

## 2. Results

### 2.1. Generation and testing of polyclonal antibodies against L-selectin

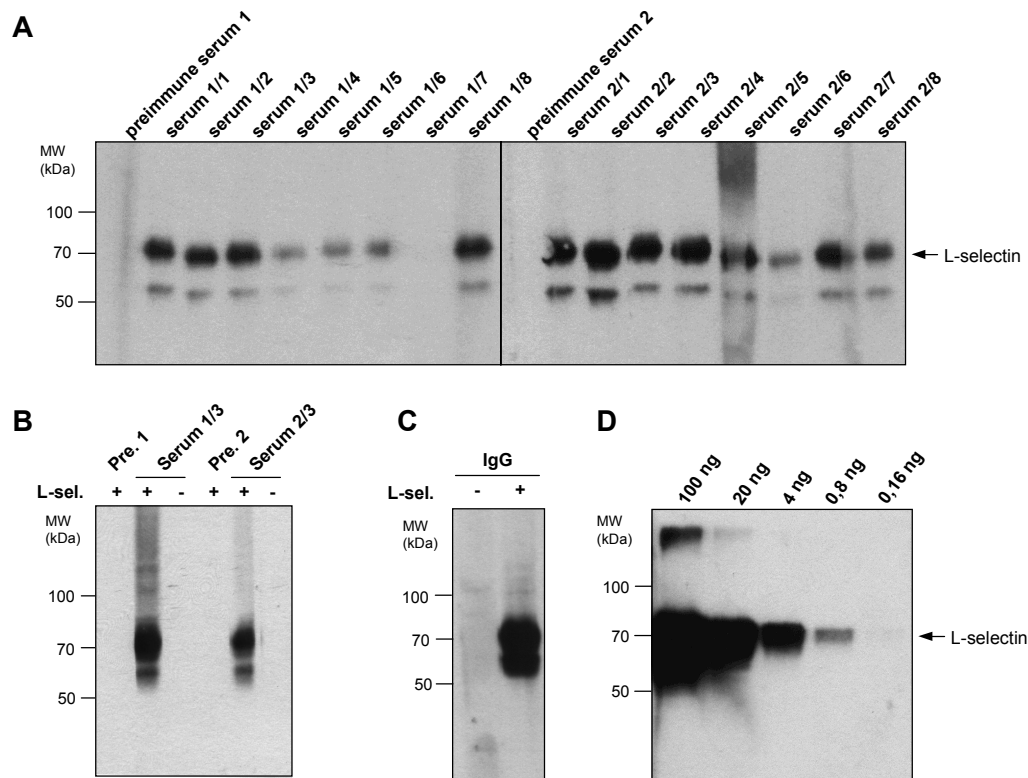
For biochemical studies of protein-protein interaction the availability of appropriate antibodies is an important prerequisite. Although a large selection of monoclonal antibodies directed against different domains of the extracellular part of human L-selectin are available commercially, they were not suitable for all the requirements of the planned investigations. For the detection of protein constructs only containing the cytoplasmic domain of L-selectin, antibodies against this part of the molecule were essential. Due to the fact that no suitable immunoglobulins directed against this L-selectin domain can be obtained commercially, polyclonal antisera were produced in rabbits. Antisera were also produced against the extracellular domain of the receptor, since for some applications polyclonal sera, which bind to epitopes along the entire length of the molecule, are advantageous compared to monoclonal immunoglobulins which only recognise one distinct epitope.

#### 2.1.1 Antibody against the L-selectin ectodomain

Polyclonal L-selectin antisera against the extracellular domain were generated by immunisation of rabbits with purified recombinant soluble L-selectin (sL-selectin). The protein was overexpressed in BHK cells (Fieger, 1997) and corresponds to the fragment generated during the physiological shedding process, which comprises most of the extracellular domain. Sera taken at different time points during the immunisation process (Tab. 3) were tested for reactivity against and specificity for L-selectin in Western blot analysis.

**Tab. 3: Immunisation protocol for generation of polyclonal antibody against sL-selectin**

time point	immunisation/ serum obtained
week 1	1 <sup>st</sup> immunisation with 75 µg sL-selectin with adjuvant preimmune serum
week 6	2 <sup>nd</sup> immunisation with 75 µg sL-selectin with adjuvant
week 8	serum 1
week 12	serum 2
week 15	3 <sup>rd</sup> immunisation with 75 µg sL-selectin with adjuvant
week 18	serum 3
week 22	serum 4
week 26	4 <sup>th</sup> immunisation with 100 µg sL-selectin with adjuvant
week 30	serum 5
week 34	serum 6
week 42	serum 7
week 44	5 <sup>th</sup> immunisation with 100 µg sL-selectin with adjuvant
week 48	serum 8



**Fig. 6: Evaluation of polyclonal sera and purified IgG fraction against sL-selectin**

Polyclonal sera and purified IgG fractions from rabbits immunised with sL-selectin were tested for specificity and sensitivity by immunoblotting against whole cell lysates and purified sL-selectin. (A) Sera from immunised rabbits obtained at different time points (see Tab. 3) were tested for reactivity with L-selectin expressed by KG1a cells. All sera were used in 1:1000 dilutions. (B) Specificity of selected sera (1:1000) was analysed using whole cell lysates from wild type K562 cells (-) and K562 cells transfected with L-selectin cDNA (+). (C) Isolated IgG (1:5000) from pooled sera 2/2 and 2/3 was tested on whole cell lysates of K562 cells expressing or not expressing L-selectin. (D) Sensitivity of isolated IgG was determined using serial dilution of purified sL-selectin.

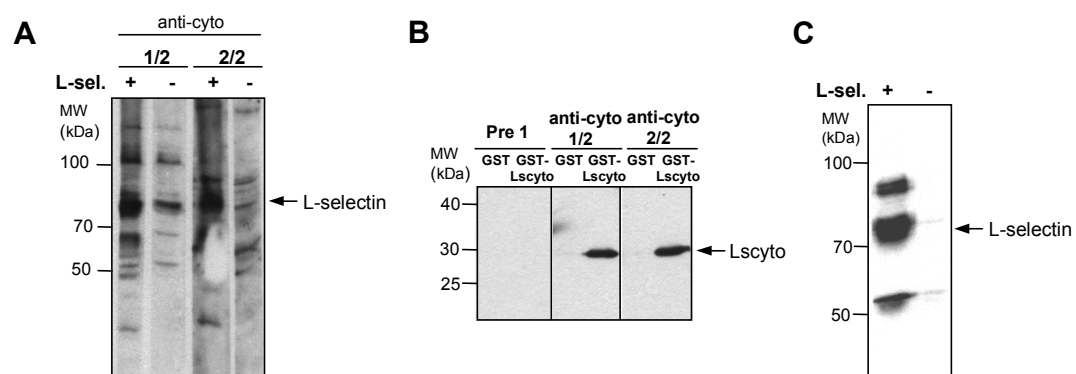
Immunised rabbits produced immunoglobulins reactive with a protein of approximately 70 kDa in Western blots from lysates of KG1a cells separated by SDS-PAGE under non-reducing conditions which was not observed with preimmune serum. This corresponded well with the expected size of L-selectin (Fig. 6A). The signal was specific for L-selectin, since it was detected in lysates from K562 cells transfected with pCR 3.1 LS, an eukaryotic expression vector encoding full length L-selectin, but not in wild type K562 cells (Fig. 6B).

To remove non-immunoglobulin serum proteins and reduce background occasionally observed on Western blots, immunoglobulin G (IgG) was isolated from the pooled sera 2/2 and 2/3 by protein A affinity chromatography. The purified IgG fraction reacted specifically with L-selectin (Fig. 6C) and detected at least 800 pg of recombinant sL-selectin in Western blots using a chemoluminescence based detection system (Fig. 6D).

The additional band detected at approximately 55 kDa is specific for L-selectin and is also observed with several monoclonal L-selectin antibodies. It is most likely due to partially glycosylated forms of L-selectin generated during glycan processing.

### 2.1.2 Antibody against the L-Selectin cytoplasmic domain

Antisera against a peptide comprising the C-terminal 10 amino acids of the cytoplasmic domain of L-selectin coupled to KLH were raised in rabbits. The complete antisera (Fig. 7A) and also a IgG fraction purified by protein A affinity chromatography (not shown) displayed strong background staining and only weak specific signals for L-selectin in Western blotting experiments using whole cell lysates. The sera reacted with a chimeric protein composed of the L-selectin cytoplasmic domain fused to glutathion S-transferase in Western blots (Fig. 6B), showing that it contained antibodies specific for this part of the L-selectin molecule. This Lscyto-specific IgG-fraction was affinity isolated using a Sepharose matrix coupled with the peptide used for immunisation.



**Fig. 7: Evaluation of polyclonal antibody against the L-selectin cytoplasmic domain**

(A) Whole cell lysates from wild type K562 cells (-) and K562 cells transfected with L-selectin cDNA (+) separated under reducing conditions and (B) purified GST and GST-Lscyto fusion protein (0.01µg/lane) were incubated with sera raised against the cytoplasmic domain of L-selectin (1:1000). (C) Whole cell lysates from wild type K562 cells (-) and K562 cells transfected with L-selectin cDNA (+) separated under reducing conditions were probed with affinity purified antibody against the cytoplasmic domain of L-selectin (1:1000).

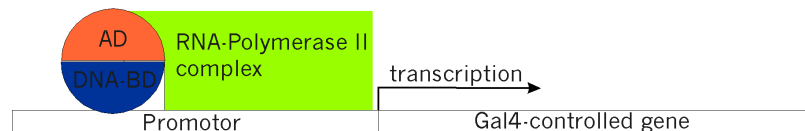
This purification step resulted in a significant reduction of unspecific binding to proteins in Western blots of whole cell lysates. The most prominent band of 80 kDa detected in lysates from L-selectin expressing cells corresponded to the size of full-length L-selectin and was not found in lysates from cells not expressing L-selectin (Fig. 6C). Additional to the 55 kDa band usually observed in L-selectin Western blots, a 90 kDa band of unknown origin also appeared in lysates from cells that express L-selectin.

This antibody directed against the cytoplasmic domain of L-selectin, in contrast to the L-selectin antibodies recognising the ectodomain, also reacts with the protein in a reduced state. Reduced L-selectin displays an apparent molecular weight of about 80 kDa, while unreduced L-selectin co-migrates with the 70 kDa marker band (compare Fig. 6).

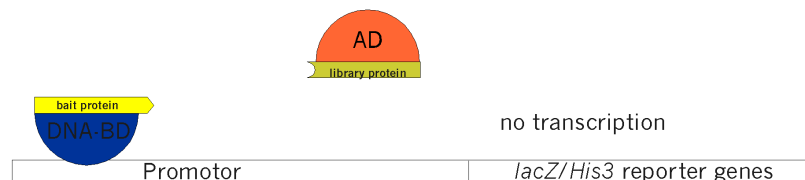
## 2.2. Yeast 2-hybrid screen

The central focus of this investigation was on the identification of novel interaction partners for the cytoplasmic domain of L-selectin. In addition to the ‘classical’ approach of affinity purification and identification by biochemical methods, several techniques have been developed during the last years which allow direct access to the DNA encoding a binding partner isolated from an expression library. This facilitates further studies once the interacting proteins are identified, e.g. for the generation of recombinant protein variants or mutation/deletion studies of interacting domains. Therefore, the yeast 2-hybrid system was chosen to attempt isolation of L-selectin interacting proteins.

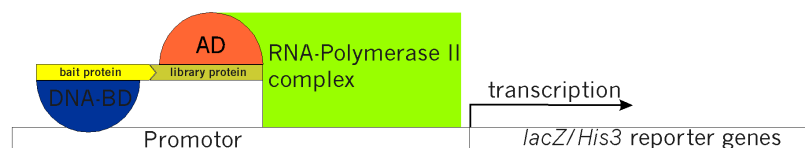
Gal4-dependent transcription in normal yeast cells



Gal4-dependent transcription in the yeast 2-hybrid system  
non-interacting proteins



interacting proteins



**Fig. 8: The yeast 2-hybrid system.**

For transcription of Gal4-controlled genes in yeast, the Gal4 transcriptional activator recruits the RNA-polymerase complex to promoters containing Gal4-binding sites. In the yeast 2-hybrid system, transcription of reporter genes containing Gal4 binding sites is initiated by reconstitution of a functional transcription factor by interaction of the proteins fused to the DNA-binding domain and the activation domain of Gal4.

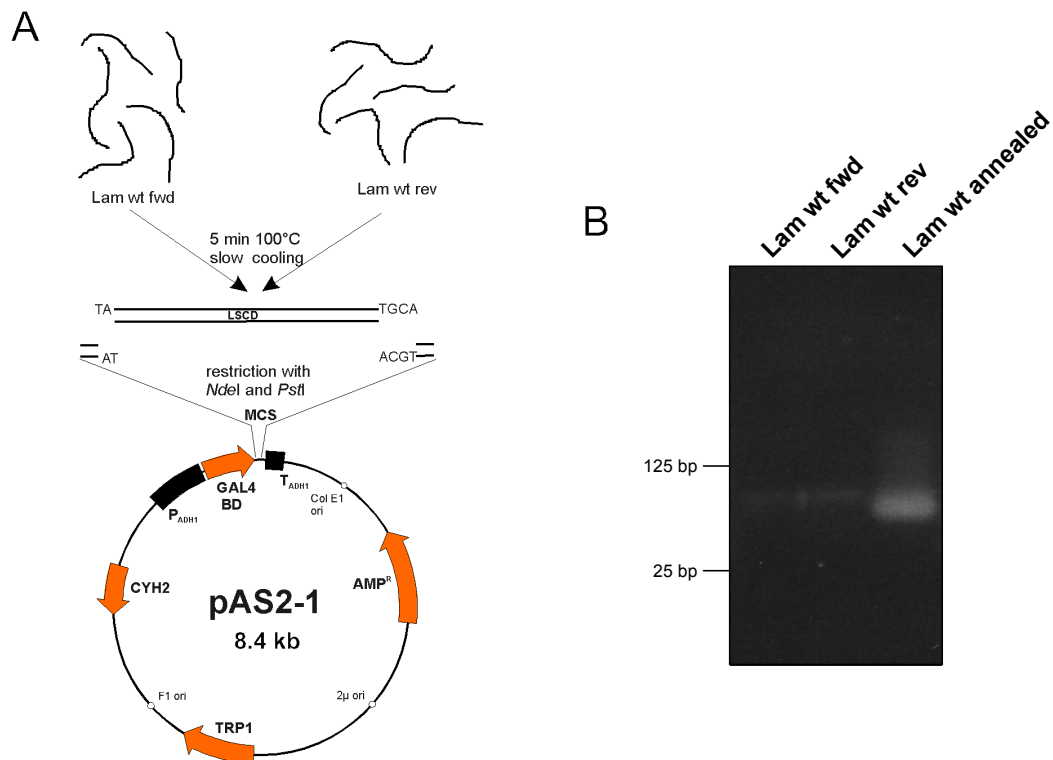
The yeast 2-hybrid assay is a powerful tool for the study of protein-protein interactions *in vivo* (Fields and Song, 1989; Chien *et al.*, 1991). All derivatives of this technology exploit the fact that transcriptional activators have a modular architecture (Fig. 8). Two physically distinct functional domains are necessary: a DNA binding domain (BD), which binds to a specific promoter sequence, and an



activation domain (AD), which directs the RNA polymerase II complex to transcribe downstream genes. The two domains need not to be covalently attached, but function as transcriptional activators when brought into close proximity. For this knowledge, a fusion protein consisting of the DNA-binding domain and the target protein (the “bait”) is used to screen a cDNA library fused to the activation domain. In yeast cells where interaction between the co-expressed bait protein and a library encoded target protein occurs, reporter genes under the control of a promoter with binding sites for the reconstituted transcription factor are activated (Fig. 8). In the Gal4-based Matchmaker 2-Hybrid system 2 (Clontech, Heidelberg), which was employed for this screen, interacting clones express the HIS3 gene, which allows positive metabolic selection, and  $\beta$ -galactosidase, which can be detected by blue staining when tested with X-Gal.

