4. Material and methods

4.1 General materials

4.1.1 Chemicals

All chemicals were obtained from Sigma (Munich) or Merck (Darmstadt), unless indicated otherwise in the text.

4.1.2 Labware

Disposable plastic labware for culture of bacteria, yeast and eukaryotic cell lines was from BD Falcon, Hamburg, Greiner, Nürtingen or Nunc, Karlsruhe.
Standard laboratory plastic ware was obtained from Eppendorf, Hamburg, BD Falcon, Hamburg, Brand, Wertheim, or Sarstedt, Nürnbrecht. All special items used are included with the methods.

4.1.3 Devices

Devices used in this work belonged to the standard equipment of biochemical laboratories. Special appliances that might be important for the outcome of experiments are stated in the method description.

4.2. Molecular biology methods

4.2.1 Special materials

Vectors

pGEX 2TK, pGEX 4T1 (Amersham Pharmacia Biotech, Freiburg)

The pGEX series of vectors is designed for prokaryotic expression of proteins as fusion products with Glutathion-S-transferase (GST) from *Schistosoma japonicum*. Since GST binds to reduced glutathione with high affinity, the recombinant protein can be easily purified by chromatography using glutathione-coupled sepharose beads.
All pGEX vectors carry a *lacI*^{q} gene coding for the *lac* repressor, which allows expression in all *E. coli* strains. The *lac* repressor ensures tight regulation of the introduced gene, preventing expression in uninduced cells. Induction of the *tac* promoter is achieved by IPTG, which, by binding to the repressor, relieves transcriptional repression.
pGEX 2TK features a thrombin cleavage site for separation of the expressed protein from the fusion tag and a PKA recognition site for *in vitro* labelling of the protein with ^{32}P-γ-ATP.
pGEX 4T1 contains a thrombin cleavage site and an expanded multiple cloning site as compared to pGEX 2TK but lacks the PKA phosphorylation sequence.

pCR 3.1 (Invitrogen, Karlsruhe)

pCR3.1 is a vector for transient or stable expression of genes in eukaryotic cells. The gene of interest is brought under the control of a Cytomegalo virus (CMV) immediate-early promoter, ensuring high level expression. The vector carries both neomycin/kanamycin and ampicillin resistance.

pMPSV –HE FL (Fieger, 1997)

Eukaryotic expression vector containing the complete coding sequence of L-selectin.

pGEMTGαA9 (Dr M. Rothkegel, Braunschweig)

Derivative of the pGEM3Z cloning vector (Promega, Mannheim) containing the full length sequence of the turkey smooth muscle α-actinin.

Oligonucleotides

Oligonucleotides were synthesized by Metabion (Munich), TIB Molbiol (Berlin) and BIG Biotech (Freiburg i. Br.). Primers for automated sequencing and PCR applications were desalted and purified by gel filtration, whereas oligonucleotides intended for mutagenesis or direct cloning were HPLC purified to ensure full length products.

Vector specific primers (for PCR and sequencing)

Primer	Size	Sequence
pACT fwd	27-mer	5'-CGATGATGAAGATACCCCACCAAACCC-3'
pACT rev	28-mer	5'-GCACGATGCACAGTTGAAGTGAACTTGC-3'
pAS fwd	20-mer	5'-TCATCATCGGAAGAGAGTAG-3'
pAS rev	19-mer	5'-AAGCAACCTGACCTACAGG-3'
pCR rev	18-mer	5'-TAGAAGGCACAGTCGAGG-3'
pGEX 5'	19-mer	5'-GCCACGTTTGGTGGTGGCG-3'
T7 primer	17-mer	5'-AATACGACTCACTATAG-3'

L-selectin specific primers (for PCR and sequencing)

Primer	Size	Sequence
Lam BE	23-mer	5'-CAAGAGGATCCATGGGCTGCAGA-3'
Lam F(R)	25-mer	5'-CCCCCTATTTAAAATGGGTCATTCA-3'
Lam F 1012	17-mer	5'-ATGATTAAGGAGGGTGA-3'
Lam F 414	17-mer	5'-GGAGGACTGCGTGGAGA-3'
Lam F 709	17-mer	5'-GCCTTCAGGTGGTCTGA-3'
Lam F142	17-mer	5'-GGAACCGACTGCTGGAC-3'
Lam R 1080	30-mer	5'-CCCAGAGAATGCAGTAACCATGACTGCCAC-3'
Lam R 237	30-mer	5'-GGCAACTAAATCTGTGTAATTGTCTCGGCA-3'
Lam R 480	17-mer	5'-GCAGGCGTCATCGTTCC-3'
Lam R 606	17-mer	5'-GGGCCCATAGTACCCCA-3'
Lam R 832	17-mer	5'-GTGCTGCTAGAGGCTCA-3'
Lam wt/Bam	30-mer	5'-CGCGGATCCCATAGGAGATTAAAAAAAGGC-3'
LS-S377/A	39-mer	5'-TTAATATGGGTCATTCATACTTCTCTTGGCTTTCTTGCC-3'
LS-S380/A	29-mer	5'-TTAATATGGGTCATTCATAGCTCTCTTGG-3'
Tri-LS1	30-mer	5'-CGCGCCATGGGTAGGAGATTAAAAAAAGGC-3'
Tri-LS2	30-mer	5'-CGCGGCGCCGGTAGGAGATTAAAAAAAGGC-3'
Tri-LS3	30-mer	5'-GCTCGGCGCCATATGGGTCATTCATACTTC-3'
Tri-LS4-2	30-mer	5'-GCTCCTGCAGTTAATATGGGTCATTCATAC-3'

Primers specific for other genes (for PCR and sequencing)

Primer	Size	Sequence
Act/Eco fwd	32-mer	5'-TCGAGAATTCCCATGGATCATCACTACGACCC-3'
Act/Xho rev	32-mer	5'-CGCCTCGAGCCCCAAGGTCACTTTCGCCATAG-3'

Oligonucleotides for direct cloning

Primer	Size	Sequence
Lam wt fwd	60-mer	5'-TAGGAGATTAAAAAAAGGCAAGAAATCCAAGAG AAGTATGAATGACCCATATTAACTGCA-3'
Lam wt rev	54-mer	5'-GTTAATATGGGTCATTCATACTTCTCTTGGATTT CTTGCCTTTTTTTAATCTCC-3'

Mutagenesis primers

Primer	Size	Sequence
4T1 S/A fwd	35-mer	5'-GGTTCCGCGTGGAGCCCATTAACCGGAATCCCCGG-3'
4T1 S/A rev	35-mer	5'-CCGGGGATTCCGGTTAATGGGCTCCACGCGGAACC-3'
pGEX-S/A fwd	24-mer	5'-GGTTCCGCGTGGAGCCCATAGGAG-3'
pGEX-S/A rev	24-mer	5'-CTCCTATGGGCTCCACGCGGAACC-3'

