FUNCTIONAL ELUCIDATION OF PAG THROUGH THE GENERATION OF TRUNCATION AND POINT MUTANTS

Dissertation

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Everybody knows who has had the possibility to experience the feeling of being a PhD student that there are days and weeks when everything goes wrong, the DNA resists the mutagenesis, Mycoplasma attack the cell culture and the assay results are everything but clear. These dark periods highlight the importance of a supportive background which luckily I had: I cannot be grateful enough for my husband, *Vilmos* for keeping my motivation alive and drawing me out each time from the depths of frustration. He was my rescuer also when my technical inability hindered me in measuring or analysing data. I am also very thankful for my *parents and brother* who always showed interest in my work giving me the impression that it has relevance and the everyday fight is worthwhile. Many thanks go to my friends, *Betty and Zsolt* who were always there for me to share good and bad news and had supportive words when I needed them.

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ABSTRACT

PAG, the phosphoprotein associated with GEMs (glycosphingolipid-enriched microdomains) is a ubiquitously expressed transmembrane adaptor protein that negatively regulates Src-family kinases via the phosphotyrosine-dependent recruitment of Csk to the GEMs. Csk is a cytoplasmic protein tyrosine kinase that phosphorylates the C-terminal inhibitory tyrosine of Src kinases. Upon TCR engagement, PAG becomes dephosphorylated by a yet unknown phosphatase thus displacing Csk from the membrane and thereby enabling the activation of Src kinases (Fyn, Lck). Since Fyn is responsible for the majority of PAG phosphorylation, this provides a feed back loop, as the rephosphorylation of PAG recruits Csk to the membrane to inhibit the Src kinases.

The aim of my thesis was to gain a better understanding of the regulatory function of PAG by studying the role of various motifs in GEM localisation and Fyn binding.

Palmitoylation at the juxtamembrane CxxC motif is thought to target transmembrane adaptors into the lipid rafts. We have therefore studied the effect of a C$\rightarrow$A mutation at this site within PAG in the Jurkat T-cell line, using both biochemical and functional assays. While the CxxC mutant is localized to the membrane, it does not target to the GEMs. However it becomes Tyr phosphorylated, binds Fyn, EBP-50 and recruits Csk to the membrane, similar to wt PAG. To monitor the effect of displacing PAG from the lipid rafts we studied the effects of the overexpressed palmitoylation mutant on CD3 and SDF-mediated signalling and as functional read outs we present proximal signalling studies, calcium flux measurements and migration assays. Migration assays show clear increase in migratory response of the cells overexpressing the CxxC mutant. Experiments obtained with siRNA further strengthen these data as suppression of PAG also leads to increased migration.

Fyn is the kinase responsible for phosphorylation of PAG, but unlike Csk and EBP-50, the site of interaction between Fyn and PAG is still unknown. To study the mechanism of Fyn binding, several truncation and site directed mutants needed to be generated. Identification of the Fyn binding site should help us to better understand how the kinase interacts with its target protein and by mutating the site of interaction; we can study whether direct association is necessary for PAG phosphorylation and whether Fyn has an additional adaptor function.

REAGENTS AND RECIPES

Vectors

pGEM-5Zf(+) (Promega)
pEF-IRES (1)
pEF-BOS (2)
pCMS3-H1p-EGFP (D.D.Billadeau, Mayo Clinic, Minnesota, USA)

Enzymes

T4 Polynucleotide Kinase 10U/µl (NEB)
DNA Polymerase I Large (Klenow) fragment 5U/µl (NEB)
T4 DNA ligase 400U/µl (NEB)
Taq DNA polymerase (NEB)
Deoxynucleotide (dNTP) Solution Mix (NEB)
Glycogene (Bioline)
Aat II (NEB)
Nco I (NEB)
Nhe I (NEB)
Not I (NEB)
Sma I (NEB)
Bgl II (NEB)
Hind III (NEB)

Chemically competent bacteria

TYM media
2% (w/v) tryptone peptone (DIFCO)
0.5% (w/v) yeast extract (DIFCO)
0.1M NaCl (Roth)
10mM MgSO4 (Roth)

TFB1 media
30mM potassium acetate (Roth)
50mM MnCl2 (Sigma)
100mM KCl (Roth)
10mM CaCl2 (Merck)
15% (w/v) glycerin (Sigma)

TFB2 media
10mM Na-MOPS pH 7.0 (Roth)
75mM CaCl2 (Merck)
10mM KCl (Roth)
15% (w/v) glycerin (Sigma)

Bacterial culture

LB Broth (Sigma)
LB Agar (Sigma)
Ampicillin (Sigma)
IPTG (bts)

X-gal (Promega)

SOC media
20g tryptone peptone (Roth)
5g yeast extract (Roth)
10ml 1M NaCl (Roth)
2.5ml 1M KCl (Roth)
900ml H_2O
Autoclave the solution and cool it down to RT. Then add the rest of the components:
10ml 1M $MgCl_26xH_2O$ (filtered, Sigma)
20ml 1M glucose (filtered, Sigma)

DNA preparation

MiniPrep Kit (Nucleo Spin Plasmid, Macherey-Nagel)
MaxiPrep Kit (Nucleo Bond PC500EF Macherey-Nagel)
Nucleo Spin Extract Kit (Macherey-Nagel) for elution of DNA fragments from agarose gel
OD was measured with: Ultrospec 3000 (Pharmacia Biotech)

Agarose gel

50xTAE buffer (for 1 l)
242g Tris base (Roth)
57.1ml acetic acid (Baker)
100ml 0.5M EDTA (Sigma) pH 8.0

Agarose gel 1% (for 100ml)
1g agarose (BioRad)
100ml TAE buffer
10µl etidium bromide (Roth)

DNA loading buffer, blue (Bioline)

Markers
HyperLadder I, V (Bioline)
GeneRuler 50bp DNA Ladder (Fermentas)

Cell culture

RPMI 1640 (Biochrom)
Dulbecco´s MEM (Biochrom)
FCS (Fetal Bovine Serum, PAN Biotech)
PBS w/ Ca^{2+}, Mg^{2+} (Biochrom)
PBS w/o Ca^{2+}, Mg^{2+} (Biochrom)
DMSO (dimethyl-sulfoxide, Fluka)

Flow cytometry

Blocking buffer
0.1% Na-aside (Roth)
0.1% saponin (Sigma)
0.2% BSA/c (1:500) (Aurion)
10mg/ml BSA/PBS

Fixative/permeabiliser
3.7% paraformaldehyde (Sigma)
0.2% saponin (Sigma)
in PBS

Washing buffer
Like blocking buffer but in 1xPBS

Microscopy

Trypan blue (Sigma)
BSA/c (Aurion)

Fixative (for 30 ml) for Jurkat cells
8.7ml 12% paraformaldehide (final: 3.5%) (Sigma)
21.3ml 1xPBS (Gibco)

Fixative (for 30 ml) for adherent cells
10ml 12% PFA (final: 4%) (Sigma)
20ml 1xPBS pH 7.4
6µl glutaraldehide (final 0.02%) (Sigma)

Permeabilisation solution
0.1% Triton X-100 (Packard) in PBS

Embedding-medium
2.45ml glycerin for fluorescent microscopy (Merck)
2.45ml Vectashield (Vector Laboratories)
100mg Dabco (= 1,4 diazabicyclo-octane, Sigma) in 100µl PBS (pH 8.9)

Lysates and IPs

Lysis buffer
500mM Hepes (N-2-hydroxyethyl-piperazin-N-ethansulfon-acid, Serva)
100mM NaCl (Roth)
1% NP-40 (Nonidet P-40, Igepal, Sigma)
5mM EDTA (Sigma)
1mM Na-monovanadate (Merck)
50mM NaF (Sigma)
10mM Na-pyrophosphate (Sigma)
1mM PMSF (=phenylmethylsulfonylfluride, Sigma)
1% LM (=lauryl maltoside, Calbiochem)

NP-40 washing buffer
1% 0.5M EDTA (Sigma)
5% 1M Tris pH7.4 (Roth)
5% NP-40 (Igepal, Sigma)
15% 1M NaCl (Roth)

5x non-reducing sample buffer-NRSB (for 10 ml)
50% glycerol (Sigma)
330mM Tris, pH 6.8 (Roth)
10% SDS (Calbiochem)

0.01% bromphenol blue (Roth)

5x reducing sample buffer
1ml NRSB
50μl 2-mercaptoethanol (Sigma)

Others
PBS Dulbecco's (Gibco)
PP2 (Calbiochem)
BSA (albumine, bovine serum, fraction V, Sigma)
Anti-FlagM2-agarose (Sigma)
Flag-peptide (Sigma)

Gel electrophoresis and Western blot

SDS-PAGE gel (for 10 ml, 10% separating gel)
4.7ml water
2.5ml 40% acrylamide/BIS (Bio-Rad)
2.6ml 1.5M Tris-HCl pH8.8 (Roth)
0.1ml 10% SDS (Calbiochem)
0.1ml 10% APS (ammoniumperoxidisulphate, Roth)
0.004ml TEMED (Roth)

SDS-PAGE gel (for 3 ml stacking gel)
2.19ml water
0.375ml 40% acrylamide/BIS
0.375ml 1M Tris-HCl pH6.8
0.03 10% SDS
0.03 10% APS
0.003ml TEMED

ProSieve gel (for 10 ml, 12%)
4.9ml water
2.4ml ProSieve 50 gel solution (Cambrex)
2.5ml 1.5M Tris-HCl pH8.8 (Roth)
0.1ml 10% SDS (Calbiochem)
0.1ml 10% APS (Roth)
0.004ml TEMED (Roth)

Transblot solution (for 1l)
5.80g Tris (Roth)
2.90g glycine (Roth)
0.37g SDS (Calbiochem)
200ml methanol (Roth)

10xTBS buffer (for 1l)
80.0g NaCl (Roth)
2.0g KCl (Sigma)
30.0g Tris (Roth)
pH 8.00

Stripping buffer (for 200ml)
40.0ml 10% SDS (Calbiochem)
1.4ml 2-mercaptoethanol (Sigma)
25.0ml 0.5 M Tris, pH 6.7

10x electrophoresis buffer for ProSieve gel (for 1l)
121g Tris Base (Roth)
179g Tricine (Roth)
100ml 10% SDS solution

Others
10xTGS - electrophoresis buffer for SDS-PAGE gel (Bio-Rad)
Ponceau S solution (Sigma)
Tween-20 (Roth)
Protein MW standard (Page Ruler Prestained Protein Ladder, Fermentas)
ECL Kit (Amersham)

Preparation of GEM fractions

Sucrose (Sigma)

Lysis buffer
50mM Hepes, pH 7.4 (N-2-hydroxyethyl-piperazin-N-ethansulfon-acid, Serva)
100mM NaCl (Roth)
3% Brij 58 (Pierce)
5mM EDTA (Sigma)
1mM Na-monovanadate (Merck)
50mM NaF (Sigma)
10mM Na-pyrophosphate (Sigma)
1mM PMSF (=phenylmethylsulfonylfluride, Sigma)

MNE buffer, pH 6.5
25mM MES (Sigma)
5mM EDTA (Sigma)
150mM NaCl (Roth)

Kinase Assay

[γ32P] ATP 3000Ci/mM (Amersham)
Enolase (Sigma)
Kinase buffer
50mM Tris-HCl pH 7.5
10mM MnCl2 (Sigma)
0.1% NP-40 (Sigma)

Migration Assay

Human SDF-1α (TEBU)
Transwell inserts 5μm pore size (Costar)

Assay medium (in RPMI)
20mM Hepes buffer (Biochrom)
1% BSA (Sigma)

METHODS

Oligo phosphorylation

Aat II-CD8/Flag-*Nco* I oligonucleotides (synthesised by Thermo electron corporation):

w: 5' catggccttaccagtgaccgccttgctcctgccgctagccttgctgctccacgccgccaggccggactacaaggac gacgatgacaaggc 3'
rev: 5' catggccttgtcatcgtcgtccttgtagtccggcctggcggcgtggagcagcaaggctagcggcaggagcaagg cggtcactggtaaggccatgacgt 3`

The oligos have been dissolved to a final concentration of 2µg/µl at 37°C, 15min. Then 12.5µl from both oligos were mixed with 3µl T4 DNA ligase buffer and 2µl polynucleotide kinase and incubated at 37°C overnight. The mixture was shortly centrifuged (10sec, 14000rpm, RT), 1 volume (30µl) dH_2O and 2 volumes (60µl) of phenol/chloroform/isoamylalcohol (25:24:1) were added to it. Following 1min centrifugation on 14000rpm, at RT the upper phase was transferred to a new tube and the DNA was precipitated by adding 1.5µl glycogen, 1/10 volume co-precipitation buffer (Bioline) and 2 volumes of cold absolute ethanol (96%, -20°C). Precipitation was carried out at –70°C for 30min and was followed by 15min centrifugation (14000rpm, 4°C). Pellet was washed with 200µl 70% ethanol, dried for 10min and resuspended in 25µl dH_2O.

