as a scaffold protein in the Ras MAPK pathway at a different cell compartment.

MP1 binds specifically to MEK1 and ERK1, thereby facilitating signaling in the MAPK cascade. However, unlike KSR, MP1 localizes to late endosomes. The adaptor protein p14 is essential to mediate the localization of MP1 to endosomes. The endosomal localization of the p14/MP1 complex is exclusively required for the activation of ERK at endosomes but not for ERK activation at the plasma membrane.

By isolating endosomal/lysosomal fractions from EpH4 cells and analyzing the membrane-associated fractions, where many known proteins described in regulating endocytic transport or scaffolding signal transducing modules are located, Wunderlich et al. [7] identified a novel protein of 14 kDa, called p14. Using this protein as bait in a yeast-two-hybrid screen, MP1 was found to interact with p14. Furthermore it was found that mislocalized p14 (targeted to the plasma membrane) resulted in mislocalization of coexpressed MP1, indicating that p14 is required for MP1 localization. The interaction of p14 with MP1 suggests that these proteins act as an endosomal adaptor/scaffolding complex, which regulates MAPK signaling.

The existence of adaptor proteins that determine the localization of scaffold proteins provides additional flexibility in regulating the efficiency and the specificity of the MAPK cascade. As shown by Teis et al. 2006 [8], p14 functions as an endosomal adaptor for MP1. p14 is required and sufficient to localize MP1 and the MP1-MAPK-signaling module to late endosomes, since depletion of p14 resulted in mislocalization of

MP1 to the cytoplasm and in defective ERK signaling.

Only endosomal ERK activation is affected by short interfering RNA of either p14 or MP1 [8]. In contrast, the p14/MP1 complex appears dispensable for the initial phase of MAPK signaling at the plasma membrane, which requires KSR. Thus KSR assembles a signaling complex at the plasma membrane and generates an ERK signal that is different from a p14/MP1-coordinated ERK signal emanating from endosomes. However, the molecular mechanisms of compartmentalized ERK signaling remains to be elucidated. [9]

Perturbation of endosomal p14-MP1–MEK1 signaling is detrimental for tissue homeostasis, and a mutation in human p14 causes a primary immunodeficiency syndrome [8] [10]. These findings support the concept that the endosomal system serves as an intracellular site for the regulation of signal transduction [11] [12].

A mechanism has been suggested by which a specific signaling unit is generated on endosomes to provide specificity as well as spatial and temporal resolution to the ERK cascade [13]. An additional means for cells to achieve specificity and increase the variation of functions of proteins at the lower level of protein structure are posttranslational modifications.

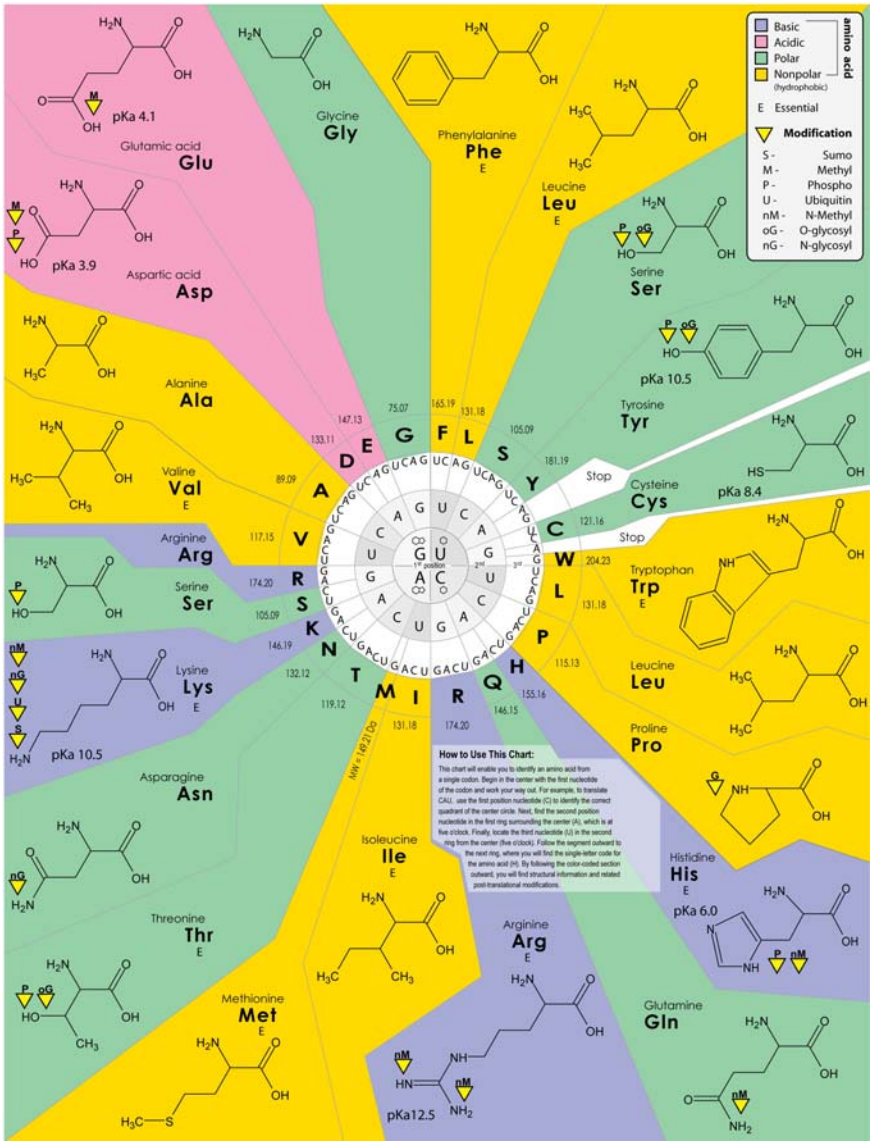
2.2 Posttranslational modification (PTM)

2.2.1 General

PTM is the chemical modification of apolypeptide/protein after its translation. It is one of the later steps in protein biosynthesis for many proteins.

During protein synthesis, the 20 different amino acids are incorporated into the proteins. After translation, the posttranslational modification of the amino acids extends the range of functions of the protein by attaching to it other biochemical functional groups such as acetate, phosphate, sulfate, various lipids and carbohydrates, by changing the chemical nature of an amino acid (e.g. citrullination) or by making structural changes, like the formation of disulfide bridges.

Also, enzymes may remove amino acids from the amino end of the protein, or cut the peptide chain in the middle. For instance, the peptide hormone insulin is cut twice after disulfide bonds are formed, and a propeptide is removed from the middle of the chain; the resulting protein consists of two polypeptide chains connected by disulfide bonds. Picture 1 depicts the genetic code diagram showing the amino acid residues as target of modification:



Picture 1

Protein post-translational modification (PTM) is an extremely important cellular control mechanism altering the proteins' physical and chemical properties, folding, conformation, distribution, stability, activity and consequently, their functions. Examples of the biological effects of protein modifications include phosphorylation for signal transduction, attachment of fatty acids for membrane anchoring and association, and glycosylation for changing protein half-life, targeting substrates, and promoting cell-cell and cell-matrix interactions. With the accelerating progress in proteomics, biological knowledgebases containing a wealth of information, in particular protein modifications, are playing crucial roles in cell regulation research.