E. coli strains

Strain	Properties	Supplier
BL21	Strain derived from *E. coli* B for expression of recombinant proteins. Deficient for the Lon and OmpT protease genes. Lack of these proteases prevents degradation of recombinant proteins.	Stratagene, Amsterdam
BL21 (RIL)	BL21 derivative which carries additional copies of the *argU*, *ileY* and *leuW* tRNA genes, which encode tRNAs that recognize the arginine codons AGA and AGG, the isoleucine codon AUA and the leucine codon CUA which are rare in prokaryotic genes, but frequently used in human sequences.	Stratagene, Amsterdam
DH5α	General purpose cloning strain.	Promega, Mannheim
HB101	This strain an auxotrophic mutation in the *leuB* gene, which can be complemented by the Leu2 selection marker on yeast 2-hybrid library plasmids and is used to separate library and bait vector.	Stratagene, Amsterdam
SURE	This strain displays increased stability of eukaryotic DNA inserts that contain inverted repeats or secondary structures, because it is deficient in the *uvrC* and *umuC* DNA repair systems as well as the SbcC and RecJ recombination proteins.	Stratagene, Amsterdam
TOP10 F'	General purpose cloning strain suitable for blue-white screening, used in the TA-cloning kit	Invitrogen, Karlsruhe
XL-1 blue	General purpose cloning strain suitable for blue-white screening, also used in the QuikChange Mutagenesis kit	Stratagene, Amsterdam

Enzymes

Enzyme	Supplier
Taq polymerase	MBI Fermentas, St. Leon-Roth
Pwo Polymerase	Roche, Mannheim
T4 ligase	MBI Fermentas, St. Leon-Roth
Restriction endonucleases	NEB, Frankfurt a. M.
	MBI Fermentas, St. Leon-Roth
Lysozyme from chicken egg white	Sigma, Munich
RNase A from bovine pancreas	MBI Fermentas, St. Leon-Roth
Calf intestine phosphatase (CIP)	MBI Fermentas, St. Leon-Roth

Markers and standards

Marker	Supplier
25 bp DNA Ladder	Gibco, Karlsruhe
100 bp DNA Ladder	Gibco, Karlsruhe
1 kb DNA Ladder	Gibco Karlsruhe
DNA Mass Ladder	Gibco, Karlsruhe
Low DNA Mass Ladder	Gibco Karlsruhe

Commenly used buffers

TAE	
40 mM	Tris
1 mM	EDTA
	adjusted to pH 8.3 with acetic acid
TE	
10 mM	Tris pH 8.0
0.1 mM	EDTA

4.2.2 Strain maintenance and glycerol stocks

LB medium		Supplier
10 g	Tryptone	Difco/BD, Heidelberg
5 g	Yeast extract	Gibco BRL, Karlsruhe
5 g	NaCl	
1 ml	1N NaOH	
ad 1l	dd H_2O	
	autoclaved and cooled to 55°C before addition of appropriate antibiotics	
LB-Agar plates		
15 g	agar	
1 l	LB-medium	
	autoclaved and cooled to 55°C before addition of antibiotics and pouring of plates	

Antibiotics
Ampicillin: 100 µg/ml final concentration Kanamycin: 50 µg/ml final concentration

For culture of bacterial strains, single colonies were obtained by scraping small amounts of bacteria from glycerol stocks and streaking onto LB agar plates containing the appropriate antibiotic, followed by incubation at 37°C over night. The plates were kept for a maximum of 2 weeks at 4°C before preparing new ones from frozen stocks.
Glycerol cultures were made by addition of 15% final concentration sterile glycerol (Roth, Karlsruhe) to overnight cultures. Glycerol stocks were frozen in sterile tubes and stored at –70°C.
All centrifugation steps involving bacteria were carried out at room temperature using a microcentrifuge (Eppendorf, Hamburg) at 5000 rpm/5 min for volumes up to 2 ml or in a cell centrifuge (Megafuge 2.0R, Heraeus, Osterrode) at 5000 rpm/15 min for larger volumes unless indicated otherwise.

4.2.3 Polymerase chain reaction (PCR)

PCR allows the specific amplification of DNA sequences from a complex mixture by using oligonucleotide primers that bind to flanking regions of the desired sequence. Exponential increase of the amount of insert is achieved by multiple cycles of template denaturation, primer annealing and extension by DNA polymerase, because newly synthesised strands can serve as templates in the next cycle.

Amplification of DNA fragments for cloning

Fragments obtained by PCR can be cloned directly by blunt-end- or TA-cloning (depending on the kind of polymerase used for amplification) Alternatively, PCR primers can be designed to introduce recognition sites for restriction enzymes, which allows directional ‘sticky-end’cloning.
Typical PCR reactions for amplification with Taq-polymerase contained 0.1-0.5 µg template DNA, 1x PCR buffer containing 20 mM $(NH_4)_2SO_4$ provided by the supplier, 2.5 mM $MgCl_2$, 200-400 µM of each nucleotide, 600 µM of each primer and 1-2.5 U Taq-polymerase in a reaction volume of 50 or 100 µl.
For amplification using the ‘proof-reading’ polymerase Pwo, reactions contained 0.1 µg template, 1x Pwo buffer, 300 µM of each primer, 200 µM of each nucleotide and 2.5 U Pwo in 100 µl volume. In this case, the enzyme had to be added last and cycling started immediately since primers may be degraded by the 3’-5’ exonuclease activity of the enzyme.
Annealing temperature was chosen 5-10°C lower than the average melting temperature of both primers calculated by the 4+2 rule (4°C for every complementary G/C, 2°C for every complementary A/T in the primer sequence).
Denaturation took place at 95°C. After an 2 min initial denaturation step, DNA was heated for 30 s during each cycle.
Elongation time was 1 min for inserts up to 2 kb plus 1 min for every additional 2 kb of amplified sequence.
For cloning purposes the number of cycles was kept to a maximum of 30 to prevent accumulation of mutations.

Another elongation step of 5-10 min was added after the last cycle to ensure full length products. 10 µl of PCR reactions were analysed for presence of the correct fragment by agarose gel electrophoresis. The remaining sample was purified and used in cloning experiments.

Colony PCR

PCR was also used to quickly analyse *E. coli* colonies for the presence of the desired insert after cloning. Colonies were picked with a pipette tip, restreaked on fresh plates and remaining bacteria adhering to the tip were transferred to 5 µl 0.5 % Triton X-100. Incubation for 5 min before addition of the PCR reagents facilitated cell disruption during the first denaturation cycle.

Colony PCR Mix:	
2.5 µl	10 x Taq-Puffer
0.5 µl	dNTPs (10 mM each)
1.25 µl	Primer1 (10 µM)
1.25 µl	Primer2 (10 µM)
1 U	Taq polymerase
14 µl	dd H_2O

Cycling conditions		
5 min	94°	
10 sec	94°	
10 sec	55°	35x
10 sec	72°	
2 min	72°	

10 µl of the PCR samples were separated on agarose gels to check for the presence of amplified product.

4.2.4 Restriction

For restriction of DNA, commercially available endonucleases were used according to the manufacturers recommendation. The reactions were carried out in the provided buffers, with one to five units of enzyme per µg DNA. Volumes were kept as small as possible, but were at least 10 times the volume of enzyme added, since glycerol concentrations above 5% can inhibit the activity of the endonucleases. Reactions were terminated by heating to 65 or 80°C (depending on inactivation temperature of enzyme) or by addition of DNA sample buffer for agarose gel electrophoresis.

4.2.5 Agarose gel electrophoresis

DNA sample buffer (6x)	
60 %	sucrose
20 mM	EDTA
0.025 %	Bromphenol blue

Ultrapure agarose for analytical gels was from Gibco BRL, Karlsruhe, for gel-purification of fragments Seakem GTG agarose (Biozym, Hess. Oldendorf) was used.