For the isolation of potential interaction partners of the cytoplasmic domain of L-selectin, cDNA fusion constructs encoding the L-selectin cytoplasmic part together with the yeast transcription factor Gal-4 DNA binding domain were generated and used to screen a human leukocyte cDNA library fused to the Gal4 activation domain.

### 2.2.1 Cloning of Yeast 2-Hybrid bait vector



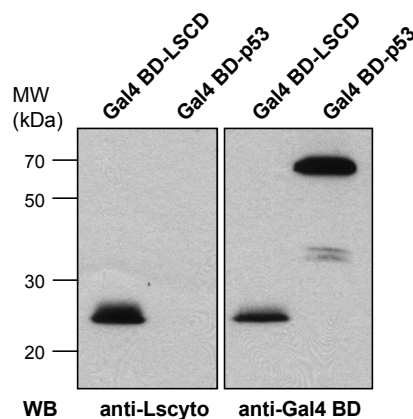
**Fig. 9: Cloning of pAS2-1/LSCD.**

(A) Schematic representation of pAS2-1/LSCD cloning. After hybridisation of the oligonucleotides Lam wt fwd and Lam wt rev the resulting fragment was ligated into pAS2-1 digested with endonucleases *NdeI* and *PstI*. Correct sequence was confirmed by sequencing of positive clones. (B) Hybridisation of inserted oligonucleotides. Single stranded DNA fragments migrate differently from double stranded fragments and show weaker staining with ethidium bromide due to the low amount of double stranded conformation that allows intercalation of the stain.

Since the cytoplasmic domain of L-selectin is very short (17 aa), complementary oligonucleotides comprising the complete coding sequence were annealed and cloned into *NdeI* and *PstI* restricted pAS2-1 using single stranded overhangs included in the oligonucleotides (Fig. 9)

### 2.2.2 Test for expression of bait protein

The pAS2-1/LSCD bait vector was introduced into yeast strain Y190 by small scale lithium acetate transformation. Since the plasmid pAS2-1 carries the TRP1 marker, positive colonies were selected on minimal (SD) medium lacking thryptophan. These colonies were analysed for expression of Gal4 BD-LSCD fusion protein. Whole cell extracts were separated by SDS-PAGE and analysed by Western blotting with antibodies directed against Gal4-BD and the cytoplasmic part of L-selectin (Fig. 10).



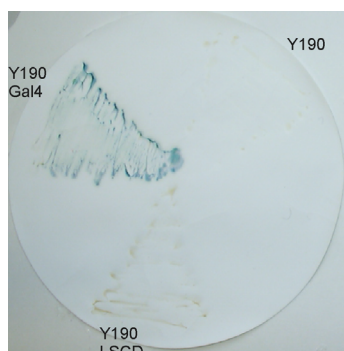
**Fig. 10: Gal4 BD-LSCD is expressed in Y190**

Yeast strain Y190 was transformed with pAS2-1/LSCD or the control vector pVA3-1 coding for GAL4 BD/p53. Whole cell lysates were incubated with antibody either specific for the cytoplasmic domain of L-selectin or the GAL4 BD.

Expression of the Gal4 BD-LSCD fusion protein in yeast cells was detected with antibodies both against Lscyto and Gal4 BD. The amount of expressed protein was significantly lower for Gal4 BD-LSCD compared to Gal4 BD-p53 protein, which was used as a control.

### 2.2.3 Test for autonomous activation of reporter genes

Some target proteins are not suitable for 2-hybrid screening since they activate transcription of Gal4-driven reporter genes without need for an interaction partner. To exclude this in the case of LSCD, Y190 expressing the Gal4 BD-LSCD fusion protein were tested for  $\beta$ -galactosidase activity (Fig. 11).



**Fig. 11: GAL4-LSCD does not autonomously activate the *lacZ* reporter gene.**

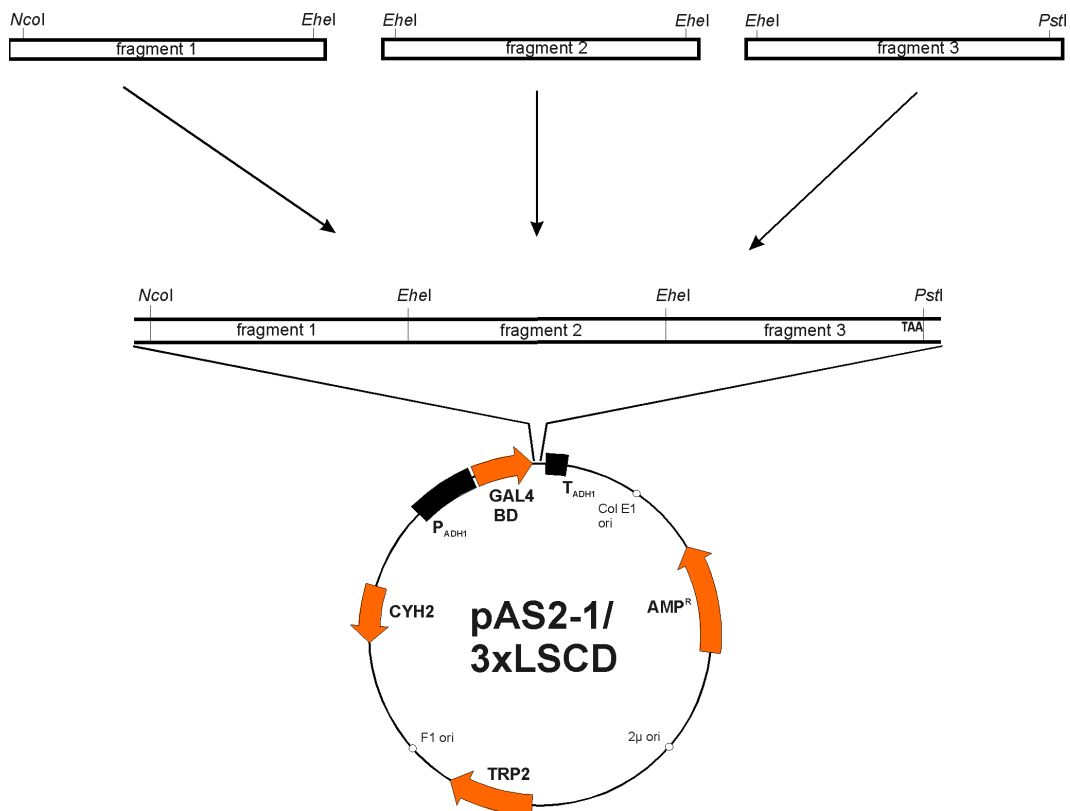
Yeast strain Y190 was transformed with pAS2-1/LSCD or pCL1 and tested for *lacZ* expression in a filter lift  $\beta$ -galactosidase assay together with untransformed cells.

Whereas colonies transformed with the control vector pCL-1, which encodes a full length Gal4 protein, developed a strong blue staining within the first hour of incubation, Y190 colonies expressing Gal4-BD/LSCD as well as untransformed Y190 remained white even after over night incubation.

#### 2.2.4 Screening with pAS2-1/LSCD

pAS2-1 Lscyto and human leukocyte cDNA library plasmids were simultaneously co-transformed into yeast reporter strain Y190. According to control plating about  $1.1 \times 10^8$  colonies were screened, corresponding to eight times the number of independent clones present in the library. Small colonies growing on SD/-HIS plates were only obtained after unusually prolonged incubation periods (two weeks). All of these colonies failed to produce a positive result when tested for  $\beta$ -galactosidase expression.

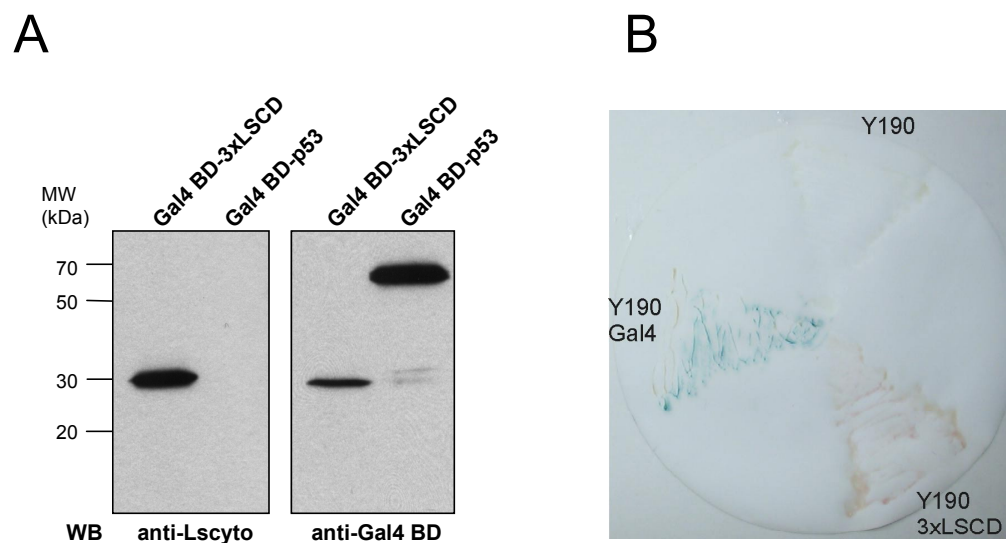
#### 2.2.5 Construction of triple bait vector



**Fig. 12: Cloning of pAS2-1/3xLSCD.**

Three fragments containing the cytoplasmic domain of L-selectin and part of the peptide linker were generated by PCR. Cloning was performed in three steps: first fragment 1 was ligated into pAS2-1 using the *NcoI* and *SmaI* (blunt end) restriction sites, then fragment 3 was added using *EheI* and *PstI* and last fragment 2 was cloned into the central *EheI* site. Fragments 1 and 2 do not contain the internal LSCD stop codon, which is included in fragment 3. Correct sequence was confirmed by sequencing.

A reason for the negative result in the library screen with pAS2-1/LSCD could be the size ratio between the small cytoplasmic domain and the much larger Gal4 BD fusion partner, possibly resulting in a steric unavailability of the L-selectin part for interacting proteins. To provide better access for interaction partners, a bait construct with three consecutive copies of the L-selectin cytoplasmic tail linked by neutral tri-peptides was made. Three fragments, each containing one copy of L-selectin CD flanked by suitable restriction sites, were generated by PCR and ligated into pAS2-1 in a stepwise manner as shown in Fig. 12. This bait vector was tested for expression and autonomous activation as described for pAS2-1/LSCD (Fig. 13).



**Fig. 13: Gal4 BD 3x LSCD is expressed in Y190 and does not activate the *lacZ* reporter gene.** (A) Whole cell lysates from yeast strain Y190 transformed with pAS2-1/3xLSCD or the control vector pVA3-1 coding for Gal4 BD-p53 were incubated with antibody directed against the cytoplasmic domain of L-selectin or the GAL4 BD. (B) Y190 transformed with pAS2-1/3xLSCD or pCL1 was tested for *lacZ* expression in a filter lift  $\beta$ -galactosidase assay together with untransformed cells.

### 2.2.6 Screening with pAS2-1/3xLSCD

pAS2-1/3xLSCD and the human leukocyte cDNA library were sequentially transformed into the yeast strain Y190. Transformation efficiencies for both plasmids were calculated by plating small aliquots on media lacking thryptophan, leucine or both amino acids. Remaining transformants were spread on plates lacking thryptophan, leucine and histidine to select for interacting proteins. After one week of growth, colonies were transferred to fresh plates and tested for  $\beta$ -galactosidase activity. Clones showing blue staining after 2 hours were subjected to three rounds of restreaking to allow segregation of multiple plasmids present in one cell. A number of clones lost expression of reporter genes during that process (Tab. 4). All colonies stably expressing  $\beta$ -galactosidase were analysed for presence of library plasmid.

