Generation of GST-p24 fusion proteins for antisera production and p24/COP binding studies

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SUMMARY

p24 proteins are type I transmembrane proteins with a size of ~24 kDa. The p24 protein family consists of four subfamilies: p24α, -β, -γ and -δ. The sequence homology between the four subfamilies is only about 30%, but the homology within a subfamily is considerably higher (~ 60%). Moreover, they share the structural characteristics of a large lumenal domain and a short cytoplasmatic tail (Kuiper et al., 2000). Their function is not exactly known at this moment, but they seem to play an important role in cargo transport, because they are abundantly present in COP-coated vesicles, which are transport vesicles that move between the ER and the Golgi. For the binding of p24 proteins to COP subunits, certain coat-binding motifs are important. These motifs are part of the cytoplasmic tail of the p24 proteins. For the retrograde transport the motif is K(X)KXX, for the anterograde transport the motif is FF (Dominguez et al., 1998). These motifs in the p24 proteins bind to COP subunits or COP subcomplexes (multiple COP subunits connected).

Here we studied the binding of p24 proteins to αCOP, γCOP and εCOP subunits. For this purpose, several fusion proteins of glutathione S-transferase (GST) and p24 cytoplasmic tails were created, bound to glutahione-Sepharose and incubated with cytosol. By using COP antibodies, the bound COP subunits could be detected. Furthermore, by creating fusion proteins of GST and p24 lumenal parts, antibodies against p24 proteins could be produced. The human α/γCOP antibody that was used in this experiment did detect some subunits bound to the cytoplasmic tail of the Xlp24γ_1, but since a similar extent of binding was observed for the GST alone, this binding is most likely nonspecific. This contradicts with the fact that both Xlp24γ_1 and Xlp24γ_2 have the K(X)KXX motif that is necessary for the binding to these subunits. The other interaction that was studied was the binding of a p24 C-tail to εCOP subunits. The εCOP subunit, with a size of 37 kDa, did not bind, but there was a band at 50 kDa in the lanes where the Xlp24γ_1 and Xlp24γ_2 cytoplasmic tails were used. This protein could be isolated and subjected to further study. This experiment could be improved by using better antibodies or by using cytosol isolated form a human cell line. Furthermore, it will be interesting to use other cytoplasmic tails of p24 proteins from other subfamilies, and antibodies for the other COP subunits.

1. INTRODUCTION

1.1. The p24 protein family

Xenopus laevis is able to adapt the colour of its skin to the colour of its environmental background. The cells that are responsible for this adaptation are the melanotrope cells of the pituitary gland. When *X. laevis* is placed on a dark background, there is an increased production of the pigment αMSH, leading to a dispersion of melanine, and the skin of the animal will get darker. The precursor of αMSH is proopiomelanocortin (POMC), which is produced by melanotrope cells in the intermediate lobe of the pituitary gland. The level of POMC mRNA is up to 30-fold higher in black-adapted animals than in white-adapted animals (Martens et al., 1987).

Several proteins have been isolated which show a coordinate expression with POMC. One of these is the p24δ_2 protein, which is a member of the p24 protein family. p24 proteins are type I transmembrane proteins (figure 1), which have a size of ~24kDa. The p24 protein family consists of four subfamilies: p24α, -β, -γ and -δ (Dominguez et al., 1998). The sequence homology between the four subfamilies is only about 30%, but the homology within one subfamily is considerably higher (~ 60%). Overmore, they share the structural characteristics of a large lumenal domain and a short cytoplasmatic tail (Kuiper et al., 2000).

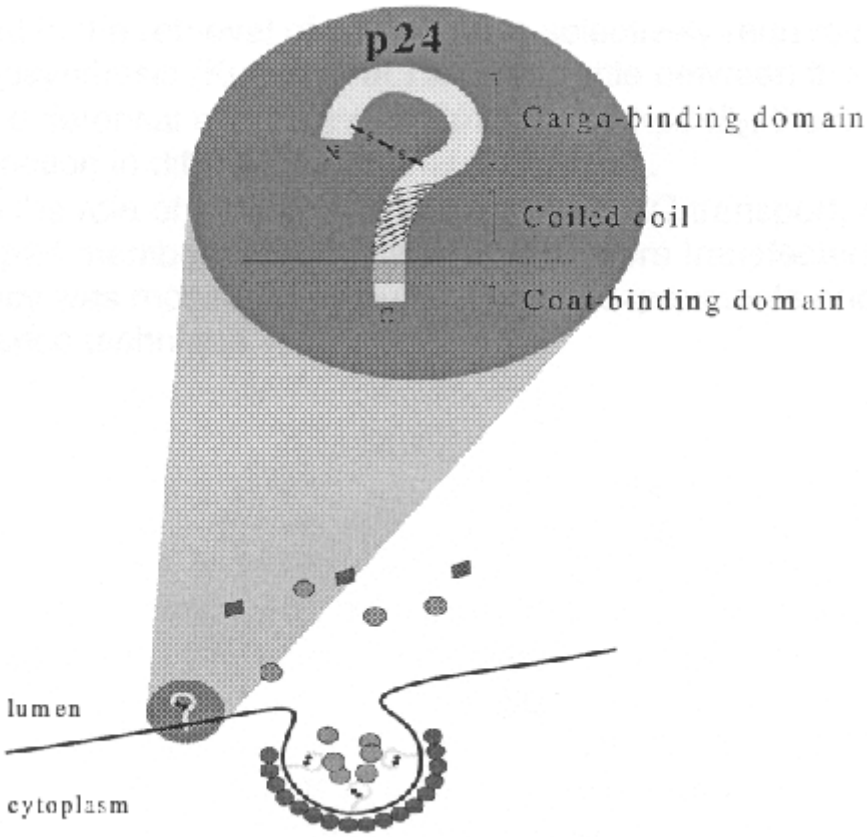

***Figure 1**: Structural characteristics of the p24 proteins.*

The lumenal part of the p24 protein could be a cargo-binding domain, which forms a loop as a result of the disulfide bridge between the two conserved cysteine residues (Stamnes et al., 1995). The coiled-coil domain, located at the more conserved membrane proximal part of the lumenal domain, structure could enable molecular interactions, not only between the same proteins, but also between different p24 proteins, since the coiled-coil domains are all at the same position relative to the transmembrane domain (Emery et al., 1999). In this way, the p24 proteins could form heteromeric complexes. For example, hp24γ_3, hp24β_1, and hp24δ_1 coprecipitated in what appeared to be stochiometric amounts. This heterocomplex was specific (Füllekrug et al., 1999). In the mammalian Golgi, p24 proteins can be found in complexes of sufficient size to contain more than 50 copies of p24 proteins (Dominguez et al., 1998).

Because members of the p24 protein family has a high presence in COP-coated vesicles (Schimmöller et al., 1995, Sohn et al., 1996, Nickel et al., 1997), they are thought to play an important role in cargo transport. COP-coated vesicles are transport vesicles that move between the ER and the Golgi. Budding of these vesicles is induced by either coat protein complex I (COPI) or coat protein complex II (COPII). COPII is involved in export of biosynthetic cargo from the ER (anterograde ER→Golgi transport; Schekman and Orci, 1996), whereas COPI is involved primarily in retrograde (Golgi→ER) transport, but probably also in anterograde transport (Allan and Balch, 1999). These COP complexes interact with signals located in the cytoplasmic tail of the p24 proteins (figure 1).

1.2. Binding of p24 proteins to coat protein COP

As already mentioned, both COPI and COPII are bound by the coat-binding domain in the C-tail of the p24 proteins. For this binding, certain coat-binding motifs are important. For retrograde transport (Golgi→ER, COPI) the motifs KKXX and KXKXX appear to be necessary (Cosson and Letourneur, 1994, Letourneur et al., 1995, Fiedler et al., 1996, Girod et al., 1999). This K(X)KXX motif, where K is lysine and X is any amino acid, has been called the ER retrieval signal. For the COPII binding, a FF (double phenylalanine) motif was found to be important (Fiedler and Rothman, 1997), which is probably just a part of a larger motif (Dominguez et al., 1998). Figure 2 shows a line-up of the C-tails of the Xlp24 proteins used in this study. Xlp24α, Xlp24β, and Xlp24γ_3 contain the FF motif, whereas Xlp24γ_1 and Xlp24γ_2 have a LF motif. This difference probably does not have a large effect on the binding affinity of the protein, because both leucine and phenylalanine are large, non-polar residues (leucine sidechain = -CH_2-CH-(CH_3)$_2$, phenylalanine sidechain = -CH_2-phenyl). The K(X)KXX motif is found in Xlp24α, Xlp24γ_1 and Xlp24γ_2. For the Xlp24β, the two lysines are replaced by two arginines. This difference makes the Xlp24β less efficient in COPI binding (Dominguez et al., 1998). Xlp24γ_3 is lacking the K(X)KXX motif.