Agarose was added to 1x TAE to give the desired concentration (0.7-2 %(w/v)), and slowly heated until fully dissolved. After stirring for 5 min, gels were poured in a horizontal gel apparatus (Biorad, München), and left to set with a comb inserted. Gels were immersed in 1x TAE and samples containing 1x DNA loading buffer were transferred into the slots. Electrophoresis was performed at 90-120 V.
After termination of the run, gels were stained by incubation in TAE containing 0.5 µg/ml ethidiumbromide for 10 min, analysed on an UV-transilluminator and photographed.

4.2.6 Generation of oligonucleotide inserts for cloning

Short inserts were cloned using complementary oligonucleotides which, after hybridisation, generated double stranded DNA fragments with terminal overhangs that allowed direct cloning in vectors restricted with suitable endonucleases.
For generation of double stranded inserts, equimolar amounts of complementary oligonucleotides were combined, heated to 95°C for 5 min and slowly cooled to room temperature in a metal heating block to allow correct annealing of the molecules. The annealed oligonucleotides were used in ligation reactions without further purification steps.

4.2.7 Purification of nucleic acids fragments

DNA fragments in solution (PCR or restriction reaction products) were purified using the Qiaquick DNA purification kit (Qiagen, Hilden). The kit was used according to the manufacturers recommendations. Purified DNA was eluted with 10 mM Tris pH 8.0.
Purification of DNA fragments from agarose gels was performed using the Qiaquick gel extraction kit or the Qiaex II gel extraction kit (both from Qiagen, Hilden) according to the manufacturers instructions.

4.2.8 Ligation

For ligation of DNA fragments, T4 ligase and the appropriate buffer supplied by the manufacturer was used. The fragments were added in approximately equimolar concentrations or with threefold excess of insert, the final reaction volume was kept at 10-20 µl. Ligations were incubated at 16°C for at least one hour or over night, heated to 65°C for 20 min to inactivate ligase and transformed into chemically competent bacteria.

4.2.9 TA cloning

The TA cloning system (Eukaryotic TA cloning kit, Invitrogen, Karlsruhe) allows easy cloning of PCR fragments into the pCR3.1 expression vector based on the fact that fragments obtained with Taq polymerase carry single deoxyadenosine overhangs at the 3' ends of the duplex molecule. The TA cloning vectors are supplied in linearised form with single deoxythymidine 5'-overhangs, which allow annealing of the insert and promotes efficient ligation. Since 3'-A overhangs at the end of fragments are removed by the 3'-5' exonuclease activity of Pwo polymerase, creating blunt ends, Taq Polymerase is used for addition of 3' adenines to blunt end fragments after PCR amplification. 1 U of Taq polymerase was added to the reaction and incubated at 72°C for 10 min. Samples

were then directly used in TA cloning or purified for further use. For ligation in TA vectors, 10 ng of vector are incubated with PCR fragment at a 1:1 molar ratio together with ligase buffer and T4 DNA ligase overnight at 14°C. 2 µl of the ligation mix are transformed into chemically cometent TOP10 F' cells according to the manufacturers guidelines.

4.2.10 DNA transformation in E.coli

Preparation of chemically competent bacteria

TSS-buffer	
85 %	LB medium
10 %	PEG 8000 (w/v)
5 %	DMSO
50 mM	$MgCl_2$
87% glycerol	
filter sterilised	

Chemically competent bacteria were prepared according to the method described by Chung *et al*. (1989). *E. coli* were streaked onto LB plates from frozen stock and resulting colonies inoculated for over night cultures in 5 ml LB. After 1:100 dilution in LB, cultures were incubated at 37°C with shaking at 220 rpm until they reached an OD578 of 0.5. Bacteria were chilled on ice for 20 minutes, transferred to centrifuge tubes and sedimented. Pellets from 500 ml suspension were resuspended in 10 ml TSS buffer. After addition of 2 ml of 87 % glycerol the competent bacteria were aliquoted (100 – 200 µl) and frozen in liquid nitrogen. Long term storage was at –80°C.

Transformation of chemically competent bacteria

SOC medium		
20 g	tryptone	
5 g	yeast extract	
0.5 g	NaCl	
ad 1 l	dd H_2O	
	autoclaved, after cooling to room temperature addition of:	
10 ml	1 M $MgCl_2$	(filter sterilised)
10 ml	1 M $MgSO_4$	(filter sterilised)
20 ml	20% glucose	(filter sterilised)

Chemically competent *E. coli* were thawed on ice, mixed with 0.1–1 µg DNA and incubated on ice for 30 min. The transformation mixture was heat shocked at 42°C for 90 sec to induce uptake of the DNA, kept on ice for 2 min. and 0.5 ml of SOC medium was added. After shaking at 37°C for 1 h, a fraction of the transformation according to the expected transformation efficiency was plated on LB plates containing the appropriate antibiotic and incubated overnight at 37° C.

Preparation of electrocompetent *E. coli* HB101

Circle Grow medium	
rich medium for e.g. protein expression; prepared according to the manufacturer's guidelines and autoclaved	Qbiogene, Heidelberg

Fresh colonies on LB-plates were used to inoculate 2 ml over-night-cultures in Circle Grow medium which were diluted to 0.5 l in the same medium the next morning. Bacteria grown to a density of OD578 of 0.5-0.6 were chilled on ice and harvested in a refrigerated centrifuge. Cell pellets were washed one in 0.5 l ice cold H2O, twice in decreasing volumes (250 and 125 ml) 10 % glycerol in dd H2O, finally resuspended in 2 ml 10 % glycerol/dd H2O and frozen in 50 µl aliquots at –80°C.

4.2.11 Transformation of electrocompetent HB101

5x M9 salt solution	
64 g	$Na_2HPO_4 \cdot 7H_2O$
15 g	KH_2PO_4
2.5 g	NaCl
5 g	NH_4Cl
ad 1 l	dd H_2O
	autoclaved before use
M9 minimal medium plates	
20 g	agar
750 ml	dd H_2O
	autoclaved, cooled to room temperature
200 ml	5x M9 salt solution
20 ml	20 % glucose
2ml	1M $MgSO_4$
0.1 ml	1 M $CaCl_2$
1 ml	1M thiamine HCl
0.67 g	amino acid mix lacking leucine (see yeast SD-medium)
1 ml	50 mg/ml ampicillin
4 ml	10 mg/ml proline
	all components filter sterilised or autoclaved

Aliquots of competent cells were thawed on ice, mixed with 1 µg of DNA, incubated on ice for 1 min. and transferred to precooled electroporation cuvettes with 0.2 cm electrode gap. Electroporation was performed using a Gene Pulser Electroporation System II (Biorad, München) set to 2.5 kV, 25 µF, 200 ΩAfter pulsing, cells were immediately mixed with 500 µl SOC medium and transferred to culture tubes. After a recovery period of 1 h at 37°C with shaking, transformants were selected on M9 minimal plates lacking leucine.