Tab. 4: Positive clones obtained in the 2-hybrid screen.

clones screened	His <sup>+</sup> clones	clones initially expressing $\beta$ -galactosidase	clones stably expressing $\beta$ -galactosidase
$4 \times 10^7$	190	58	20

### 2.2.7 Analysis of positive clones

To test for the multiple presence of the same insert in different clones, PCR and restriction analysis of the library plasmids was performed. PCR with primers pACT fwd and pACT rev, binding to regions in the library vector flanking the multiple cloning site, yielded products with five different sizes between 0,15 and 1.6 kb. These PCR products were digested with the restriction enzyme *AluI* which has a four base recognition sequence, resulting in frequent cutting sites. Restriction patterns confirmed the presence of five different inserts in the positive clones (Fig. 14 and Tab. 5)

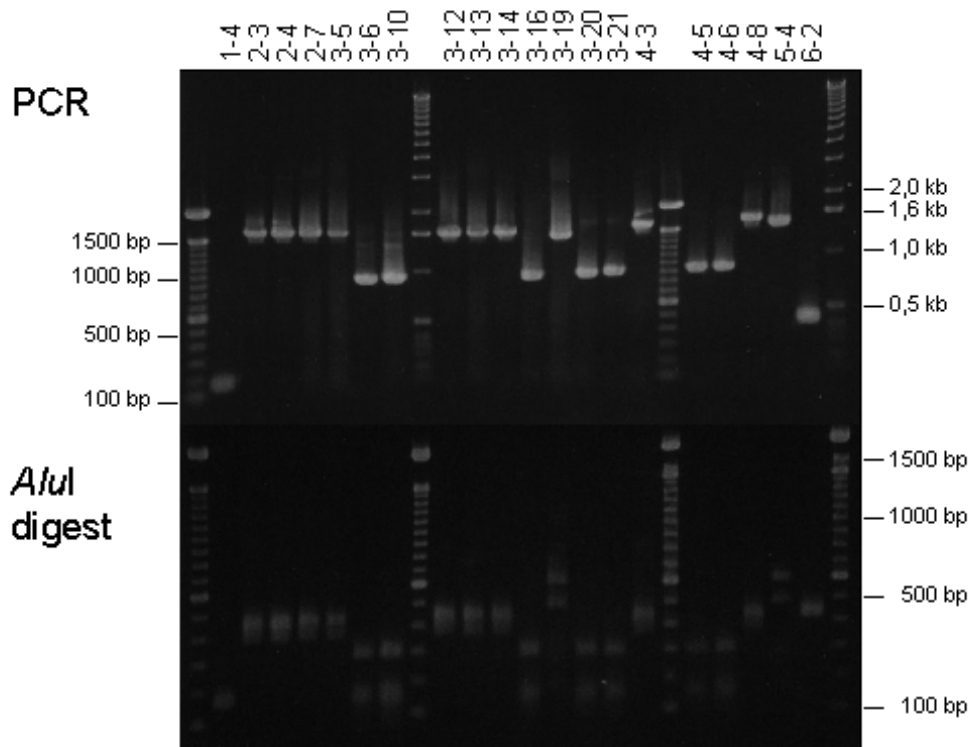


Fig. 14: Analysis of 2-hybrid clones.

DNA was isolated from yeast clones positive in both His- and  $\beta$ -galactosidase assay. Primers binding to the vector pGAD10 close to the multiple cloning site was used to determine size of inserts by PCR (upper panel). Resulting fragments were digested with *AluI* to distinguish between inserts with similar sizes (lower panel).

**Tab. 5: Results of DNA analysis of positive 2-hybrid clones.**

Fragment sizes of PCR-products and fragments from AluI-digests of these PCR fragments are given. Some AluI bands may contain more than one digestion product since they are diffuse and staining intensities are different.

size PCR fragment	fragment sizes AluI digest	clones
1,6 kb	0.45, 0.4, 0.3 kb	2-3; 2-4; 2-7; 3-5; 3-12; 3-13; 3-14; 4-3; 4-8
1,5 kb	0.6, 0.5 kb	3-19; 5-4
0,9 kb	0.3, 0.15 kb	3-6; 3-10; 3-16; 3-20; 3-21; 4-5; 4-6
0,45 kb	0.45 kb	6-2
0,15 kb	0.15 kb	1-4

DNA was extracted from one representative yeast clone for each insert and retransformed into the *E. coli* strain HB101, which carries a leucine deficiency that can be complemented by the LEU2 marker present on the library vector pGAD10, thereby allowing selection for this plasmid separating it from the bait vector also present in the DNA preparation. Plasmid DNA isolates from this strain can be used to test for the presence of false positive library insert, i.e. sequences, that activate reporter expression without interaction with the bait protein.

Sequences of the identified clones were obtained by sequencing the DNA obtained after transformation into HB 101 (Tab. 6). Sequences were analysed by Blast database searches.

**Tab. 6: Proteins deduced from the DNA clones isolated from positive 2-hybrid clones.**

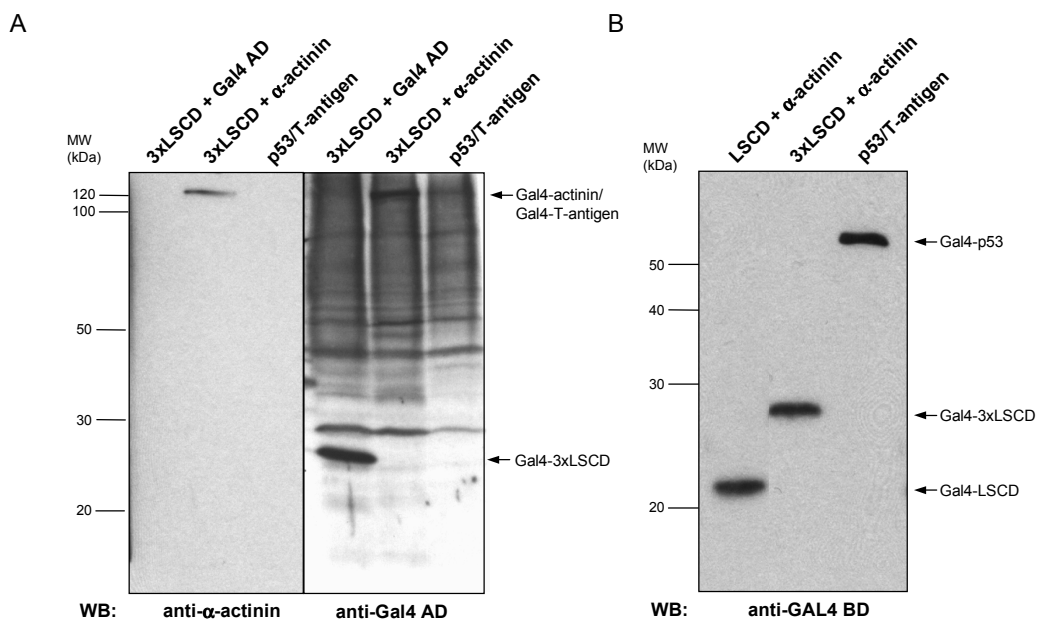
Clone No.	Gene description	Accession number	Region contained in isolated clone	Features and function of identified protein
3-14	CCCTC-binding factor	XP007875	coding region aa 120-621	This protein contains a 11-zinc-finger motif and acts as a transcriptional repressor regulating c-myc expression (Filippova et al., 1996)
5-4	hypothetical protein KIAA0117	P42696	coding region from aa 13 to the end and 3' UTR	Putative protein containing a RNA recognition motif (RRM) similar to the ones found in heterogeneous nuclear ribonucleoproteins and proteins involved in RNA splicing.
4-6	hypothetical protein FLJ20596	XP007779.1	coding region for C-terminal 124 aa and 3' UTR including the poly-A tail.	Protein shows homologies to ATP-dependent RNA helicases and contains a DEAD/DEAH box.

Clone No.	Gene description	Accession number	Region contained in isolated clone	Features and function of identified protein
6-2	Methyl-CpG binding domain protein 2	NM015832	coding region aa 18-147	Protein binds to methylated DNA sequences and acts as transcriptional repressor (Hendrich and Bird, 1998)
1-4	$\beta$ -2-microglobulin mRNA	NM004048	part of the 3' untranslated region inserted with wrong orientation	Translation did not reveal ORFs in the inserted sequence

All of the isolated sequences encode DNA- or RNA-binding proteins which are known to occur frequently as false positives in yeast 2-hybrid screens and are not likely to interact with L-selectin in a physiological context. For this reason, no further analysis of these clones was performed.

### 2.2.8 Direct testing for interaction with $\alpha$ -actinin

Since the 2-hybrid screen was not successful in isolating binding partners for L-selectin from an expression library, it was determined whether this system is applicable for the detection of known L-selectin interaction with alpha-actinin (Pavalko *et al.*, 1995).



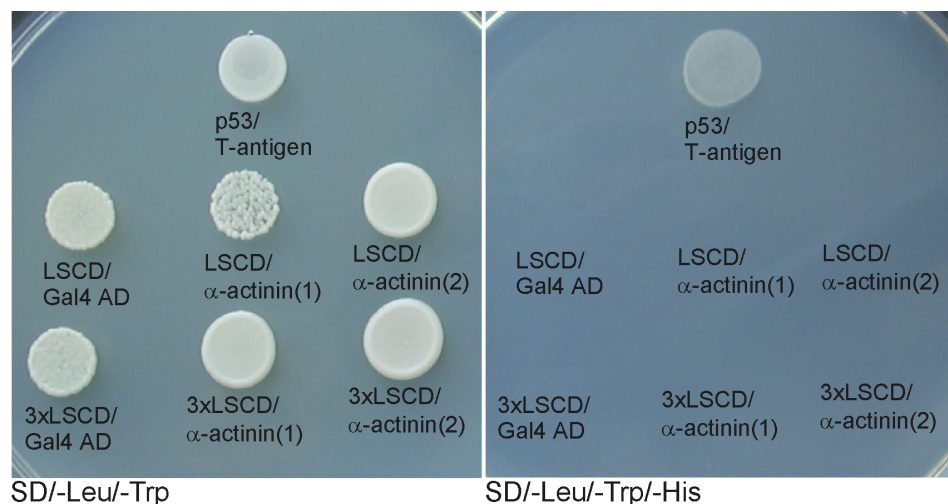
**Fig. 15: Gal4 BD-LSCD fusion proteins and Gal4 AD- $\alpha$ -actinin are expressed in yeast**

(A) Whole cell lysates from yeast transformed with pAS2-1/3xLSCD together with pACT2 or pACT2/ $\alpha$ -actinin or were tested with antibodies against alpha-actinin or the Gal4 activation domain. (B) Yeast cells carrying pACT2/ $\alpha$ -actinin together with pAS2-1/LSCD constructs or pVA3-1 and pTD1-1 were analysed for expression of target proteins by immunoblotting with antibody directed against Gal4 BD.

For this purpose, turkey smooth muscle alpha-actinin, which differs in only one amino acid from the chicken gizzard smooth muscle alpha-actinin used in the

original study, was cloned into pACT2 following PCR amplification with primers Act/Eco fwd and Act/Xho rev, using pGEMT $\alpha$ A9 as a template. To minimise the risk of inactivating mutations during PCR, two different clones of pACT2/ $\alpha$ -actinin were analysed for interaction. The Gal4 AD- $\alpha$ -actinin construct was transformed into yeast strain Y190 together with pAS2-1/ LSCD and pAS2-1/3LSCD and tested for expression (Fig. 15) and growth on SD/-Leu/-Trp/-His plates (Fig. 16). As a positive control, yeast containing the vectors pVA3-1 and pTD1-1, which code for the interacting proteins p53 and T-antigen, were analysed in the same way. Expression of the Gal4 fusion proteins containing the cytoplasmic domain of L-selectin as well as  $\alpha$ -actinin was detected in these transformants by Western blotting against the Gal4 BD, the Gal4 AD and  $\alpha$ -actinin (Fig. 15).

All transformed cells grew on plates lacking leucine and thryptophan, indicating presence of both plasmids in the yeast cells (Fig. 16, left picture). No growth on plates lacking histidine, which would require interaction of the expressed proteins, was observed even after two weeks of incubation. In contrast, control cells expressing p53 and T-antigen grew well without histidine (Fig. 16, right picture). This indicates that no interaction of L-selectin and  $\alpha$ -actinin takes place in the yeast cell nucleus, although this interaction has been shown *in vitro* and in cell lysates. This finding implies that the 2-hybrid system is not a suitable tool for the identification of L-selectin binding partners. For this reason, alternative methods for the isolation of binding proteins from cellular extracts were considered (see chapter 2.4).



**Fig. 16: Gal4- $\alpha$ -actinin does not induce growth by interaction with LSCD.**

Yeast strain Y190 was co-transformed with different combinations of pAS2-1/LSCD, pAS2-1/3xLSCD, pACT2 and pACT2/ $\alpha$ -actinin clone 1 and 2 or pVA3-1 and pTD1-1 as positive control and spotted onto SD/-Leu/-Trp and SD/-Leu/-Trp/-His plates at identical densities.



### 2.3. Cloning and expression of GST-Lscyto fusion constructs

Creating fusions of a target protein with polypeptide tags provides a technique for recombinant protein expression which allows easy one-step purification and detection. Protein tags used for this purpose range from short peptides, like the 6xHIS sequence, to small proteins as in the case of glutathion S-transferase (GST).

Since the cytoplasmic domain of L-selectin was intended to be used in various applications, including *in vitro* phosphorylation assays, pull-down experiments and binding studies, an expression construct of this domain fused to glutathion S-transferase was created for production of the recombinant protein in *E. coli*. This allows more easy handling and detection of the very small cytoplasmic tail in the planned experiments compared to the use of peptides.