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Xlp24α      216 RHLKSFFAKKLV
Xlp24β      189 YYLKRFFEVRRVV
Xlp24γ1     215 YMLKSLFDDKRKIRT
Xlp24γ2     212 YMLRSLFEDKRKSRL
Xlp24γ3     201 FLLKSFFSDKRTTTTRVGS
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***Figure 2**: Line-up of the cytoplasmic tails of three Xlp24 proteins. The coat-binding motifs are underlined.*

When looking at binding of p24 proteins to COP proteins, one should also take into account the different COP subunits. The COPI coatomer contains several subunits, called α-, β-, β'-, γ-, δ-, ε- and ζCOP (Hudson and Draper, 1997). Some subunits form subcomplexes: for example a γ/ζ and a α/β'/ε subcomplex (Gu et al., 1997). The α/β'/ε subcomplex was shown to interact with membranes and with cytoplasmic K(X)KXX motifs (Lowe and Kreis, 1995). Fiedler et al. (1996) suggested that different COPI subunits bind to different members of the p24 protein family.

1.3. The function of p24 proteins

The exact function of p24 proteins is yet not clear. As already mentioned, they are probably involved in cargo transport, because they are major membrane components of COPI or COPII coated vesicles. Moreover, the cytoplasmic tails contain motifs for binding to COP subunits, as discussed in paragraph 1.2.

One approach to define the physiological function of the p24 proteins is to mutate the corresponding genes and then look for associated defects in vesicular trafficking (Kaiser, 2000). Such an experiment has been carried out with the yeast *Saccharomyces cerevisiae*. Yeast strains carrying mutations in p24 genes grow normally, and overt defects in either COPI or COPII vesicle functions are not seen (Schimmoller et al., 1995, Belden et al., 1996). However, this could also be a consequence of the redundancy of p24 genes, since the *S. cerevisiae* genome carries eight p24 homologues, which all have to be knocked out to see the consequences of elimination of the p24 function. This octuple mutant was constructed and showed no detectable defect in the rate of both ER to Golgi transport and Golgi to ER transport (Springer et al., 2000). So p24 proteins are not essential in yeast for the function of either COPI or COPII vesicles.

However, p24 gene mutations or deletions do have some effect. For example, deletion of p24 genes has no observable effect on carboxypeptidase Y export (from the ER), but slows export from the ER of invertase and proteins linked to glycosylphosphatidylinositol (GPI) anchors (Schimmoller et al., 1995, Belden et al., 1996). This difference in the rates of export for different protein cargo molecules suggests that p24 genes might encode cargo receptors for a subset of secretory proteins. However, none of the attempts to crosslink p24 proteins to cargo molecules has succeeded yet. This could be an indication for an indirect connection between p24 function and cargo loading. Kaiser (2000) suggested that p24 has a role in the budding process of the transport vesicles.

In this process, they could increase the fidelity of cargo loading by temporal control of the budding process, or they might sterically exclude proteins that do not belong in the vesicle. The p24 proteins also might define a subcompartment that would contain cargo proteins and exclude resident proteins. This final view of p24 function finds support from the observations that multiple p24 proteins associate with another within the membrane (Marzioch et al., 1999, Füllekrug et al., 1999) and that p24 proteins contribute to the formation of vesiculotubular clusters (also called the ER Golgi intermediate compartment), a subcompartment of the ER responsible for much of the vesicular sorting between the ER and Golgi in mammalian cells (Lavoie et al., 1999).

1.4. Creating GST fusion proteins

If one wants to do binding studies with (parts of) a p24 protein, it is necessary to create fusion proteins, because the parts of a p24 protein (especially the C-tails) are very small. The pGEX expression system (figure 3) is often used in this context. In this system, the polypeptides are expressed as a fusion protein with Glutathione S-Transferase (GST). This method has a number of advantages. First of all, the fusion protein is, in most of the cases, soluble, whereas 'free' polypeptides are mostly insoluble. A second advantage is the possibility to purify the expressed proteins in only one step, with glutathione-Sepharose. Large amounts of proteins can be purified rather fast in this way. A further advantage of the pGEX-expression system is the possibility to cleave off the GST fusion part (after purification of the protein) with a protease (like factor Xa).

1.5. Research goal

The goal of this research was to study the binding of the cytoplasmic tails of various p24 proteins from *X. laevis* to subunits of the coatomers COPI and COPII, in order to determine their role in protein transport in the secretion pathway. Therefore, fusion proteins of GST and the cytoplasmic tail of different *X. laevis* p24 proteins were created. Fusion proteins of GST and the lumenal parts of some p24 proteins were also created to use them as antigens for the production of antisera.

2. MATERIAL & METHODS

2.1. General protocols

2.1.1. Polymerase Chain Reaction (PCR)

First 15 µl of master mix 1 (see appendix, paragraph 5.3) and 5 µl template DNA (~20 ng) were put together in a PCR tube. Then 20 µl of master mix 2 (appendix, paragraph 5.3) was added and the PCR tube was placed in a PCR apparatus which was programmed in the following way: 2 min. 94°C, 10 cycles 30 sec. 94°C, 20 sec. 58°C and 20 sec. 72°C, 15 cycles 30 sec. 94°C, 20 sec. 63°C and 30 sec. 72°C, 4 min. 72°C and finally ∞ 4°C.

2.1.2. Ethanol precipitation

In a 1.5 ml microcentrifuge tube 0.1 volumes of 3 M sodium acetate (pH 5.2) were added to the solution of DNA. Then 2 volumes of ice-cold 100% ethanol were added. The sample was mixed by vortexing and placed at –20°C for 5 minutes or longer. Then it was spinned for 5 minutes and the supernatant was carefully removed. Then 1 ml of 70% ethanol (room temperature) was added. The sample was spinned and the supernatant was removed again. Finally, the pellet was dried and stored in TE buffer.

2.1.3. Restriction enzyme digestion

2.1.3.1. EcoRI restriction enzyme digestion

The following was pipetted into a microcentrifuge tube (for 1 ml total): x µg DNA (depending on DNA fragment size) in 885 µl H_2O and 100 µl 10x restriction buffer H. Then 15 µl of the restriction endonuclease EcoRI (10 U/µl) was added and the reaction mixture was incubated at 37°C for 5 hours.

2.1.3.2. BamHI restriction enzyme digestion

The following was pipetted into a microcentrifuge tube (for 1 ml total): x µg DNA (depending on DNA fragment size) in 890 µl H_2O and 100 µl 10x restriction buffer B. Then 10 µl of the restriction endonuclease BamHI (75 U/µl) was added and the reaction mixture was incubated at 37°C for 5 hours.

2.1.4. Gel electrophoresis

2.1.4.1. Agarose gel electrophoresis

Either 0.8% (for larger DNA fragments) or 2.5% agarose (for smaller DNA fragments) was added to TBE. This mix was heated and then cooled down again to approximately 50°C. Then 0.001% ethidiumbromide was added and the whole mix was put in a agarose gel cassette. 1/6 volume of 6x sample buffer was added to the samples, which were then loaded on the gel.

2.1.4.2. SDS-PAGE electrophoresis

The samples were mixed with ½ volume 3x sample buffer and heated for five minutes at 95°C. Then the proteins were analyzed on a 10% SDS gel (for larger proteins) or a 12.5% gel (for smaller proteins) at a maximum of 200V.

2.1.5. Ligation

In a 1.5 ml eppendorf tube was mixed: 4 µl of the vector pGEX2T (figure 3), 10 µl of the insert (in the case of the negative control H_2O) and 3 µl H_2O. The tube was placed in a 37°C water bath for 5 minutes. Then 2 µl 10x ligation buffer + ATP (Boehringer) and 1 µl ligase (1u/µl) were added. The ligation mix was incubated o/n at 18°C.