2.2.2 Phosphorylation

Post-translational phosphorylation is one of the most common protein modifications that occurs in animal cells for controlling the behavior of a protein, for instance activating or inactivating an enzyme. The vast majority of phosphorylations occur as a mechanism to regulate the biological activity of a protein and as such are transient. The reversible protein phosphorylation, principally on serine, threonine or tyrosine residues, plays critical roles in the regulation of many cellular processes including cell cycle, growth, apoptosis and signal transduction pathways. To understand the molecular basis of these regulatory mechanisms, it is necessary to identify and characterize these phosphorylation sites in proteins.

The enzymes that phosphorylate proteins are termed kinases and those that remove phosphates are termed phosphatases. Protein kinases catalyze reactions of the following type:

ATP + protein <----> phosphoprotein + ADP

The largest group of kinases are those that phsophorylate either serines or threonines and as such are termed serine/threonine kinases. The ratio of phosphorylation of the three different amino acids is approximately 1000/100/1 for serine/threonine/tyrosine. (source: wikipedia)

Although the level of tyrosine phosphorylation is minor, the importance of phosphorylation of this amino acid is profound. As an example, the activity of numerous growth factor receptors such as the EGF receptor is controlled by tyrosine phosphorylation.

2.3 Aim of this study

The aim of this study was to establish expression and purification conditions for the purification of p14 and MP1 from insect cells using the Baculovirus expression system. Furthermore, we aimed in investigating the interaction of p14 and MP1 both in vitro by biochemical binding assays and in vivo in Hela cells, and analyze the potential involvement of phosphorylation in this interaction and localization of p14 and MP1.

3 Materials and Methods

Standard molecular biology techniques were performed as described in Molecular Cloning [14].

3.1 HeLa cell culture

HeLa cells were grown at 37ºC in the presence of 5% CO2 in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FCS, 0.3µg/ml L-glutamine, 100 units/ml penicillin and 100µg/ml streptomycin. Cells were arrested in mitosis by the addition of 100 ng/ml nocodazole for 18 hours and S-phase arrest was induced by an 18 hour treatment with 2mM hydroxyurea.

3.2 HeLa cell transfection

Cells were transfected using Lipofectamin Plus Reagent (Invitrogen). 4□g of DNA were prediluted in 750□l medium and mixed with 20□l Plus Reagent. The mixture was incubated for 15min at room temperature and then combined with 30□l of Lipofectamin reagent diluted in 750□l medium. After an additional incubation for 15min the solution was added onto a 10cm dish of Hela cells with 5ml medium (FCS-free). After 4h the transfection medium was replaced with normal medium and cells were lysed after 48h.

3.3 HeLa cell extract preparation

HeLa cells were harvested and centrifuged at 1'200 rpm at 4 ºC for 5 minutes in a Heraeus Biofuge. The cell pellet was washed twice with ice cold PBS and resuspended in one volume of ice cold extraction buffer (20 mM Tris pH 7.5, 100 mM NaCl, 20 mM β-glycerophosphate, 5 mM MgCl2, 0.2 % NP-40, 10 % glycerole, 1 mM NaF, 0.5 mM DTT and 10µg/ml each of chymostatin, leupeptin and pepstatin A). Cells were broken open by dounce homogenization, 20 minutes on ice, and the extract was spun at 20'000 x g at 4 ºC for 7 minutes to pellet the DNA. The supernatant was used for immunoprecipitation and western blotting (see below).

3.4 Immunoprecipitation

Cell extracts were pre-cleared with Ultralink protein A beads (Pierce). The resulting supernatant was subjected to immunoprecipitation using the anti-Xpress or the anti-FLAG antibody. Cell extracts were first incubated with the antibody for two hours at 4°C on a rotating wheel. Then Ultralink A beads were added for 1 hour. Beads were washed four times with IP-buffer.

3.5 Protein expression in Sf9 and Hi5 insect cells

Baculoviruses allow high levels of recombinant gene expression in insect cells. Commonly used insect cell lines are Sf9, Sf-21, Tn-368 and High-FiveTM BTI-TN-5B1-4. All four cell lines can be grown as monolayers or in suspension cultures. An advantage over protein expression in bacteria is the ability of the insect host to post-translationally modify the expressed protein in a manner similar to that of mammalian cells, which in many cases is important for the generation of active protein.

GST-MP1, His-MP1, GST-p14 and His-p14 Baculoviruses were used for the infection of Sf9 cells. Baculoviruses are generated using the BAC-to-BAC expression system (Invitrogen, Carlsbad California, USA). The virus was amplified by sequential infections of Sf9 cells followed by harvesting the virus containing supernatant after 48h. For protein expression, 2 ml virus was used to infect a 175-cm2 filter-capped tissue culture flask of 80% confluent monolayer Hi5 cells. 48 hours after infection, cells were harvested and washed twice in PBS. A cell pellet from 10 175-cm2 flasks is resuspended in 6 ml lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, 0.1% TritonX-100, 1 μM okadaic acid and 10 μg/ml each of chymostatin, leupeptin and pepstatin A (Sigma-Aldrich)). After 20 minutes on ice with occasional douncing, the lysates are centrifuged for 1 hour at 20,000 x g at 4 °C. This supernatant was then used for the purification of the GST- or His-tagged proteins.

3.6 Protein purification from Hi5 insect cells

3.6.1 Affinity Chromatography:

Glutathion S-transferase (GST) fusion proteins can be purified directly from cell lysates using Glutathion Sepharose 4B. Employing this affinity chromatography, proteins can be eluted under mild, non-denaturating conditions which preserve protein antigenicity and functionality.

Activation: GST Sepharose 4B-beads have to be activated and rid of the ethanol which they are stored in by three washing steps in ice cold PBS (10xVol) (centrifuge at 700rpm).

Binding: GST-fusion proteins containing cell lysates were incubated with the equivalent volume of GST Sepharose 4B-beads and 1mM DTT for 45min at room temperature. The beads were then centrifuged at 700rpm and washed three times with ice cold PBS (10xVol).

Elution: GST-tagged proteins ere eluted by incubation of the beads with elution buffer (20mM Glycerin in Tris/HCl pH 8,2, 1% Triton X-100; final pH: 7,7) for 30 min at room temperature.

3.6.2 Immobile Metal Affinity Chromatography (IMAC):

The principle of this purification method is based on the reversible interaction between various amino acid side chains and immobilized metal ions. To purify His6-tagged proteins Talon® IMAC resins were used to gain the native protein.

Activation: The resins are activated by washing with 10xVol of ice cold PBS (centrifuge at 700rpm).

Binding: The insect-cell-lysates were incubated for 45min at room temperature with 1xVol of resins. Then the beads were washed three times with 10xVol of ice cold IP-buffer and once in cold PBS.

Elution: His6-fusion proteins were recovered by incubation of the beads with elution buffer (50mM NaPi; 300mM NaCl; 150mM Imidazol) for 30min at room temperature.

3.7 Interaction Studies

GST-tagged protein was immobilized on Glutathion Sepharose 4B following the binding procedure described above, washed three times in cold PBS and then incubated for 2h at 4˚C with the putativly interacting protein. After three washing steps in cold IP-buffer and one in PBS (10xVol each) the proteins were eluted in elution buffer at 4˚C for one hour.

3.8 Coomassie Staining

The gel was incubated in previously heated up staining solution for 10min and then destained. Destain solution was changed several times and and also heated up.