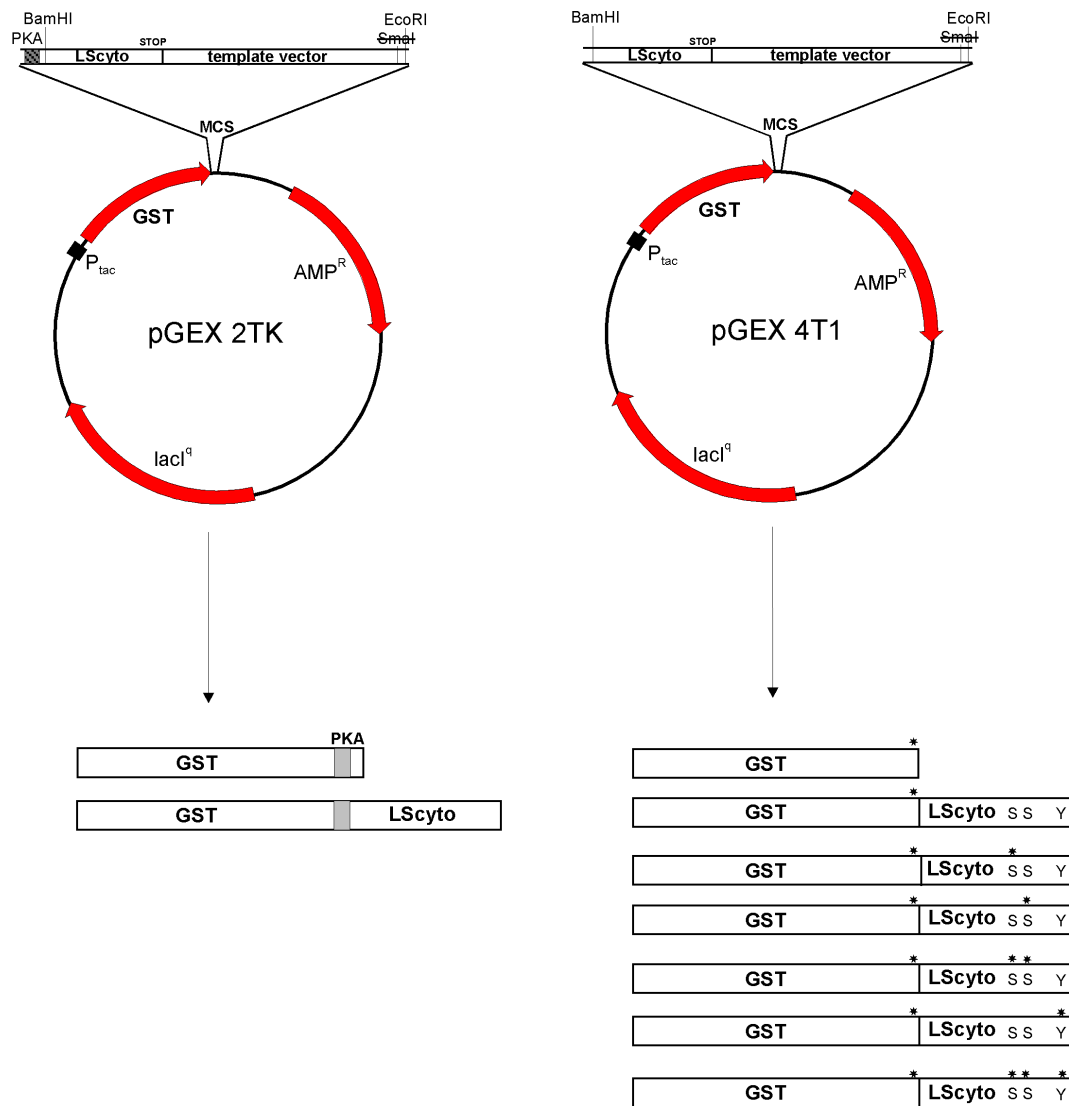
The cytoplasmic domain of L-selectin was cloned into vectors of the pGEX series which are designed for the expression of proteins fused to the tag. For construction of Lscyto fusion proteins two vectors were used: pGEX2TK which contains the GST sequence followed by a Protein kinase A (PKA) consensus site allowing *in vitro* radioactive labelling of expressed protein by phosphorylation, and pGEX4T1 which lacks this phosphorylation site. Additional to expression constructs of the wild type intracellular domain, a series of mutations was introduced into the sequence, thereby inactivating the potential phosphorylation sites of L-selectin.

#### 2.3.1 Construction of GST-Lscyto

pGEX2T1-Lscyto was generated by PCR amplification with Pwo polymerase of the desired sequence using pAS2-1/LSCD as a template. To obtain a fragment which is easier to isolate, part of the 3' untranslated region of the vector was included in the sequence. By use of primers LAMwt/BAM and pAS rev a 228 bp fragment was amplified, digested with *Bam*HI and ligated into pGEX2TK restricted with endonucleases *Bam*HI and *Sma*I. The *Sma*I restriction creates blunt ends allowing ligation to the also blunt end of the PCR product. The Lscyto insert was shuttled into pGEX4T1 by restriction of pGEX2TK-Lscyto with *Bam*HI and *Eco*RI which released the complete inserted sequence and ligation into the vector treated this the same enzymes.

#### 2.3.2 Construction of Lscyto mutants

The cytoplasmic domain of L-selectin contains three potential phosphorylation sites. For use in phosphorylation studies, mutations of these sites, serine 377 and 380 and tyrosine 385, were introduced into GST-Lscyto. A diagram of all pGEX expression constructs generated and the resulting GST-Lscyto fusion proteins is shown in Fig. 17.



**Fig. 17: pGEX constructs for expression of GST-Lscyto fusion proteins.**

This schema shows all constructs generated for prokaryotic expression of GST-tagged cytoplasmic domain of L-selectin. Asterisks indicate mutated serine or tyrosine residues. In pGEX 4T1 constructs an additional serine→alanine mutation was introduced, eliminating a putative phosphorylation site encoded in the MCS.

Inactivating mutations of the two serine residues contained in the cytoplasmic domain were first introduced in the full length sequence of L-selectin. The coding sequence was amplified from a construct containing the complete cDNA in the vector pMPSV –HE (pMPSV –HE FL, Fieger, 1997), using primers LAM BE and LS380/A and LS377/A, respectively, which change the indicated serine residue to alanine. PCR was carried out using Pwo polymerase which possesses proofreading activity to minimise errors. Single stranded A-overhangs necessary for TA cloning into pCR3.1 were added by incubation with Taq polymerase. The purified fragment was ligated into pCR3.1 and transformed into chemically competent TOP10F' *E. coli* cells. Resulting colonies were tested for presence and correct orientation of insert by restriction digestion. Positive clones were

completely sequenced and, correct sequence ensured, used as template for amplification and cloning of the cytoplasmic domain into pGEX4T1 as described above for the wild type sequence.

The 377+380/A double mutant was made by using pCR3.1-LS377/A as a template for amplification with primers Lam BE and LS380/A to obtain the full length construct. Cytoplasmic tail fusion genes in pGEX4T1 were created as described for the single mutants.

A fusion construct containing phenylalanine instead of the C-terminal tyrosine residue was created as described for the serine mutants, first cloning the mutated full length construct obtained by PCR with primers Lam BE and LAM F(R), followed by amplification of the cytoplasmic domain and ligation into pGEX4T1 vector.

A mutant lacking all three putative phosphoryl acceptor sites was generated by PCR amplification with primers Lam BE and LAM F(R) using pGEX4T1-377+380/A as a template and ligated into the *Bam*HI and *Sma*I restriction sites of pGEX4T1.

### 2.3.3 Modification of GST as a control

GST expressed from unmodified pGEX vectors contains a stretch of amino acids which do not belong to the glutathion S-transferase sequence but are encoded by the multiple cloning site (MCS). For use of GST as a control in various experiments it was desired that the control protein only contained sequences which were also present in the GST-Lscyto fusion protein to prevent artefacts originating from the unrelated sequence. To accomplish this, a stop codon was introduced at the site of transition from GST to the MCS by site-directed mutagenesis using primers 4T1 S/A fwd and 4T1 S/A rev.

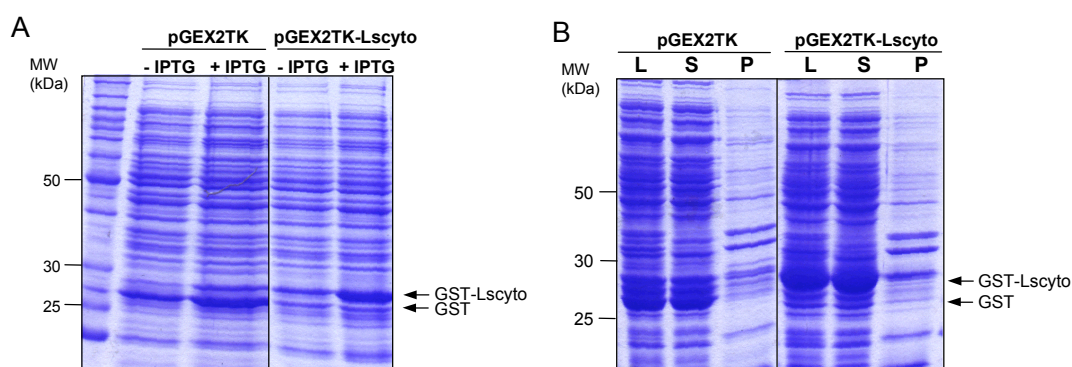
### 2.3.4 Mutagenesis of potential phosphorylation site in the MCS

Fusion of Lscyto to GST created a potential phosphorylation site for kinases with basic recognition motifs at the transition between the two fusion partners, because a serine encoded in the *Bam*HI restriction site was followed by two arginine residues at the start of the cytoplasmic sequence of L-selectin. To prevent false positive results in phosphorylation studies, this serine was mutated to alanine in all pGEX-Lscyto fusion constructs and the control GST vector. The mutation was introduced by site-directed mutagenesis with primers pGEX-S/A fwd and pGEX-S/A rev.

### 2.3.5 Expression of recombinant fusion proteins

The pGEX series of expression vectors carries a gene for the lac repressor (*lacI<sup>q</sup>*), allowing IPTG-inducible expression in any *E. coli* strain without requirement of intrinsic *lacI<sup>q</sup>*. First attempts for expression of the GST fusion constructs were undertaken in the *E. coli* strain BMH 71-18. BMH 71-18 cells were transformed with pGEX 2TK and pGEX 2TK-Lscyto and tested for GST fusion protein expression (Fig. 18A). After induction, expression of proteins of approximately 27 kDa and 29 kDa was observed, corresponding well to the expected sizes of GST and GST-Lscyto respectively. After disruption of the cells, the large majority of recombinant protein was found in the supernatant, which

shows that both GST and GST-Lscyto are expressed in a soluble form (Fig. 18B). After purification of this proteins by GSH-Sepharose affinity chromatography, both samples displayed identical sizes of about 27 kDa, indicating a proteolytic degradation of the Lscyto fusion part. To reduce degradation, induction of fusion protein was carried out at 30°C and chromatography was performed in the cold with protease inhibitors added to the all buffers used in the isolation process. This procedure did not improve recovery of full length fusion protein (data not shown). Expression in the *E. coli* host XL-1 blue showed similar results, not yielding full length products after protein purification.



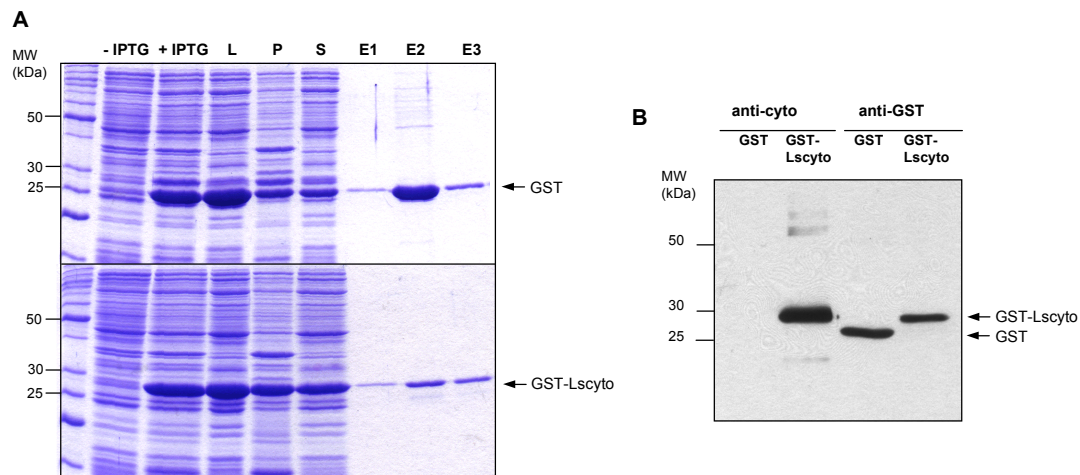
**Fig. 18: Expression of GST fusion proteins in BMH 7118**

BMH 71-18 transformed with pGEX 2TK and pGEX 2TK-Lscyto were grown in small scale culture over night and induced with 0.5 mM IPTG for 2h. (A) Whole cell lysates were made by boiling cells in 1x SDS sample buffer. Lysates were subjected to SDS-PAGE to check for fusion protein expression by Coomassie staining. (B) Cells were disrupted by sonication and insoluble material sedimented by ultracentrifugation. Equal volumes of the different fractions were analysed by SDS-PAGE. L, whole cell lysate; S, supernatant; P, pellet.

To overcome the degradation problem, the *E. coli* strain BL21, which lacks the intrinsic proteases encoded by the *ompT* and *lon* genes, was used to express the pGEX constructs. This strain is described as being well suited for expression of recombinant proteins which are sensitive to degradation (Grodberg and Dunn, 1988, Phillips *et al.*, 1984).

When GST-Lscyto was purified from induced cultures of BL 21, a protein of the expected size of 29 kDa was observed, which showed only small amounts of degradation products. For subsequent expression of all GST fusion constructs, a derivative of BL21, BL21 (RIL) was used. This strain carries additional copies of tRNA genes for certain arginine, isoleucine and leucine codons that are very rare in prokaryotes, but frequently used in human sequences. If several of these codons are present in a sequence to be expressed, shortened protein products are possible due to premature translation termination. The use of this strain improved expression levels of recombinant GST-Lscyto significantly, probably due to a more efficient translation of the two arginine codons present at the start of the L-selectin sequence. Furthermore, the occurrence of truncated products was reduced, indicating that, in BL21, they were caused by inefficient translation rather than proteolytic degradation.

A typical purification procedure for GST and GST-Lscyto is shown in Fig.19A. GSH-Sepharose affinity purification yielded highly purified protein (>90% as estimated from gels). The presence of both the L-selectin cytoplasmic domain and the GST portion in the expressed protein was confirmed by immunoblotting with antibodies against both fusion partners.(Fig. 19B).



**Fig. 19: Expression of GST-Lscyto in BL21**

(A) BL21 (RIL) transfected with pGEX 4T1(upper panel) or pGEX 4T1-Lscyto (lower panel) were induced to express recombinant protein with 0.5 M IPTG for 2 h. Whole cell lysate from uninduced and induced cultures, complete lysozyme-digested extracts (L), soluble (S) and insoluble (P) cellular fractions and samples from the start (E1), middle (E2) and end (E3) of elution were analysed by SDS-PAGE and Coomassie staining. (B) purified protein was tested for the presence of both fusion parts with antibodies against Lscyto and GST.

The amount of recovered protein in all purifications performed was significantly lower for GST-Lscyto than for GST alone, although the expression levels in whole cell lysates appeared similar. This points towards a reduced binding of the GST-Lscyto fusion protein to GSH, probably caused by increased misfolding of the GST moiety in the presence of the fusion partner.

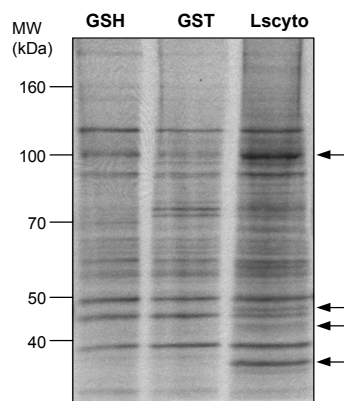
## 2.4. Affinity isolation of interacting proteins ("Pull-down" experiments)

Since the yeast 2-hybrid system proved not to be suitable in the search for intracellular binding partners of L-selectin, affinity isolation of interacting proteins from cell lysates using recombinant GST-Lscyto fusion protein was attempted.