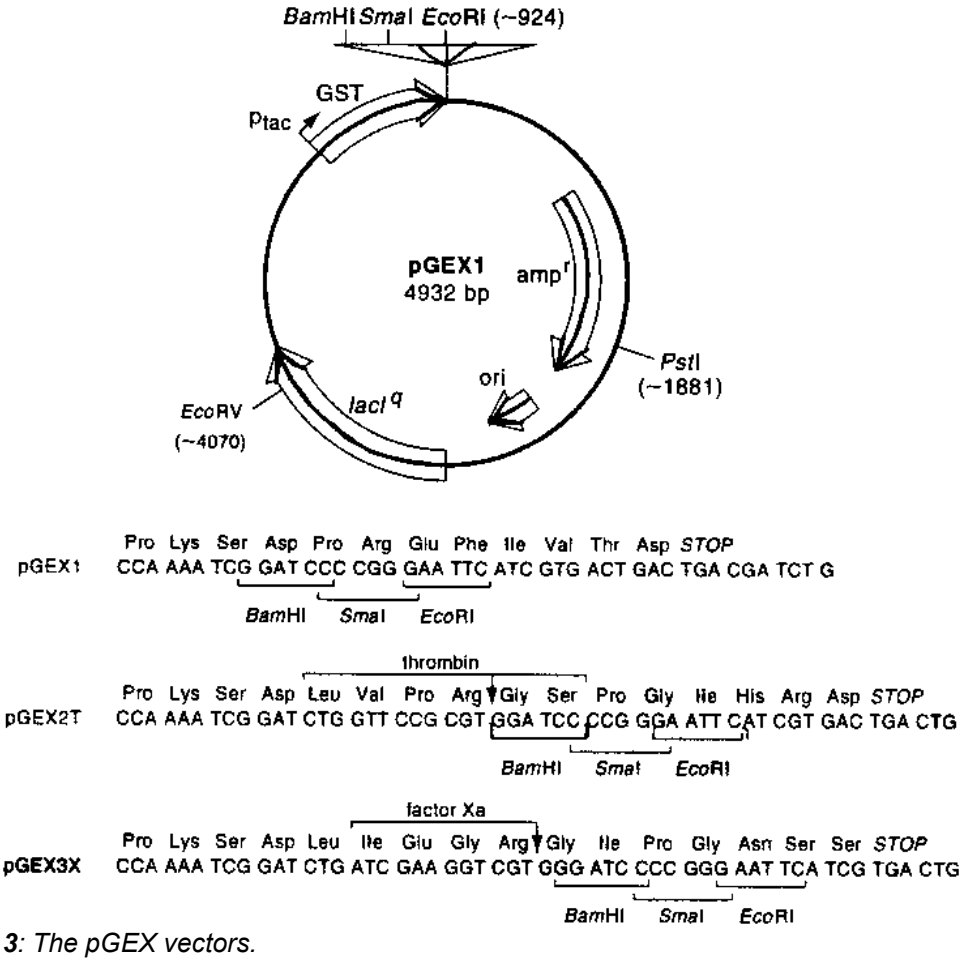


***Figure 3**: The pGEX vectors.*

After the o/n incubation, a transformation was carried out, using either $CaCl_2$ competent or electrocompetent *Escherichia coli*.

2.1.6. Transformation

2.1.6.1. Transformation with $CaCl_2$-competent *E. coli*

To 100 µl $CaCl_2$ competent XI1-blue cells was added 5 µl of the ligation mix. The cells were put on ice for 30 minutes and then heat-shocked for 1 minute at 42°C. After the heat-shock they were put on ice for exactly 2 minutes and 900 µl of SOC media was added. The cells were incubated at 37°C, shaking, for 1 hour. After the incubation the mix was centrifuged for 1 minute at 10,000 rpm. The supernatant was discarded, while the pellet was resuspended in 200 µl SOC media. Finally, the cells were spread on LB-amp. plates and incubated o/n at 37°C.

2.1.6.2. Transformation with electrocompetent *E. coli*

To 50 µl electrocompetent XI1-blue cells was added 0.5 µl of the ligation mix. The cells and the DNA were mixed well and put on ice for 1 minute and then transferred to the bottom of a cold, 0.1 cm electroporation cuvette. This cuvette was placed in a Gene Pulser apparatus at 1,8 kV. A pulse with a time constant of 4 to 5 msec was given, after which 1 ml of SOC^c media was added immediately. The cells were quickly resuspended in the media with a pasteur pipette. The cell suspension was transferred to a 17x100 mm polypropylene tube and incubated for 1 hour at 37°C, shaking with 225 rpm. The supernatant was discarded and the cells resuspended in 200 µl SOC media. Finally, the cells were spread on LB-amp. plates and incubated o/n at 37°C.

2.1.7. DNA sequencing

The DNA was diluted with H_2O to a concentration of 100 ng/µl. Then the following was pipetted into a PCR tube: 1 µl Terminator Ready Reaction Mix (from ABI Prism kit), 3.5 µl Big Dye Buffer, 1.5 µl DNA (template), 1 µl primer 5' pGEM-T (3.2 mM, see appendix for sequence) and 13 µl H_2O. Then the PCR tube was placed in a PCR apparatus which was programmed in the following way: 1 min. 94°C, 25 cycles 30 sec. 96°C, 15 sec. 50°C and 4 min. 60°C and finally ∞ 4°C. An isopropanol precipitation was carried out to remove residual dye terminators from the sequencing reaction. First the extension products (20 µl) were transferred to 0.5 ml microcentrifuge tubes and 80 µl of 75% isopropanol (2-propanol) was added. The samples were vortexed briefly and left at room temperature for 15 minutes to precipitate the extension products. Then the tubes were spinned for 20 minutes at maximum speed. The supernatant was carefully removed with a pipet and the pellet was rinsed with 250 µl of 75%

isopropanol (2-propanol). The samples were vortexed briefly again and centrifuged for 5 minutes at maximum speed. Then the supernatant was removed and the pellet was dried at room temperature for 10-20 minutes. Finally, the pellet was dissolved in 15 µl TSR, heated for 3 minutes at 95°C and the samples were sequenced in an ABI Prism 310 sequencing apparatus.

2.1.8. Expression of proteins

LB media with 100 µg/ml ampicillin was inoculated 1/50 with an overnight culture of cells containing the fusion plasmid. The cells were grown at 30°C to A600 ~ 0.5-1. IPTG was added to a final concentration of 0.2 mM. The cells were incubated at 30°C, shaking, for 2-4 hours. Then the cells were harvested by centrifugation at 5,000 g for 10 minutes. The supernatant was discarded. The pellet was stored at -80°C.

2.1.9. Coomassie staining

The gels were stained for 30 minutes with 0.1% Coomassie blue R-250 in fixative (40% MeOH, 10% HOAc) and then destained with several changes of 40% MeOH/10% HOAc (1 to 3 hours).

2.2. Generation of GST-Xlp24 fusion proteins

2.2.1. Preparation of insert DNA

First a Polymerase Chain Reaction (PCR) was carried out to amplify the DNA of the cytoplasmic tails or the lumenal parts. To check the size of the PCR fragments, they were loaded on a 2.5% agarose gel. The PCR fragments, having the correct size, were digested by an EcoRI restriction enzyme digest (~5 µg DNA in 1 ml reaction mixture). This was followed by an ethanol precipitation, and a BamHI restriction enzyme digest (~1 µg DNA in 500 µl reaction mixture). After each digestion, the size of the PCR fragments were checked once more. After this preparation, the fragments were ready to be inserted in the vector.

2.2.2. Preparation of vector DNA

Because there was no ‘empty’ pGEX-2T vector available, a pGEX-2T vector with an insert was used. This insert had to be cut out before the vector could be used again. Therefore, a EcoRI/BamHI restriction digest had to be carried out.

The vector was cut first with EcoRI (~5 µg in 300 µl reaction mixture), followed by ethanol precipitation. The size of the fragment was checked on a 0.8% agarose gel. After the BamHI digestion (~1 µg in 300 µl reaction mixture),

the DNA was loaded on a 0.8% agarose gel again. These bands were extracted from the gel by QIAEX II agarose gel extraction.

2.2.2.1. QIAEX II agarose gel extraction

The DNA band was cut from the agarose gel with a razor blade. The agarose was put into 1.5 ml microcentrifuge tubes (250 mg agarose per tube). Then Buffer QX1 was added (<100 bp: 6 volumes, 100 bp – 4 kb: 6 volumes, >4 kb: 3 volumes + 2 volumes H_2O). Then 3 µl/µg DNA of QIAEX II was added. The samples were incubated at 50°C for 10 minutes (to solubilize the agarose and bind the DNA) and vortexed every 2 minutes. The samples were centrifuged for 30 seconds and the supernatant was carefully removed with a pipet. Then the pellet was washed with 500 µl of Buffer QX1 (1x) and with 500 µl of Buffer PE (2x), by resuspending, centrifuging for 30 seconds and removing the supernatant. Then the pellet was air-dried for 10-15 minutes and the DNA was eluted by resuspending it in H_2O and incubating it at room temperature for 5 minutes. Finally, the samples were centrifuged for 30 seconds and the supernatant (=purified DNA) was pipetted into clean tubes.

2.2.2.2. Fill-in of vector with Klenow fragment

For the binding studies, a negative control was needed. The 3' recessed ends of the pGEX-2T vector fragment, cut at the BamHI and EcoRI restriction sites, were filled in by using the Klenow-fragment. The following was pipetted into a microcentrifuge tube: 8 µl pGEX-2T BamHI/EcoRI (0.2-5 µg), 2 µl 10x restriction buffer B, 1 µl 1.25 mM dNTP, 1 µl Klenow fragment (1 u/µg DNA) and 8 µl H_2O. This mixture was incubated at 25°C for 15 minutes. The reaction was stopped by adding EDTA to 10 mM final concentration and heating for 10 minutes at 75°C.

2.2.3. Creating and checking the fusion proteins

First the vector and the inserts were ligated. After the ligation, a transformation was carried out, using either $CaCl_2$ competent or electrocompetent *Escherichia coli*. Then the produced contructs were checked by sequencing. After the expression, the proteins were checked on a 12.5% SDS-PAGE gel, followed by Coomassie staining.