Ligation

a) Vector (pGEM-5Zf *Aat* II/*Nco* I digested, alkaline phosphatase treated): 40ng
Insert (phosphorylated CD8-Flag oligonucleotide): 1.5µg
b) Vector (CD8-Flag/pGEM *Nco* I/*Not* I digested, alkaline phosphatase treated): 40ng
Insert (PAG *Nco* I/*Not* I digested): 80ng
c) Vector (pEF-IRES *Sma* I/*Not* I digested, Klenow and alkaline phosphatase treated): 60ng
Insert (CD8-Flag/PAG *Aat* II/*Not* I digested, Klenow treated): 120ng

T4 DNA ligase (NEB): 1µl (400U)
T4 DNA ligase buffer (NEB): 1/10 volume
BSA: 0.3µl
The mixture was incubated for 10min at RT.

When blunt end ligation was carried out hexamino-cobalt-cloride (Sigma) was added to the reaction up to 10µM final concentration and was incubated for 2 hours at RT.

Site directed mutagenesis

QuikChange Mutagenesis Kit (Stratagene) was used according to the manufacturer's instructions.

Primers used to generate C37,40S double mutant of N-terminally Flag-tagged PAG:

SxxS fw: 5´cttcctcatcttcctgt**c**ctctagtt**c**tgacaggg 3´
rev: 5´ccctgtca**g**aactagag**g**acaggaagatgaggaag 3´

Primers used to insert stop codons into the sequence of N-terminally Flag-tagged PAG:

STOP1 fw: 5´acagcatagtggggaccat**t**agaacctgatgaacgtgc 3´
rev: 5´gcacgttcatcaggttct**a**atggtccccactatgctgt 3´

STOP2 fw: 5´gatctgctggattcc**t**aggacagcacagggaaac 3´
rev: 5´gtttccctgtgctgtcct**a**ggaatccagcagatc 3´

STOP3 fw: 5´cgtcaaagtgttaatgta**t**agagtatccttggaaattc 3´
rev: 5´gaatttccaaggatactct**a**tacattaacactttgacg 3´

STOP4 fw: 5´gctactgttaaagacttc**t**aaaaaactccaaacagcac 3´
rev: 5´gtgctgtttggagttttt**a**gaagtctttaacagtagc 3´

Primers used to generate P134A, P137A double mutant at the membrane proximal proline rich region of PAG:

PxxP fw: 5´cgggagctg**g**ccagaatc**g**ctcccgagagc 3´
rev: 5´gctctcgggag**c**gattctgg**c**cagctcccg 3´

Primers used to generate Y105F mutant of PAG:

Y105F fw: 5´ggacgtacgtcgtaa**a**gctccttcaggtctg 3´
rev: 5´cagacctgaaggagc**t**ttacgacgtacgtcc 3´

Primers used to generate T430A mutant within the EBP-50 binding motif:

T→A fw: 5´gcaaggcagagatatt**g**ccaggctctagcaacc 3´
rev: 5´ggttgctagagcctgg**c**aatatctctgccttgc 3´

Digestion of DNA with restriction enzymes

Single digestions were carried out in 20µl end volume, double digestions in 50µl. Reactions were supplemented in each case with 0.3µl or 0.6µl BSA, respectively. The optimal amount of restriction enzymes for proper digestion of certain DNAs was adjusted: 4U *Aat* II/µg DNA, 2U *Nco* I/µg DNA and 3U *Not* I/µg DNA).

In the case of double digestions of the pGEM vector, 4U / µg DNA was used from both enzymes (*Aat* II, *Nco* I). For cutting pEF-IRES 1U *Sma* I and *Not* I / µg DNA was sufficient.

Klenow treatment of DNA fragments

The DNA fragments have been extracted from the gel before blunt end ligation and alkaline phosphatase treatment. 440ng of digested and gel extracted DNA or 5µg of digested DNA was treated with Klenow enzyme. 33µM dNTP and 1/10 volume buffer 2 (NEB) was added and the reaction was carried out at 25°C for 15min. The enzyme activity was blocked by incubating the reaction for 20min at 75°C.

When alkaline phosphatase treatment was required, 2µl (2U/µl) alkaline phosphatase was added to the Klenow treated DNA (following the inactivation of the Klenow enzyme) and was incubated for additional 1 hour at 37°C.

Preparation of chemically competent cells

A single bacterial colony was picked and grown in 20ml TYM media for 5 hours (OD 0.2-0.8). Then 2.5ml of bacterial suspension was inoculated into 100 ml TYM media and cultured until OD 0.2-0.8 was achieved (ca.1-2 hours). 500ml media was added and the bacteria were further cultured to OD 0.6 (ca.1 hour). Prior to pelleting the bacteria (20min, 4200rpm, 4°C) the culture was cooled down to 4°C with constant shaking. Bacteria were carefully resuspended in 100ml ice-cold TFB1 and centrifuged for 8min. The pellet was resuspended in 20ml ice-cold TSB2 and aliquoted. Aliquots were stored at -70°C.

Transformation of chemically competent E.coli

50-100µl chemically competent bacteria were added to the ligation reaction and the mixture was kept on ice for 15min. This was followed by the heat shock step at 42°C for 1min and 10min on ice. After adding 250µl SOC media, bacteria were grown for at least 1 hour at 37°C, under constant shaking at 300rpm. Then they were spread onto prewarmed LB agar amp^+ plates and grown overnight at 37°C.

PCR (for screening the colonies after ligation)

Primers

Primer 1: 5'- ccgatgtctggacttcctcg -3'

Primer 2: 5'- gctgctccacgccgccag -3'

Primer 3: 5'- tgtgacagggaaaagaagccgcga -3'

Reaction:

primer 1	2µl (100µM)
primer 2	1µl (100µM)

primer 3	1µl (100µM)
Taq polymerase	0.2µl (4U/µl)
buffer	1/10 volume
dNTP	1µl (10mM stock)
DMSO	1/10 volume
H_2O	up to 50µl

To each reaction 1 bacterial colony or 100ng DNA was added.

Program: 95°C	5’	
30 x	95°C	1’
	50°C	1’
	72°C	1’
72°C	10’	
4°C	∝	

Constructs

The following constructs were used in this study: FLAG-TRIM/pEF-BOS (3), FLAG-PAG/pEF-BOS (4), and FLAG-C37,40A-PAG/pEF-BOS. The PAG CxxC mutant construct was generated using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufactures instructions with wild type C-terminally FLAG tagged PAG as the template. Lck-CFP was kindly provided by Dr. Wolfgang Paster (Medical University, Wien, Austria).

Cell culture

Jurkat T-cells (human peripheral T-cell leukaemia cell line) (Kab14 and JCam1.6) were maintained in RPMI 1640 medium supplemented with 10% FCS at 37°C, 5% CO_2 in a humidified atmosphere. HEK 293T cells (human transformed primary embryonal kidney cell line) were maintained in Dulbecco´s modified Eagle medium (DMEM) supplemented with 10% FCS.

Transfection

Jurkat T-cells ($20x10^6$ at a density of $2\text{-}4x10^5$ cells/ml) were centrifuged (1200rpm, 5min, RT) and the pellet washed once in PBS w/ Ca^{2+}, Mg^{2+}. Following washing, the cells were resuspended in PBS w/ Ca^{2+}, Mg^{2+}. DNA (30µg) was added to the cell suspension and electroporated (BTX cuvette gap size 4mm, Qbiogene) (230V, 950µF) using a Bio-Rad Gene Pulser II. After removing the DNA aggregates from the cuvette, transfected cells were added to 40ml medium and cultured for 20-24 hours at 37°C.

Flow cytometry

Intracellular staining: $0.5x10^6$ cells were centrifuged for 5min, 1200rpm, at 4°C. The pellet was resuspended in 100µl Fix/Perm solution and incubated for 15min at RT. Fixed and permeabilised cells were washed. Following a blocking step (15min in blocking buffer) the cells were incubated with primary antibody for 45min at RT (dilutions made in blocking buffer). After two additional washing steps, secondary antibody was added (dilutions made in blocking buffer) and incubated for 30min at RT. Again the samples were washed twice and finally the cells were resuspended in 400µl PBS and measured with FACS Calibur machine [BD]. Data were analyzed using the Cell Quest Pro Software package.

Extracellular staining: $0.5x10^6$ cells were washed with PBS as described above, without fixation and permeabilisation the cells were incubated with primary antibody (dilutions made in 1xPBS) for 20min at 4°C. Following two washing steps with PBS, samples were incubated with secondary antibody for 20min at 4°C. The final steps were carried out as described for intracellular staining.

Microscopy

$1.4x10^5$ cells were washed in RPMI 1640 medium. Pellets were resuspended in the same media to $7x10^6$ cells/ml. 20µl of cell suspension was pipetted per spot onto a 12 spot slide (Marienfeld) and incubated at 4°C for 10min to allow the cells to attach. The remaining cell suspension was then washed away in PBS. Cells were fixed (20min) and permeabilised for 1min. The slide was washed 3 times in 1xPBS. Before staining a blocking step was included with 5% BSA/PBS or horse serum/PBS for 15min, at RT to avoid unspecific binding of the antibody. Cells were stained for 60min in a wet chamber at RT. After 3x5min washing the cells were stained with FITC labelled secondary antibody for 60min in a wet chamber. Following three additional washing steps the samples were embedded in mounting media (Vectashield, Glycerol, 2% Dabco™ [1:1:1]) and the cover slip fixed to the slide with nail polish.

The cells were visualized using a Leica DMRE-7 white-field fluorescence microscope with a 1.4/63x objective using a Spot RT camera [Diagnostic Instruments] and the Spot RT acquisition software. Deconvolution was performed using Metamorph (version 6.1, Universal Imaging). Images were processed using the InfranView software (version 3.95).

Confocal images were acquired using a Leica DMIRE2 microscope and processed with the LSM Image browser software (Zeiss).

Cell lysis

After washing with 1ml cold PBS, $1x10^6$ cells were lysed in 30µl lysis buffer for 30min on ice. Samples were centrifuged at 13000rpm for 15min at 4°C. The post-nuclear supernatant was then heated for 5min at 95°C with 5x reducing sample buffer.