For this purpose, T-cell lines were metabolically labelled with  $^{35}\text{S}$ -containing methionine and cysteine, lysed, and proteins binding to Lscyto were isolated using an indirect "pull-down"-approach. Fusion protein was added to the cell lysates, incubated to allow binding of cellular proteins, and protein complexes were recovered with glutathione-Sepharose.

This approach yielded several protein bands that specifically associated with GST-Lscyto, but not with GST or GSH-Sepharose alone (Fig. 20). However, high

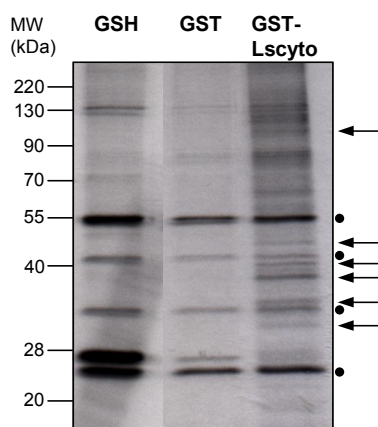
levels of unspecific adherence of cellular proteins to Sepharose beads posed difficulties in analysis of specific bands.



**Fig. 20: Indirect pull-down isolation of L-selectin binding proteins from metabolically labelled cells.**

A.301 T-cells were grown with  $^{35}\text{S}$ -labeled amino acids overnight, lysed in RIPA buffer and Lscyto-binding proteins isolated by an indirect pull-down approach. Proteins were eluted in SDS-sample buffer, separated by SDS-PAGE and visualised by autoradiography. Arrows indicate proteins that specifically bind to Lscyto.

To achieve lower background binding, direct pull-downs assays were performed, i.e., GST fusion protein was bound to the GSH-Sepharose beads prior to addition of cell lysates. Fusion protein was used in saturating amounts, thereby limiting unspecific binding sites on the Sepharose. Furthermore, conditions for cell lysis were altered. By using a less stringent detergent (1% Brij 58) as compared to the SDS present in the RIPA lysis buffer, cell nuclei remained largely intact and proteins present in the solubilised fraction mainly originate from the cytosol and plasma membrane, where presence of physiological L-selectin interaction partners is expected. These changes in the experimental procedure reduced significantly the diffuse background binding to Sepharose beads observed in indirect pull-down experiments (Fig. 21). Some cellular proteins strongly bound GSH-Sepharose and were found consistently in pull-down eluates. GST coated GSH-Sepharose beads display the same pattern of bands as GSH Sepharose alone, indicating that GST binds no cellular protein.



**Fig. 21: Direct pull-down isolation of L-selectin binding proteins from metabolically labelled cells.**

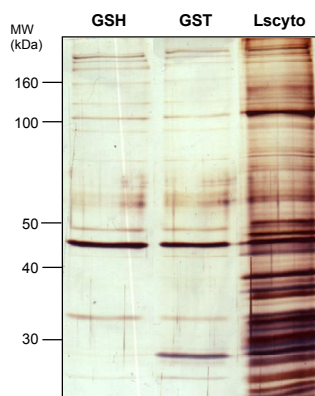
A.301 T-cells were grown with  $^{35}\text{S}$ -labeled amino acids overnight, lysed in 1% Brij 58 buffer and subjected to direct pull-down isolation of Lscyto-binding proteins. Proteins were eluted in SDS-sample buffer, separated by SDS-PAGE and visualised by autoradiography. Cellular proteins adhering to GSH-Sepharose are marked with dots, proteins specifically binding to GST-Lscyto are indicated by arrows.

Several proteins exclusively binding to GST-Lscyto are present in the eluates at approximate molecular weights between 30 and 45 kDa and around 100 kDa.

To prepare these L-selectin binding proteins for analysis by mass spectrometry, large scale preparations were performed from lysates of unlabeled cells and proteins associated with GST-Lscyto were analysed by silver staining of



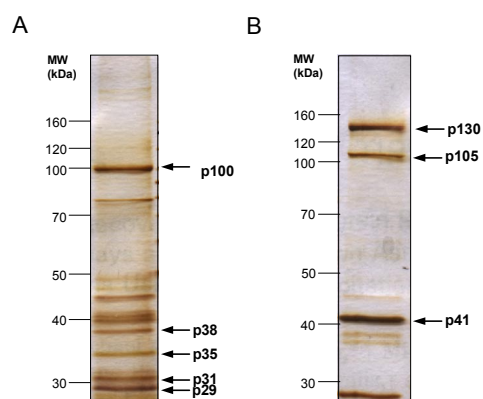
SDS-PAGE gels. Since the large amounts of fusion protein dissociating from the beads by elution in SDS sample buffer made analysis of weaker band difficult due to over-staining, proteins were eluted with 1 M sodium chloride. This high salt elution yielded a similar pattern of bands compared to experiments using metabolically labelled cells (Fig. 21) but these conditions did not affect the binding of GST-Lscyto to GSH-Sepharose.



**Fig. 22: Direct pull-down from unlabeled cells.**

Direct pull-down isolations from A.301 lysates were performed using GST-Lscyto, GST or uncoated GSH-Sepharose beads. Bound proteins were eluted with 1 M NaCl and analysed by SDS-PAGE and silver staining.

These pull-down experiments from whole cell lysates yielded several Lscyto-associated proteins in the molecular weight range of 30 to 50 kDa as seen before in isolations from metabolically labelled cells (Fig. 22). These proteins migrated close together in SDS-PAGE gels, making identification of distinct band difficult. To achieve better discrimination between these proteins for further analysis, experiments were performed with fractionated cell lysates. Cytosolic and membrane fractions (including membrane associated proteins) were obtained by differential centrifugation and use as a starting material for affinity isolation as described above. Moreover, to obtain highly purified proteins for analysis by mass spectrometry, eluates from fractionated pull-downs were subjected to a second round of precipitation with GST-Lscyto after dialysis to remove sodium chloride.



**Fig. 23: proteins purified for mass spectrometry.**

Lscyto-binding proteins were purified by two consecutive rounds of pull-down precipitation and high salt elution from (A) membrane and (B) cytoplasmic fractions of A.301 T-cells.

Analysis of proteins isolated from fractionated cells (Fig. 23) revealed that the majority of bands observed in pull-downs from whole cell lysates were found in eluates obtained from membrane fractions. These include most of the bands between 30 kDa and 50 kDa and the prominent band at 100 kDa.

Eluates from cytosolic fractions displayed three major bands. p130 and p105 are also found in isolates from whole cell eluates, whereas p41 is only seen as a weak band in pull-downs from unfractionated cells, but shows strong association when precipitated from the cytosolic fraction.

Remaining eluates from this large scale preparation were separated by SDS-PAGE and Coomassie stained. Bands corresponding to the ones indicated on the silver stained gel shown in Fig. 23 were excised from the gel and analysed by MALDI-TOF and ESI mass spectrometry (Tab. 7).

**Tab. 7: Results from mass spectrometry for Lscyto binding proteins.**

(A) Protein data obtained from the membrane fraction

Approx. mol. weight	Identified protein	Identified peptide mass	Theoretical peptide mass	Peptide sequence
p100	Nucleolin	2200.02	2200.025	577- 596 GLSEDTTEETLKESFDGSR
p38	Nucleophosmin	1568.72	1568.730	033-045 VDNDENEHQLSLR
		931.42	931.473	240-248 GPSSVEDIK
		1819.82	1819.843	278-291 MTDQEIQLDLWQWR
p35	RL5	1241.60	1241.602	058-067 DIICQIAYAR
		1533.69	1533.678	209-220 YLMEEDDAYKK
		913.46	913.478	221-227 QFSQYIK
p31	RS6	1284.70	1284.679	120-131 DIPGLTDTTVPR
		1620.80	1620.757	032-042 MATEVAADALGEEWK
p29	RS4	1886.94	1885.925	077-093 TDITYPPAGFMDVISIDK
		1445.74	1445.748	155-167 VNDTIQIDLETGK
		990.60	990.598	221-229 LSNIFVIGK

(B) Protein data obtained from the cytosolic fraction

Approx. mol. weight	Identified protein	Identified peptide mass	Theoretical peptide mass	Peptide sequence
p130	-	-	-	-
p105	Nucleolin	1178.56	1178.569	410-419 EVFEDAAEIR
		1322.64	1322.632	577-588 GLSEDTTEETLK
p41	PHAPII	2194.99	2195.021	027-044 EQQEAIHIDEVQNEIDR
		1273.64	1273.663	045-055 LNEQASEEILK
		1063.56	1063.605	063-070 LRQPFFQK
		1208.56	1208.604	110-119 VEVTEFEDIK

#### 2.4.1 Proteins isolated from the membrane fraction

##### p100–nucleolin

Nucleolin was contained both in the 100 kDa band from membrane fractions and in the 105 kDa band from cytosol (see below). This protein has been originally



described as a component of nucleolar rRNA/protein complexes (ribonucleoprotein complexes, RNPs), involved in ribosome biogenesis and maturation. It has since been found involved in a large number of cellular functions, e.g. as regulator of transcription factors and binding partner for transmembrane receptors and signalling molecules (reviewed in Srivastava and Pollard, 1999).

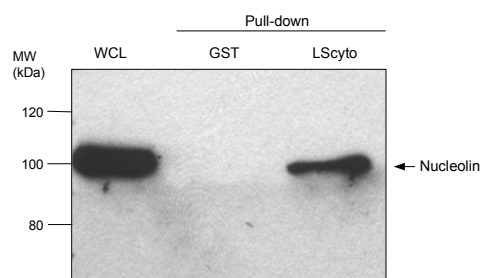
### **p38–nucleophosmin**

Nucleophosmin, also known as B23, is like nucleolin a constituent of the RPP pre-ribosomal complexes and is also involved in ribosome assembly.

### **p35, p31, p29–ribosomal proteins**

The remaining three bands analysed all contained ribosomal proteins derived both from the large and small subunit.

The large number of different protein bands in the pull-down eluates from membrane fractions suggested, that not all of these proteins bind directly to Lscyto. In this regard, it was interesting, that all of the bands identified contained proteins that are known to assemble into the large pre-ribosomal complexes. This gave rise to the assumption that only one or few of these candidate proteins interact with the cytoplasmic domain of L-selectin, thereby carrying further associated proteins into the precipitate. The most probable choice of direct binding partners was nucleolin, which has been implicated in signal transduction events and was found as a binding partner of transmembrane receptors and nucleophosmin. The presence of nucleolin in the eluates from Lscyto-pull-downs was confirmed by immunoblotting (Fig. 24). Several reports indicate that association of the protein constituents of the ribosomal precursors mainly occurs through bridging RNA molecules and that the complex can be dissociated by RNase treatment (Yanagida *et al.*, 2001, Pinol-Roma 1999).

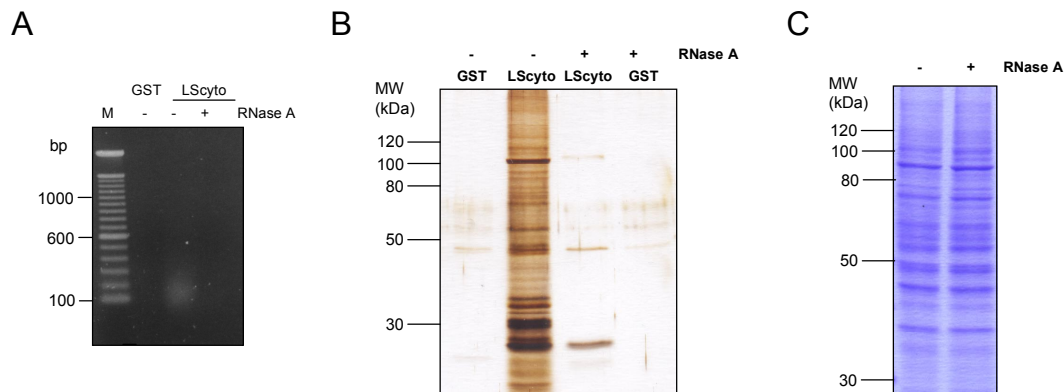


**Fig. 24: Nucleolin is present in pull-down eluates.**

GST and GST-Lscyto pull-downs from A.301 cells were eluted with 1 NaCl. Proteins were separated by SDS-PAGE, transferred onto PVDF membrane and probed with antibody directed against nucleolin.

To test the hypothesis that RNA-containing preribosomal complexes bound to Lscyto, eluates from pull-downs were analysed by agarose gel analysis, which confirmed the presence of nucleic acids (Fig. 25A). These were sensitive to digestion with RNase A, indicating that the signal indeed derived from RNA present in the preparation. When membrane fractions were digested with RNase A prior to addition of Lscyto-coupled Sepharose, no NaCl-elutable associated proteins were detected (Fig. 25B). Membrane fractions were analysed by SDS-PAGE and Coomassie staining before and after digestion with enzymes to exclude degradation of proteins by contaminating proteases in the nuclease preparation. No alteration in the protein pattern was observed after this treatment (Fig. 25C).

This findings lead to the conclusion, that binding of the pre-ribosomal complexes to Lscyto was not mediated by protein-protein-interactions but rather by electrostatic adherence of the negatively charged RNA molecules present in the complex to the highly basic L-selectin cytoplasmic domain.



**Fig. 25: Binding of pre-ribosomal proteins to Lscyto is abolished by RNaseA treatment.**

(A) Pull-down eluates were analysed by agarose gel electrophoresis and ethidium bromide staining for the presence of nucleic acids. (B) Pull-down eluates from untreated or RNase A-treated cell lysates. (C) Coomassie-stained SDS-PAGE gel showing membrane fractions either untreated (-) or RNase A-digested membranous fractions.

## 2.4.2 Proteins isolated from the cytosolic fraction

### p130

The protein contained in this band could not be identified by mass spectrometry due to insufficient purity although analysis of two independent preparations was undertaken. Since this protein displayed an irregular migration behaviour on SDS-polyacrylamide gels, resulting in broad, poorly defined bands, it is probable that a number of contaminating proteins or differently modified forms are present which impair analysis. Identification of this band was not further attempted.