2.3. Purification of GST-Xlp24-lumenal part fusion proteins to use as antigens

2.3.1. Isolation of insoluble fusion proteins

To allow solubilization of the fusion proteins, the insoluble fusion proteins had to be isolated. The volumes in the following protocol are appropriate for a 1000 ml culture. After harvesting the cells, the cells were resuspended in 80 ml of lysis buffer (50 mM Tris pH 8.0, 25% sucrose, 1 mM EDTA). The cells were lysed by addition of 200 mg lysozyme dissolved in 20 ml lysis buffer (final conc. 2 mg/ml) and rocked for 30 minutes at 4°C. Then $MgCl_2$ and DNase I (Sigma, D5025) were added to final concentrations of 10 mM and 25 µg/ml respectively. The solution was incubated for 20 minutes at 4°C, rocking. Then 200 ml of detergent buffer (0.2 M NaCl, 1% deoxycholic acid, 1% Nonidet P-40, 20 mM Tris pH 7.5, 2 mM EDTA) was added. The lysate was centrifuged at 5000 g for 10 minutes. The supernatant was carefully removed and the pellet completely suspended in Triton-EDTA solution and centrifuged at 5000 g for 10 minutes. The milky supernatant was carefully removed and the pellet completely suspended again in the Triton-EDTA solution and centrifuged (this step was repeated two times). The final pellet contained all of the insoluble fusion proteins.

2.3.2. Solubilization and dialysis

The pellet containing the insoluble proteins was resuspended in a solubilization reagent (8M urea, 1 mM DTT, 1 mM EDTA, 50 mM Tris-HCl pH 8.0) and then transferred to a Spectro/Por 6 dialysis tube with a molecular mass cutoff value of 1 kDa. Then it was dialyzed in a cold room (8°C) against 1 liter of 8 M urea, 100 mM NaCl and 50 mM Tris-HCl (pH 8). The urea concentration was reduced by 1 M every 3 hours. Finally, the sample was recovered from the dialysis tube.

2.4. Binding studies with GST-Xlp24-cytoplasmic tail fusion proteins

First the insoluble fusion proteins were isolated, solubilized and dialyzed (see paragraph 2.3). Then the proteins were bound to glutahione-Sepharose and incubated in cytosol to study the binding of p24 proteins to COPI and COPII.

2.4.1. Binding to glutathione-Sepharose

100 mg of glutathione sepharose in 1.5 ml of NETN (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.5% Nonidet P-40, 5 mM DTT) was incubated for 1 hour at 4°C. After the incubation the beads were washed 5x with NETN. The beads were blocked for 60 minutes in NETN + 5 mg/ml BSA. Then the beads were washed again 4x with NETN. The beads were stored as a 1:1 slurry in NETN + 0.01% methiolate. 30 µl of the 1:1 slurry was added to 5 µg of fusion protein (in 200 µl H_2O) and incubated for 20-30 minutes at 4°C. After spinning, the supernatant was stored and the pellet was washed 3x with NETN.

For checking on gel, the fusion proteins were eluted by boiling in 50 µl 1x SDS sample buffer.

2.4.2. Binding to COP subunits

2.4.2.1. Binding to cytosol

100 µl of frozen cytosol (COS monkey kidney) was thawed and spinned for 15 minutes at >20,000 g. The cytosol was added to 5 µg of bound fusion and incubated for 3 hours at 4°C (rotating). After centrifugation, the supernatant was saved and the pellet was washed 3x with NETN buffer. The bound proteins were eluted by boiling in 50 µl 1x SDS sample buffer and the samples were loaded on a 12% SDS-PAGE gel, which was Western blotted and incubated in COP antibodies afterwards.

2.4.2.2. Western blotting

The proteins were then transferred onto a nitrocellulose membrane using a Western blot device. Transfer was at 200 mA for 1 hour in a cold 1x blot buffer with 20% methanol.

2.4.2.3. *Super Signal West Pico*™ detection

After transfer, the blots were blocked for 20 minutes in blocking buffer (PBST), followed by a 1 hour incubation in the primary antibodies (human α/γCOP and εCOP), 1:5,000 diluted in blocking buffer.

The blots were washed 6 x 5 minutes in 1x PBST. The secondary antibodies (goat-anti-rabbit/HRP) were diluted 1:10,000 in blocking buffer. The blots were incubated in diluted conjugate solution for 45 minutes and afterwards washed 6 x 5 minutes with 1x PBST. The blots were drained and placed on Saran wrap on a flat surface. They were incubated for 5 minutes in 1 ml working solution (0.5 ml lum./enh. solution, 0.5 ml stable perox. solution). The excess working solution was drained and the membrane was placed in a development folder and exposed to film for a short period (5 sec. - 1 min.).

3. RESULTS

3.1. Generation of GST-p24 fusion proteins

3.1.1. Preparation of insert DNA

The C-tails of Xlp24α, Xlp24β, Xlp24γ_1 and Xlp24γ_2 were amplified by PCR (for C-tail sequences see figure 2), resulting in DNA fragments having an engineered BamHI site at the 5' end and an EcoRI site at the 3' end. Figure 4 shows the amplified C-tails of Xlp24γ_1 and Xlp24γ_2 (for primers see appendix). Products of the correct size (~46 bp) were formed (marked with an arrow). The negative control (H_2O in stead of template) showed no amplified products.

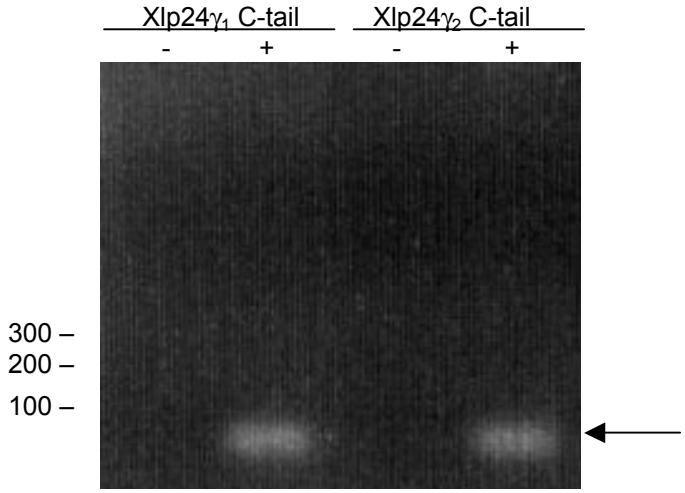


***Figure 4**: PCR products of Xlp24γ_1 and Xlp24γ_2 cytoplasmic tail on a 2.5% agarose gel. '-' = H_2O used in stead of template (negative control). '+' = correct template used. Size of marker DNA fragments in bp.*

The PCR products were further digested with the restriction enzymes EcoRI and BamHI. Figure 5A shows the PCR products (cytoplasmic tail Xlp24γ_1 and Xlp24γ_2) before and after the EcoRI restriction digest. The PCR products (marked with an arrow) are smaller after digestion with EcoRI. In a second restriction enzyme digest, the PCR products were digested with BamHI (figure 5B) and then ethanol precipitated.

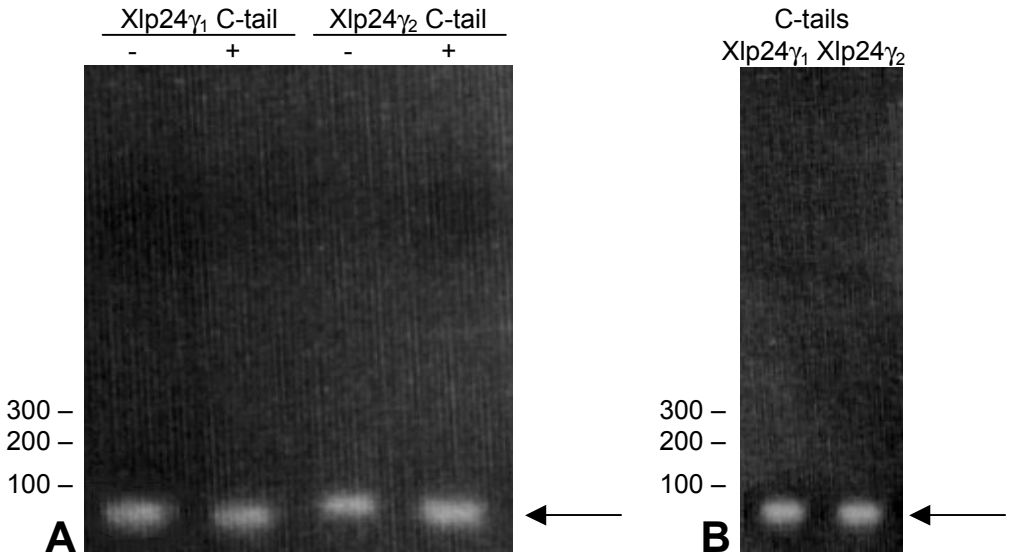


***Figure 5**: **(A)** Restriction enzyme digest of the inserts with EcoRI. '-' = before digest. '+' = after digest. **(B)** BamHI restriction enzyme digest of the EcoRI digested PCR product. Both lanes after digest. Size of marker DNA fragments in bp.*

3.1.2. Preparation of vector DNA

Figure 6 shows the restriction digest of a pGEX-2T-based construct. Its insert had to be cut out with BamHI and EcoRI before the PCR products of the Xlp24 cytoplasmic tails could be inserted. Figure 6A shows the pGEX-2T-based construct before ('-') and after ('+') the restriction digest with BamHI. Before the digest, there are two bands visible. The upper band is the normal, "relaxed" form, while the lower band is the "coiled" form. The coiled DNA has the same weight as the relaxed DNA, but because of its larger density, it runs faster through the gel. After the digest, there is only one band visible, representing the single-cut pGEX-2T-based construct (marked with an arrow). Figure 6B shows the DNA pattern after digestion with BamHI and EcoRI. The upper band is the fully digested vector (marked with an arrow). This band was was cut out of the gel by QIAEX II agarose gel extraction.