3.9 Silver Staining

Silver staining was performed according to the modified Blum's protocol:

The gel was fixed in a solution containing 40% ethanol and 10% acetic acid and then washed twice for twenty minutes in 30% ethanol and once for twenty minutes in H2O.The gel was then sensitized in 0,02% Na2S2O3 for 1min, washed three times in water for 20sec and then incubated in a cold solution of 0,1% AgNO3 for 20min at 4C. It was subsequently washed three times in water for 20sec and for another minute after changing the gel. The gel was developed in a solution with 3% Na2CO3 and 0,05 formalin (change solution when developer turns yellow). After the developer has been washed away with water for 20sec the staining was terminated by incubation in 5% acetic acid. The gel was then washed subsequently in water three times for 10min and stored at 4˚C in 1% acetic acid.

3.10 In vitro dephosphorylation assay

17 µl	protein solution (ca 2 □g protein) (or bead volume)
2,5µl	10x λ-PPase buffer
2,5µl	20mM $MnCl_2$
3µl (800U)	λ-PPase

The mixture was incubated at 30ºC for 1h and then washed three times with 10xVol of ice cold PBS.

3.11 In Vitro Kinase assays

For in vitro phosphorylation, GST-p14 was incubated with aPKCζ kinase at 30 ºC for 45 minutes in 10 µl buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgCl2, 10 mM β-glycerophosphate, 1 mM EGTA, 1 mM DTT, 1 µM ocadaic acid and 0.2 mM cold ATP and 1 µCi/µl γ-[32P]ATP. Phosphorylation was detected by SDS-PAGE followed by phosphorimaging.

3.12 Immunofluorescence microscopy

24h after transfection cells were split onto cover slips in 6-well dish. After another 24h, medium was taken off, the cells were washed twice in PBS, fixed with 4% PFA in CB for 10min and permeabilise ith 0,25 Triton for 2min. Cells were blocked in IF-blocking-buffer for 30min and then incubated in prim. antibody (1:500 in IF-blocking-buffer). After 1h coverslips were washed 5x in WQ-buffer and incubated in sec. antibody (1:1000 in blocking-buffer, spin down at 13000rpm for 2min) for 30min. The cells were again washed 5x in WQ and then washed once with WQ containing Dapi (1:10000). The coverslips were mounted in Moviol, which was dried for 1h at room temperature.

3.13 Reagents

4% PFA:		160mg	PFA
		2ml	H_2O
		60µl	2N NaOH
		heat up to 80˚C for 10min	
	add	2ml	2xCB
IF-blocking-buffer:		0,5g	gelatine
		10ml	H_2O (warm up until dissolved)
		12,5ml	2xCB
		2,5ml	NH_4Cl (0,5M)

1pinch BSA

2xCB pH 6,8:	20mM	PIPES pH6,8
	300mM	NaCl
	10mM	EGTA
	10mM	glucose
	10mM	$MgCl_2$
WQ:	50ml	NH_4Cl (0,5M)
	250ml	2x CB
	200ml	H_2O

Dapi-solution: 1mg/ml DAPI in H_2O

Moviol-solution:	5g	MOWIOL
	20ml	PBS
	stir over night at 4˚C	
add	10ml	glycerol
	centrifuge for 15min at 14000rpm and freeze aliquots at –20˚C	

IP-buffer:	50mM	Tris pH 7,5
	150mM	NaCl
	1%	Triton X-100
	0,5mM	EDTA
	0,5mM	EGTA
	50mM	NaF
	5mM	NaP_i
	10%	Glycerol

Protease inhibitors: 10µg/ml aprotinin, 1µg/ml pepstatin, 10µg/ml leupeptin, 1mM Pefabloc SC

Phosphatase inhibitors: 2mM Na_3Ov_4, □-Glycerophosphate Disodium Salt, Ocadaic Acid

λ-PPase kit (BioLabs)

λ-PPase buffer:	50mM	Tris-HCl
	0,1mM	Na_2EDTA
	5mM	dithiothreitol
	0,01%	Brij 35
	(pH 7,5)	

Coomassie Brillant Blue R 250	0,24g	Coomassie Brillant Blue R
	45ml	Methanol Abs.
	45ml	H_2O
	10ml	Acetic Acid 99.5 %

Stear ON
Store working solution at RT in the **dark**

Destain solution	150 ml	Methanol Abs.
	70 ml	Acetic Acid 99.5 %
	780ml	H_2O
Amido black	0,5g	Amido Black
	125ml	Isopropanol
	50 ml	Acetic Acid 99.5 %
	325ml	H_2O
	keep light protected	

4 Results

4.1 Purification of p14 from insect cells

To be able to biochemically analyze the function of p14, we aimed to purify this protein in high amounts. We chose the insect cell sytem for our protein expression as it is known that insect cells, in contrast to E.coli, are able to perform many secondary protein modifications, e.g. phosphorylation, which is known to facilitate proper protein folding. We used the bac-to-bac baculovirus insect cell system (invitrogen) for the expression of p14 and MP1 in Sf9 and Hi5 insect cells. p14 was fused to an N-terminal GST- or His6-tag and coexpressed with MP1. First I established a purification method to obtain high purity and high protein amounts. The GST purification method from Hi5 cell lysates resulted in the highest amount and best purity of p14 protein, which efficiently copurified MP1 (Figure 1A and data not shown). Therefore we decided to use the GST purification method and Hi5 insect cells for further experiments.

The amount of purified GST-p14 and copurified MP1 was quantified by comparison to BSA standard protein on a coomassie SDS-PAGE gel (Fig1B). In the first elution step we obtained a protein yield of 1 mg/ml GST-p14 and 0.1 mg/ml MP1, whereas in the second elution step only 0.15 mg/ml GST-p14 and less than 0.02 mg/ml MP1 were obtained. Also the remaining protein on beads after the elution steps was very low, indicating that the first elution step efficiently recovered most of the bound protein.

In addition to GST-p14 and MP1, four additional bands were detected by silver staining. These bands were analyzed by mass spectrometry. In addition to MP1 (5,6) and p14 (2), additional proteins GST (4) and a chitinase (3) were identified, the latter most likely copurified non-specifically. Interestingly, we found that in addition to the major p14 containing band (2) a second band, which showed retarded mobility (1,7), also contained p14 protein, indicating that the retarded mobility on a SDS-PAGE might come

from secondary modifications of this protein.