Immunoprecipitation

Cells ($5x10^6$) were lysed in ice-cold lysis buffer for 30min on ice. After centrifugation (13000rpm, 15min, 4°C), the post-nuclear supernatant was taken and 60µl anti-FLAG M2 affinity gel (50% slurry) was added (murine IgG1 monoclonal antibody covalently attached to agarose) that was washed before usage according to manufacturer's instructions. To reduce non-specific binding, 1/10 volume of 10mg/ml BSA was added. The samples were incubated on a rotating wheel minimum 2 hours or overnight at 4°C. After washing 5x in 1ml ice cold NP-40 washing buffer, the FLAG-tagged constructs were competitively displaced from the anti-FLAG agarose by incubating with 30µl 100µg/ml FLAG peptide for 30min at 37°C. The agarose was pelleted by centrifugation and the supernatant cooked with 5x reducing sample buffer for 5min at 95°C.

Gel electrophoresis and Western blotting

Lysates and IPs were separated on 10% SDS-PAGE gel at 130V and transferred onto nitrocellulose membranes (Hybond-C Extra, Amersham) with 140mA (2.2 mA/cm^2), 75min. After blocking in 5% milk for 1 hour and washing 1x in TBS/0,1% Tween-20, membranes were probed with primary antibody and the appropriate horseradish peroxidase-conjugated secondary antibody (dilutions indicated in Appendix). Membranes were washed 3x5min in TBS/Tween between and after staining with secondary antibody. Samples were visualized with an enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions.

12% ProSieve gels were prepared with ProSieve®50 gel solution and transferred onto PVDF Westran membrane (Schleicher & Schuell). Prior transfer the membrane was methanol treated according to the manufacturer's instructions.

Quantification of scanned blots was done with Kodak 1D Image Analysis Software.

GEM fractionation

GEM fractions were isolated by sucrose gradient centrifugation as previously described (4). Briefly, $20\text{-}30x10^6$ cells were lysed in 0.5ml lysis buffer for 10min on ice. The lysate was then mixed (1:1) with 80% sucrose in MNE buffer and dounced 10 times. The suspension was transferred to ultracentrifuge tubes [Sorvall] and overlaid with 2ml 30% sucrose in MNE and 1ml 5% sucrose in MNE. Samples were centrifuged in Sorvall

Centrifuge rotor TH 660 on 200000xg, 4°C, for 20 hours and the centrifuge was stopped without brake. Fractions (9 x 400µl) were collected from the top of the gradient. All solutions were ice cold and all steps were performed on ice.

In vitro kinase assay

IPs were washed 5x in NP-40 washing buffer (as described above) and the samples divided into two equal aliquots. Both were pelleted and one cooked in 1xRSB for 5min at 95°C and used for Western blotting, the other was resuspended in kinase buffer supplemented with acid-denatured enolase as substrate and P^{32} and incubated for 5min at RT. 5x reducing sample buffer was added and after cooking for 5min at 95°C samples were separated on 10% SDS-PAGE gels. After drying, the gels were exposed to autoradiography film (Kodak, BioMaxLight Film).

Migration assay

The cells were washed (900rpm, 5min) in migration media and resuspended at 5 x 10^6 per ml. 600µl migration media was pipetted into the wells of a 24-well plate. Transwell-inserts of 5µm pore size containing 100µl (5x10^5 cells) of cell suspension were placed into the media containing wells and equilibrated for 1 hour at 37°C. The transwell inserts were removed carefully and SDF-1 chemokine was added to the lower chamber to 100ng/ml final concentration and the plate was incubated for 4 hours at 37°C. Migrated cells were collected from the lower chamber and counted with a FACS Calibur (for 60sec).

Calcium measurements

Jurkat T-cells were transfected with either empty vector or the appropriate Flag-tagged PAG construct together with a vector containing GFP. 24 hours after transfection, 2x10^6 cells were washed in RPMI 1640 without phenol red and loaded with Indo-1/AM (Molecular Probes/Invitrogen) for 45min at 37°C. The cells were briefly washed and incubated an additional 30min before measuring the FL4 (510/20nm) vs. FL5 (400/40nm) ratio on a flow cytometer [BD LSR1]. The measurement was gated upon the GFP positive population. During the measurement, the cells were stimulated first with either anti-TCR [C305] (1:50) or SDF-1 [150ng/ml] and finally with ionomycin [2µg/ml].

siRNA

The following oligos were used for siRNA design:

Fw: 5’ gatccccgcgatacagactctcaacattcaagagatgttgagagtctgtatcgctttttggaaa 3’

Rev: 5’ agcttttccaaaaagcgatacagactctcaacaagagaactttgttgagagtctgtatcgcggg 3'

The 19 nt long target sequence corresponds to the sense strand of the generated siRNA (gcgatacagactctgaaca), which in turn corresponds to a 19 nt sequence within the mRNA

of PAG. The oligos (1µl) were annealed in 48µl annealing buffer (100mM NaCl, 50mM HEPES pH5.4) at 90°C for 4min and then kept on 70°C for 10min. The annealing was followed by stepwise cooling to 37°C (within 15-20min) and then further to 10°C or RT. The annealed oligos were ligated into pCMS3-H1p-EGFP vector that was previously linearised by *Bgl* II/*Hind* III digestion and gel purified on 1% agarose gel. The ligation reaction contained 2µl oligos, 1µl vector, 1µl T4 DNA ligase, 1µl T4 DNA ligase buffer and 5µl H_2O. It was carried out overnight at RT. Afterwards the plasmids were treated with *Bgl* II for 30min at 37°C. Upon successful cloning the *Bgl* II site is destroyed, so that the insert containing plasmids will be resistant to the digestion reaction. The recombinant vector was transformed into competent cells as described above and the clones were tested for the presence of the right plasmids via digestion with *EcoR* I and *Hind* III.
Jurkat T-cells were transfected with the siRNA containing plasmid as described above.

INTRODUCTION

Multicellular organisms are shielded against pathogens by the immune system. The innate immune system is the first level of defence, which is immediate and non-specific. The specific immune answer is provided by the cellular and humoral elements of the acquired immune system and takes days. During phylogenesis, acquired immunity developed in higher Eukaryotes based upon the innate immunity. The elements of the innate and acquired immune system are interwoven and strongly cooperate with each other. The innate immune cells (macrophages, dendritic cells) that recognise, process and present antigen are initiating the specific answer of the acquired immunity. The innate immunity is also involved in the effector phase of immune reaction via the complement system, macrophages and granulocytes. (5)

Generation of an effective adaptive immune response involves two major groups of cells: professional antigen presenting cells (APC: dendritic cells, monocytes, macrophages, B-cells) and lymphocytes. Antigen presentation is a process in which peptides (derived from proteins) are bound to MHC molecules and presented on the cell surface. Whether an antigen will be processed and presented together with class I or class II MHC molecules is determined by the route that the antigen takes to enter the cell.(5, 6)

Specificity, diversity, and memory within the immune system are provided by the lymphocytes. Three groups of lymphocytes can be distinguished according to their morphology and function: T lymphocytes (T-cells), B lymphocytes (B-cells) and natural killer (NK) cells. The latter plays a role in the innate (non-specific) immune response, while T- and B-cells (T stands for thymus, B for bursa Fabricii, the organ in birds, where B-cells were first isolated) are the cellular components of acquired (specific) immunity. While B-cells are able to recognise a variety of antigens, from native proteins, lipids, carbohydrates, artificial compounds and metals, T-cells are specialised, recognising only peptides presented in the context of MHC-peptide complexes. (5)

After they arise from haematopoietic stem cells in the bone marrow, T lymphocyte precursors migrate to the thymus to mature. Maturation into immunocompetent cells involves the random rearrangement of a series of gene segments encoding the cell's antigen receptor. Each matured T-cell expresses approximately 10^5 T-cell receptor (TCR) on its surface with each of these having the identical specificity for antigen. The process of random rearrangement of the genes

encoding the TCR is capable of generating enormous diversity: 10^{15} unique receptor specificities. This huge diversity is later diminished through selection in the thymus, which ensures that only T-cells with functional receptors possessing low recognition of self antigens presented by MHC molecules will be able to mature. (6) Identification of the genes encoding the TCR occurred in the early 1980s. Since that time the structure of this protein complex has been described, and the events that occur following engagement of the TCR have been extensively studied.

1. T cell signalling

1.1. The T cell receptor

Based upon the structure of the T-cell receptor, one can distinguish two T-cell populations: $\alpha\beta$ and $\gamma\delta$ T-cells. The latter have a different expression pattern (gut, skin) and role within the immune system and will not be discussed further. $\alpha\beta$ T-cells (90-95% of human T-cell population) can be further divided into subpopulations based upon the expression of cell surface markers ($CD4^+$ T helper cells and $CD8^+$ T cytotoxic cells) and effector functions (T_H1 cells - that mediate inflammatory responses and activate T_C cells; T_H2 cells - that are responsible for antibody production and involved in the fight against extracellular bacteria, parasites and toxins; T_C cells - that eliminate viruses and tumour cells; and T_{reg} cells – that suppress T-cell mediated immune responses). An activated T helper cell becomes an effector cell secreting various cytokines, which play a role in activating B- cells, T_C cells and macrophages that participate in the immune response. In contrast to T_H cells, cytotoxic T-cells do not secrete many cytokines, but instead exhibit cytotoxic activity against cells that are virus infected, tumour cells or foreign tissue graft. (5, 7)

The TCR heterodimer (α, β chains) is responsible for antigen recognition. The positively charged amino acids within the transmembrane domain of the α and β chains destabilise the heterodimer, so that it cannot exist in the membrane without associating with the chains of the CD3 complex (γ, δ, ε). Beside the CD3 chains, two ζ chains are also a central part of the TCR/CD3 complex. These and the α and β chains are bonded with a disulphide bridge (5). With its short cytoplasmic domains the TCR is unable to initiate signal transduction. Thus, the CD3 complex not only regulates the surface expression of the TCR, but also mediates signal transduction (8). The cytoplasmic tails of its associated chains contain 10 specific motifs, which were described by Reth (9) and later named immunoreceptor tyrosine-based activation motifs (ITAMs). The consensus

sequence of these motifs is: YXX(L/I/V)X_{6-8}YXX(L/I/V) where X represents any amino acid. Tyrosines embedded in this motif are both necessary and sufficient for the initiation of downstream signalling. The ITAM mediated signals are critical at distinct stages of T-cell development and for activation of effector functions (10).

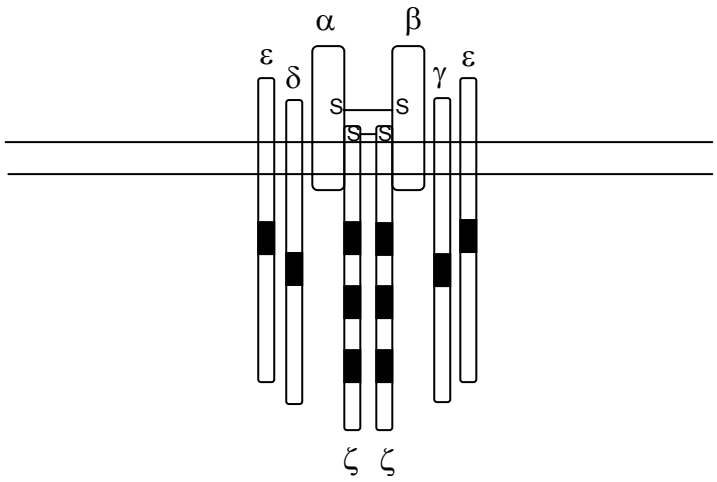


Figure I. The TCR/CD3 complex
The TCR heterodimer consists of the α and β chains that associate with the chains of the CD3 complex (γ, δ, ε). The two ζ chains are in central position and bind the α and β chains with a disulphide bridge. Black boxes represent ITAMs.