### p105–nucleolin

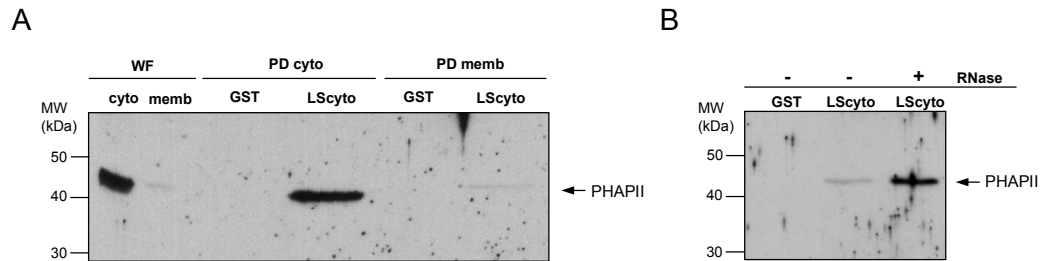
Although the band migrated at a slightly different size on SDS-polyacrylamide gels compared to the nucleolin isolated from the membranous fraction, it also contained nucleolin as a main constituent. This might be due to different posttranslational modifications of the protein located in different cellular compartments, since nucleolin is known to be a target for phosphorylation, methylation and partial degradation.

Although eluates from the cytosolic fraction only contained minor amounts of other members of pre-ribosomal complexes, binding of nucleolin to Lscyto was also sensitive to RNase A digestion. This might be due to the fact that nucleolin can be bound to RNA in the absence of large complexes (Wu *et al.*, 2000, Zaidi and Malter, 1995).

### p41–PHAPII

p41 was identified as PHAPII (putative HLA-associated protein 2), also known as SET protein or I2PP2A (inhibitor 2 of protein phosphatase 2A). This protein has

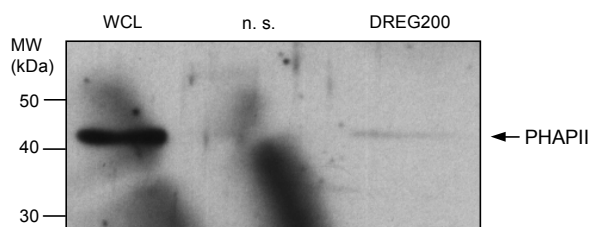
been described as a binding partner for the cytoplasmic domain of the HLA class II  $\alpha$ -chain and as a specific, non-competitive inhibitor of protein phosphatase 2A, one of the major serine/threonine phosphatases in the cell (Adachi *et al.*, 1994a; Vaesen *et al.*, 1994; Li *et al.*, 1995).



**Fig. 26: PHAPII is present in pull-down-eluates.**

Proteins eluted with 1 M NaCl from pull-downs using subcellular fractions were separated by SDS-PAGE, transferred onto nitrocellulose and visualised by immunodetection with polyclonal antiserum directed against PHAPII. WF, whole fraction; PD, pull-down eluate; cyto/memb, cytosolic and membrane fraction, respectively.

Presence of PHAPII in eluates from pull-downs using cytosolic cell fractions was confirmed by immunoblotting. Consistent with the distribution seen on silver-stained gels, only minor amounts of PHAPII were detectable in whole membrane fractions or in pull-downs from these fractions (Fig. 26A). RNase A treatment of cell fractions did not interfere with binding of this protein to Lscyto, but rather enhanced the amount of associated PHAPII, probably due to increased number of binding sites available (Fig. 26B).



**Fig. 27: PHAPII co-immunoprecipitates with L-selectin from T-cells.**

Immunoprecipitation of Jurkat whole cell lysates (WCL) were performed with DREG200 or non-specific, isotype-matched antibody (n. s.) and tested for the presence of PHAPII by immunoblotting with polyclonal antiserum.

To test for interaction between PHAPII and L-selectin in a cellular environment, co-immunoprecipitations were performed using Jurkat T-cells that have a higher expression level of L-selectin compared to the A.301 cell line. These experiments showed that PHAPII is detectable in immunoprecipitations with mAb DREG200 directed against the lectin domain of L-selectin (Fig. 27), but not with IgG<sub>1</sub> of irrelevant specificity. This finding demonstrates that PHAPII can associate with L-selectin in a cellular environment.

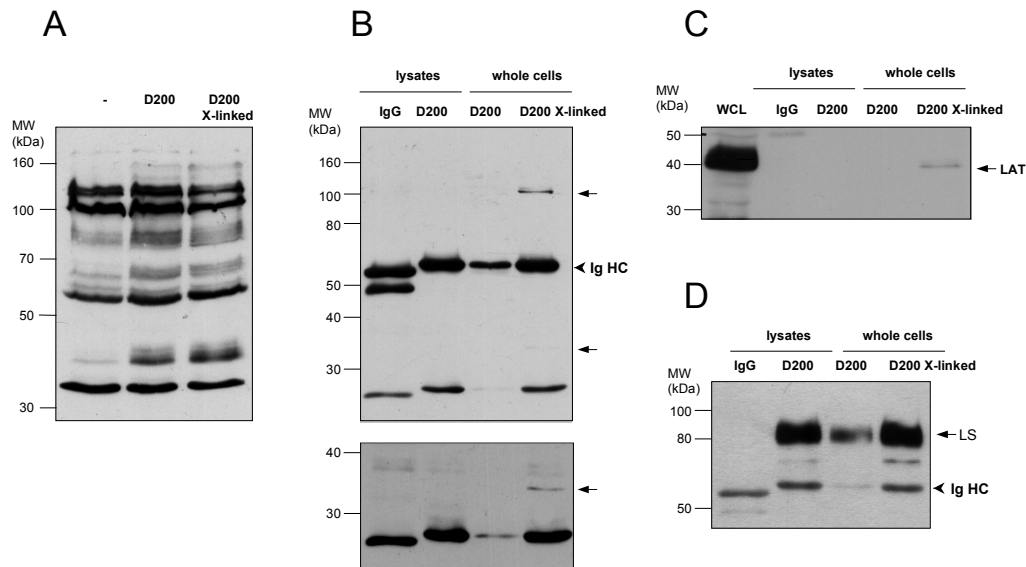
## 2.5. Isolation of L-selectin-associated membrane complexes

Many transmembrane receptors assemble large signalling complexes containing co-receptors, adaptor proteins, cytoskeletal linkers and signal transduction molecules like kinases and phosphatases following stimulation. L-selectin has been shown to initiate numerous signal events, including increased tyrosine kinase activity, and to become linked to the cytoskeleton (Crockett-Torabi, 1998; Evans *et al.*, 1999). To investigate association of L-selectin with intracellular complexes in Jurkat T-cells, a method was employed that induces receptor crosslinking by binding of intact cells to paramagnetic beads coated with mAb against the lectin domain of L-selectin, followed cell lysis with mild detergent. This procedure has proven suitable for isolation of sub-membrane complexes associated with the cytoplasmic domain of cell surface receptors and has been used for the preparation of focal adhesions and E-selectin associated proteins (Plopper and Ingber, 1993; Yoshida *et al.*, 1996). For comparison with proteins isolated from cells where L-selectin was crosslinked, L-selectin was also precipitated from untreated cell lysates and from cells stimulated with soluble (non-crosslinked) antibody against L-selectin. Analysis of the whole cell lysates (Fig. 32A) showed, that an increase in overall tyrosine phosphorylation is induced after treatment with non-crosslinked and crosslinked anti-L-selectin mAb in comparison with untreated cells as reported before (Waddell *et al.*, 1995; Brenner *et al.*, 1996). When L-selectin becomes crosslinked before cell lysis, the presence of two tyrosine-phosphorylated proteins with apparent molecular weights of approx. 100 kDa and 35 kDa in the immunoprecipitates is observed (Fig. 23B). These bands are not present in L-selectin immunoprecipitates from untreated cells, L-selectin stimulated cell without antibody crosslinking or in precipitates using a control antibody. Therefore, association of these proteins with the receptor is either induced by L-selectin crosslinking or they are constitutively bound, but only become phosphorylated upon L-selectin mediated signalling events.

L-selectin has been reported to co-localise with the T-cell receptor and have a costimulatory function for this receptor (Murakawa *et al.*, 1992). When the isolated membrane complexes were examined for the presence of the T-cell specific transmembrane receptor LAT (Linker of activated T-cells), which has a major role in linking the TCR to downstream signalling cascades, a LAT-signal was observed specifically in cells where L-selectin has become crosslinked before cell lysis (Fig. 23C). This indicates, that LAT is present in close proximity of L-selectin after crosslinking of the receptor.

Although the level of overall tyrosine phosphorylation in the cells treated with L-selectin antibody is increased, phosphorylation of L-selectin itself following ligation with either soluble antibody or crosslinking of the receptor by antibodies immobilised on magnetic beads could not be detected in these experiments. This is in contrast to a study reporting tyrosine-phosphorylation of the receptor after treatment of Jurkat cells with L-selectin mAb (Brenner *et al.*, 1996). Tyrosine phosphorylation of L-selectin could also not be reproduced in many experiments using various L-selectin mAb, time frames, stimulating agents and cell lines. Co-precipitation of a tyrosine phosphorylated protein with L-selectin antibodies, which migrates at approximately the same size as L-selectin, was sometimes observed, but was also present in samples originating from cell

that do not express L-selectin (data not shown). Therefore, it is debatable whether this tyrosine phosphorylation of L-selectin indeed occurs.



**Fig. 28: L-selectin crosslinking induces association with tyrosine-phosphorylated proteins and the transmembrane adaptor LAT.**

(A) Jurkat T-cells were left untreated or were stimulated with soluble Dreg 200 mAb or Ab coupled to anti-mouse magnetic beads. Cells were lysed, proteins were separated by SDS-PAGE, blotted and probed with antibody against phospho-tyrosine. (B) L-selectin was precipitated from unstimulated cell lysates using mAb Dreg 200 and unspecific isotype-matched mAb as a control, or soluble or bead-bound Dreg 200 was bound to intact cells and cells lysed afterwards. Washed precipitates were analysed by Western blotting with antibody mixture against phospho-tyrosine (4G10 and PY20). The lower panel shows part of the upper blot exposed for a longer time to detect the 35 kDa band, which is only faintly visible after short exposition. (C) Western blots prepared as described in B were probed with antibody against LAT. (D) Western blot used in B was stripped and reprobed with polyclonal antibody against the cytoplasmic domain of L-selectin.

In these experiments, the amount of precipitated L-selectin was lower in samples where soluble mAb was added to the intact cells than for isolation from unstimulated cell lysates, or cells that were crosslinked with antibody-bound beads (Fig. 23D). This was due to the separation of the cells from unbound antibody by centrifugation prior to cell lysis (note that the amount of antibody-derived signal is also lower in these samples). L-selectin was precipitated with antibodies immobilised on magnetic beads in the other samples, enabling further association of L-selectin with previously unoccupied antibody molecules after cell lysis.

## 2.6. *In vitro* phosphorylation assays with GST-Lscyto

It has been reported that L-selectin is phosphorylated at serine residues following chemokine treatment (Haribabu *et al.*, 1997), but the physiological significance of this phosphorylation and the kinase(s) involved in the process are still unknown.

The two serine residues contained in the cytoplasmic tail of L-selectin are surrounded by several positively charged residues, that hind towards the involvement of serine/threonine kinases that target basic consensus sequences (Tab. 8).

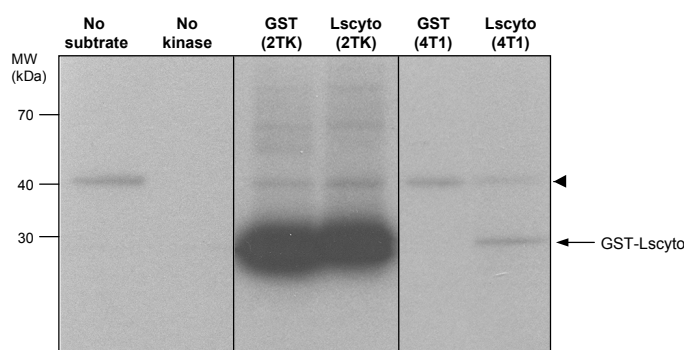
**Tab. 8: Consensus sequences of kinases.**

X indicates residues irrelevant for recognition. Sequences present in Lscyto are underlined.

Kinase	Consensus sequences
PKA	R-X-S; R-K/R-X-S; R-XX-S
PKC	<u>S-X-K/R</u> ; K/R-X-X-S; <u>K/R-X-S</u>
PKG	<u>R/K-X-S</u> ; R/K-X-X-S; <u>R/K-X-X-X-S</u> ; <u>S-X-R/K</u>

To test the capability of PKA, PKC and PKG, which all prefer sequences related to the ones found in the cytoplasmic domain of L-selectin, for phosphorylation of this protein, *in vitro* kinase assays were performed.

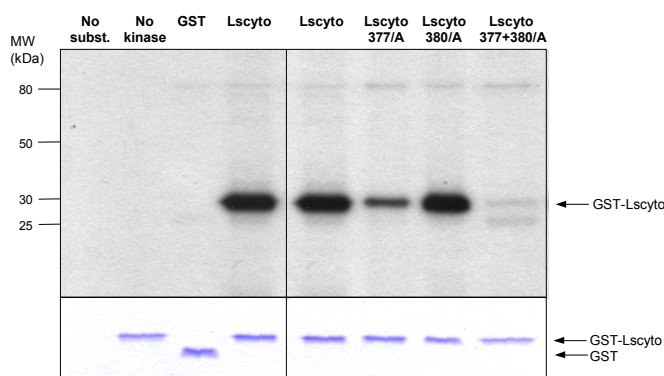
Recombinant fusion proteins were incubated together with purified kinase and  $^{32}\text{P}$ - $\gamma$ -ATP and tested for incorporation of radioactivity by SDS-PAGE and autoradiography.