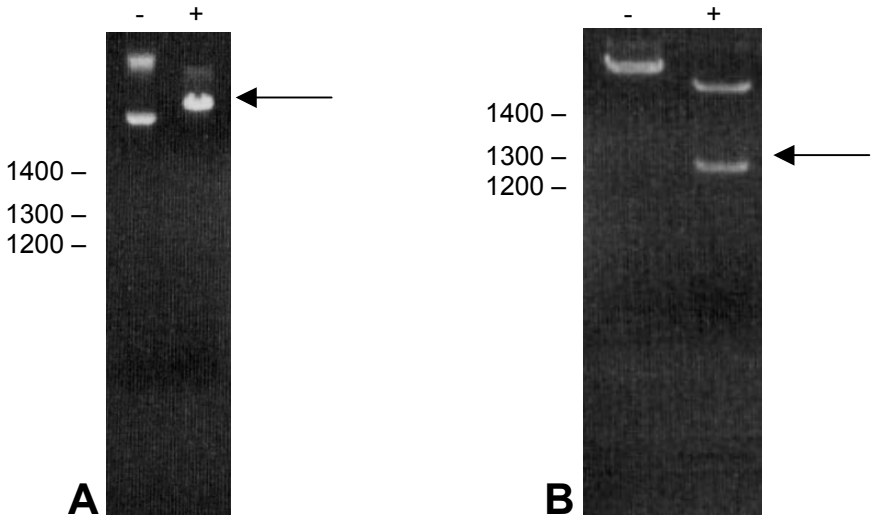


***Figure 6**: Restriction enzyme digest of the vector with **(A)** EcoRI and **(B)** BamHI. '-' = before digest. '+' = after digest. Size of marker DNA fragments in bp.*

3.1.3. Expression of the fusion proteins

Figure 7 shows the expression of the fusion proteins GST-α-C and GST-β-C, that were created after ligation and transformation (for the created fusion proteins see table 1). Figure 8 shows the expression of the fusion proteins GST-γ_1-L and GST-γ_2-L (see also table 2). The GST fusion proteins were only produced after induction with IPTG ('+'). These proteins (marked with an arrow) have the correct size of ~30 kDa. The expression of the fusion proteins was also checked by western blotting and a detection with specific antibodies (Hulsen, 2000). However, the produced fusion proteins were insoluble, and the yield was low. Therefore the insoluble fusion proteins were isolated as inclusion bodies.

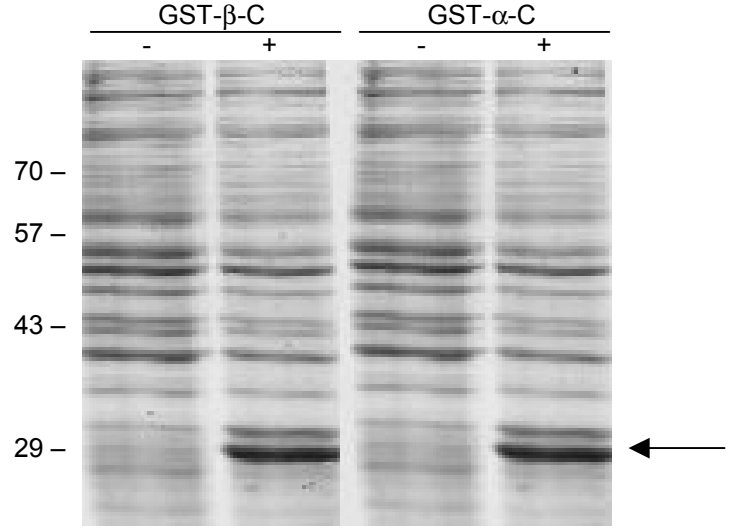


***Figure 7**: The expression of the GST fusion proteins GST-β-C and GST-α-C. '-' = not induced with IPTG. '+' = induced with IPTG. Marker sizes are in kDa.*

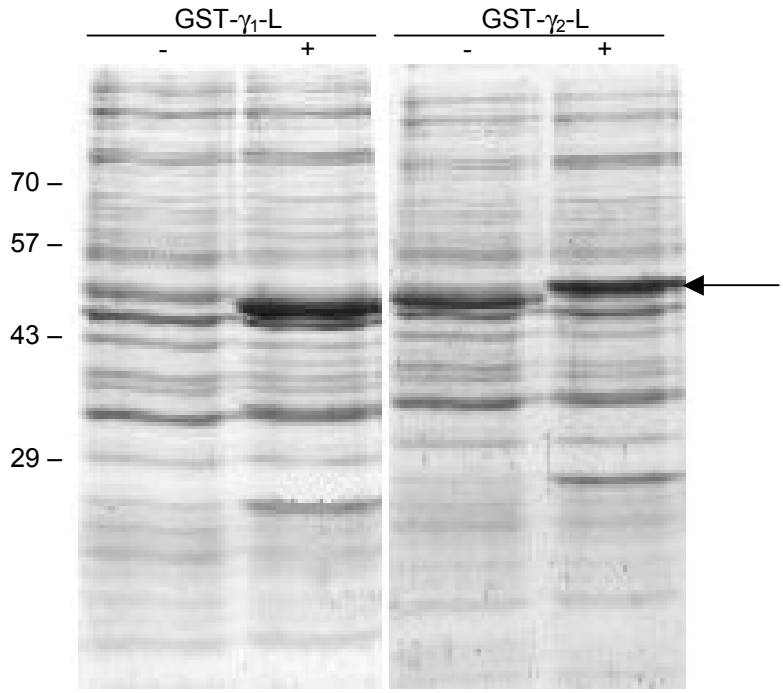


***Figure 8**: The expression of the GST-γ_1-L and the GST-γ_2-L fusion proteins. '-' = not induced with IPTG. '+' = induced with IPTG. Marker sizes are in kDa.*

3.2. Creation and purification of GST-Xlp24-lumenal part fusion proteins to use as antisera

3.2.1. The constructs for production of GST-Xlp24-lumenal part fusion proteins

Two different constructs were formed, which are listed in table 1, together with some additional information. The constructs were checked by sequencing.

***Table 1:** The created GST-Xlp24 lumenal part fusion proteins.*

Protein	Vector	Insert	Size	Comments
GST-γ_1-L	pGEX-2T BamHI/EcoRI	Lumenal part Xlp24γ_1 BamHI/ EcoRI (PCR, primer Xlp24E-s12/as13, temp. pcDNA3-Xlp24γ_1)	insert: 548 bp protein: 47.8 kDa	Cloned by Rötter, J. on 16-12-1999
GST-γ_2-L	pGEX-2T BamHI/EcoRI	Lumenal part Xlp24γ_2 BamHI/ EcoRI (PCR, primer Xlp24F-s14/as15, temp. pcDNA3-Xlp24γ_2)	insert: 497 bp protein: 45.9 kDa	Cloned by Rötter, J. on 16-12-1999

3.2.2. Isolation of insoluble fusion proteins

Figure 9 shows a SDS gel loaded with samples taken during the isolation of insoluble GST-γ_2-L fusion proteins. The final pellet indeed contained the fusion proteins (marked with an arrow), although it also contained some

contaminating proteins. The inclusion bodies were suspended in PBS and will be used as antigen for making antisera against Xlp24γ_1 and Xlp24γ_2.

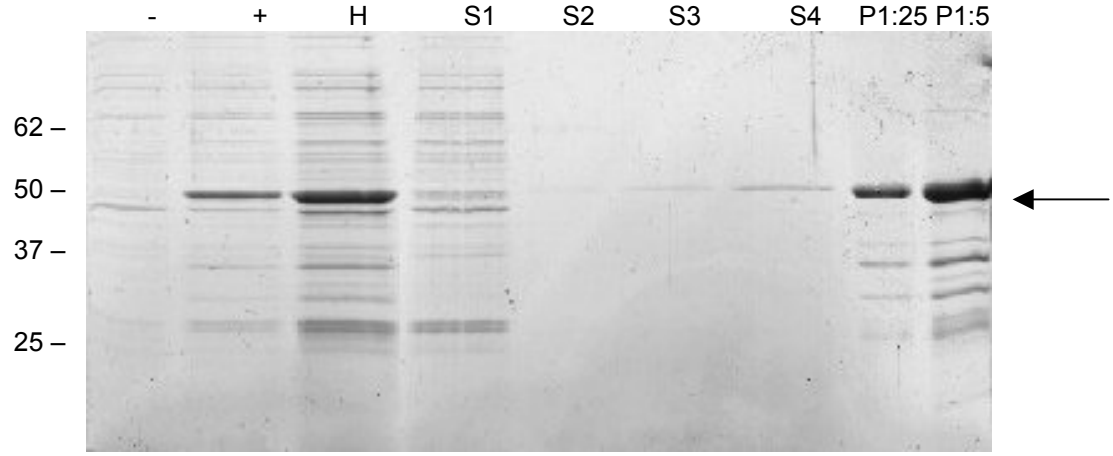


***Figure 9**: The isolation of insoluble GST-γ_2-L fusion proteins. '-' = not induced with IPTG. '+' = induced with IPTG. 'H' = homogenate. 'S#' = supernatant #. 'P' = pellet, diluted 1:5 and 1:25. Marker sizes are in kDa.*

3.3. Binding studies with GST-Xlp24-cytoplasmic tail fusion proteins

3.3.1. The constructs for production of GST-Xlp24-cytoplasmic tail fusion proteins

Six different constructs were formed, which are listed in table 2, together with some additional information. The constructs were checked by sequencing.