1.2. Signal transduction from the TCR

Following interaction of the TCR/CD3 complex with the antigen-derived peptide:MHC complex presented on the surface of an antigen presenting cell, receptor aggregation occurs. The receptor aggregates (TCRs, CD4 or CD8) formed after interaction with the ligand move to the lipid rafts (also known as glycosphyngolipid-enriched microdomains - GEMs) (11). Co-receptor dimerisation occurs (12), and plays a role in mediating prolonged cell contact with the APC (13). The Src kinase Lck (lymphocyte-specific cytoplasmic protein tyrosine kinase) is constitutively associated with CD4/8, and is more active when associated with a CD4/8 receptor dimer than compared with the monomer (13). Thus, beside the recruitment of pre-activated Lck, CD4/8 dimerisation plays a role in initiating and maintaining the active signalling machinery (13). Activated Lck phosphorylates the ITAMs providing binding sites for the tandem SH2 (Src homology 2) domains of the Syk kinase ZAP-70 (Zeta-associated protein of 70 kDa) and it activates the other Src kinase Fyn (14), ZAP-70 and the Tec family kinase Itk (interleukin-2 inducible T-cell kinase) through phosphorylation (15). These kinases propagate the signal by phosphorylating and activating their substrates: various adaptor proteins and enzymes. The transmembrane adaptor protein LAT (linker for activation of T-cells) is phosphorylated by ZAP-70. This leads to the recruitment of Grb2 (growth factor receptor

bound protein 2) - SOS (son of sevenless homologue) a complex that is involved in the activation of the Ras-Erk (extracellular signal-regulated protein kinase) pathway. GADS (Grb2 related adaptor protein) binds to LAT via its SH2 domain and to SLP-76 (SH2 domain containing leukocyte protein of 76 kDa) via an SH3 domain bringing these two key signalling proteins together. The inositol phospholipid specific phospholipase C-γ1 (PLCγ1) also binds to phospho-LAT via its SH2 domain. LAT bound SLP-76 undergoes phosphorylation by ZAP-70. The phosphorylated SLP-76 then binds Itk, which in turn activates PLCγ1 by phosphorylating it. The activated PLCγ1 enzyme hydrolyses PIP2 (phosphatidylinositol 4,5-biphosphate) to IP3 (inositol 1,4,5-triphosphate) and DAG (diacylglycerol). IP3 induces calcium release from the intracellular stores via interaction with a specific receptor located on the surface of the endoplasmatic reticulum, which leads to activation of calcium dependent enzymes like calcineurin. Calcineurin dephosphorylates NFAT (nuclear factor of activated T-cells) allowing its translocation into the nucleus. DAG activates PKCθ (protein kinase C), which is involved in NFκB (nuclear factor κB) activation, and RasGRP (Ras guanyl-releasing protein), which is a guanine-nucleotide exchange factor responsible for Ras activation that in turn leads to AP-1 (activator protein 1) activation via the MAPK (mitogen-activated protein kinase) pathway. The transcription factors NF-AT, AP-1 and NFκB initiate the transcription of IL-2 and the IL-2R genes. Beside PLCγ1 activation, SLP-76 is involved in regulation of the actin cytoskeleton and adhesion via its associating partners ADAP (adhesion and degranulation promoting adaptor protein), VAV (guanine nucleotide exchange factor) and the Nck (non-catalytic region of tyrosine kinase)-PAK (p21 activating kinase)-WASP (Wiskott-Aldrich syndrome protein) complex. (reviewed in (15))

1.3. Costimulation

T-cell responses require not only signals generated via the TCR, but costimulatory signals are also crucial for full activation. CD28 is the major positive costimulatory molecule. Its ligands (B7.1 and B7.2 also known as CD80 and CD86, respectively) are highly expressed on activated APCs. CD28 mediated signals contribute to the generation of stable T cell-APC conjugates by recruiting crucial signalling elements to the contact area (16).

There are two models for the mechanism of costimulation. In one CD28 has a unique signalling “voice” as it is able to activate several molecules (e.g. PI3K, Itk, p62Dok), initiates actin cytoskeletal rearrangement, plays a role in adhesion and controls

early and late effectors of anti-apoptotic programs. The other model considers CD28 as an amplifier of TCR signalling (17, 18) via strengthening the T cell-APC contact (the APC expresses 100-1000 times more B7 then pMHC) (18), via contribution to full activation of PLCγ1 through the PI3K/PIP3/Itk activation pathway (19), via amplifying Vav-mediated processes (18) and via contributing to strong activation of the main transcription factors involved in immediate and early gene expression. As one can see there are several places in the molecular network where the signals mediated via TCR and the costimulatory signals interweave leading to a synergised effect on gene transcription and effector functions of the T-cell reviewed in (20). *Figure II.* presents some elements of these two pathways and highlights the meeting points between them.

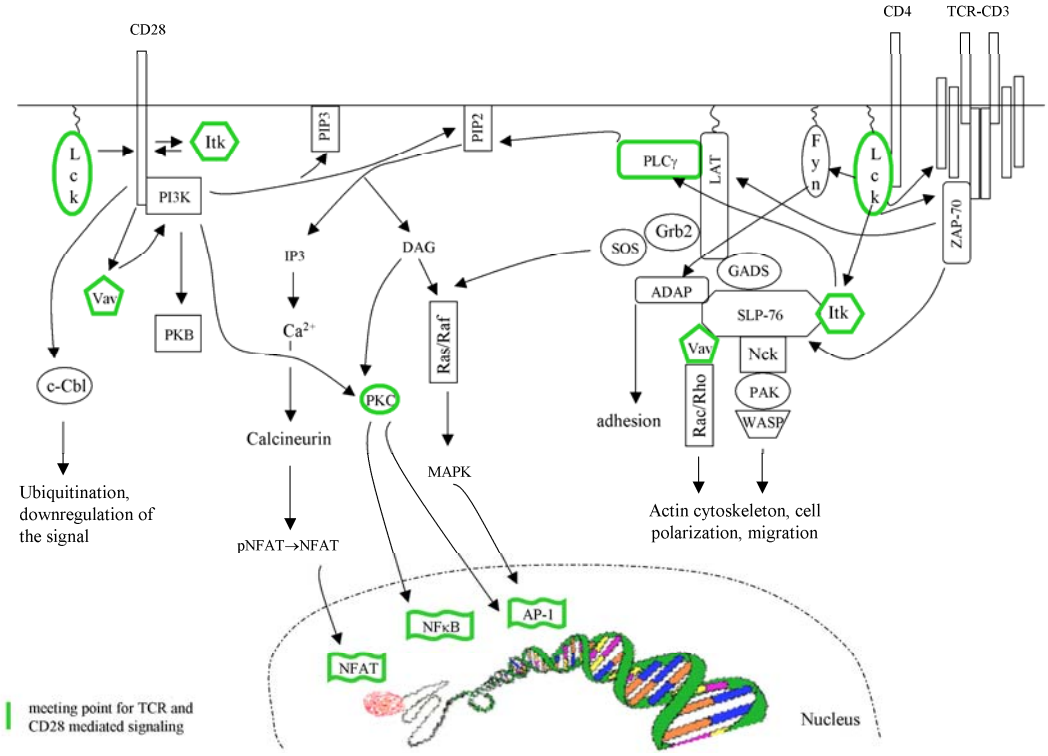


Figure II. TCR and CD28 signalling with meeting points

The signalling cascades induced by peptide stimulation and costimulation interweave leading to a synergised effect on gene transcription and effector functions of the T-cell. The costimulatory signal contributes to T-cell activation by activating PI3K and Itk thus playing a role in full activation of PLCγ1. Via amplifying Vav mediated processes it plays a role in actin cytoskeleton rearrangement, adhesion and migration. Signals generated via TCR and CD28 interact also on transcriptional level. Both pathways induce NFAT translocation to the nucleus (via PLCγ1 activation and inducing calcium mobilisation), NFκB activation (via PKCθ) and AP-1 activation (via PKCθ and the MAP kinases).

2. Lipid rafts

The fluid mosaic model of Singer and Nicolson (21) describes the plasma membrane as a phospholipid bilayer. They realised that lateral diffusion of membrane components (i.e. lipids and proteins) occurs within the bilayer and that these components are also able to aggregate. The biggest breakthrough of this model compared to the preceding view was that it considered the membrane as a dynamic structure. The weak point of it was that it models the membrane as a random two-dimensional liquid. In the last three decades biological and model membranes were extensively studied and the results led to the need for modification of the fluid mosaic model. The data indicated that the plasma membrane has a heterogeneous structure with functional compartmentalisation (protein complexes, lipid compositional areas) in which components are not randomly distributed. The current view is that the thickness and patchiness of the membrane are variable and the protein occupancy is higher than previously thought. (22)

Regarding the lipid rafts, the currently accepted model is the raft hypothesis by Simons and Ikonen (23) which defines the rafts as “dynamic clustering of sphingolipids and cholesterol that move within the fluid bilayer” and function as “platforms for the attachment of proteins during signal transduction˝.

Lipid rafts were found to play a role in various biological processes, including several signalling pathways (e.g. TCR, BCR, IgE receptor, neurotropic factors, growth factors, chemokines, interleukins and insulin), vesicle trafficking (endocytosis), apoptosis, adhesion and migration. They were also described as entry sites for some viruses (e.g. HIV- human immunodeficiency virus)**,** bacteria (e.g. *E. coli* to phagosomes, mycobacteria to macrophages) and toxins (e.g. cholera toxin) (24). Rafts also support the assembly (e.g. measles virus (25)) and budding (e.g. HIV (26)) of some viruses, the formation of prions and Alzheimer amyloids (27) were also localised to the lipid microdomains. Lipid rafts either serve the pathogen’s interest by providing site for receptor clustering (pore forming toxins) thereby increasing binding affinity or promoting toxin oligomerisation, or by providing link to the actin cytoskeleton (*E. coli*) (24).

2.1. Structure of the lipid rafts

The lipid composition of the plasma membrane differs from that of the intracellular membranes as it contains high levels of sterols (mainly cholesterol) and sphingolipids. Sphingolipids (originating from the Golgi) have a ceramide backbone and depending upon the attached head group, one can distinguish sphingomyelin (with a phosphocholine head group) from glycosphingolipids (with a carbohydrate head group).