4.2.12 Isolation of plasmid DNA

Small scale isolation

Buffer 1
TE containing 10 µg/ml RNase
Buffer 2
0.2 M NaOH 1 % SDS
Buffer 3
3 M potassium acetate pH 5.5

Bacteria from a 1.5-2 ml overnight cultures in LB, containing the appropriate antibiotic to maintain selective pressure on the plasmid, were harvested and resuspended in 300 µl buffer 1. For cell lysis, 300 µl buffer 2 were added and cells lysed for a maximum of 5 min at room temperature. Neutralisation with 300 µl buffer 3 for 10 min lead to precipitation of proteins and denatured genomic DNA, which was removed by centrifugation at 20 000 xg and 4°C for 15 min. The supernatent containing the plasmid DNA was transferred to a new tube containg 700 µl isopropanol and the precipitated DNA sedimented by centrifugation at 20 000 xg for 15 min. After washing with 70 % ethanol, the pellet was dried and resuspended in 10 mM Tris pH 8.0.

Large scale isolation

Larger amounts of DNA were isolated using the Plasmid Midi Prep Kit (Qiagen, Hilden). Alkaline lysis and purification with anion exchage resin columns were performed according to the manufacturers guidelines from 100 ml overnight cultures.

4.2.13 DNA sequencing

DNA sequences were obtained by cycle sequencing using the ABI Big Dye Terminator kit (Applied Biosystems, Weiterstadt), containing AmpliTaq® DNA polymerase, fluorescence-labelled dideoxynucleoside triphosphates and deoxynucleoside triphosphates in an appropriate buffer. Cycle sequencing samples consisted of 1 µg of DNA, 4 µl of the terminator mix and 1 pM sequencing primer in a volume of 10 µl. Cycling conditions were 96°C 1 min, 25x (96°C 10 s, 50°C 5 s, 60°C 4 min). Reaction products were separated from unincorporated nucleotides using Centriflex gel filtration cartridges (Edge Biosystems/Mobitec, Göttingen), and analysed with an ABI prism 310 Genetic Analyser (Applied Biosystems, Weiterstadt)

4.2.14 Mutagenesis of plasmids

Site-specific base changes in DNA constructs were introduced using the QuikChange kit (Stratagene, Amsterdam). For this mutagenesis technique, complementary oligonucleotides containing the desired sequence change and the flanking regions were used to amplify the complete plasmid sequence. Non-mutated parental strands were then digested with *Dpn*I, an enzyme recognising a

4 bp sequence with methylated residues that are not present in the newly synthesised strands. Following transformation in *E. coli*, the nicks present in the plasmids were ligated and the mutagenised vector was isolated and analysed by DNA sequencing.

4.2.15 Expression of recombinant GST-fusion protein

The expression of recombinant GST fusion proteins was performed in the *E. coli* strain BL21(RIL) after initial experiments with other strains. Colonies that carried a vector coding for recombinant protein were inoculated for overnight culture in 10 ml of Circle Grow medium containing the appropriate antibiotic, and incubated at 37°C with shaking at 220 rpm. After dilution to an optical density (578 nm) of 0.1 in Circle grow medium, cultures were grown to an OD_{578} of 0.7-0.8. Expression of GST fusion genes from pGEX vectors, that are under the control of the *tac* promoter, was induced by addition of 1 mM IPTG. After induction, cultures were further grown for two hours before harvesting the bacteria for protein isolation.

4.2.16 Purification of GST-fusion protein

Lysis buffer	
50 mM	Tris pH 8.0
50 mM	NaCl
5 mM	EDTA,
2x	Complete protease inhibitors cocktail
Lysozym	
10 mg/ml in lysis buffer	
Elution buffer	
100 mM	Tris pH 8.0
20 mM	glutathione

Bacterial cultures (0.5-1 l) were harvested by centrifugation. The resulting pellet was washed once with PBS to completely remove medium and resuspended in 1/10 of the original volume in lysis buffer. Lysozyme and Triton X-100 were added to a final concentration of 100 µg/ml and 0.1 % respectively. The lysis mixture was incubated at 30°C for 15 min to destabilise bacterial cell walls by enzymatic digestion and the cells were disrupted by sonication (Branson sonifier, settings: output control: 5, duty cycle: 50 %, 2x 30 s). Insoluble material was removed by ultracentrifugation (120 000 xg, 30 min, 4°C). The supernatant was used as the starting material for protein isolation.
Purification of GST fusion proteins was performed using sepharose beads coated with reduced glutathione (GSH), which binds glutathione S-transferase. 1-2 ml GSH sepharose (Amersham Pharmacia Biotech, Freiburg) were transferred to Econo-Pac columns (Biorad, Munich) and chromatography performed by gravity flow or using a peristaltic pump with flow rates of 0.6-2 ml/min. The matrix was washed with at least 10 vol. TBS to remove ethanol-containing storage buffer before applying the bacterial lysate. After binding of the GST moiety of expressed proteins to GSH the column was washed with TBS-B until no protein was detected in the flow through. Fusion protein was then eluted with 20 mM reduced

glutathione, which competes with the immobilised GSH. Eluted proteins were collected in 2 ml fractions, protein content determined by BCA protein assay and purity was judged by SDS-PAGE and coomassie staining. Fractions containing sufficient amounts of purified protein were pooled and dialysed against TBS to remove glutathione.

4.3. Yeast methods

4.3.1 Special materials

Yeast 2-Hybrid vectors

pAS2-1 (Clontech, Heidelberg)

High-level expression vector for construction of Gal4 DNA-binding region fusion proteins used as a bait in yeast 2-hybrid screening. It confers ampicillin resistance for cloning and propagation in *E. coli* and contains the nutritional marker TRP1 for selection of *Saccharomyces cerevisiae* transformants.

pVA3-1 (Clontech, Heidelberg)

pAS2-1 with murine p53 fused to Gal4 BD. Used as a positive control together with pTD1-1.

pACT 2 (Clontech, Heidelberg)

High level expression vector for generation of fusion proteins with the Gal4 transcription activation domain. It can be used for insertion of known genes for direct interaction testing or for construction of cDNA libraries for interaction screening. This vector contains an Ampr cassette and the LEU2 marker for metabolic selection.

pTD1-1 (Clontech, Heidelberg)

Encodes a Gal4 AD/SV40 large T antigen fusion protein that interacts with p53. Used as a positive control with pVA3-1.

Human Leukocyte Matchmaker cDNA library (Clontech, Heidelberg)

This library was used for conducting 2-hybrid interaction screening. It contains approximately 1,6 x 106 independent clones fused to Gal4 activation domain in the vector pGAD10. The library was supplied in *E. coli* DH10B and was titered and amplified as described by the manufacturer. DNA was isolated with a Plasmid Mega Prep Kit (Quiagen, Hilden)

Yeast strains

Strain	Properties	Supplier
Y190	This *S. cerevisiae* strain is designed for use in 2-hybrid assays. It is deficient for *TRP* and *LEU,* allowing metabolic selection of pAS2-1 and pACT 2 plasmids. The reporter genes *HIS*3 and lacZ are under the control of the Gal4-dependent upstream activating sequence of the Gal1 gene.	Clontech, Heidelberg