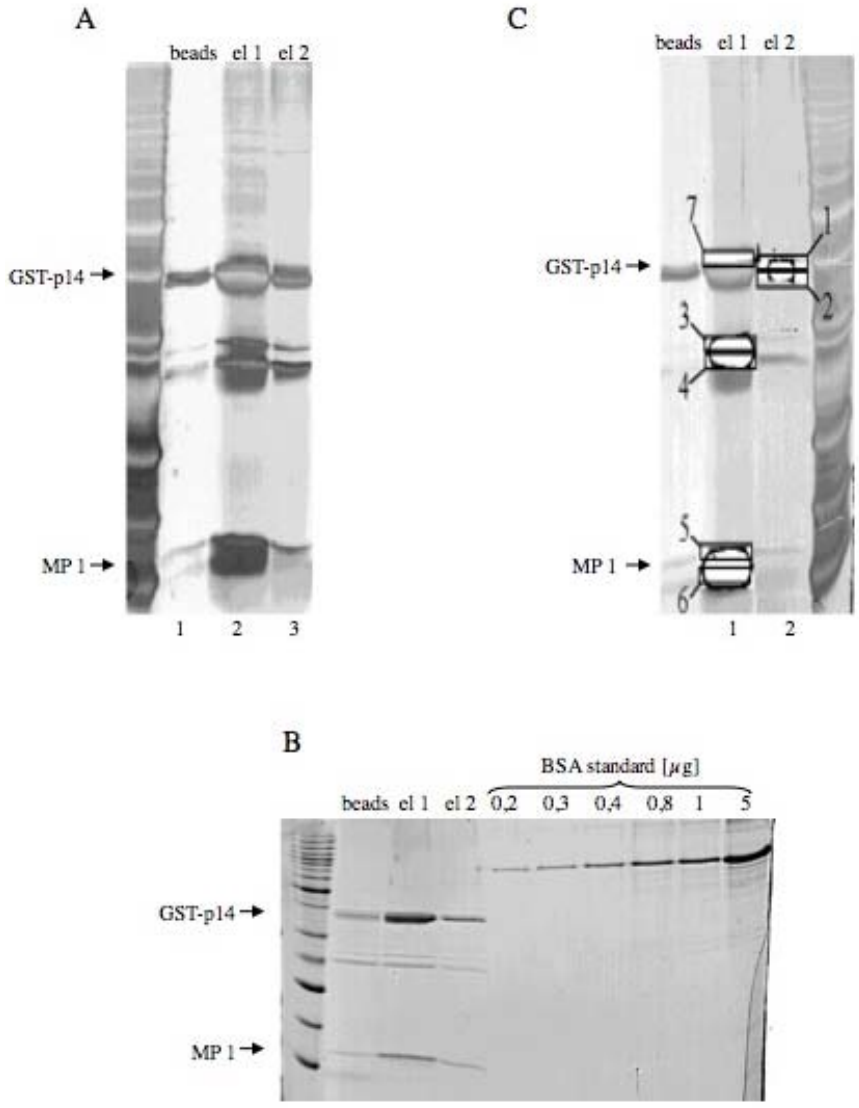


Figure 1. Purification of p14 from Hi5 insect cells. (A) P14 was N-terminally tagged with GST and co-expressed with MP1 in Hi5 insect cells. The protein was isolated on GSH sepharose and eluted twice with glutathione. The remaining protein on beads as well as both elution steps were analyzed by silver staining. (B) 5 µl of both elution steps of (A) were analyzed by SDS-PAGE and coomassie staining and compared to different amounts of BSA protein run on the same gel. (C) The samples from (A) were run separated by SDS-PAGE followed by silver staining, and bands indicated were excised for mass spectrometry analysis, which resulted in the identification of MP1 (5,6), p14 (1,2,7), GST (4) and a chitinase (3).

4.2 Purified p14 is phosphorylated

The identification of a mobility retarded p14 protein from the GST pulldown suggested that this protein is subjected to secondary modification in insect cells. To analyze if this mobility shift is a result from phosphorylation of GST-p14, the purified protein eluates were treated with λPhosphatase (λPPase). Incubation of GST-p14 with λPPase resulted in the loss of the mobility shifted band (Figure 2, lane 2), whereas in samples which were treated without λPPase (lane 1) or with λPPase and an inhibitor of this phosphatase (lane 3) the mobility shifted band was still observed. These results strongly suggest that GST-p14 isolated from insect cells is phosphorylated. In addition, αp14 western blotting of HeLa cell extracts also showed a second, mobility retarded band for p14 (David Teis, personal communication), suggesting that this phosphorylation is also present on endogenous p14 in HeLa cells.

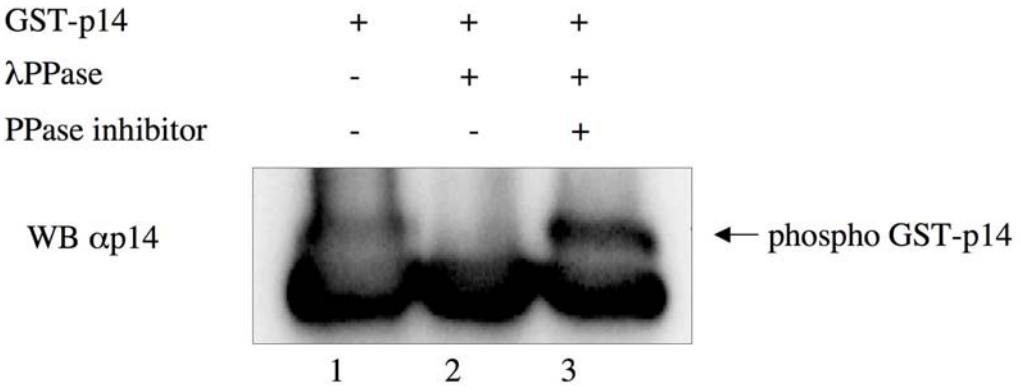


Figure 2: GST-p14 purified from Hi5 insect cells is phosphorylated. GST-p14 was purified from Hi5 insect cells and incubated with (2) and without (1) λPPase, followed by SDS-PAGE and αp14 immunoblotting. As a control, one sample was incubated with λPPase and a λPPase inhibitor (3).

We then mapped phosphorylation sites on p14 by mass spectrometry. Unfortunately we only resulted in a sequence coverage of 40%, because p14 turned out to

be very difficult to digest for mass spectrometry analysis. Attempts to purify the mobility-shifted p14 in higher amounts by upscaling the purification and concentration of the protein failed. However, we still obtained two phosphorylation sites on the sequence covered by the mass spectrometry analysis, S31 and T41. We furthermore used bioinformatical phosphosite prediction (www.profteispleasehelp.com), which resulted in the identification of an additional potential phosphorylation site, T125 (see also Figure 5).

4.3 Phosphorylation of p14 is not required for its interaction with MP1

It is known that p14 functions as an adaptor protein, which is required and sufficient to localize MP1 to endosomes (Teis et al., 2002). The finding that p14 is phosphorylated, opens the possibility that the secondary protein modification of p14 might be involved in regulating its binding to MP1. Therefore we asked if the interaction of p14 with MP1 was dependent on p14's phosphorylation in vitro. GST-p14 was bound to GSH sepharose beads and incubated with or without λPPase. After washing the beads to remove the phosphatase, the p14 bound beads were incubated with purified His6-tagged MP1 (Figure 3). In a second experiment, GST-p14 and MP1 were co-expressed and purified with GSH sepharose, followed by λPPase treatment. The amount of bound MP1 was analyzed by western blotting. In both cases the amount of MP1 bound to GST-p14 was not altered after λPPase treatment, suggesting that phosphorylation of p14 is not required for its interaction with MP1.