Sphingolipids are present in the exoplasmic leaflet of the plasma membrane and possess two saturated chains. They associate with each other via weak interactions of their head groups. The head groups occupy larger areas than the chains. Thus, the forming interspaces are filled with cholesterol. Sterols are synthesised in the endoplasmatic reticulum (ER) and have the capability of flipping between the outer and inner leaflet of the membrane. According to the cholesterol content of the membrane two types of liquid phases exist: liquid-ordered (l_o) and liquid-disordered (l_d). The third group of lipid components of the plasma membrane are glycerophospholipids composed of a glycerol backbone with two acyl chains, one of which is saturated. Some of them, like phosphatidil inositol are restricted to the cytoplasmic leaflet. Raft assembly can occur at the earliest in the Golgi apparatus where sphingolipids are synthesised. Transmembrane proteins and GPI-anchored proteins incorporate into the sphingolipid-cholesterol complex in the Golgi. Protein interaction with the rafts can be ensured by different mechanisms: glycosylphosphatidylinositol (GPI)-anchored proteins have two saturated fatty acyl chains, membrane associated proteins are lipid modified, for transmembrane proteins transmembrane domains, in the case of intracellular proteins special lipid-binding domains (e.g. pleckstrin homology-PH, C2) or acylation (palmitoylation, myristoylation, isoprenylation) provide raft associating capability. (23, 28)

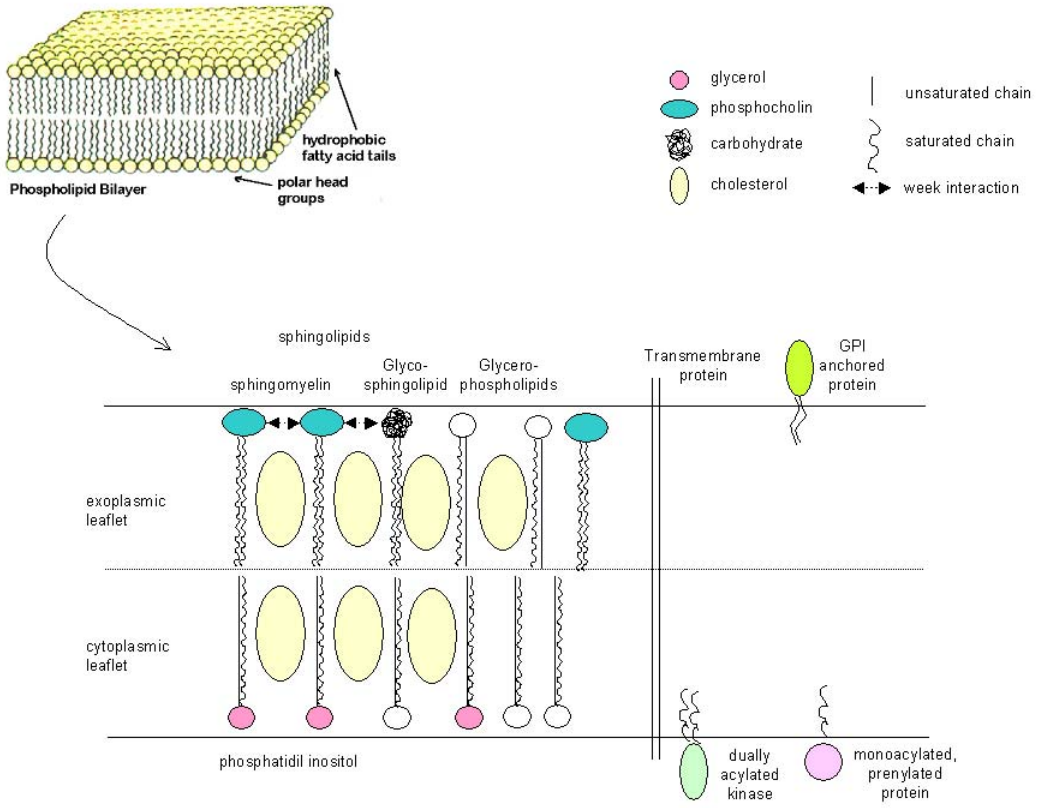


Figure III. Structure of the lipid rafts

Lipid components of the plasma membrane can be divided to three groups: sphingolipids, glycerophospholipids and sterols. Sphingolipids can be distinguished based on their head groups: sphingomyelin contains a phosphocholin, glycosphingolipids a carbohydrate group. Sphingolipids localise to the exoplasmic leaflet. Glycerophospholipids are found in both leaflets, but some of them (like phosphatidil inositol) are restricted to the cytoplasmic leaflet. Cholesterol fills the interspaces formed in between the sphingolipid and phospholipid chains. Protein interaction can be ensured by saturated fatty acyl chains (GPI-anchored proteins), transmembrane domains (transmembrane proteins), lipid-binding domains or acylation (intracellular proteins).

2.2. S-acylation

Lipid modification of proteins may occur co-translationally like N-terminal myristoylation or post-translationally like C-terminal prenylation of cytoplasmic proteins and palmitoylation of integral and peripheral membrane proteins (by the membrane associated palmitoyl acyltransferase (PAT)). Fatty acids are esterified to the free thiol of membrane-proximal cysteines. Post-translational lipid modification may occur by adding various fatty acids: e.g. stearic, oleic, arachidonic acid. The importance of S-acylation in T-cell signalling can be demonstrated by the row of S-acylated key signalling proteins: CD4, CD8β, the Src family kinases Fyn and Lck, Ras, the transmembrane adaptor proteins LAT, PAG (phosphoprotein associated with glycosphingolipid enriched

microdomains), LIME (Lck-interacting membrane protein) and NTAL (non-T-cell activation linker). In the case of CD8, palmitoylation was shown to be necessary for co-receptor function since this modification enables it to associate with the lipid rafts and thereby recruit its binding partner Lck to the site of activation (29, 30). Lck has also to be palmitoylated in order for this interaction to occur. Both Lck and Fyn are primarily myristoylated on their amino-terminal glycin through a stable amide bond, but for stable membrane localization and lipid raft association they must be dually acylated. The position of the 16-carbon fatty acid palmitate and the charge of surrounding amino acids influence the protein distribution. For Lck it has been shown that a lack of palmitoylation due to mutations of Cys-3 and Cys-5 not only decreases its affinity for membrane localization but also makes it biologically inactive (31). From studies dealing with the relevance of palmitoylation sites it is known that the membrane proximal cysteines are more crucial than the distal ones for lipid raft targeting of Fyn (Cys-3), Lck (Cys-3) and LAT (Cys-26). In the case of Fyn the half-life of palmitoylation was also studied and was shown to be 1,5-2 hours while 8 hours were measured for the polypeptide backbone. This indicates that palmitoylation is a reversible modification and most probably plays a role in the regulation of membrane targeting. (S-acylation reviewed in (32)). Indeed, 50% of Fyn was found to be non-palmitoylated and localised to intracellular membranes and non-raft fractions in a mouse fibroblast cell line (33). The transmembrane adaptor protein LAT is palmitoylated at Cys-26 and Cys-29. Palmitoylation is essential for the targeting of LAT to the lipid rafts and for its tyrosine phosphorylation (34). As proper localization of LAT is required for efficient signalling and T-cell activation (35) one can conclude that S-acylation is crucial for T-cell function. Note that one aim of this study is to investigate the role of palmitoylation in PAG function.

Inhibition of S-acylation by fatty acids or their analogues (e.g. 2-bromopalmitate) interferes with the raft localization of lipid modified proteins and T-cell activation. Inhibitors of palmitoylation influence Lck activity thereby suppressing signal transduction from the TCR. Polyunsaturated fatty acids (PUFAs) interfere with raft integrity by replacing saturated fatty acid residues in sphingolipids (36) and they were also shown to inhibit S-acylation. This gives a possible explanation for why the PUFAs are successful in immunosuppression when applied against inflammatory diseases like rheumatoid arthritis and Crohn´s disease (37).

2.3. Lipid rafts or DRMs?

It has long been known that under certain conditions some membrane components are not solubilised by detergents. GPI-anchored proteins were shown to be resistant to Triton-X100 and lipid rafts were identified as detergent insoluble membrane domains. Incorrectly, rafts also called glycosphingolipid-enriched microdomains (GEMs) and detergent-resistant membranes (DRMs) appeared as synonyms in the literature. The main difference between these structures is that DRMs can be obtained at 4°C, but not at 37°C. Detergent resistance means that incomplete solubilisation occurs and large membrane fragments remain instead of micelle formation even at high detergent concentrations. One should not forget the observations that different detergents (Triton-X100, Lubrol, Brij) revealed different "rafts". DRM association of a protein does not mean raft localisation and vice versa. DRMs are evoked only after detergent treatment and they do not necessarily correspond to any pre-existing membrane structure (reviewed in (38)). Heerklotz gives us a very strict conclusion in his paper that summarises all the critics against associations between DRMs and rafts: "detergent resistant membranes should not be assumed to resemble biological rafts in size, structure and composition or even existence" (39).

2.4. Enigmas surrounding the lipid rafts

During the past few years "raftology" became a field full of controversial studies and discussions about the existence and properties of membrane domains. Due to the wide range of methods and technical limitations, the size, protein composition, appearance and function of lipid microdomains still remains obscure and the subject of debate. One has to be critical with the results obtained from biochemical approaches as they may reveal artefacts. Detergent solubilisations are usually carried out at 4°C. This non-physiological temperature may alter the lipid organisation. As mentioned already (see *section 2.3.*) detergent resistance does not necessarily correlate with raft association of a protein but this does not mean that we should forget about detergents. In contrast to Triton-X-100, Brij 98 (polyoxyethylene) was found to preserve the composition and the protein interactions in the resistant membrane fractions. In contrast to other detergents (28) Brij 98 did not induce the appearance of non-plasma-membrane proteins (nuclear, ribosomal etc.) in DRM fractions. Importantly the same DRMs were isolated at 37°C with Brij 98 and with detergent free methods (40, 41). The difference in protein composition detected by various detergents can be also explained by the structure of the rafts. It was suggested that rafts may have a core which is resistant to strong detergents, like Triton-

X-100 (contains ‘typical’ raft proteins, like LAT). On the other hand the peripheral region is more sensitive and gets dissolved by strong detergents and is preserved by Brij 98 and Brij 58 (contains ‘atypical’ proteins CD45, MHC) (36). Another biochemical approach to study rafts is cholesterol depletion with cyclodextrins or synthesis inhibitors. But, since cholesterol is essential for the proper function of the cells, its depletion can cause complex effects: alteration of the membrane permeability, calcium dependent responses, ERK signalling and was shown to induce domain formation within the plasma membrane (28). Thus results obtained with this method may be misleading.

The most reliable approaches are those, which observe living cells without manipulating them. However, it soon turned out that the resolution and sensitivity of light microscopes was not sufficient to detect the membrane rafts. New techniques were developed with increasing sophistication in order to gain more precise insight into the structure and dynamics of plasma membrane microdomains. The first direct visualisation of liquid ordered (lo) domains in living cells was obtained using Laurdan staining of macrophages by two-photon microscopy (42). Laurdan is a fluorescence probe with a red shifted profile when higher local hydration is detected in the membrane, and with a blue shift when low hydration is detected. Laurdan staining of living neutrophils also verified the existence of gel phase lipid rafts, as the lamellipodia (leading edge) of the intact cells contained blue shifted regions that spatially corresponded to ganglioside (e.g.GM3 lipid raft marker) enriched domains and was sensitive to cholesterol depletion (43). Recently another dye, the di-4-ANEPPDHQ has been applied to visualise lo phases at the plasma membrane. Fluorescent life time imaging (FLIM) together with this dye even detected varying levels of ordered phases (44).

Fluorescence resonance energy transfer (FRET) is a widely used method to study the proximity of two proteins. Although it has high resolution (1-10nm between the donor and acceptor) it has provided mixed results. In some cases the raft markers showed random distribution (45), in others co-clustering was observed (46). Inefficient FRET may be caused by various physical factors (e.g. steric constraints between the donor and acceptor), which are independent of the observed biological system. Therefore the absence of evidence is not evidence of absence. For a long time, rafts were identified only at the outer leaflet of the plasma membrane. The first direct support for rafts in the inner leaflet was obtained by FRET analysis of S-acylated proteins (46).

Biophysical approaches enabled researchers to observe membrane structure and components not only spatially but also temporally. Electron-paramagnetic-resonance spectroscopy highlighted that rafts are very dynamic structures. The exchange rate of

raft components between the rafts and their environment is on a submillisecond scale (47). Diffusion also contributes to the constant renewal of microdomain components. Photonic force microscopy and single fluorophore tracking are useful methods to follow the diffusion and dynamics of single proteins within the membrane (48). Nevertheless, fluorescence correlation spectroscopy seems to be the most powerful technique for diffusion analysis thanks to its high temporal resolution and accuracy (40). Despite technical problems researchers have produced a large amount of evidence for the existence of lipid rafts and the current view is that they are small (1-10nm, (36, 47, 49)), highly dynamic structures. In unstimulated cells the life time of small, unstable rafts is on the scale of milliseconds, while following ligand binding, the oligomerised GPI-anchored receptor molecules form stabilized rafts with lifetime of few minutes (50). Subczynski et al. suggested that the stabilized rafts promote forming of larger, temporal rafts which serve as signalling modules and include the inner leaflet of the plasma membrane (50). One should take in consideration the possibility that membrane associated proteins may also have organiser function. Maybe the lipid components serve only as platforms for the protein-protein interactions (51, 52) (T. Harder, M.J. Bijlmaker personal discussion). A recent paper (53) shows, what was already predicted by Munro (28), that protein-protein interactions and not detergent insolubility are responsible for assembly of signalling complexes.