Enzymes

Enzyme	Supplier
Lyticase from *Arthrobacter luteus*	Sigma, Munich

4.3.2 Yeast culture and glycerol stocks

YPAD		Supplier
20 g	peptone	Difco/BD, Heidelberg
10 g	yeast extract	Gibco, Karlsruhe
0.1 g	adenine hemisulfat	
ad 0.9 l	dd H_2O	
	pH adjusted to 5.8; autoclaved and cooled to room temperature	
100 ml	20% glucose, sterile	
SD (Synthetic dropout) medium		
7 g	yeast nitrogen base (YNB)	Difco/BD, Heidelberg
0.7 g	amino acid mix	
ad 0.9 l	dd H_2O	
	pH adjusted to 5.6, autoclaved and cooled to room temperature	
100 ml	20% glucose, sterile	
Amino Acid Mix (complete)		
1.2 g	uracil	
2.0 g each	adenine hemisulphate arginine histidine isoleucin lysine methionine serine threonine tyrosine	
3.0 g each	phenylalanine thryptophan	
4.0 g	leucine	
9.0 g	valine	
	For dropout media, one or more of the amino acids were omitted as required	

Agar plates	
15 g	agar
1 l	YPAD or SD- medium
	autoclaved and cooled to 55°C before pouring of plates SD/-Trp/-Leu/-His plates also contained 40 mM 3-AT

Yeast strains were stored long term as glycerol stocks. For generation of stock cultures, colonies were taken from plates and resuspended thoroughly in 0.5 ml YPAD or the appropriate drop-out medium. After addition of 0.5 ml of 50 % glycerol cultures were stored in sterile tubes at –80°C
For recovery from frozen cultures, a small amount was taken from the frozen stock and streaked onto appropriate plates. Plates were incubated at 30°C until colonies appeared (2-4 days). Yeast cultures were stored at 4°C for a maximum of one month before preparation of new plates from glycerol stock.
Yeast liquid cultures were grown at 30°C with shaking at 250 rpm.

4.3.3 Transformation of yeast

Small scale transformation (Gietz and Woods, 1994)

Approximately 25 µl yeast culture per transformation were taken from plates not older than one week and suspended in 1 ml sterile H2O. After pelleting the cells by short centrifugation at top speed in a microcentrifuge, they were resuspended in 1 ml of 0.1 M Lithium acetate and incubated for 5 min at 30°C. Following short centrifugation and removal of the supernatant, 240 µl of 50 % PEG, 36 µl of 1 M Lithium acetate, 5 µl ss-DNA (10 mg/ml sonicated single stranded salmon sperm DNA, (Sigma, Munich)), 1 µg plasmid DNA and water up to 350 µl were added to the pellet. After addition of all components, the pellet was resuspended in the transformation mix by vigorous vortexing and incubated at 42°C for 20 min. Cells were centrifuged, the pellet resuspended in sterile H2O, plated onto appropriate selection plates and incubated at 30°C until colonies appeared.

Large scale library transformation (Gietz and Schiestl, 1995)

For transformation of 2-hybrid cDNA library plasmids into yeast cells already carrying the bait vector, large scale transformation was used. Cells were inoculated in 50 ml SD/-Trp medium for over night culture, diluted to an OD578 of 0.2 in 150 ml YPAD the next day and grown for 3 to 4 hours until cells had doubled twice. Cultures were harvested by centrifugation at 3000 xg for 5 min, washed with dd H2O, and pelleted again. After removal of supernatant, 7.2 ml 50 % PEG 3350, 1.08 ml 1 M lithium acetate, 150 µl ss-DNA, 50 µg library plasmid and 2.05 ml dd H2O were added to the pellet. After resuspension of the cells in the transformation mix by vigouros vortexing, the sample was incubated at 30°C for 30 min followed by a heat shock at 42°C for 30 min. Cell were sedimented by centrifugation, resuspended in dd H2O, transformation efficiency controls plated on SD/-Leu and SD/-Trp/-Leu plates in 1:10 to 1:1000 dilutions and the remaining cells were spread on SD/-Trp/-Leu/-His plates to select for interacting proteins.

4.3.4 β-galactosidase assay

Z buffer	
60 mM	Na_2HPO_4
40 mM	NaH_2PO_4
10 mM	KCl
1 mM	$MgSO_4$, pH 7.0
β-galactosidase assay buffer	
10 ml	Z buffer
27 µl	β-mercaptoethanol
167 µl	X-Gal (20 mg/ml in DMF)

Agar plates containing transformed colonies to be tested for β-galactosidase were covered with a nitrocellulose membrane (Schleicher & Schuell, Dassel). Membrans with adhering colonies were transferred to liquid nitrogen for 10 s, then left at room temperature until completely thawed. The membranes where then placed with colonies facing up on filter paper (Whatman, Rotenburg a. d. Fulda) soaked with β-galactosidase assay buffer containing the β-galactosidase substrate X-Gal, which produces a blue product when metabolised, and incubated at 30°C until colonies turned blue (30 min to over night)

4.3.5 Whole cell lysates of yeast cells

Yeast sample buffer	
60 mM	Tris pH 6.8
10% (v/v)	glycerol
2% (w/v)	SDS
5% (v/v)	β-mercaptoethanol
0.0025%	bromphenol blue

For confirmation of target protein expression in yeast, whole cell lysates were prepared for western blotting according to the method described by Horvath and Rietzman (1994). 1.5 ml of overnight culture were collected by centrifugation and washed once in 1 ml H2O. The pellet was resuspended in 100 µl yeast sample buffer, boiled for 5 min and centrifuged at 20 000 xg for 5 min to remove unsoluble material. 25 µl of cell lysate were used per lane for SDS-PAGE and Western blotting.

4.3.6 Isolation of plasmid DNA from yeast

Plasmid DNA was extracted from 1 ml overnight cultures in SD/-Leu/-Trp/-His. Cells were sedimented by centrifugation at full speed in a microcentrifuge for 5 s, supernatant was removed leaving ca 50 µl, 10 µl lyticase solution added (5 U/µl in TE) and the cell incubated at 37°C with shaking for 1 h to digest cell walls. For lysis of membranes, 10 µl 20 % SDS were added, and disruption was assisted by 1 min of vigorous vortexing. After one freeze/thaw cycle, lysates were brought to a volume of 200 µl with TE and extracted with phenol/chloroform/isoamylalcohol (25:24:1). After thorough mixing and 10 min centrifugation at 20 000 xg the upper aqueous phase was transferred to a new tube and DNA precipitated by addition of 8 µl 10 M ammonium acetate and 500 µl ethanol. After one hour at –

80°C, the DNA was pelleted by centrifugation at 20 000 xg for 20 min, washed once with 70 % ethanol, dried and resuspended in 50 µl TE.