**Fig. 29: PKA does not phosphorylate Lscyto *in vitro*.**

Recombinant proteins derived from expression vectors pGEX2TK or pGEX4T1 (1  $\mu\text{g}/\text{sample}$ ) were incubated with 10  $\mu\text{U}$  PKA and 5  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP. Reaction products were separated by SDS-PAGE and analysed by autoradiography. Arrow indicates recombinant fusion protein, arrowhead shows position of autophosphorylated PKA

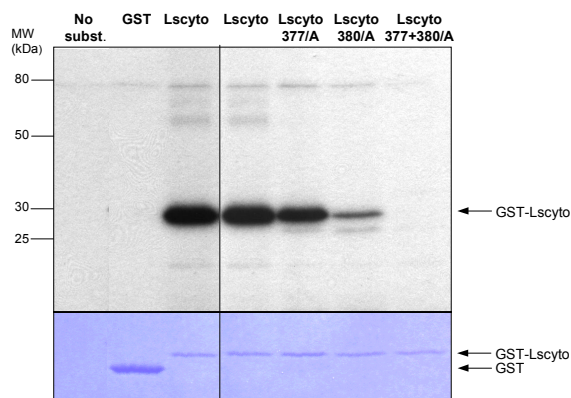
As shown in Fig. 29, PKA strongly phosphorylates proteins expressed from pGEX2TK plasmids, which encode a PKA consensus site and served as a positive control for the function of PKA. pGEX4T1-encoded GST or GST-Lscyto, which lack this vector derived phosphorylation site, are poor targets for PKA. GST shows no phosphorylation at all, while GST-Lscyto displays a very weak signal as compared to the positive control. This demonstrates that the cytoplasmic sequence of L-selectin is not a good target for PKA phosphorylation. The band observed at 40 kDa is due to autophosphorylation of the catalytic subunit of PKA.



**Fig. 30: PKC can use Lscyto as a substrate *in vitro*.**

Recombinant fusion proteins (1 µg/sample) were incubated with 10 µU of PKC. Reaction products were separated by SDS-PAGE and analysed by autoradiography. Upper panel: autoradiography of SDS-gel, lower panel: Coomassie staining of same gel to ensure comparable amounts of protein.

In contrast to PKA, PKC effectively phosphorylated GST-Lscyto, indicating that the cytoplasmic part of L-selectin is a good target for PKC (Fig. 30). GST alone and controls lacking either recombinant fusion protein or the kinase show no incorporation of radioactivity. Further analysis of the phosphorylation site used by PKC, using Lscyto with inactivating mutations of the serine residues in the cytoplasmic domain, show that both serines present in the sequence can be phosphorylated by PKC. However, the enzyme displayed a strong preference for serine 377, since fusion protein lacking this residue showed a significantly lower incorporation of radioactivity than the 380/A mutant. When both serines are changed to alanine, phosphorylation is abolished completely, indicating that these residues constitute the only target sequences present in the protein (Fig. 30).



**Fig. 31: PKG phosphorylates Lscyto *in vitro*.**

Recombinant proteins (1 µg/sample) were incubated with 40 U PKG and 5 µCi [ $\gamma$ - $^{32}$ P]ATP. Reaction products were separated by SDS-PAGE and analysed by autoradiography. Upper panel: autoradiography of SDS-gel, lower panel: Coomassie staining of same gel to ensure comparable amounts of protein.

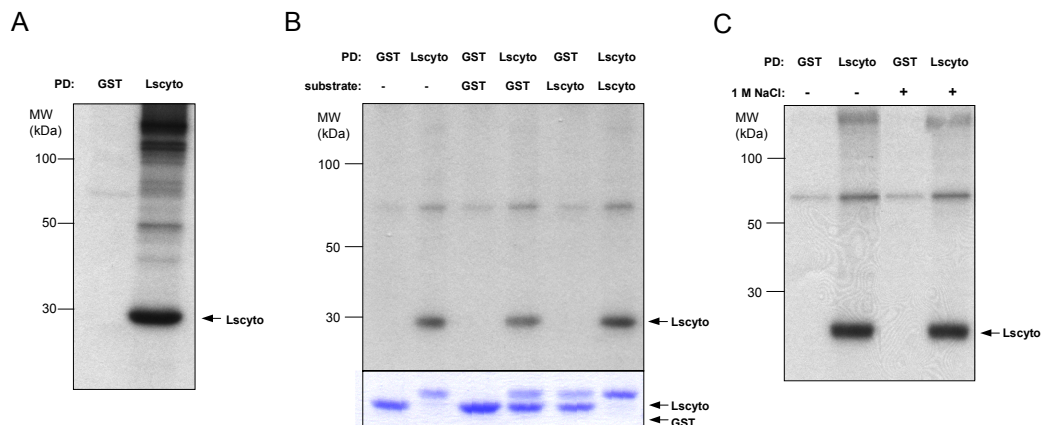
GST-Lscyto is also a good substrate for phosphorylation by PKG, as shown in Fig. 31. This kinase, in contrast to PKC, exhibits a strong preference for serine 380 over serine 377 for phosphorylation. Inactivating mutation of both serines completely abolishes phosphorylation by PKG.

This results show that PKC and PKG, serine/threonine kinases with a strong inclination for target sequences containing a number of positively charged amino acids, are capable to phosphorylate the cytoplasmic domain of L-selectin *in vitro* and therefore are good candidates for being the cellular L-selectin kinase. In contrast, PKA, which also recognises residues in the context of basic sequences, although slightly differing from the ones found in L-selectin, does not phosphorylate this protein.

## 2.7. *In vitro* kinase assays with Lscyto-associated kinases from cell lysates

Having shown that purified kinases can phosphorylate Lscyto on serine residues *in vitro*, further investigations aimed at the identification of the kinases responsible for L-selectin phosphorylation in human T-cells. By using the previously described pull-down approach it was tested whether kinases from cellular extracts associated with the cytoplasmic tail of L-selectin.

To investigate this, pull-down-precipitates of GSH-Sepharose bound fusion proteins together with associated proteins from Jurkat T-cell lysates were subjected to phosphorylation assays by incubation with [ $\gamma$ - $^{32}$ P]ATP to allow phosphorylation by kinases present in this complex. Reaction products were analysed by SDS-PAGE and autoradiography of the stained and dried gels.



**Fig. 32: Lscyto associates with a kinase activity from cell lysates.**

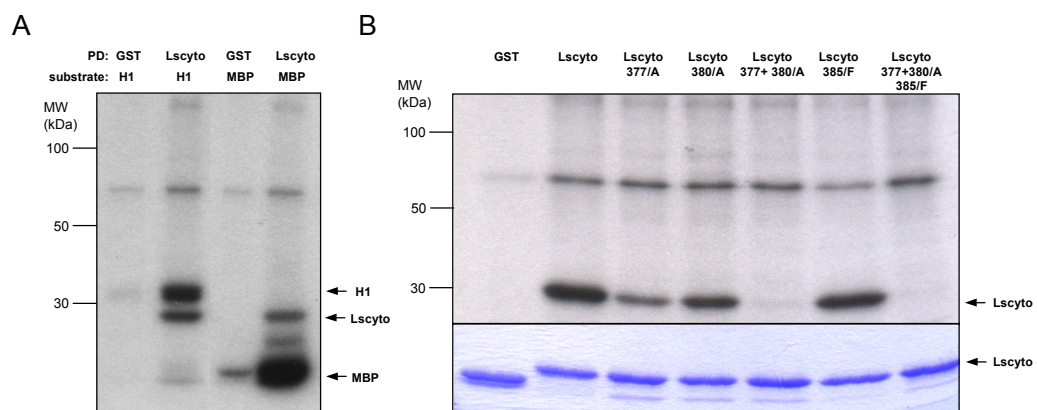
(A) Washed GST and GST-Lscyto pull-down precipitates from Jurkat T-cell lysates were incubated with 5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP in kinase buffer to allow phosphorylation by associated kinases. Proteins were eluted in SDS-sample buffer, separated by SDS-PAGE and dried gels were analysed for incorporation of radioactive phosphate by autoradiography. (B) Additional substrates were added as indicated to pull-downs from Jurkat cell lysates treated with 100 U/ml RNase A prior to the phosphorylation assay. (C) Where indicated, pull-downs from RNase A treated Jurkat cell lysates were subjected to two additional washes with lysis buffer containing 1 M NaCl preceding the phosphorylation assay. Arrows indicate GST-Lscyto fusion protein

These experiments show that Lscyto is strongly phosphorylated (Fig. 32A), whereas GST alone shows no incorporation of radioactivity when assayed in this way, indicating that kinase(s) derived from the cell extract are co-precipitated with the cytoplasmic domain.

Apart from Lscyto, several other co-precipitated proteins are phosphorylated in pull-downs from cell lysates as shown in Fig 32A. Treatment of cell extracts with RNase A greatly reduced the presence of non-Lscyto kinase substrates present in the pull-down eluates (Fig. 32B and C), as expected from the results described in chapter 2.4. Phosphorylation of Lscyto itself was not impaired by RNase A, showing that the interaction of the associated kinase was not dependent on RNA.



An additional band of unknown origin migrating at about 60 kDa on SDS-PAGE gels was seen in pull-downs with both GST alone and GST-Lscyto, although it always appeared stronger in Lscyto samples. This could either be due to an additional kinase activity present in GST pull-downs or by adherence of an ATP-binding protein to GST. To exclude the possibility that a kinase activity able to phosphorylate Lscyto was associated with the GST portion of the fusion protein, GST pull-downs were subjected to a phosphorylation assay with Lscyto fusion protein as a additional substrate (Fig. 32B). No phosphorylation of the L-selectin fusion part was observed in this assay. Phosphorylation of the fusion protein in the GST sequence by an Lscyto-binding kinase was as well excluded, since no incorporation of radioactivity in GST was observed after incubation with Lscyto pull-downs. The above experiments prove that a kinase activity from T-cell extracts binds to the cytoplasmic domain of L-selectin during pull-down experiments and is able to phosphorylate residues contained in this sequence. The kinase(s) associating with Lscyto are tightly bound to the fusion protein since high salt washes of the pull-down precipitates prior to the addition of [ $\gamma^{32}$ -P]ATP with concentrations up to 1 M NaCl did not reduce phosphorylation (Fig. 32C).



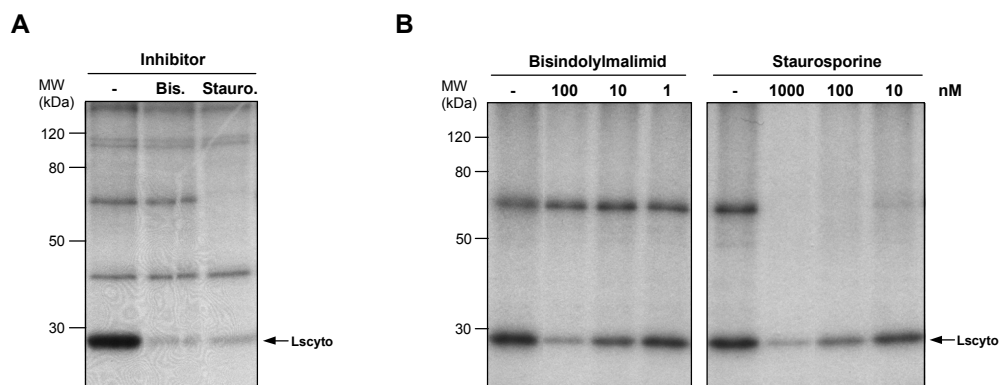
**Fig. 33: The associated kinase activity phosphorylates Lscyto on serine residues.**

(A) Phosphorylation assay using external substrates. Pull-downs from RNase A-treated Jurkat cell lysates were used in phosphorylation assays with additional substrates histone H1 and myelin basic protein (MBP) (5  $\mu$ g/sample). (B) Pull-down precipitates using Lscyto variants that carry inactivating mutations of putative phospho-acceptor sites were subjected to kinase assays. Proteins were separated by SDS-PAGE and analysed by autoradiography (upper panel) and Coomassie staining to ensure comparable amounts of fusion protein (lower panel).

To characterise the specificity of the co-isolated kinase, experiments with external kinase substrates and Lscyto variants that lack the putative serine and/or tyrosine phosphorylation sites were performed (Fig 33). As shown in Fig. 33A, the typical serine/threonine kinase substrates histone H1 and myelin basic protein (MBP) were phosphorylated strongly when added to Lscyto pull-down precipitates, whereas GST samples contain little reactivity towards these substrates.

When Lscyto lacking both serine residues of the cytoplasmic tail were tested in pull-down/phosphorylation assays (Fig. 33B), no phosphorylation of the fusion protein was observed, whereas lack of the C-terminal tyrosine did not significantly reduce phosphorylation. Inactivation of single serine residues demonstrated phosphorylation of both serines, with a preference of the kinase for serine 377, since this mutated fusion protein displays strongly reduced incorporation of radioactive phosphate. Taken together, this results indicate that the kinase activity from cell lysates is exclusively directed against the serine residues in the cytoplasmic sequence of L-selectin and that no tyrosine kinase capable for phosphorylation of L-selectin is present in the pull-down complexes.

To obtain further indications to the identity of the kinase involved in phosphorylation of Lscyto, inhibitor studies were performed using the broad range serine/threonine kinase inhibitor staurosporine and the specific PKC inhibitor bisindolylmaleimide.



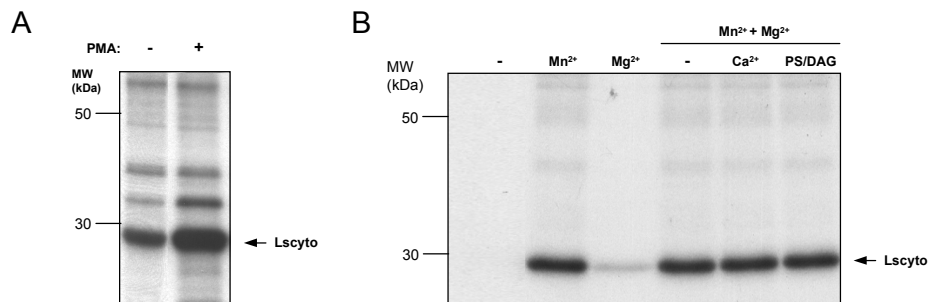
**Fig. 34: The Lscyto kinase is blocked by PKC inhibitors.**

(A) Kinase assays with Lscyto pull-down precipitates was performed in the presence of 1  $\mu$ M bisindolylmaleimide or 50 nM staurosporine or (B) indicated concentrations of staurosporine and bisindolylmaleimide as indicated.

The inhibition studies showed that the phosphorylation of Lscyto was blocked to a large extent by the broad range serine/threonine kinase inhibitor staurosporine as well as the PKC-specific inhibitor bisindolylmaleimide (Fig 34B). The tyrosine kinase inhibitor genistein did not influence the co-precipitated kinase activity (data not shown), consistent with the finding the phosphorylation of the cytoplasmic domain occurred exclusively on serine residues. The PKG inhibitor Rp-8-pCPT-cGMPS also had no effect on Lscyto phosphorylation, showing that this kinase is not the activity associating with the fusion protein, although purified PKG can phosphorylate Lscyto *in vitro*. Further investigation of the effects of staurosporine and bisindolylmaleimide using a range of concentrations (Fig.34C) showed that half-maximal inhibition by bisindolylmaleimide occurs at concentrations between 10 and 100 nM. At this concentration, the inhibitor is thought to be acting exclusively on PKC. Different PKC isozymes display an  $IC_{50}$  ranging from 8 nM (PKC $\alpha$ ) to 200 nM (PKC $\delta$ ) for inhibition by bisindolylmaleimide, whereas an effect on other serine/threonine kinases tested can only be

observed at concentrations in the micromolar range (Toullec *et al.*, 1991; Martiny-Baron *et al.*, 1993). This results was a strong indication, that PKC isozymes are involved in the phosphorylation of the L-selectin cytoplasmic tail.