Table 2: *The constructs which produce GST-Xlp24 cytoplasmic tail fusion proteins (plus a negative control).*

Protein	Vector	Insert	Size	Comments
GST-β-C	pGEX-2T BamHI/EcoRI	C-tail Xlp24β BamHI/EcoRI (PCR, primer Xlp24A-s11/as12, temp. pGalAD4-Xlp24A3)	insert: 76 bp protein: 30.3 kDa	Cloned by Rötter, J. on 16-12-1999
GST-α-C	pGEX-2T BamHI/EcoRI	C-tail Xlp24α BamHI/EcoRI (PCR, primer Xlp24D-s8/as9, temp. pGalAD4-Xlp24D-C5)	insert: 68 bp protein: 30.0 kDa	Cloned by Rötter, J. on 16-12-1999
GST-γ_1-C	pGEX-2T BamHI/EcoRI	C-tail Xlp24γ_1 BamHI/EcoRI (PCR, primer Xlp24E-s14/as15, temp. pcDNA3-Xlp24γ_1)	insert: 46 bp protein: 29.2 kDa	Cloned on 12-05-2000
GST-γ_2-C	pGEX-2T BamHI/EcoRI	C-tail Xlp24γ_2 BamHI/EcoRI (PCR, primer Xlp24F-s16/as9, temp. pcDNA3-Xlp24γ_2)	insert: 46 bp protein: 29.2 kDa	Cloned on 12-05-2000
GST	pGEX-2T BamHI/EcoRI, Klenow fill-in, religation	-	insert: - protein: 27.5 kDa	Cloned on 12-05-2000
GST-γ_3-C	pGEX-2T BamHI/EcoRI	C-tail Xlp24γ_3 BamHI/EcoRI (PCR, primer Xlp24B-s13/as14, temp. pAD-Gal4-p24γ_3)	insert: 70 bp protein: 30.1 kDa	Cloned by Rötter, J. on 06-06-2000

3.3.2. Isolation of insoluble fusion proteins

Figure 10 shows a SDS gel loaded with samples taken during the isolation of insoluble GST-β-C fusion proteins. The final pellet indeed contained the fusion proteins (marked with an arrow). After the insoluble fusion proteins were purified, they were solubilized and dialyzed. After the solubilization and dialysis, the proteins can be used for the binding studies.

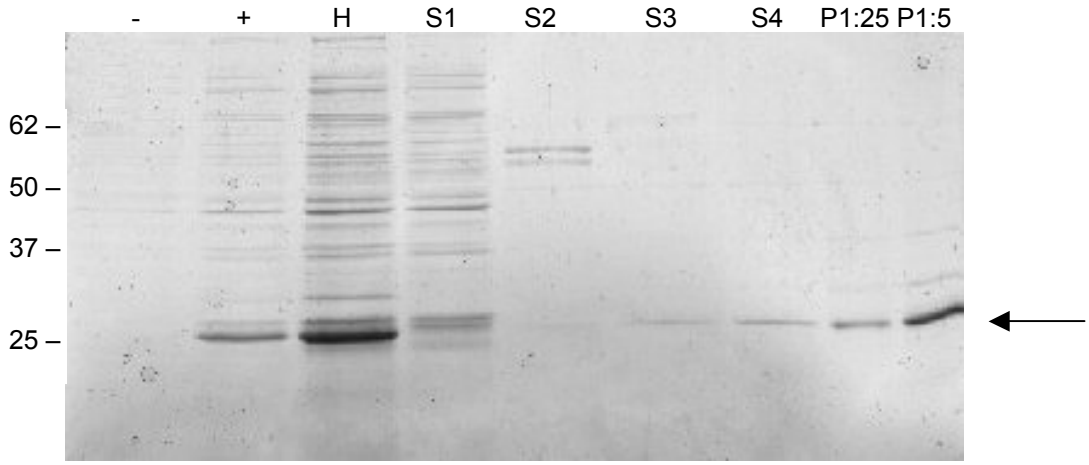
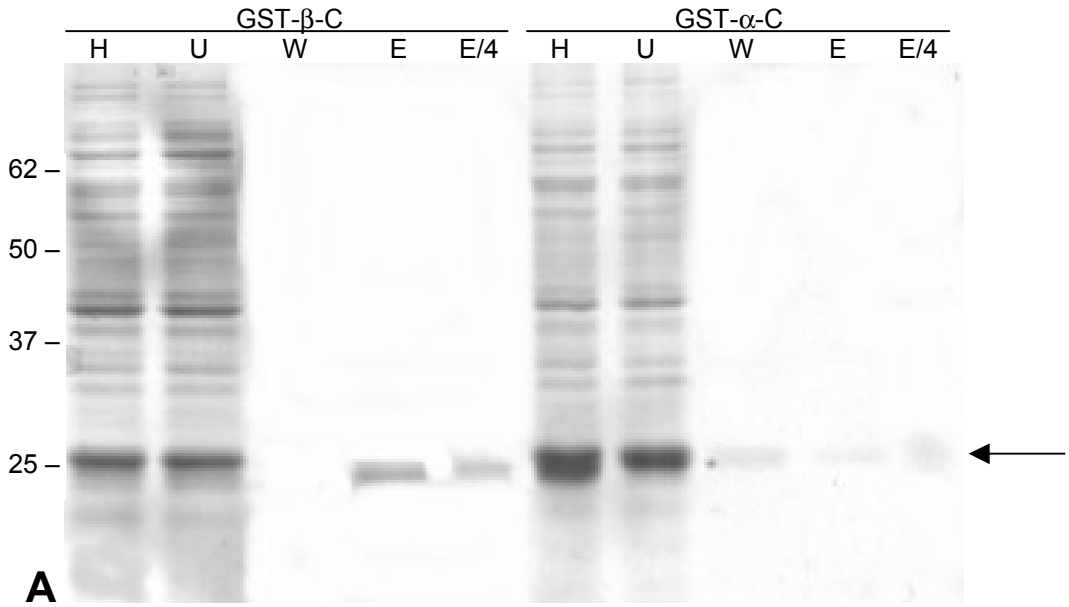
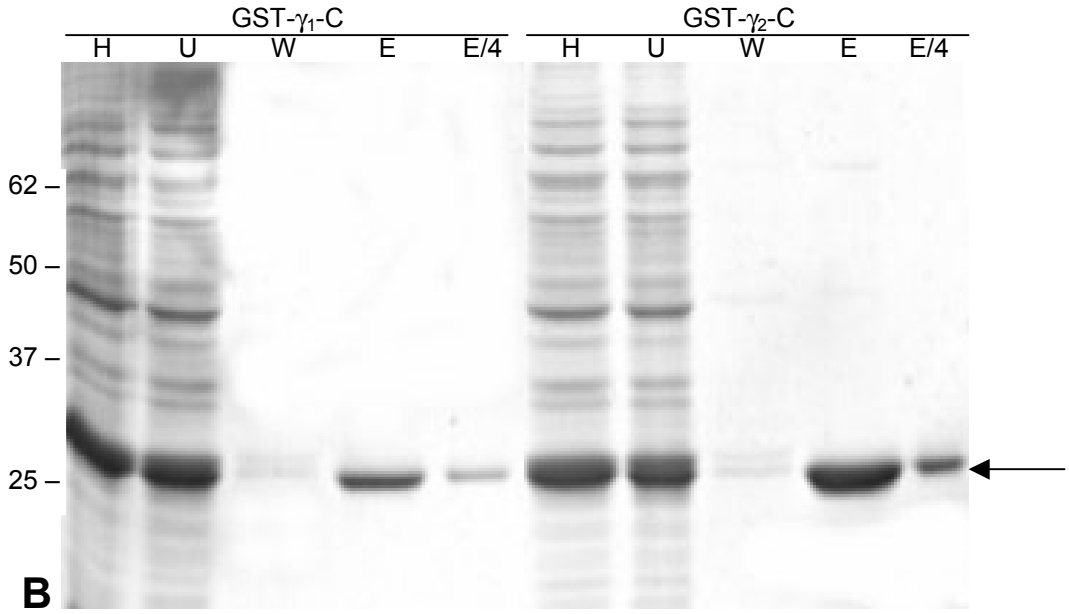


Figure 10*: The isolation of insoluble GST-β-C fusion proteins. '-' = not induced with IPTG. '+' = induced with IPTG. 'H' = homogenate. 'S#' = supernatant #. 'P' = pellet, diluted 1:5 and 1:25. Marker sizes are in kDa.*

3.3.3. Binding of GST-Xlp24-cytoplasmic tail fusion proteins to glutathione-Sepharose

The fusion proteins were bound to glutathione-Sepharose. The homogenate, the unbound material, the washing material and the eluate were loaded on a gel to check if the eluate indeed contained the fusion proteins (figure 11). This seems to be the case (marked with arrows), although the amount of fusion proteins in the eluate is a bit low for both GST-β-C as GST-α-C, because the fusion proteins were largely insoluble. Therefore, an isolation of insoluble fusion proteins was necessary.