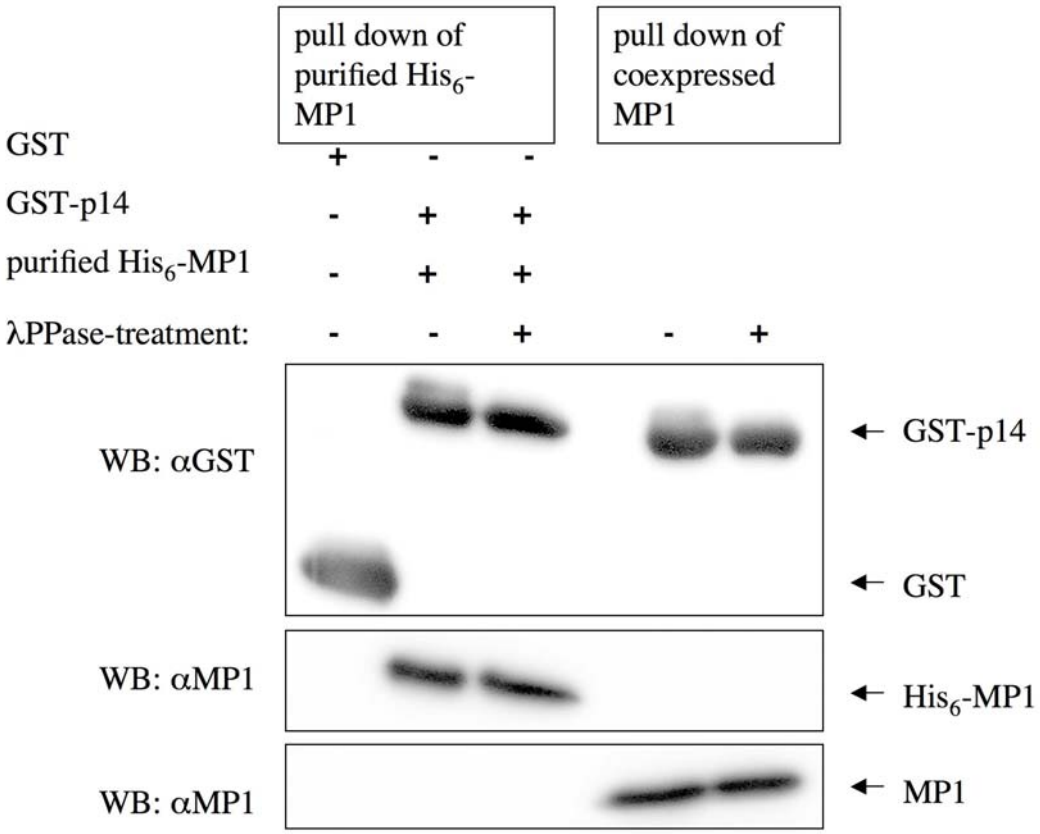


Figure 3: Phosphorylation of p14 is not required for MP1 binding *in vitro*. GST-p14 was bound to GSH sepharose beads and subjected to λPPase treatment as described in Figure 2. After removal of the phosphatase by washing, the beads were incubated with purified His_6-MP1 (lanes 2 and 3). As a control, one pulldown was performed from insect cells expressing GST alone, and beads were treated as for 2 and 3. GST-p14 and MP1 were also coexpressed and purified using GSH sepharose, followed by λPPase treatment (lanes 4 and 5). The amount of MP1 bound to the GST-p14 pulldown was analyzed by immunoblotting.

4.4 Characterization of potential p14 phosphorylation sites

As aPKCζ is a serine-/threonine kinase and might be involved in p14 phosphorylation (and consensus sights for this kinase were found in the sequence of

p14???), we tested if this kinase was able to phosphorylate p14 in vitro. GST-p14 was incubated with recombinant aPKCζ in the presence of [32P]-ATP and the incorporation of radioactive phosphate was analyzed by SDS-PAGE and phosphorimaging. As a control, maltose-binding protein (MBP), which is known to be phosphorylated by aPKCζ, and GST, which should not be phosphorylated by aPKCζ were used. Figure 4 shows that both in the presence and absence of MP1, p14 is not phosphorylated by aPKCζ, suggesting that another kinase(s) than aPKCζ is responsible for p14 phosphorylation.

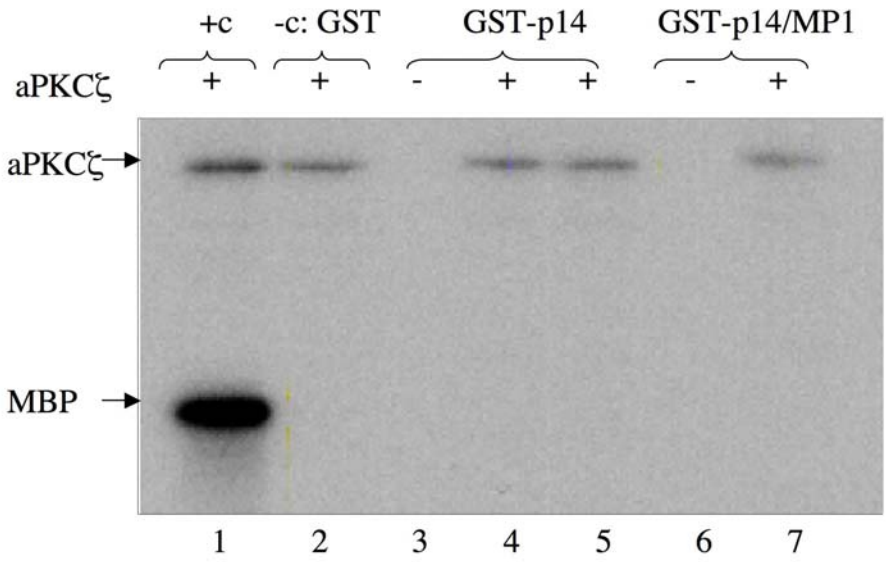
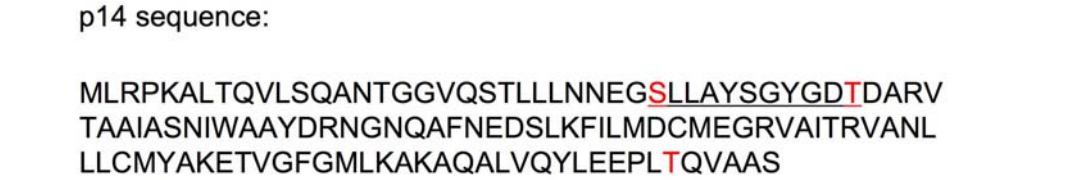


Figure 4: aPKCζ is not able to phosphorylate p14 *in vitro*. GST-p14 was purified from insect cells and incubated with recombinant aPKCζ in the presence of [^{32}P]-ATP. As a positive control the known aPKCζ substrate maltose-binding protein was used. Samples were analyzed by SDS-PAGE and phosphorimaging.

Analyzing p14 sequence for the existence of potential kinase consensus sites raised our interest for threonine 125 in its C-terminal region. This residue is surrounded by a sequence similar to a threonine in Chk2 protein, which is phosphorylated by ATM

kinase. We therefore analyzed the ability of this kinase in phosphorylating p14 in vitro. However, whereas ATM was able to phosphorylate a control substrate in vitro, p14 was not phosphorylated under these conditions (data not shown), suggesting that also this kinase is not the p14 phosphorylating kinase.

As shown in Figure 3, phosphatase treatment of p14 indicates that phosphorylation of p14 is not required for its interaction with MP1. However, phosphorylation might be important for the subcellular localization of p14 to endosomes. We therefore asked if mutation of the potential phosphorylation sites altered localization of p14 in the cell. Single mutations of S31, T41 and T125 on p14 to alanine, which resembles the non-phosphorylated state, or to glutamate, which resembles the phosphorylated state, were performed, as well as the simultaneous mutation of S31 and T41 (Figure 5).



	point mutations			double mutations	deletion mutant
neutral charge	S31A	T41A	T125A	S31AT41A	S31-T41 Δp14
negative charge	S31E	T41E	T125E	S31E413E	

Figure 5: Mutational analysis of potential phosphorylation sites on p14. (A) The serine and threonine residues mutated to alanine ("unphosphorylated") or glutamate ("phosphorylated") are indicated in colour. S31 and T41 were identified by mass spectrometry, whereas T125 was found by phosphosite prediction. (B) S31 and T41 of p14 were mutated to alanine or glutamate and transiently expressed as Xpress-tagged fusion proteins together with myc-tagged MP1. The localization of these proteins was analyzed by αXpress and αmyc staining and fluorescence microscopy. (C) As (B) using T125 p14 mutations to alanine or glutamate.