4.4. Biochemical and immunological methods

4.4.1 Special materials

Eukaryotic cell lines

Cell line	Properties
A3.01	Human T cell line, derivative of the CD4+ CCRF-CEM cell line (Folks et al. 1985)
Jurkat	human T cell line established from peripheral blood of an acute lymphoblastic leukemia patient (Schneider et al. 1977).
KG1a	human haematopoietic progenitor cell line established from bone marrow of an acute myelogenous leukemia patient (Koeffler et al. 1980).
K562	human myeloid leukemia cell line (Lozzio and Lozzio, 1977)
K562 LS	K562 stably transfected with pCR3.1 LS (Fieger, 1997)

Antibodies

Primary antibodies

Name/specificity	isotype	Supplier
Dreg 200 anti-L-selectin	mouse IgG1	hybridoma cell line from E.C. Butcher, Dep. of Pathology, Stanford University, USA
Lam1-116 anti-L-selectin	mouse IgG2b	Ancell, Bayport MN, USA
anti-Gal4 AD	rabbit polyclonal	Heike Göhler, MPI für Molekulare Genetik, Berlin
anti-Gal4 BD	mouse IgG	Clontech, Heidelberg
anti-GST	rabbit polyclonal	J. Wienands, MPI für Immunbiologie, Freiburg
4G10 anti-phospho-tyrosine	mouse IgG2b	Upstate Biotechnology, Waltham MA, USA
PY20 anti-phospho-tyrosine	mouse IgG2b	BD Transduction Laboratories, Heidelberg
BM-75.2 anti-α-actinin	mouse IgM	Sigma, Munich
isozyme-specific anti-PKC mAb	mouse IgG1/IgG2	BD Transduction Laboratories, Heidelberg
anti-LAT	rabbit polyclonal	Upstate Biotechnology, Waltham MA, USA

Name/specificity	isotype	Supplier
4E2 anti-nucleolin	mouse IgG1	StressGene, Victory BC, Canada
anti-PHAPII	rabbit polyclonal	Dr. T. Copeland, NCI Frederick, Maryland, USA

Secondary antibodies

Name/specificity	isotype	Supplier
anti rabbit IgG-HRP	goat polyclonal	Dako, Hamburg
anti mouse IgG-HRP	goat polyclonal	Dako, Hamburg
anti mouse IgM-HRP	goat polyclonal	Sigma, Munich

Enzymes

Enzyme	Supplier
Protein kinase A from bovine heart, catalytic subunit	Promega, Mannheim
Protein kinase C from rat brain, mixture of isoforms α, βI, βII and γ	Roche, Mannheim
Protein kinase G from bovine lung, α-isozyme	Promega, Mannheim

Inhibitors

Inhibitor	Target	Supplier
Genistein	tyrosine kinases	Alexis, Grünberg
Staurosporine	serine kinases	Alexis, Grünberg
Bisindolylmaleimide	PKC	Calbiochem, Bad Soden
Rp-8-pCPT-cGMPS	PKG	BioLog, Bremen
Okadaic Acid	PP2A, PP1	Calbiochem, Bad Soden
sodium orthovanadate	tyrosine phosphatases	Sigma, Munich
sodium fluoride	serine/threonine phosphatases	Sigma, Munich
Complete protease inhibitor cocktail	serine and cysteine proteases	Roche, Mannheim

Markers and standards

Marker	Supplier
Benchmark protein marker	Gibco, Karlsruhe
Benchmark prestained protein marker	Gibco, Karlsruhe
Prestained SDS molecular weight standard mixture	Sigma, Munich

Radiochemical

Compound	Specific activity	Supplier
Redivue [γ-^{32}P]ATP	1175,0 Ci/mmol	Amersham Pharmacia Biotech, Freiburg
Express ^{35}S Protein labeling mix	>5000 Ci/mmol	NEN, Cologne

Matrices for affinity Isolation

Matrix	Supplier
GSH-sepharose	Amersham Pharmacia Biotech, Freiburg
Affi-10	Biorad, Munich
High Trap Protein A prepacked column	Amersham Pharmacia Biotech, Freiburg

Commonly used buffers

PBS	
138 mM	NaCl
2.67 mM	KCl
1.47 mM	KH_2PO_4
8.1 mM	$Na_2HPO_4 \cdot 7\ H_2O$
	pH 7.2
PBS+/+	
PBS containing	
0.9 mM	$CaCl_2$
0.5 mM	$MgCl_2$
TBS	
50 mM	Tris
150 mM	NaCl
	adjusted to pH 7.6 with HCl
TBS-B	
TBS containing	
0.2%	Brij 58

4.4.2 Culture of eukaryotic cells

All cell lines were cultured in tissue culture flasks containing RPMI-1640 culture medium supplemented with 1x Glutamax, 100 U/ ml penicillin/streptomycin (all from Gibco, Karlsruhe) and 10% foetal bovine serum (Biochrom, Berlin).
Cultures were grown in a humidified 37°C, 5 % CO2 incubator. Cells were maintained in culture by diluting the suspension every 2 to 3 days with complete RPMI culture medium at a ratio of 1:3 to 1:5.
For long term storage, cells were frozen and kept in liquid nitrogen. Cell suspensions were centrifuged (5 min, 800 rpm), resuspended in 0.5 ml FCS and transferred to sterile cryo vials. After cooling on ice for 30 min, 0.5 ml freezing medium (FCS/20 % DMSO) was added and the vials were frozen in a Qualifreeze freezing device (Nalgene, Karlsruhe), which ensures constant cooling when placed at –70°C over night. Cells were then transferred to a liquid nitrogen storage freezer.
Cryo-conserved cells were thawed rapidly in a 37°C water bath and subsequently resuspended in prewarmed culture medium. After washing with medium to remove residual DMSO, cells were kept at high density for the first passage to ensure good recovery.
Cell number and viability was determined by trypan blue staining. Equal volumes of trypan blue and cell suspension were mixed. The number of unstained (viable) cells was counted in an improved Neubauer Haematocytometer (Brand, Wertheim), which allowed calculation of cell density in the original cell suspension.

4.4.3 Metabolic labelling of eucaryotic cells

Cells in logarithmic growth phase were washed once in methionine- and cysteine-free RPMI-1640 and suspended at 5x 10^6 cells/ml in the same medium containing 10 % FCS dialysed against PBS. After 3 h of culture to deplete endogenous stores of the missing amino acids, 5 µl/ml Express 35S Protein labeling mix containing ^{35}S-labelled cysteine and methionine were added and cells grown over night at 37°C/5 %CO2. Cells were washed three times with PBS before lysis and use in experiments to remove radioactive compounds that were not incorporated.

4.4.4 Whole cell lysate

Lysis buffer	
1 %	detergent (NP-40, Triton X-100 or Brij58)
50 mM	Tris pH 7.6
150 mM	NaCl
1x	Complete protease inhibitors
1 mM	sodium orthovanadate
10 mM	sodium fluoride

Whole cell lysates were prepared by lysis of PBS-washed cells in detergent lysis buffer (1 % in TBS, 1x Complete protease inhibitors, 1 mM sodium orthovanadate and 10 mM sodium fluoride) at a density of 1-5x 10^7 cells /ml. After incubation on ice for 15 min, unsoluble cell debris was removed by centrifugation at 20 000 xg/4°C for 15 min and 4x SDS sample buffer was added to the

supernatant. After heating to 95°C for 5 min, 10-20 µl of the samples were used per lane for SDS-PAGE and Western blotting experiments.

4.4.5 Immunoprecipitation

For immunoprecipitation of L-selectin, 30 µl Protein G Dynabeads (Dynal, Hamburg) were washed twice with PBS+/+/0.1 % BSA and coated with 20 µg primary antibody over night. Unbound antibody was removed by washing as above. Lysates were prepared from 2-5x10^7 Jurkat cells by detergent lysis as described above and centrifuged 2x at 20 000 xg for 15 min to remove unsoluble material. Antibody-coated beads were added to the cell lysates and immunoprecipitation was carried out for 2 h at 4°C on a head-over-tail rotating device to allow antibody binding. Immunocomplexes were washed four times with lysis buffer using a magnetic side-pull device (Dynal, Hamburg) to collect beads after washing steps. Proteins bound to the antibodies were eluted by heating in 1x SDS sample buffer to 80°C for 5 min.