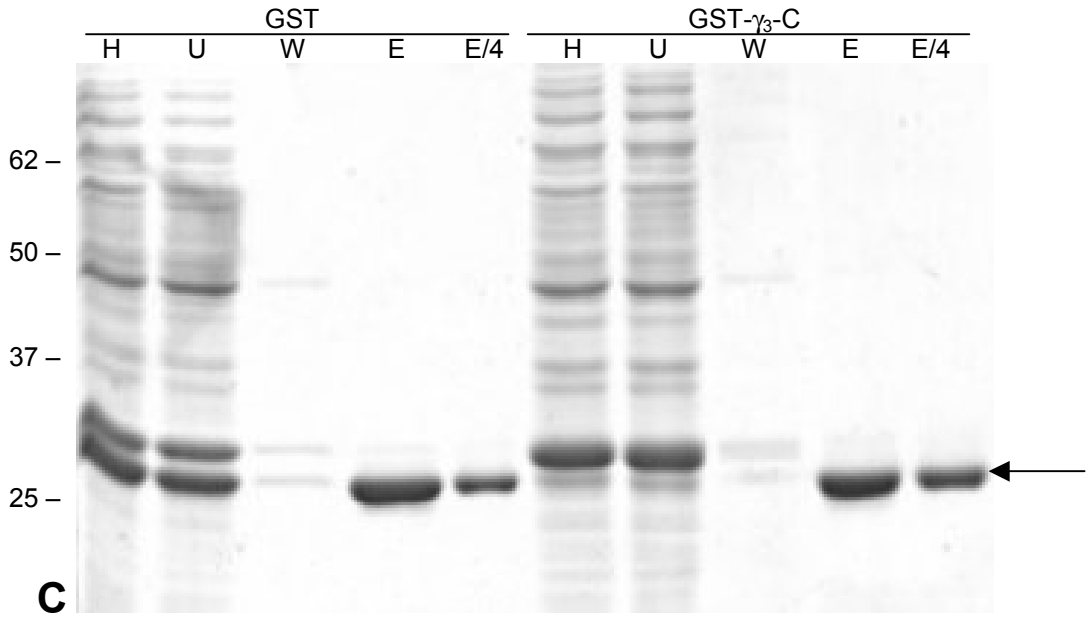


***Figure 11**: The binding of the GST-Xlp24 fusion proteins to glutathione-sepharose. **(A)** GST-β-C and GST-α-C. **(B)** GST-γ1-C and GST-γ2-C. **(C)** GST and GST-γ3-C. 'H' = homogenate. 'U' = unbound. 'W' = wash. 'E' = eluate. Marker sizes are in kDa.*

To determine the protein concentrations, the different eluates were loaded on a SDS-PAGE gel and stained with Coomassie (figure 12). The protein gels were scanned in and the protein bands were compared to a 29 kDa marker protein with a known protein concentration (table 3). Because of the low amounts of bounded fusion proteins for the GST-β-C and the GST-α-C, these eluates were not loaded here.

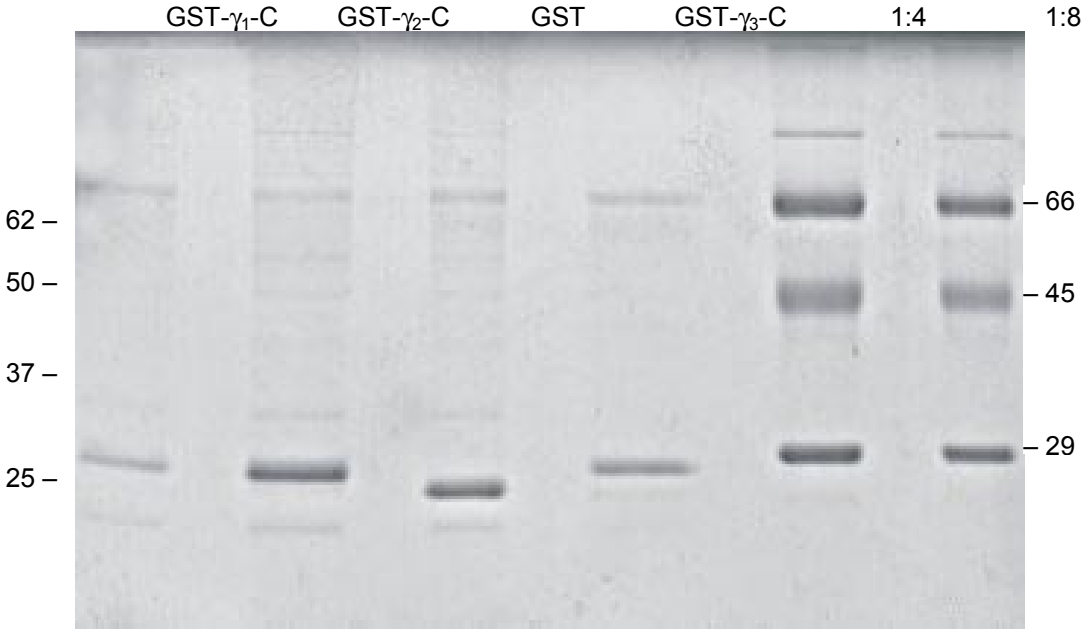


***Figure 12**: Eluted GST-Xlp24 fusion proteins loaded on a gel to determine the concentration. Marker sizes are in kDa.*

Table 3: *The concentrations of the bound fusion proteins, measured by the intensity of the bands on a SDS-PAGE gel.*

Protein	MW 1:8	GST-γ_1-C	GST-γ_2-C	GST	GST-γ_3-C
rel. value	18.05	3.25	22.87	18.47	11.08
conc(µg/ml)	62.5	8.11	29.59	30.17	26.42

3.3.4. Binding of GST-Xlp24-cytoplasmic tail fusion proteins to COP subunits

Equal amounts of the bound fusion proteins were bound to cytosol. The samples were loaded on a gel and Western blotted. Figure 13 shows a film made of these blots, which were incubated in COP antibodies, according to the Super Signal West Pico detection protocol. The blots were cut horizontally at the 50 kDa marker band. The upper halfs were incubated in human α/γCOP antibodies (expected size of αCOP+γCOP subunit: 90 kDa), while the lower halfs were incubated in human εCOP antibodies (expected size εCOP subunit: 36 kDa). No binding of α/γCOP to GST-γ_2-C and GST-γ_3-C was observed. A small fraction of α/γCOP from the cytosol was binding to GST-γ_1-C (90 kDa band), but binding is most likely unspecific since a similar extent of binding was observed for GST alone. The antibody directed against the human εCOP protein was unable to detect its ortholog in the monkey kidney cell line COS. It was therefore not useful to study εCOP binding (see cytosol fraction). However, the eCOP antibody was weakly cross-reacting with a protein band of approximately 48 kDa in size. This protein was clearly enriched in the bound fraction of GST-γ_1-C and to a lower extent of in GST-γ_2-C. No binding of this protein was observed for GST-γ_3-C and GST alone. Although it is not clear what kind of protein the 48 kDa band could be, it seems that this protein specifically interacts with GST-γ_1-C and GST-γ_2-C.