These mutated proteins were transiently expressed as Xpress-tagged fusion proteins in HeLa cells together with myc-tagged MP1. The localization of these proteins was analyzed by αXpress and αmyc staining and fluorescence microscopy (Figure 6). All

p14 mutants showed a tipical endosomal staining, indicating that mutation of the potential phosphorylation sites did not influence the localization of p14 . Furthermore, the overlay of the staining pattern of Xpress-p14 and myc-MP1 showed that these proteins still colocalized, supporting the previous finding that phosphorylation is not required for the interaction of these two proteins. In summary, these findings indicate that phosphorylation of p14 is not required for the proper subcellular localization of p14 and MP1.

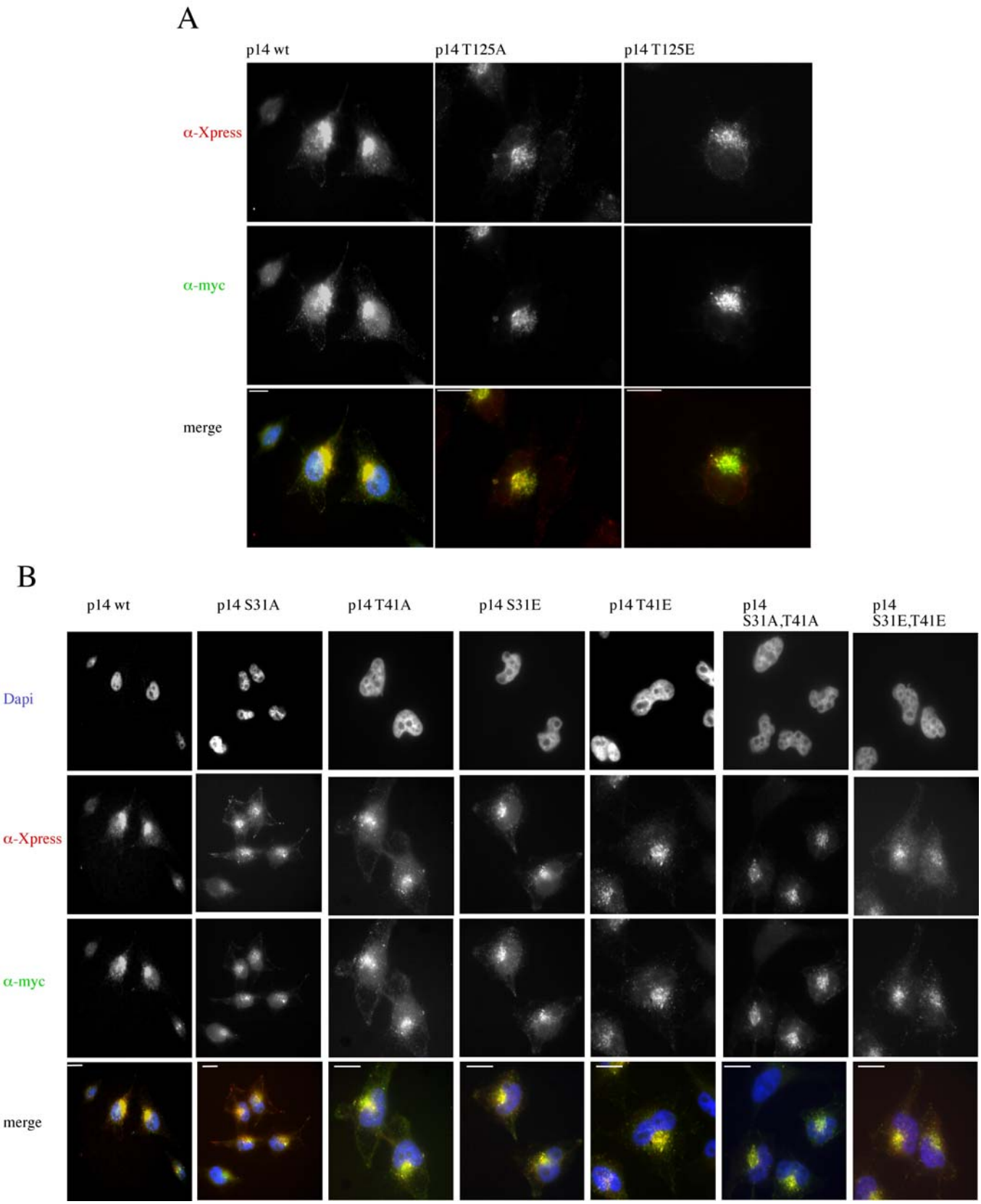


Figure 6: Interaction between p14-mutants and MP1 *in vivo*: Hela cells were transiently cotransfected with MP1-myc and various Xpress-tagged point-mutants of p14. After 24h, the cells were fixed and processed for immunofluorescence using anti-Xpress and anti-myc antibodies. The nucleus was visualized with Dapi-staining.

We then asked if phosphorylation of p14 has an influence on the binding to MP1. For this reason, we co-expressed MP1-myc and various Xpress-tagged point-mutants of p14 in Hela cells, and immunoprecipitated p14 using anti-Xpress antibodies. MP1 bound to precipitated p14 was then detected by anti-myc westernblotting. As shown in Figure 7, one phospho-mimicing mutant, S31E, showed a strong reduction in MP1 binding, whereas another phospho-mimicing mutant, T41E showed an enhanced binding to MP1, indicating that these phosphorylation sites might be involved in regulating MP1 binding. On the other hand, the double mutant of S31A and T41E reversed the effect to wild type levels.

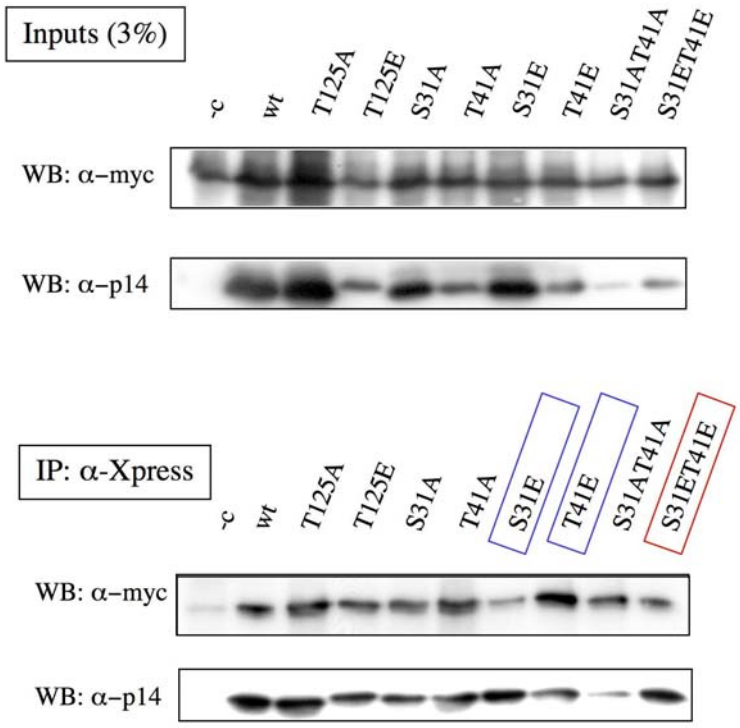


Figure 7: Analysis of p14 point-mutants for interaction with MP1 in vtiro: Extracts from Hela cells that had been transiently cotransfected with myc-MP1 and various Xpress p14 mutants were subjected to immunoprecipitation with anti-Xpress antibodies. Input and immunoprecipitates were immunoblotted after SDS-PAGE with anti-myc and anti-p14.