2.5. Lipid rafts and the TCR signalling

In the majority of cells the lipid bilayer is continuously renewed. Phospholipids at the inner leaflet are hydrolysed by phospholipases and resynthetized by other enzymes. The products of hydrolysis (IP3, DAG, arachidonic acid) play a role in signalling processes and in regulating cellular functions. The decrease in the phosphatidylinositol concentration serves as an important signal for actin binding proteins and for those proteins possessing lipid binding domains e.g. PH and C2 domains through which they associate with the membrane lipids (54).

The TCR/CD3 complex was thought to be excluded from lipid rafts in resting cells. Horejsi (36) and others (55) speculated about the existence of two-step T-cell signalling in which two types of lipid rafts are involved. One set would preassociate with the TCR and may serve as a source of kinases necessary for signal initiation. The other would translocate to the aggregated TCR from cytoplasmic stores upon stimulation and could be involved in signal amplification. Recently it was shown that 10-20% of TCR/CD3 constitutively associates with a subset of lipid rafts which becomes the site of

signal initiation upon stimulation (40). This subset also contains CD4/8, Lck and CD45. Upon activation, the TCR complex is highly enriched in lipid microdomains (40). Raft association of Lck is ensured by myristoylation at Gly2 and palmitoylation at Cys3 and Cys5 (56). Additionally both CD4 and CD8 are palmitoylated which is necessary not only for their lipid raft association, but also for their efficient co-receptor function, and for association with Lck (57). The membrane phosphatase, CD45 positively regulates Lck activity. It was shown to be excluded from the rafts, but there are also groups, which could detect it inside the microdomains (58). The contradiction regarding the localisation of the TCR and CD45 in lipid rafts may emerge from different technical approaches. As mentioned already in *section 2.4.* lipid raft preparation with Brij 98 at physiological temperature preserves the interactions that are sensitive to stronger detergents. Therefore, studies using Triton-X-100 would not see raft association of the TCR/CD3 complex and CD45. (The regulatory role of kinases and phosphatases is discussed in *section 3*.). Beside the Src kinases and co-receptors, adaptor proteins (LAT, PAG, NTAL, LIME) are also present in lipid rafts of resting cells (36). The cytoplasmic kinase Csk (C-terminal Src kinase) is recruited by PAG and its presence in proximity to the Src kinases may contribute to setting the threshold for TCR activation (59, 60). Upon TCR stimulation, Csk is released. PAG serves also as an anchor for the rafts via binding to EBP-50 (ezrin-radixin-moezin binding phosphoprotein of 50kDa) and thereby to ezrin-radixin-moezin proteins and through them to actin (61, 62). (For more details about structure and function of adaptor proteins see *section 4.*). Werlen and Palmer (8) have suggested the term TCR signalosome for the complex that contains all elements necessary for signal initiation. It is enriched in cholesterol and sphingolipids and is highly dynamic. In the resting state, only weak associations occur among the signalosome members (40). Based upon the structural studies of the TCR/CD3 complex it has been speculated that upon stimulation, in addition to horizontal movements, vertical displacement between modules also occurs: transmembrane helices of CD3$\varepsilon\gamma$ and CD3$\varepsilon\delta$ move up-and downward relative to one another and the TCR$\alpha\beta$ chains. The horizontal movements lead to aggregation of lipid signalosomes that bring their protein components to the TCR signalosome. As fourth dimension one can consider the highly dynamic temporal feature of these changes (8). These multidimensional changes lead to expansion of the TCR signalosome and formation of scaffolds, like LAT/Grb2/SOS, LAT/GADS/SLP-76/Itk/Vav/PLCγ1, and SLP-76/Nck/PAK/WASP. These signalling pathways are discussed in *sections 1.2.* and *1.3.*

2.6. Further roles of lipid rafts

2.6.1. Costimulation and cytoskeletal rearrangement

The importance of costimulation via CD28 was discussed in *section 1.3*. Are the membrane microdomains involved in this function? Several papers have shown that upon costimulation lipid rafts are mobilised, move from the cytoplasmic stores to the cell surface and take part in immunological synapse (IS) formation (63, 64). Lipid raft accumulation at the IS is dependent upon Vav-1, Rac, WASP, the SLP-76/Nck/Fyn/PAK complex and other molecules. Association of rafts with the actin cytoskeleton can be mediated by the adaptor protein PAG via the ezrin-radixin-moesin protein family and also by the PIP2/WASP/Arp2/3 complex. The actin-raft interaction may contribute to maintaining the resting state without stimulation. The reason and cause of the movement inhibition is still not clear: either the actin filaments anchor the rafts thereby inhibiting their aggregation at the plasma membrane or the rafts themselves inhibit the cytoskeletal rearrangements which would lead to receptor aggregation and signalling initiation. (36)

2.6.2. Curing via lipid rafts

Given that lipid rafts play a crucial role in T-cell signalling and they are involved in pathogenic attack against immune cells, they may be potential therapeutical targets. As mentioned already in *section 2.2*, polyunsaturated fatty acids are used clinically as immunosuppressants. Their effect can be explained by the observation that they disrupt the structure of lipid rafts (65). However, it is not only PUFAs that are able to modify the lipid composition of the inner leaflet of the rafts. Glucocorticoids are widely used steroids with immunosuppressive and anti-inflammatory effect. Recently they have been reported to inhibit T-cell activation by selectively altering the lipid raft localisation of Src kinases and LAT (66).

2.6.3. Role of lipid rafts in other immune cells

The importance of lipid rafts is not restricted to T-cells. They play a key role in NK and mast cell activation and in regulating B cell responses. They are also involved in the spatial organisation of peptide-MHCII complexes on the APC surface, this way ensuring the optimal arrangement for recognition by the T-cell. (36, 48)

3. Regulation of lymphocyte activation by kinases and phosphatases

Maintaining T-cells in the resting state is an active process that requires equilibrium between protein tyrosine phosphatases (PTP) and protein tyrosine kinases (PTK). The balance of these enzymes resembles the harmony of yin and yang. Imbalance between PTPs and PTKs has pathological consequences in the form of autoimmunity, immunodeficiency and malignancy.

The subcellular localisation, activity and substrates of the PTPs and PTKs change during development and depend upon the activation status of the T-cell (67). As mentioned in *section 1.2.,* several different kinase families take part in proximal (Src, Syk, Tec kinases) and distal (e.g. serine/threonine kinases, MAP kinases) TCR signalling. Here I shall focus on the proximal signalling events and upon Src family kinases (SFKs) in particular, as they are more relevant to the topic of this thesis.

3.1. Src family kinases

The Src family of tyrosine kinases (SFKs) include 11 members, of which 4 are expressed in T-cells: Lck, Fyn, Yes and Src (68). SFKs are present in all metazoan cells and are involved not only in antigen receptor induced signalling but also in growth factor, cytokine and adhesion signalling (69). SFKs possess a conserved structure consisting of a myristoylated N-terminal segment (SH4 domain, which is responsible for membrane targeting), an SH3 and SH2 domain (for protein-protein interactions), a linker region, a tyrosine kinase domain (SH1, possessing the enzymatic activity) and a short C-terminal tail with regulatory properties. The kinase is held in an inactive state by intramolecular interactions between the SH3 domain and the linker region and the SH2 domain and the C-terminal inhibitory tyrosine. These interactions can be competed out only by specific binding partners excluding the possibility of autophosphorylation that would lead to unregulated T-cell activation. How SFKs recognise their substrates is not known. The SH2 and SH3 domains have been suggested to play role in it either by directing the binding partner to the kinase domain or by ensuring spatial proximity (68, 69).

The SH4 domain is followed by a unique region, which has a role specific to each kinase. In Lck this region mediates its association with the cytoplasmic tail of CD4/8, in Fyn it may be responsible for interaction with the TCR. Beside the structural and post-translational modification similarities, initial experiments showed a common substrate panel for these two kinases. However, this view was changed by the results obtained from Lck deficient cell lines and knock out mice. It is now clear that Lck is the kinase responsible for initiating the TCR signalling cascade, but it is dispensable for

CD3-induced stimulation (70, 71), for the latter Fyn was shown to be crucial (72). Following TCR engagement Lck not only phosphorylates the ITAMs of TCRζ chains and CD3 but also ZAP-70, LAT and PLCγ. Targets of Fyn are the adaptor protein PAG, Pyk2 (proline-rich tyrosine kinase 2) and it mediates formation of the Fyn/SLP-76/ADAP complex (14). In resting cells 75-95% of Lck localises in the non-raft membrane fraction while the majority of Fyn (98%) is detected within the lipid rafts (73). It is still unclear how Lck becomes activated but it is known that upon stimulation Lck translocates to the lipid rafts to mediate Fyn activation (73, 74). Brdičková et al (75) offers a nice model in which Fyn is phosphorylated and thereby activated by LIME-bound Lck. LIME is a raft-associated adaptor protein that upon phosphorylation by activated Lck binds both Csk and Lck itself. Csk phosphorylates the inhibitory tyrosine of Lck but since the SH2 domain is bound to LIME the closed conformation cannot be formed. This way the phospho-tyrosine remains free and is a potential binding site for an SH2 domain containing protein, which happens to be Fyn.

3.2. Regulation of Src family kinases

Many of the SFKs were identified as cellular oncogenes. They were found to contribute to the metastatic spread of carcinoma cells (76) and to be implicated in breast, colon and pancreatic cancers (Src), in malignant melanoma (Yes) and in acute lymphocytic leukaemias (Lck) (77, 78). These highlight the importance of a multilevel regulation of these crucial but also potential oncogenic kinases.

The kinase activity is regulated by tyrosine phosphorylation. It is believed that the activatory tyrosine (Y-394 in Lck and Y-418 in Fyn) within the activation loop of the kinase domain becomes phosphorylated by autophosphorylation (79). The C-terminal inhibitory tyrosine (Y-505 in Lck and Y-529 in Fyn) is phosphorylated by Csk (80), a distant relative of Src kinases (40% sequence identity) with different substrate specificity and regulation. Structurally Csk differs from SFKs in that it does not have an N-terminal lipid anchoring domain, an activation loop tyrosine or C-terminal inhibitory tyrosine (81). For proper function of Csk, both of its noncatalitic (SH2 and SH3) domains are important. Probably these are not only required for membrane targeting via binding to a membrane associated adaptor protein (PAG in resting, G3BP in activated cells, see details in *section 4.1.2.*) but they may help the enzyme to take the optimal shape for kinase activity (82). The recognition of the substrate tyrosine probably requires complex interactions dependent on the three-dimensional structure of both proteins (81) and involves the substrate docking site (SRSRRF) of Csk (83). Csk associates with the phosphatase PEP

(PEST enriched PTP) via its SH3 domain so that PEP is recruited to the plasma membrane together with Csk, where it can dephosphorylate its target proteins, Lck and Fyn at their activatory tyrosine. By tonic repression of Lck, Csk sets a threshold for TCR signalling and avoids hyperactivity (79). Csk is regulated by PKA and cAMP both in a spatial and enzymatic manner. Phosphorylation of its S-364 by PKA increases 2-4 fold the activity of Csk (84). The question, how PKA can regulate Csk when it is dissociated from lipid rafts following TCR triggering was answered by Vang et al (85). It is cAMP that stimulates targeting of Csk to the rafts where it is further activated via binding to PAG and by PKA. The phosphotranspherase activity of PKA-phosphorylated and PAG-bound Csk is 6-8 fold higher than that of cytosolic, unphosphorylated Csk. The indispensable role of Csk as a negative regulator of Src kinases is underlined by the fact that lack of Csk is incompatible with life (86). As a crucial negative regulator of Src kinases Csk could be a potential target in cancer therapy. It was shown that up-regulation of Csk function suppresses the metastatic potential of colon cancer cells (87).