4.4.6 Isolation of L-selectin complexes

Lysis buffer	
0.1 %	Brij96
25 mM	Hepes pH 7.8
150 mM	NaCl
20 mM	EDTA
1 mM	EGTA,
1x	Complete protease inhibitors
1 mM	sodium orthovanadate
10 mM	sodium fluoride

L-selectin-associated protein complexes were isolated using a method adapted from Plopper and Ingber, 1993. It combined subsequent receptor crosslinking and immunoextraction of the receptor and associated proteins by antibodies immobilised on paramagnetic beads. This technique has been used for isolation of focal adhesion complexes, ligation-dependent E-selectin-associated proteins (Yoshida *et al*., 1996) and laterally associated transmembrane receptors (TCR/CD45) (Biffen *et al*., 1994).
For isolation of proteins that complex with L-selectin after receptor crosslinking, 20 µg Dreg200 mAb were immobilised on 100 µl Dynabeads coated with anti-mouse secondary antibody (Dynal, Hamburg). Dreg200-Dynabeads were incubated with 2x10^7 Jurkat cells in RPMI-1640/5 % FCS at 4°C for 1 h with slow rotation to allow antibody binding and stimulated for 5 min at 37°C to elicit cellular responses of receptor crosslinking. Bead-bound cells were recovered using a magnetic side-pull device (Dynal, Hamburg), and lysed in a buffer containing 0.1 % Brij96, an non-ionic detergent, that is known to preserve protein-protein interactions. Immunocomplexes were washed four times in lysis buffer and eluted by heating in 1x SDS sample buffer to 80°C for 5 min.

4.4.7 Preparation of subcellular fractions

Hypotonic buffer	
20 mM	Tris-HCl pH 7.5
70 mM	NaCl
Homogenisation buffer	
20 mM	Tris-HCl pH 7.5
150 mM	NaCl
0.5 mM	EDTA
1x	Complete protease inhibitors
1 mM	sodium orthovanadate
10 mM	sodium fluoride

$1x10^8$-$1x10^9$ A.301 T-cells were harvested by centrifugation and washed once with $PBS^{+/+}$. After resuspension in 1/100 of the original volume hypotonic buffer they were kept on ice for 20 min to cause cell swelling and facilitate mechanical disruption. The cell suspension was sedimented, resuspended in 1/500 of the original culture volume in homogenisation buffer and cells were ruptured by 30 strokes in a dounce homogenizer. The homogenate was first centrifuged at 500 xg for 5 min to remove unbroken cells and nuclei, then at 10 000 xg for 20 min to sediment insoluble cell debris e.g. larger cytoskeletal complexes. The supernatant was subjected to ultracentrifugation at 100 000 xg to separate the membrane microsomes from the cytosolic proteins that remain in the supernatant. The membrane fraction was resuspended in detergent-containing lysis buffer (as in 4.4.4) and sonicated for 10 sec to solubilise membrane vesicles. Insoluble aggregates were removed by centrifugation at 20 000 xg to prevent interference with further experiments. Where indicated, fractions were treated with 100 µg/ml RNase A to dissociate RNA-dependent complexes.

4.4.8 Pull down experiments

Cells were lysed in detergent lysis buffer (as in 4.4.4) containing Complete protease inhibitors to prevent protein degradation, 10 mM NaF and 1 mM Na_3VO_4 to preserve protein phosphorylation. Cell lysates were cleared by centrifugation at 14 000 rpm (Eppendorf) to pellet nuclei and cell debris. Cleared lysates were used as material for pull down experiments.

Indirect method

Preclearing of lysates was performed by addition of 50 µl washed GSH beads and incubating at 4°C for 1 h with constant agitation. After removal of the beads by short centrifugation, GST fusion protein (2.5 µg) was added to the supernatants and rotated for 1 h at 4°C. GST fusion proteins and bound cellular proteins were recovered by addition of 40 µl washed GSH beads and incubation for an additional hour. After washing four times with lysis buffer, bound proteins were eluted by boiling in 50 µl 1x SDS sample buffer.

Direct method

GST fusion proteins were coupled to TBS-washed GSH beads (30-40 µg protein/10 µl beads) by incubation for at least two hours with constant rotation.

After washing once with TBS to remove unbound protein, beads were added to cleared cell lysates (10 µl beads/1x10^7 cells). Binding of cellular proteins was permitted for two hours at 4°C on a rotating device. After washing four times with lysis buffer, proteins were eluted with the desired elution buffer. For elution in small volumes, beads were transferred to disposable spin columns (Mobitec, Göttingen) during washing and elution was performed by incubation with elution buffer for 5 min and short centrifugation to completely recover proteins.

4.4.9 SDS polyacrylamid gel electrophoresis (SDS-PAGE)

4x Stacking gel buffer	
0.5 M	Tris pH 6.8
4x Separating gel buffer	
1.5 M	Tris pH 8.8
Rotiphorese Gel 30 (Roth, Karlsruhe)	
30 %	acrylamide
0.8 %	bisacrylamide
Electrophoresis buffer	
25 mM	Tris
192 mM	glycine
0.1 %	SDS
1x SDS sample buffer	
2 %	SDS
80 mM	Tris pH 6.8
10 %	glycerol
0.005 %	bromphenol blue
2 %	β-mercaptoethanol

Discontinuous SDS polyacrylamid gel electrophoresis was performed according to Lämmli (1970). Seperating gels containing 7.5-15% acrylamide, 1x seperating gel buffer, 0.1% SDS, and 0.05% APS/0,1 % TEMED to induce polymerisation, were poured in a vertical gel apparatus (CBS, Del Mar, CA, USA) and overlayed with isopropanol. After complete polymerisation, isopropanol was removed, a 4.5 % stacking gel was added, a comb inserted and the gel left to set. Following removal of the comb, samples in 1x SDS sample buffer were transferred to the slots and runs were carried out at 90 V for the passage through the stacking gel and 120-150 V for the seperating gel.

4.4.10 Coomassie staining

Staining solution	
0.25 % (w/v)	Coomassie R-250
50 %	methanol
5 %	acetic acid
Destaining solution	
50 %	methanol
10 %	acetic acid

For complete protein staining, SDS-polyacrylamide gels were incubated in the staining solution of at least 30 min or overnight and destained until desired protein bands were clearly visible.

4.4.11 Silver staining

Fix I	
50 %	ethanol
10 %	acetic acid
Fix II	
30 %	ethanol,
0.5 M	sodium acetate
0.2 %	sodium thiosulphate
0.5 %	glutardialdehyde, added freshly
Staining	
0.1 %	silver nitrate
0.01 %	formaldehyde, added freshly
Developing	
2.5 %	sodium carbonate (pH 11.2 with acetic acid)
0.01 %	formaldehyde, added freshly
Stop	
0.05 M	EDTA pH 8.0

SDS-polyacrylamide gels were fixed successively in fixing solution I and II for 25 min. After extensive washing (3x 10 min) gels were incubated with staining solution for 45 min, rinsed twice in dd H_2O and developed until protein bands were clearly visible. Developing was stopped by addition of 0.05 M EDTA and protein gels were dried and kept for further analysis.