4.5 *P14 and MP1 form homo-oligomeres in vitro*

During these studies, we noticed that in the presence of His6-p14, a GST-p14 pulldown co-purified the His6-tagged p14. This suggests that p14 forms oligomeres in vitro. To investigate this possibility in more detail, we performed pulldown experiments, where GST-p14 (4-6) or GST (1-3) as a control were bound to GSH sepharose followed by incubation of the beads with insect cell extracts containing His6-p14, His6-MP1 or both proteins. The amount of His6-tagged protein bound to GST-p14 was analyzed by αhis western blotting. Whereas in a GST control pulldown neither MP1 nor p14 co-purified (Figure 8A, lanes 1-3), in a GST-p14 pulldown, as expected, His6-MP1 bound (lanes 5 and 6), but also some His6-p14 was recovered on the beads in the absence of MP1 (lane 4). As GST-p14 is able to co-precipitate His6-p14 also in the absence of MP1, these findings strongly indicate that p14 is able to oligomerize in vitro.

We then asked if also MP1 was able to form homo-oligomeres and performed experiments with GST-MP1 and His6-MP1 as described above. As found for p14, also GST-MP1 was able to co-purify His6-MP1, indicating that also this protein is able to oligomerize in vitro (Figure 8B).

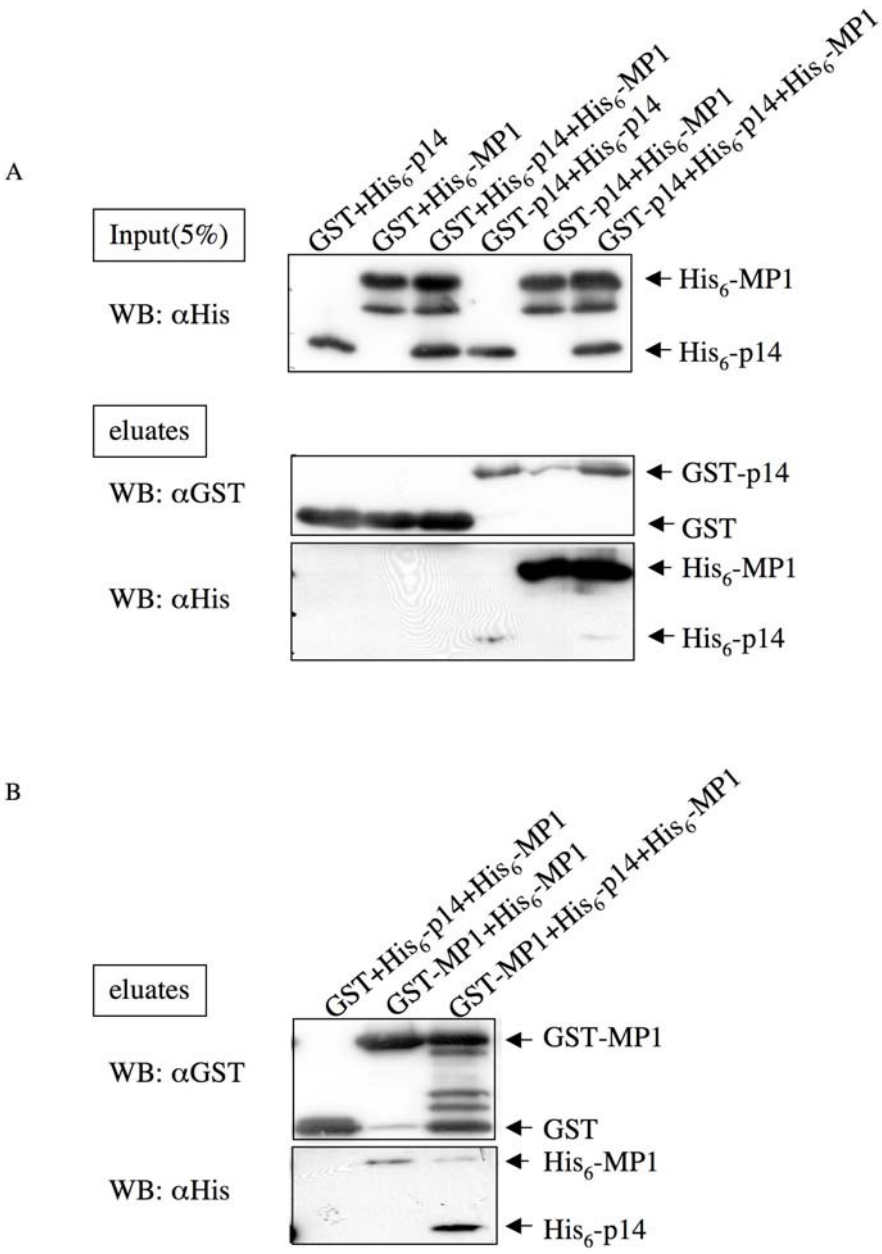


Figure 8: P14 and MP1 homo-oligomerize *in vitro*. (A) GST-p14 was bound to sepharose beads and incubated with insect cell lysates containing His_6-p14, His_6-MP1 or both proteins. After extensive washing, the protein bound to the beads was analyzed by SDS-PAGE and western blotting. As a control, the incubation was also performed with beads bound to GST alone (lanes 1-3). (B) As (A), with the difference that GST-MP1 was immobilized on the sepharose beads followed by incubation with insect cell lysates containing His_6-MP1 or His_6-MP1 and His_6-p14.

5 Discussion

Signal transduction through the MAPK pathway requires spatial and temporal specification. The signaling specificity is regulated by scaffold proteins, which coordinate the assembly of MAPK-signaling units (Catling et al. 2001 and Kolch 2000). Spatial information is provided by adaptor proteins, which define the localization of scaffold complexes (Pawson and Scott, 1997). p14 acts as an adaptor protein that is required and sufficient to localize the MP1-MAPK-signaling module to late endosomes. The late endosomal localization of the p14/MP1-MAPK complex is specifically required for proper signaling in the ERK cascade (Teis et al., 2002). A further mechanism to provide spatial and/or temporal specificity is post translational protein modifications such as phosphorylation. This may be applicable to the adaptor protein p14. In this work the possible involvement of phosphorylation on p14 in regulating its localization and its interaction with MP1 has been analyzed

In order to study these proteins, p14 and MP1 were expressed and purified from insect cells. Both GST- and His-tagged versions of both proteins were efficiently purified. We found that expression in Hi5 cells yielded in higher amounts of protein than in Sf9 cell (data not shown), therefore all proteins were expressed in Hi5 insect cells.

GSTp14 and MP1 were functionally active with respects to their interaction, as both the individual purification followed by coincubation, as well as the coexpression of the two proteins resulted in their interaction.

In addition to these two proteins, two impurities were found in the eluates, GST and a chitinase, originating from the cell system. Furthermore, another band containing GST-p14 was detected by MS, which was retarded in its mobility on SDS-PAGE,. This lead to the hypothesis, that this retarded mobility results from secondary modification on GST-p14. This band disappeared upon treatment with λPPase, strongly suggesting that GST-p14 is phosphorylated. In deed, two phosphorylation sites could be mapped by MS, and sequence homology predictions suggested the existence of a third phosphorylation

site.

Attempts to identify the kinase were taken by identification of potential consensus sites on the p14 protein and two different candidate kinases were chosen to perform an in vitro radioactive kinase assay: aPKCc, a serine-threonine kinase as well as ATM. None of the two kinases could phophorylate GST-p14 purified from the insect cells in vitro, indicating that another kinase is responsible for GST-p14 phosphorylation in insect cells.