CD45 is the phosphatase that opposes the function of Csk by dephosphorylating the C-terminal tyrosine of SFKs. This leads to a primed conformation that can be readily activated. CD45 is able to dephosphorylate the activatory tyrosines as well, but less efficiently. This dual function is regulated by spatial separation during TCR activation as CD45 is excluded from the immunological synapse and its effect on Src kinases remains net positive (67).

Protein tyrosine phosphatase α (PTPα), a widely expressed transmembrane enzyme is another member of the SFK regulatory network. It plays an important role in regulating tyrosine phosphorylation of Fyn and PAG in resting thymocytes: it negatively regulates the kinase activity of the raft-localised Fyn (88). The mechanism is similar to the one observed for CD45: both regulatory tyrosines of the Src kinases are dephosphorylated by these phosphatases with an overall negative effect. In the absence of PTPα, Fyn activity is increased and PAG is hyperphosphorylated (see also *section 4.1.3.*) in resting thymocytes (88).

Src family kinases are not only regulated by kinases and phosphatases but also via subcellular localisation mediated by the lipid environment or by binding partners. The importance of S-acylation for localisation and proper function was already described in *section 2.2*. Here examples for the binding partners will be discussed. Lck is probably maintained outside of lipid rafts by CD4. Another candidate for this function is Cbl (Casitas B-lineage lymphoma) that was found to associate with Fyn and Lck via their SH3 domains in resting cells. This suggests that Cbl may express its inhibitory role in

two levels: in resting cells it depletes SFKs from the lipid rafts and in activated cells it mediates their degradation (89).

3.3. Phosphatases

Kinases are tightly regulated by phosphatases. Of the 95 human PTPs, 30 are expressed in T-cells. Some of them are transmembrane (e.g. CD45), others are cytoplasmic (e.g. PEP, PTP-PEST, SHP-1,2, LMPTP). The Csk associated phosphatases, PEP and PTP-PEST (phosphatase with PEST sequence) are related but have different roles. PEP regulates Src kinases (*3.2.*), while PTP-PEST substrates are the members of focal adhesion complex (see *section 5.*). SHP-1 (SH2 domain containing PTP) plays a negative regulatory role during thymic development by dephosphorylating ZAP-70. In contrast SHP-2 is a positive regulator as it facilitates the MAPK pathway. MAP kinases are very strictly regulated by a large number of phosphatases that control their activation spatially and temporally on levels of basal activity, peak activity and duration of activity.

4. Adaptor proteins

Adaptor proteins connect receptors with the intracellular signalling elements. As they possess modular domains and lack enzymatic activity they play a role in organising macromolecular complexes and recruiting cytoplasmic molecules to the plasma membrane (90, 91). According to their subcellular localisation one can distinguish two populations: cytoplasmic (e.g. SLP-76, GADS, ADAP, Cbl, Grb2) and transmembrane adaptor proteins (TRAPs). The latter can be further divided into lipid raft-associated (LAT, PAG, LIME, NTAL) and non-raft proteins (SIT-SHP2 interacting transmembrane adaptor protein, TRIM-T cell receptor interacting molecule, LAX-linker for activation of X cells) (11). The typical structural components of TRAPs are the tyrosine based motifs that are phosphorylated by Src or Syk family kinases and offer binding sites for SH2 domain containing proteins, which are thereby localised to the right place at the right time. GEM-associated TRAPs are type III transmembrane proteins with a short extracellular and single transmembrane domain and a long cytoplasmic tail. They all contain a juxtamembrane positioned palmitoylation motif (CxxC). The non-GEM TRAPs are usually disulphide-linked homodimers, the exception being LAX. The cytoplasmic adaptor proteins are able to build up large protein complexes thanks to their diverse (e.g. SH2, SH3, PDZ, PH-pleckstrin homology) domains (11, 92). From functional point of view adaptor proteins may be positive, negative or bifunctional regulators (11, 93).

The role of different adaptor proteins is discussed in the various chapters of this introduction (*1.2., 1.3., 2.5., 2.6., 3.1., 5.*) but since PAG is the focus of this thesis the next section shall be dedicated to it.

4.1. PAG (phosphoprotein associated with GEMs)

The protein was simultaneously identified by two groups. One described it as a member of the Fyn complex (94) and named it PAG (4). The other group found it as a Csk binding protein (Cbp) (59).

4.1.1. Structure of PAG

PAG/Cbp (hereafter referred to as PAG) is a 432aa protein in humans (429aa in mouse) with a short extracellular domain (16-18aa), a transmembrane domain (20aa) and a long cytoplasmic tail (387-396aa). The intracellular domain contains 10 tyrosine residues nine of which are potential target sequences for Src kinases (YxxL/I/V). Six of them show ITAM-like structure (YxxL/I/V$(x)_n$YxxL/I/V) with values of *n* larger than in typical ITAMs. In addition there are 10 threonine, 12 serine residues and two proline rich regions that are potential binding sites for other signalling molecules. Some of the tyrosines and serines have been shown to be phosphorylated by mass spectrometry: Y-163, Y-181, Y-227, S-229, S-239, Y-317, Y-341, Y-359, Y-387, Y-417 (http://www.phosphosite.org). Although the predicted molecular weight was 46,9 kDa, a retarded migration was observed on SDS-PAGE gel with an apparent size of 68-85 kDa. This anomaly can be explained by the acidic character of PAG and the level of tyrosine phosphorylation. In unstimulated Jurkat T-cells the suboptimally phosphorylated 68 kDa form could be detected while following pervanadate treatment a shift was observed to 80-85 kDa. (4, 59)

PAG has been localized to the plasma membrane and within that to the lipid rafts. It is believed that palmitoylation is responsible for the GEM targeting as it was shown that PAG is palmitoylated (4). However PUFA treatment that is known to interfere with lipid raft localisation of LAT and Lck, did not displace PAG (95). This may be due to an additional mechanism that links PAG to the GEMs i.e. a particular structure of the extracellular or transmembrane domain or a constitutively GEM associated yet unidentified binding partner of PAG (92). It has been suggested that together with other TRAPs, PAG may have a function in coupling the lipid microdomains in the outer leaflet with those in the inner leaflet (96, 97).

4.1.2. Function of PAG

PAG is ubiquitously expressed, with the highest mRNA levels detected in peripheral blood leukocytes, heart, lung and placenta. Within PBLs it is strongly expressed in lymphocytes and monocytes and weakly in neutrophils and platelets (4). In most tissues, 7 and 10kb transcripts were found, in heart and placenta an additional 3kb appeared and in brain only the 10kb form was detected. This suggests tissue-specific splicing (92). The broad expression of PAG is unique among the TRAPs and suggests that this protein has a general function in regulating cellular responses throughout the body.

Although pull down assays showed that PAG potentially binds a number of signalling molecules (e.g. Lck, Fyn, Vav, SLP-76, ZAP-70, Csk), *in vivo* only Fyn and Csk were associated (4, 60, 98). Csk binds Y-317 (Y-314 in the mouse) via its SH2 domain. This interaction is necessary but not sufficient for Csk binding, indicating that other tyrosine(s), probably Y-299 (Lindquist, unpublished data), are also involved in it (99). Via targeting Csk to the lipid rafts, PAG actively takes part in increasing the activation threshold and in maintaining the resting state of the cell as Csk phosphorylates the negative regulatory tyrosine of Src kinases thereby keeping them in an inactive state. Accordingly, it was observed that overexpression of PAG in human peripheral T-cells (4) in Jurkat T-cell line (100) and also in a basophil leukaemia cell line (101) suppressed cellular responses mediated by Src kinases. Following T-cell activation PAG becomes dephosphorylated, Csk is released and Src kinases become activated. The activated Fyn in turn rephosphorylates PAG and it retargets Csk to the plasma membrane. This way the second function of Csk targeting realizes, namely that Csk down-regulates Src kinase activity and prevents the cell from overreacting the stimuli.

The interaction with Fyn is non-covalent (4). Description of the exact mechanism of Fyn binding and localisation of the binding site is one aim of my work. Fyn was shown to be the kinase responsible for PAG phosphorylation but as the phosphorylation level of PAG was not completely reduced in the T-cells of Fyn-/- mice, other kinase(s) must also be involved (98). Davidson et al recently showed that the role of Fyn is not only PAG phosphorylation that enables recruitment of Csk, but it also transduces biochemical signals during TCR stimulation that lead to increased calcium flux. These opposing effects attribute PAG with a dual function what is reminiscent of other transmembrane adaptor proteins such as LAT, SIT and NTAL (99).

According to the current functional model (11, 15, 99) PAG recruits Csk (20-30% of total cellular Csk, (14)) to the proximity of Src kinases in resting cells, in this way ensuring their suppression. Upon stimulation PAG becomes dephosphorylated, PAG-

Fyn association is rapidly lost and is followed by Csk release that allows activation of the kinases that initiate the signalling cascade (99). As PAG is one of the Fyn substrates in turn it rephosphorylates PAG enabling it to bring Csk back to the lipid rafts. In this way the negative regulatory feed back loop closes and the signalling process is silenced. The question, where does Csk locate when it is released from PAG, was recently answered (102). G3BP binds Csk upon TCR stimulation in a similar time course as it dissociates from PAG.

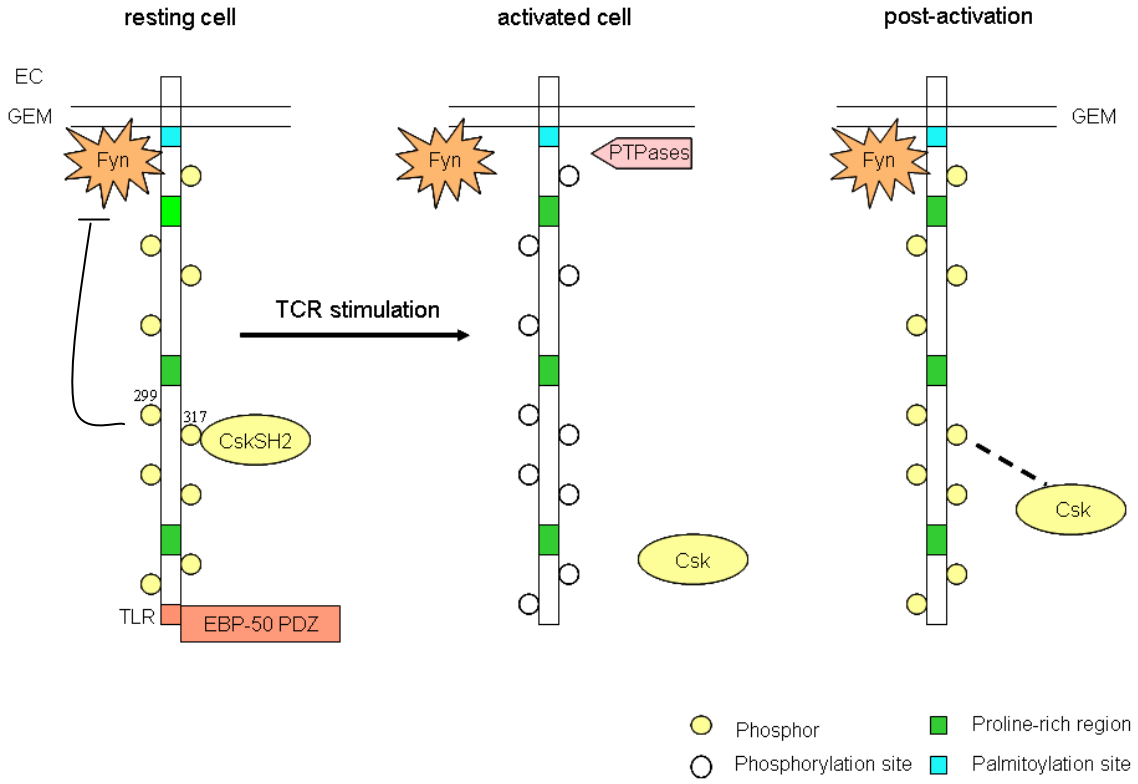


Figure IV. Model of PAG function

PAG recruits Csk to the proximity of Src kinases (e.g. the PAG associated Fyn) in resting T-cells, in this way ensuring their suppression. Upon stimulation PAG becomes dephosphorylated by an unknown phosphatase and Csk is released, what allows the activation of Src kinases. The association with EBP-50 is also resting cell specific. The activated Fyn rephosphorylates its substrate PAG, enabling it to bring Csk back to the lipid rafts. The negative regulatory feed back loop closes and the signalling process mediated by the Src kinases is silenced.