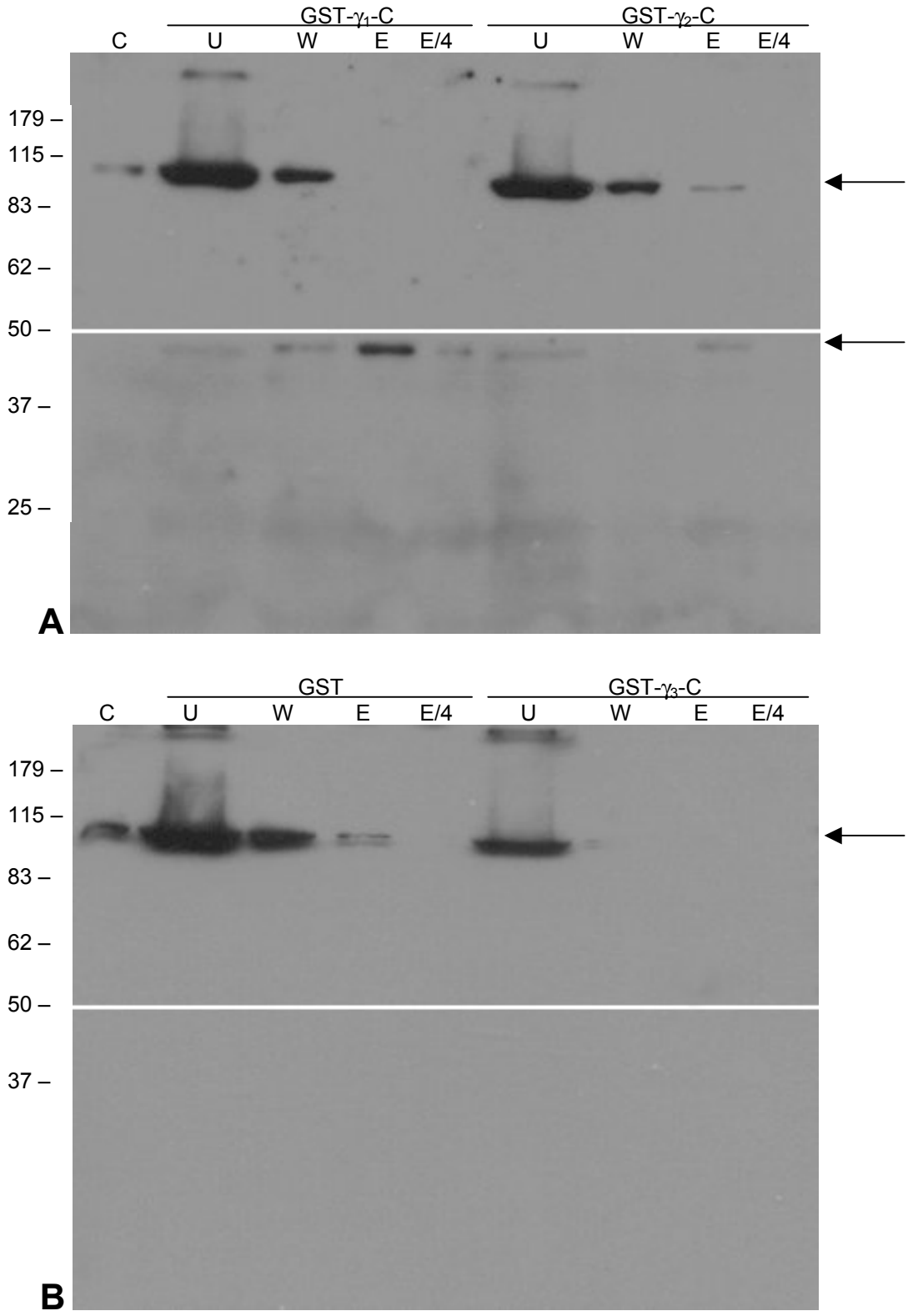


***Figure 13**: Binding of GST-Xlp24 fusion proteins to α/γCOP and εCOP subunits, detected with antibodies. **(A)** GST-γ1-C and GST-γ2-C. **(B)** GST and GST-γ3-C. The blots were cut at the 50 kDa border. The upper half was incubated in α/γCOP antibodies, while the lower half was incubated in εCOP antibodies. 'C' = cytosol. 'U' = unbound. 'W' = wash. 'E' = eluate. Marker sizes are in kDa.*

4. DISCUSSION

4.1. Using GST-p24 fusion proteins as antigens and for COP binding studies

To study the binding of p24 proteins to COP subunits, fusion proteins were made of p24 cytoplasmic tails and the protein GST. A possible consequence of this fusion is that the COP-C-tail binding is affected, since the three-dimensional structure of the fusion protein is a lot different from the 3D structure of a p24 protein. The 50 kDa band in the bound fraction of the GST-γ_1-C and GST-γ_2-C (εCOP detection, figure 14) shows that the method of using GST-p24 fusion proteins to check the binding of COP subunits to p24 C-tails, principally works. This was also demonstrated by Wu et al. (2000). In their research on the Ras-related GTP-binding protein Cdc42, they used GST-Cdc42 fusion proteins and COP antibodies to study the binding of Cdc42 to αCOP and γCOP. Of course, there is also the possibility to just use the actual p24 proteins and let them bind to the COP subunits. The advantage of the beads method however, is that it is very easy to separate the bound material from the unbound material; spinning is enough to leave the beads with the bound material in the pellet and the unbound material in the supernatant. Moreover, the fusion protein method has the advantage already mentioned in the introduction: it makes possible easy purification of the p24 (fusion) proteins. These advantages make this method also useful for the creation of antibodies. The only difficulty with the GST-p24 fusion protein method is the (partial) insolubility of the proteins, which makes it necessary to isolate these proteins as inclsuion bodies, solubilize and renature them before they can be bound to Sepharose beads. For further research, maybe a different type of beads should be used in stead of glutathione-Sepharose, in combination with a different type of vector, in stead of pGEX-2T.

4.2. Binding of p24 proteins to COP subunits

One of the interactions that was studied here, is the binding of a p24 C-tail to either αCOP subunits or γCOP subunits. The human α/γCOP antibody that was used in this experiment did detect some subunits bound to the cytoplasmic tail of the Xlp24γ_1, but since a similar extent binding was observed for the GST alone, this binding is most likely unspecific. The observation that there is no specific binding of the Xlp24γ proteins to either αCOP or γCOP subunits, contradicts with the fact that both Xlp24γ_1 and Xlp24γ_2 have the K(X)KXX motif that is necessary for the binding to these subunits (see paragraph 1.2). This could be due to the use of human antibodies in combination with monkey cytosol.

The binding of αCOP and γCOP subunits to p24 proteins has been demonstrated in several studies. For example, Letourneur et al. (1994) showed that mutations in αCOP, β'COP and γCOP result in defective retrograde transport of membrane proteins displaying the K(X)KXX signal, and it has also

been shown that a partial coatomer complex containing αCOP, β'COP and εCOP interacts with KKXX-containing proteins in vitro (Cosson and Letourneur, 1994, Lowe and Kreis, 1995). The binding of other p24 proteins to αCOP and γCOP could be investigated in another experiment, using the same method, but with other GST-p24 fusion proteins.

The other interaction that was studied was the binding of a p24 C-tail to εCOP subunits. The εCOP subunit, with a size of 37 kDa, did not bind, but there was a band at 50 kDa. This band is probably the result of a εCOP-like subunit, because it is detected by the human εCOP antibodies. This 50 kDa band was only enriched when the cytoplasmic tails of the Xlp24γ_1 and the Xlp24γ_2 were used. These p24 protein family members should have a motif in its C-tail that the Xlp24γ_3 does not have, and which makes possible binding to the εCOP-like subunit. It is interesting to know what kind of protein it is and isolation by binding to GST-γ_1-C and GST-γ_2-C should be possible. The interacting proteins could be seperated on a SDS-PAGE gel, with reversible protein stain. The 50 kDa band could be detected within the gel, cut out and this material could be used for protein sequencing.

4.3. Conclusions and suggestions for further research

In this experiment, only human α/γCOP and εCOP antibodies were available which had difficulties to detect their counterpart in monkey kidney cytosol. This problem could be solved either by using better antibodies or by using cytosol isolated form a human cell line. For a complete overview of the binding of p24 proteins to COP subunits, one should also use other cytoplasmic tails from p24 proteins from other subfamilies, and antibodies for the other COP subunits (like βCOP, β'COP, δCOP and ζCOP) could be used. The 50 kDa protein that was detected by the human εCOP antibodies could be isolated and studied (as described in paragraph 4.2.). Finally, maybe a different type of beads and vector should be used in stead of glutathione-Sepharose and pGEX-2T to produce more soluble fusion proteins.

5. APPENDIX

5.1. Primers

Xlp24E-s14	5’-CGCGGATCCTATATGCTGAAAAGCCTCTTC-3’
Xlp24E-as15	5’-CCGGAATTCAAGCTTAAGTCCGGATCTTGCGCTTG-3’
Xlp24F-s16	5’-CGCGGATCCTATATGCTAAGAAGTCTTTTTGA-3’
Xlp24F-as9	5’-CCGGAATTCAGAGCCTACTTTTCCTTTTG-3’
5’ pGEM-T	5’-GTAATACGACTCACTATAGGGC-3’

5.2. Media

LB medium:

10 g/l NaCl
10 g/l bacto-tryptone
5 g/l bacto-yeast extract
+ deionized H_2O

LB-amp. medium:

LB medium
+ 50 mg/l ampicillin

SOB medium:

20 g/l bacto-tryptone
5 g/l bacto-yeast extract
0.5 g/l NaCl
2.5 mM KCl
10 mM $MgCl_2$
+ deionized H_2O

SOC medium:

SOB medium
+ 20 mM glucose

YT medium:

8 g caseïne (pepton)
5 g yeast extract
2.5 g NaCl
+ deionized H_2O

5.3. Solutions

PBS:

8 g/l NaCl
0.2 g/l KCl
0.24 g/l KH_2PO_4
1.8048 g/l $Na_2HPO_4 \cdot 2H_2O$
+ deionized H_2O

PBST:

PBS
+ 0.05% Tween-20

TE buffer:

10 mM Tris-HCl (pH 8.0)
1 mM EDTA

PCR master mix 1:

2 µl	10x PCR buffer (+ 2 mM $MgSO_4$)
0.8 µl	dNTP mix (10 mM)
1.2 µl	sense primer (10 mM)
1.2 µl	antisense primer (10 mM)
9.8 µl	H_2O
(15 µl total)	

PCR master mix 2:

2 µl	10x PCR buffer (+ 2 mM $MgSO_4$)
17.8 µl	H_2O
0.2 µl	Pwo-polymerase (5 u/µl)
(20 µl total)	

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