Interestingly, phosphorylation of the protein migrating close to the 60 kDa marker band displayed a different inhibition pattern compared to Lscyto in the experiments shown in Fig. 34. It was inhibited almost completely even by low concentrations of staurosporine (10 nM) but was not affected by bisindolylmaleimide at concentrations where strong inhibition of Lscyto phosphorylation was observed. This further indicates that the occurrence of this band is not connected to the kinase activity responsible for L-selectin phosphorylation.

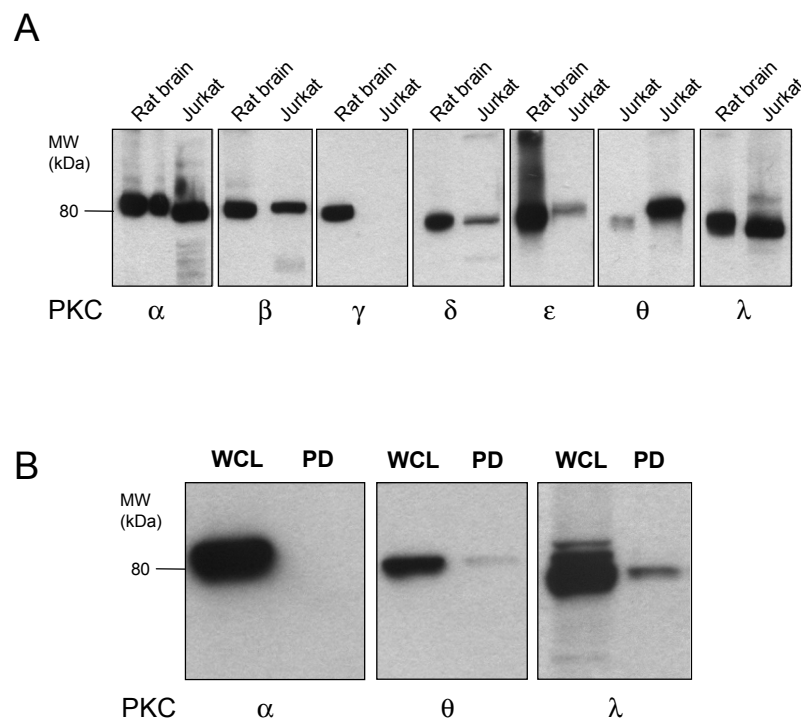


**Fig. 35: Kinase activity is enhanced by pretreatment with PMA, but independent of  $\text{Ca}^{2+}$  and DAG.**

(A) Jurkat T-cells were stimulated with 20 ng/ml PMA for 5 min at 37°C, lysed and used in pull-down experiments. Precipitates were subjected to kinase assays as described before (B) Pull-down precipitates were tested for phosphorylation in the presence of the indicated divalent cations and PKC activators.

The assumption that the L-selectin associated kinase activity is PKC was supported by the increase of Lscyto phosphorylation observed when pull-down experiments were performed from PMA-stimulated cells (Fig. 35A). When the dependence of the L-selectin associated kinase activity for divalent cations was tested,  $\text{Mn}^{2+}$  proved to be a better cofactor for phosphorylation. The activity of the kinase was not enhanced by  $\text{Ca}^{2+}$ , an activator for conventional PKCs, or by the lipid cofactors phosphatidylserine (PS) and diacylglycerol (DAG) (Fig. 35B). This point toward an involvement of atypical or novel PKCs (some of which have been shown to be largely DAG-insensitive) rather than conventional PKCs in L-selectin phosphorylation.

Since the kinase assay experiments using pull-down precipitates pointed towards a role of PKC isozymes in the phosphorylation of the serine residues of the cytoplasmic tail of L-selectin, pull-down eluates were examined for the presence of different isozymes by immunoblotting (Fig. 36).



**Fig. 36: PKC isozymes associate with the cytoplasmic tail of L-selectin.**

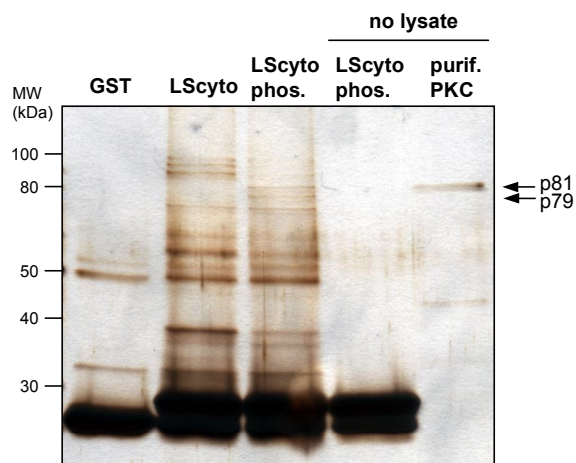
(A) Cell lysates from rat brain and Jurkat cells provided as a positive control by the supplier and Jurkat T-cell lysates were probed for the presence of PKC isozymes with isozyme-specific antibodies. (B) Lscyto pull-downs (PD) from Jurkat cell lysates were washed twice in lysis buffer containing 1 M NaCl. Proteins were eluted in SDS sample buffer, separated by SDS-PAGE together with whole cell lysates (WCL), blotted onto PVDF membrane and tested with antibodies directed against different PKC isozymes.

This assay showed that PKC isozymes theta and lambda were present in eluates of pull-downs with GST-Lscyto. Apart from PKC alpha, theta and lambda, which are highly expressed in the Jurkat T-cell line, blots were also probed with antibodies against PKCs beta, delta and epsilon, which are all detected in the cell lysates examined (Fig. 36A), but are not found in the Lscyto pull-down eluates (not shown). Therefore it can be concluded that the cytoplasmic domain of L-selectin specifically binds to the novel PKC theta and the atypical PKC lambda from cell lysates and that these isozymes are likely to be the Lscyto kinase activity identified by phosphorylation assays with pull-down precipitates. This assumption is supported by the fact, that association of the PKCs is resistant to high salt washing steps as shown for the kinase associated with the fusion protein.

## 2.8. Isolation of phosphorylation-dependent L-selectin binding partners

The previously described results clearly demonstrate that the cytoplasmic domain of L-selectin can be phosphorylated by kinases present in cell lysates. Since this phosphorylation is likely involved in signal transduction events mediated by the adhesion receptor, it was interesting to test whether this

phosphorylation influences protein-protein interactions. To identify protein specifically interacting with the phosphorylated form of the intracellular domain, GST-Lscyto fusion protein was *in vitro* phosphorylated by purified PKC and used in pull-down experiments as described in chapter 2.4. When proteins isolated with non-phosphorylated and phosphorylated Lscyto were compared (Fig. 37), binding of two proteins migrating at approximately 79 and 81 kDa was observed. These were present neither in pull-downs with GST alone or unphosphorylated GST-Lscyto nor in Lscyto *in vitro* phosphorylation samples incubated and washed in parallel to the pull-downs, but without cellular protein added. This indicated that the bands indeed originated from cell lysates rather than from the enzyme preparation added for phosphorylation. Further examinations revealed that the proteins were not eluted by washing with 1 M NaCl, as seen in Fig. 37, and that the association was not sensitive to treatment with RNase A (not shown), indicating that the binding occurred by protein-protein interaction and was not mediated merely by electrostatic interaction.



**Fig. 37: Proteins associate specifically with the phosphorylated cytoplasmic domain of L-selectin.**

Whole cell lysates from Jurkat T-cells were incubated with GSH-Sepharose coated with GST, Lscyto or Lscyto that was phosphorylated *in vitro* with purified rat brain PKC. Beads were washed with 1 M NaCl and eluted with 50 mM ethanolamine pH 11.5. Eluted proteins were analysed by SDS-PAGE and silver staining.

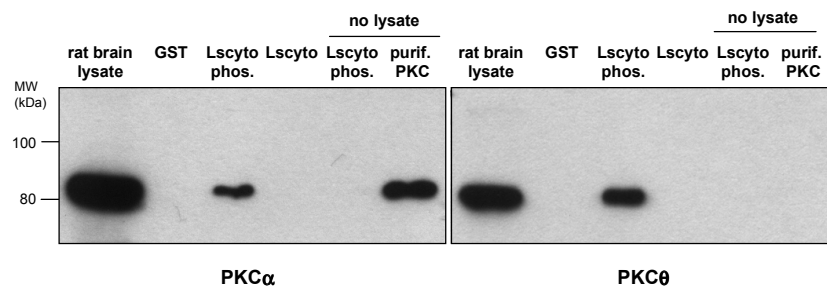
**Tab. 9: Analysis of p82 from pull-down with phosphorylated Lscyto**

Approx. mol. weight	Identified protein	Identified peptide mass	Theoretical peptide mass	Peptide sequence
81 kDa	PKC $\alpha$	1905.78	1905.858	1-18 acADVFPGNDSTASQDVANR
		1772.80	1772.889	181-196 NLIPMDPNGLSDPYVK
		1318.60	1318.643	239-248 LSVEIWDWDR
79 kDa	PKC $\theta$	1896.876	1896.841	115-129 MLMNARYFLEMSDTK
		1777.859	1777.869	287-302 LMAEALAMIESTQQR
		969.547	969.560	367-374 ERPSLQIK
		1038.460	1038.459	593-600 MDNPFYPR

When the 81 kDa band was subjected to electrospray-ionisation (ESI) mass spectrometry (Tab. 9), it was identified to be human PKC alpha. This human enzyme is highly conserved compared to rabbit PKC alpha, which was present in the enzyme preparation used for *in vitro* phosphorylation. However, sequencing of the N-terminal peptide unequivocally showed the human sequence, which differs from the rat isozym in two residues.

The results from mass spectrometry was verified by Western blotting of pull-down eluates which show that PKC alpha is not present in control samples phosphorylated and washed in the same way as the fusion protein used in the pull-down experiment, but without added cell lysates (Fig. 38). This shows that the PKC alpha found in pull-down eluates with phosphorylated Lscyto derived from cell lysates, not from the previous phosphorylation reaction. It also indicates that some additional component, which is present in the cell lysate, but absent from the phosphorylation sample, seems to be important for tight binding of this PKC isoform to Lscyto.

The 79 kDa band was identified as the PKC theta isoform by MALDI-TOF mass spectrometry and peptide mass fingerprinting. As shown by immunoblotting in Fig. 38 and also observed in analysis of silver stained gels (not shown), binding of PKC theta to phosphorylated Lscyto is strongly enhanced as compared to the unphosphorylated protein, where it was only detectable after prolonged exposition (compare Fig. 36). In Fig. 38, exposition time was not sufficient to visualise this signal.

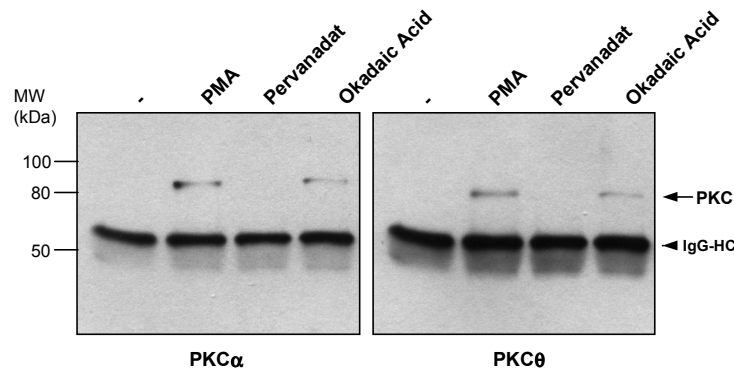


**Fig. 38: Phosphorylated Lscyto interacts with PKC isozymes alpha and theta.**

Pull-down eluates prepared as described for were analysed by Western blotting with antibody specific for PKC $\alpha$  together with whole cell lysate from rat brain and purified rat brain PKC as positive controls.

The assay shown in Fig. 38 also proved that PKC theta was not present in the purified rat brain PKC preparation used for phosphorylation of the fusion protein, excluding carry over of the protein.

*In vivo* phosphorylation of L-selectin on serine residues has been shown by Haribabu *et al.* (1997) to occur in intact cells after stimulation with PMA. To test whether phorbol ester treatment of T-cells leads to an association of cellular PKC isozymes with L-selectin, co-immunoprecipitation experiments were performed (Fig. 39).



**Fig. 39: PKC associates with L-selectin *in vivo*.**

Jurkat cells were stimulated with 100 ng/ml PMA, 100  $\mu$ M pervanadate or 2  $\mu$ M okadaic acid for 3 min at 37°C, lysed in TBS containing 1% NP-40 and L-selectin was immunoprecipitated with 10  $\mu$ g Dreg200 mAb coupled to Protein G Dynabeads. Immunocomplexes were washed four times in lysis buffer and eluted in SDS sample buffer. Proteins were separated by SDS-PAGE, transferred onto PVDF membrane and probed with mAb directed against PKC alpha and theta, respectively. IgG-HC, heavy chain of the immunoprecipitation-mAb.

When L-selectin was immunoprecipitated from PMA-stimulated Jurkat T-cells, both PKC $\alpha$  and PKC $\theta$  could be detected, whereas neither of both isozymes were present in immunoprecipitates from untreated cells. This indicates that, in agreement with the results obtained from pull-down experiments, PKC tightly associates with the cytoplasmic tail of L-selectin in an isotype-specific manner after phosphorylation on serine residues. Interestingly, this association was also observed following treatment with the serine/threonine phosphatase inhibitor okadaic acid, which has been shown to activate cellular PKCs. Phosphatase inhibitors are thought to induce an accumulation of phosphorylated protein, since constitutively occurring phosphorylation events in the cell can not be reversed by the corresponding phosphatase. The finding that PKC associates with L-selectin upon phosphatase inhibition indicates the presence of serine-phosphorylated receptor after treatment with okadaic acid. In contrast, the tyrosine phosphatase inhibitor pervanadate did not effect interaction of the proteins.