4.4.12 Western blot

Blotting buffer		
25 mM	Tris base	
192 mM	glycine	
Ponceau S staining solution		
0.2 %	Ponceau S	
3 %	acetic acid	
Blocking solutions		**Supplier**
1x	Rotiblock	Roth, Karlsruhe
5 %	dry milk in TBS-B	Glücksklee, Frankfurt a. M.
3 %	BSA in TBS-B	PAA, Cölbe

For immunological detection, proteins were transferred electrophoretically (Mini Trans-Blot, Biorad, Munich) to Hybond-C extra nitrocellulose (Amersham Pharmacia Biotech, Freiburg) or Polyscreen PVDF (NEN, Cologne) membranes following SDS-PAGE. Transfer conditions were 250 V, 1 h for 0.75 mm gels, 2 h for 1.5 mm gels. Efficiency of blotting was checked by reversible Ponceau S staining. Nonspecific binding sites on the membranes were saturated by incubation with blocking solution (choice of blocking solution was dependent on the Ab used) for at least 1 h, followed by addition of primary antibody in blocking

solution. After incubation for 2 h or over night, blots were washed 3 times with TBS-B to remove unbound antibody and incubated with secondary Ab coupled to horseradish peroxidase (HRP) for 45 min. After washing, bound antibody was detected using the Enhanced Chemoluminiscence (ECL) kit (Amersham Pharmacia Biotech, Freiburg). Emited light signals were detected by exposure to Biomax X-Ray films (Kodak, Cedex, France)

4.4.13 Generation of polyclonal antibodies

Immunisation

For induction of a polyclonal antisera against the extracellular domain of L-selectin, affinity purified protein from BHK cell supernatants was used. 75 µg of protein in 150 µl of PBS was mixed with the same volume of MPL+TDM+CWS (Monophosphoryl lipid A+Trehalose dicorynomycolate+cell wall skeleton; Sigma, Munich) as adjuvant and injected subcutaneously. Further immunisations were performed at approximately two month intervals using 75-100 µg protein with adjuant. Preimmunsera and small volumes of blood were drawn from the ear vene, larger volumes of blood were obtained by heart punction.

Antisera against the cytoplasmic domain were generated at the Institut für Zell- und Molekularbiologie, Schering AG, Berlin, against a KLH-coupled peptide containing the ten C-terminal amino acids of the L-selectin sequence and a N-terminal cystein residue for coupling.

Purification

Serum was obtained from whole blood by coagulation over night at 4°C and centrifugation at 16 500 xg. Supernatant containing the immunglobulins was aliquoted and stored at –20°C.

For isolation of the IgG fraction from serum, low pressure affinity chromatography with a protein A column (5 ml High trap protein A prepacked column, Amersham Pharmacia Biotech, Freiburg) was performed. 30 ml serum was dialysed against 20 mM phosphate buffer pH 7.0, diluted to 50 ml and applied to the equilibrated column at a flow rate of 0.6 ml/min. After extensive washing with phosphate buffer bound IgG was eluted with 200 mM glycine pH 2.8 and neutralised with 60 mM Tris pH 9.0. Fractions were analysed by SDS-PAGE, pooled where appropriate, and dialysed against PBS. Protein content was determined by BCA (Pierce, Bonn) and specificity of IgG fractions was checked by Western blotting using whole cell lysates from L-selectin expressing cells.

Lscyto specific antibodies were further purified from protein A-isolated IgG fractions by affinity chromatography with a matrix displaying the peptide used for immunisation. 15 µM (18 mg) peptide solubilised in DMSO was coupled to 1 ml Affi-10 matrix (Biorad, Munich) for 4 h at room temperature. The matrix was transferred to a Econo-Pac column (Biorad, Munich) and washed extensivly with isopropanol and PBS before use. The purified IgG fraction was applied to the column, washed with PBS, eluted and analysed as described above.

4.4.14 Determination of protein concentrations

Protein concentrations were determined using the BCA (Pierce, Bonn) or Bradford assay (Sigma, Munich), depending on the buffer composition of the protein solution. BCA is more compatible with detergents, whereas Bradford tolerates higher concentrations of reducing agents. All tests were performed in 96-well format with 10 µl of protein solution and 200 µl reagent, which yields a range of 0.1-1 mg/ml. Absorptions were read using a Spectramax (Molecular Devices, München) at 562 nm for BCA and 595 nm for Bradford.

4.4.15 Mass-spectrometric analysis of proteins

Protein band were excised from coomassie-stained SDS-polyacrylamide gels and tryptic digests of the proteins were subjected to analysis by MALDI-TOF (matrix-assisted laser desorption/ionisation-time of flight) or ESI (electrospray ionisation) mass spectrometry. Mass spectrometry was performed by G. Grelle and Dr. E.-C. Müller at the Max-Delbrück-Center, Berlin-Buch and Dr. I. Bahr at the Department of Protein Chemistry at Schering AG, Berlin.

4.4.16 In vitro kinase assays

With purified kinases

10x PKA buffer	
200 mM	Tris pH 7.5
1 M	NaCl
120 mM	$MgCl_2$
10x PKC buffer	
200 mM	Tris pH 7.5
100 mM	$MgCl_2$
10 mM	$CaCl_2$
10x Lipid Mix	
1 mg/ml	1,2-dioleoyl-sn-glycerol in chloroform
5 mg/ml	L-α-phosphatidyl-L-serine in chloroform
	were dried under vacuum and resuspended in
10 mM	Chaps,
10 mM	Tris pH 7.5
	at a concentration of 0.2 and 1 mg/ml respectively.
10x PKG buffer	
400 mM	Tris pH 7.4
200 mM	Mg-acetate
Phosphorylation stop buffer	
10 mM	sodium phosphate buffer pH 8.0
10 mM	sodium pyrophosphate
10 mM	EDTA

Kinase assays with purified kinases were performed in 50 µl volume containing 1 µg substrate protein, 1x kinase buffer, 5 µCi [γ-^{32}P]ATP and 10-50 U kinase. Additional components were added depending on the requirements of the

individual kinase for optimal activity: 1.2 mM DTT (PKA), 5 µl lipid mix (PKC) and 2 µM cGMP (PKG).
Phosphorylation was allowed to take place for 20 min at room temperature and was stopped by addition of 450 µl phosphorylation stop buffer. GST fusion proteins were recovered by binding to 30 µl washed GSH sepharose beads at 4°C for 30 min with gentle agitation. The beads were washed four times with stop buffer containing 1 % Triton X-100 and eluted by addition of 30 µl 2x SDS buffer.

Kinase assays with pull down complexes

Universal kinase buffer	
20 mM	Hepes pH7.4
100 mM	NaCl
5 mM	$MgCl_2$
5 mM	$MnCl_2$

To test for kinases associated with GST fusion protein, the bound proteins from direct pull down experiments were subjected to kinase assays by providing the appropriate buffer for kinase activity and radioactively labelled ^{32}P-γ-ATP. Phosphorylation was performed in 50 µl kinase puffer containing 5 µCi ^{32}P-γ-ATP for 20 min at room temperature. Reactions were terminated by addition of 450 µl phosphorylation stop buffer and washing several times with stop buffer containing 0.1 % Triton X-100, until negative controls showed no residual radioactivity. Protein complexes were dissociated by addition of 50 µl 1x SDS-buffer or elution buffer, resolved by SDS-PAGE and phosphorylated proteins were detected by autoradiography.