Both spatial as well as functional impact of this potential phosphorylation were targeted for further investigation. Whether the phosphorylation has an impact on the interaction of p14 with MP1 was assessed by two approaches. First we investigated the importance of p14 phosphorylation for its interaction with MP1 in vitro. The addition of λPhosphatase to GST-p14 to both co-purifications of GST-p14 and MP1 as well as to immobilized GST-p14 prior to incubation with eluates containing His6-MP1 purified from Hi5 insect cells resulted in no difference in the interaction between p14 and MP1, indicating that the phosporylation of p14 does not play a role in the interaction between the two proteins, at least in vitro. It seems unlikely that the phosphorylation is required for the binding of the two proteins in vitro, since only a low amount of the GST-p14 is modified and still a strong interaction between the two proteins occurs, regardless of phosphatase treatment. For the same reason a positive influence is rather unlikely. It is however possible that phosphorylation of p14 partially inhibits the interaction of p14 and MP1, which would most likely not be seen in this setup of experiment as the amount of modified GST-p14 is very low compared to its unmodified form.

Therefore we took a second approach where we mutated the phosphorylation sites on p14, first to analyze their potential influence on interaction in vivo, as well as to analyze the involvement of individual sites on the localization pattern of p14, and on the ability of the mutants to colocalize with MP1.

Visualizing the localization of the p14 mutants in Hela cells via immunofluorescence, no change of the localization compared to wt p14 could be observed. Also MP1 localized normally to endosomes in these cells. As it has been

shown in the mean time that reduction of p14 protein levels by p14 siRNAi causes mislocalization of MP1 and that the localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14, (Teis et al, 2002) our results suggest that phosphorylation of p14 is not required nor inhibitory for its proper localization and interaction with MP1.

However, pull down assays of p14 mutants revealed that the single point mutations S31E and T41E, mimicking phosphorylation, changed the interaction of p14 with MP1. While the mutant S31E displayed reduced interaction with MP1, the mutant T41E lead to an increased interaction between p14 and MP1. This effect was neutralized by the immunoprecipiation of MP1-myc with the double point mutant S31E;T41E

This supports the previous assumption that phophorylation of p14 may have an inhibitory effect on its binding capacity of MP1. However, this observation could not be confirmed visually, which might be due to the lower sensitivity of fluorescence microscopy compared to immunoblotting, where a partial loss of colocalization is difficult to be observed under the microscope. However the use of more sensitive fluorescence methods as confocal microscopy or real time imaging and quantification could address this difference in a more sensitive manner.

While there is indication that phosphorylation of p14 can be observed in Hela cell extracts (data not shown/David Teis), and we were able to detect two phosphorylation sites by MS, the appropriate kinase that targets GST-p14 remains unkown. It is furthermore possible that this modification only occurs in insect cells, and therefore is not important in vivo. This could be addressed by mapping phosphorylation sites on p14 isolated from Hela cells. It is also possible that in addition to the phosphorylation sites we found by MS, there are additional sites in the remaining 60% of p14 protein not covered in the MS as well as on the GST tag itself, which might be responsible for the mobility shift. This could be clarified by analyzing the mobility of p14 tagged with another tag than GST on SDS-PAGE. Furthermore, in the course of these pull down assays and the required additional incubation times, degradation of the proteins was observed on the immunoblots through an increased band pattern. Also a faint double band could for the first time be detected for the GST protein, which would be in favor of

the possibility that additional secondary modification of GST-p14 occurs on the GST-tag. Up to these experiments the GST band appeared in quantities, which may have lead to an overlay or fusion of the two bands on the immuno blots. It is important to rule out this possilibty for instance by repeating the dephosphorylation experiment with λPhosphatase on purified GST in quantities and a gel concentration, which allows to separate the two bands and distinguish a potential modified form.

During the investigations p14 as well as MP1 were found to be able to form homo-oligomers in vitro when purified in their tagged forms from insect cells using the Baculovirus expression system and either a GST-tag or a His6-tag.

Recently, the crystal structure of the p14-MP1 complex revealed that the overall topology of the individual MP1 and p14 proteins is almost identical (Kurzbauer et al, 2002). It is thus possible that the observed homo-oligomerization of p14 and of MP1 is due to the similarity of these proteins and only occurs in vitro. It is therefore unclear if this homooligomerization plays a role in vivo.

In summary, we were able to detect phosphorylation on p14 but were not able to assign a function to the sites so far, however, this modification needs to be assessed deeper for its role in vivo and its potential to provide specificity to the adaptor protein p14 in its function in the MAP-kinase signaling pathway.

6 References

[1] Wunderlich, J. Cell Biol. 2001

[2] Robinson et al., Curr.Op.Cell Bio 1997

[3] Catling et al. 2001

[4] Pawson and Scott, Science 1997

[5] Teis & Huber, Cell. Mol. Life Sci. 2003

[6] Schaeffer et al., Science 1998

[7] Wunderlich et al., J. Cell Biol. 2001

[8] Teis et al., J Cell Biol., 2006

[9] Kurzbauer et al, 2004

[10] Bohn et al., Nat Med. 2007

[11] Cavalli V et al., Mol. Cell. 2001

[12] Di Fiore et al., Cell, 2001

[13] Teis et al, Dev. Cell 2002

[14] Sambrook and Russell 2001

7 ANHANG – Curriculum Vitae

Personal data

Name:	Sonja Hinterndorfer
Date of birth, -place:	29/Jan/1979, at Linz, Austria
Languages:	German (native speaker) English (fluent) French (fluent) Spanish (basic skills) Latin (basic skills)

Professional background

Oct 2005 – current:	Qualitymanagement at Baxter AG, Vienna/Austria.
May 2004 – June 2005:	Internship at Novartis: Laboratory technician in the Research Laboratory of Univ.-Doz. Dr. T.Baumruker in the department for autoimmune-diseases.
Nov 2001 – Nov 2002:	Research for the Diploma thesis at the Institute for Molecular Pathology in the Laboratory of Dr. L.A.Huber
July/Aug 2001:	Internship at the Institute for Molecular Pathology in the Research Laboratory of Dr. L.A.Huber
01/Jan/2001 – 30/June/2001:	Erasmus exchange program of the European Union at the University Joseph Fourier in Grenoble, France in the laboratory for organic-chemical synthesis of Prof.Dr.Y.Vallee.
2000 – 2001:	Enrolled for courses in Human Medicine at the University of Vienna
Nov 2000:	Tutor for students of Pharmacy/Nutrition in the course of their experimental laboratory course of Prof. F Vierhapper.
Oct 2000:	Internship at Novartis: in the Research laboraty of Univ.-Doz. Dr. T.Baumruker the department for autoimmune-diseases.
Aug/Sep 2000:	Internship at Baxter/Highland/Immuno: in the production department for Diagnostica
June 2000:	Internship at Castrol: in the Quality Control laboraty for production of fuel/gasoline etc.

01/Oct/1997- current:	Unversity of Vienna: Student for Chemistry; main subject: Biochemistry, second subject: Organic Chemistry
13/June/1997:	Final high school degree (Matura) with distinction
1989 – 1997:	High school at Linz, Austria 3. Bundesrealgymnasium Ramsauerstrasse

Training courses and additional skills

2010:	Training for Workshop-Moderation and presentation skills
2007:	3 weeks Training as 6-sigma Green Belt for problem solving investigation techniques
2006:	Project Management basic skills
29/June – 03/July/2002:	Participant at the ELSO-Conference of the European Life Science Organization in Nice, France
Oct 1999 – Jan 2000:	Writing and Speaking Scientic English
1997:	Drivers Licence category B (automobiles)