Hermiston et al (67) predicted that eliminating PAG would have consequences on thymic development (similarly to the $Csk^{-/-}$ thymocytes, (103)) and could possibly lead to autoimmune diseases or lymphomas due to hyperactive Src kinases. However, detailed studies on mice having a disrupted PAG gene did not fulfil this prediction. Embryogenesis, thymic development, vital functions (growth, development, fertility), TCR induced proliferation, signalling events, humoral immune response and peripheral B-and

T-cell tolerance were all normal, resembling wt mice (104, 105). This makes the impression that the function of PAG in regulating Src kinases is so important that a compensatory mechanism developed in these mice, possibly provided by another adaptor protein with redundant functions. The potential candidates of Csk binding proteins are LIME, SIT, Dok-3, paxillin, FAK. Some of these translocate into the lipid rafts upon activation but others like SIT remain outside. It is still an open question whether Csk localisation to GEMs is altered in the absence of PAG. One group could clearly show reduced Csk levels within the lipid raft fractions (104) but the other found it comparable with the wild type cells (105). In the background of this inconsistency methodological differences in lipid raft isolation may be suspected such as iodixanol gradient vs. sucrose density gradient, eight vs. eleven fractions (104, 105). The finding that PAG can function both as a negative and as a positive regulator may also explain the lack of phenotype in PAG-deficient mice since removal of opposing signals may remain without net effect on cellular functions (99).

PAG interacts with the adaptor protein EBP-50 that binds to the C-terminal TRL sequence of PAG via one of its PDZ (postsynaptic density/disc-large/ZO1) domains. The role of this interaction in anchoring rafts to the cytoskeleton was already mentioned in *section 2.5*. It was reported to negatively regulate immune synapse formation in unstimulated cells. Following stimulation the association is broken. The mechanism regulating the dynamics of this interaction is still unknown. (61, 62, 92)

Recent work from our group describes a novel protein complex consisting of PAG, Fyn, Sam68 and RasGAP and suggests that this complex may play a role in development and maintenance of the anergic phenotype (an unresponsive state of T-cells that can be provoked by a strong TCR mediated signal without costimulation) via inhibitory effects on proximal signalling (zeta chain- and LAT phosphorylation), block in Ras activation and block in cell cycle (Smida, submitted to Blood).

4.1.3. Regulation of PAG

As mentioned earlier the reversible protein phosphorylation is a widely employed mechanism for regulating various cellular functions, as protein localisation, assembly of protein complexes, enzyme activity and gene transcription. The phosphorylation of PAG appears to be controlled by Src kinases, primarily Fyn (4, 98). The phosphatase responsible for PAG dephosphorylation is still unknown. It would be important to identify it since dephosphorylation of PAG precedes and leads to Src kinase activation, which belongs to the very first steps of T-cell activation that is still veiled in mist. Possible

candidates are CD45 (100), SHP-2 (106), SHP-1, PTP-PEP, PTP-H1 (92) but none of them causes complete dephosphorylation. Davidson et al. have found that CD45 was involved in dephosphorylation of PAG upon TCR stimulation. Studies on CD45, PEP and SHP-1 deficient cells revealed that unlike CD45, PEP and SHP-1 were dispensable for dephosphorylation of PAG (100). As it has never been proved that CD45 directly acts on PAG, it can be speculated that CD45 regulates PAG phosphorylation via an indirect mechanism e.g. by inactivating Fyn via dephosphorylating its activatory tyrosine and thereby inhibiting phosphorylation of PAG (88, 100). In non-haematopoietic cells, where CD45 is not expressed, PAG still becomes dephosphorylated (100). Therefore it seems to be more correct to talk about PAG phosphatases instead of a phosphatase.

PTPα was excluded to be a PAG phosphatase but from studies on PTPα deficient mice we know that it has an indirect effect on the phosphorylation status of PAG. In the absence of PTPα the kinase activity of Fyn is increased and in turn causes hyperphosphorylation of PAG. This effect was observed only in resting tymocytes of PTP$\alpha^{-/-}$ cells indicating that PTPα is involved in regulation of tyrosine phosphorylation in thymocytes before TCR activation (88).

4.1.4. PAG in non-T cells

PAG functions as a negative regulator also in mast cells. Following aggregation of the FcεRI receptor, PAG becomes phosphorylated by the Src kinase Lyn, recruits Csk and in turn suppresses Lyn activity (101). The main difference in appearance of PAG in mast cells compared to T-cells is that it is not constitutively phosphorylated. The same was described for fibroblasts. To explain the difference in phosphorylation status of PAG in various cell types one has to think about the development and function of these cells. Peripheral T-cells have already undergone positive and negative selection and they constantly receive week stimulation in the periphery in order to stay alive. This keeps them in a kind of preactivated state in which the low level of activated Src kinases is sufficient to phosphorylate PAG and ensure that the presence of Csk prevents activation by low affinity MHC-TCR interactions (92).

In B cells Lyn has a dual function: via phosphorylating ITAMs it contributes to the initiation of BCR mediated signalling and by phosphorylating the ITIMs (inhibitory motifs) it can activate inhibitory molecules. In these cells the role of PAG is still not clear. Via Csk recruitment it may potentially inhibit both functions of Lyn that could lead either to down-regulation of B cell signalling or to temporal suppression of the inhibitory signals (15, 107).

The first study showing a connection between PAG and a pathogen agent was published recently. Bovine T-and B-cells infected with the intracellular parasite *Theileria parva,* behaved as invasive tumours. As this kind of transformation is not connected with genomic alterations, it can be reverted by drug treatment. In infected cells, the expression of PAG was down-regulated and the lack of Csk in the plasma membrane resulted in increased Hck (haematopoietic-cell kinase) activity and proliferation. Following drug treatment the amount of PAG and Csk markedly increased at the plasma membrane and suppressed the oncogenic effect of the hyperactive kinase (108). In my opinion the fact that a pathogen evolutionary developed a mechanism to target PAG shows that PAG is an important actor in the cellular theatre and contributes to the fate of the cell. The fact that there was no observable phenotype in PAG-/- mice does not mean that PAG is unimportant, in contrary its function has to be ensured by redundant molecule(s) to avoid uncontrolled processes that could have fatal consequences for the whole organism.

4.2. Perspectives for clinical application

TRAPs were found to be useful markers in hematopathology as their expression can be connected to different cell types and therefore lymphomas of different origin. PAG, LAT and SIT are expressed both in T- and B-cells, LIME and TRIM are T-cell specific and NTAL is typical for B cells. Accordingly LIME and TRIM were found to be associated with T-cell lymphomas and LAT was the best marker for peripheral T-cell lymphoma. NTAL was the only TRAP expressed in hairy-cell leukaemia and in lymphocyte predominant Hodgkin disease. It was a clear marker together with PAG for follicle center lymphoma. NTAL, PAG and SIT showed complementary patterns of expression in B-cell lymphomas. SIT could be used as a marker for chronic lymphocytic leukaemia and mantle-cell lymphoma while it is not expressed in follicle-center lymphoma (109). PAG was earlier shown to be expressed in germinal centers of lymphoid follicules, but not in mantle cell lymphoma what may reflect role in regulation of proliferation and differentiation of germinal center B-cells. PAG could be a suitable positive marker for follicular lymphoma (together with NTAL) and a negative marker for mantle-cell lymphoma (110).

5. SDF-1 induced migration

In the fight against pathogens lymphocyte migration plays a crucial role. For effective immune response naive T-cells have to be mobilised from the lymphoid tissues to the sites of antigen exposure. The induction of an immune response is mediated by the continuous migration of naive T-cells through the secondary lymphoid organs in response to chemoattractant gradients provided by chemokines (111). The chemokine superfamily of small, secreted proteins can be divided into four groups based on their N-terminal cysteine motif: CXC (α), CC (β), C (γ) and CX_3C (δ). Chemokine receptors are members of the seven transmembrane domain G protein coupled receptor family. The receptors CXCR1-5 bind CXC chemokines. CXCR4 was described in 1996 as a co-receptor playing role in HIV-infection and therefore became the focus of many research fields. It is expressed on wide range of cells, including lymphocytes. In T-cells it can be found predominantly on unstimulated, naive cell population. It has a single ligand, the widely expressed stromal cell-derived factor 1 (SDF-1, also called CXCL12), that exists as two splicing forms: SDF-1α and SDF-1β. The mRNA of SDF-1α is more abundant than SDF-1β. They differ in amino acid sequence by the presence of additional 4 amino acids at the C-terminus of SDF-1β (112). CXCR4 and SDF-1 were found to be important not only for the homing of T-cells and recruitment to the sites of inflammation but also for haematopoiesis, organogenesis, vascularisation and embryogenesis (113). Therefore it is not surprising that disruption of either the SDF-1 or CXCR4 gene is lethal in mice (114).

Several groups have shown that chemokine receptors localise to the lipid rafts after stimulation (115, 116). Like in the case of participants in TCR signalling there is still a debate about the raft-association, but it appears that the reasons for this are the same as described in *section 2.4*, namely the different methodological approaches. As shown for CD3 and CD28, cross-linking of chemokine receptors also causes lipid raft aggregation and polarisation that is critical for processes such as migration (116). Knowing that chemokine receptors possess large transmembrane domains it is not surprising that upon cholesterol depletion CXCR4 undergoes conformational changes and SDF-1 binding is altered (117). CXCR4 is present in the non-raft region of the plasma membrane, but from yet unknown reasons SDF-1 is able to bind only to the raft associated pool of the receptor. It was suggested that in circulating T-cells, CXCR4 is mainly outside of the lipid rafts and following an activation signal (e.g.: CD4 engagement) it becomes recruited to the rafts (116, 117) where SDF-1 can induce the

signalling pathway leading to chemotaxis. Rafts are probably acting as spatial organisers and amplification centres during chemotaxis (51).

The crucial role of Src kinases in the migration of haematopoietic cells is well established within the literature. A physical interaction between Lck and CD4 and the kinase activity of Lck are both required for the SDF-1 induced chemotaxis (118). Lck associates with a number of proteins that play a role in actin cytoskeleton reorganisation, which is necessary for the migratory response: Vav, PKCθ, Itk (118, 119), PI3K (120), c-Cbl (121, 122). In T-cells not only Lck but also Fyn is implicated in this process (121, 122). ZAP-70, which is a substrate for Lck, was also found to be involved in migration signalling (123), playing role in Erk activation (124). ZAP-70 may provide a link to PI3K, which showed increased tyrosine phosphorylation upon SDF-1 stimulation (125). Studies using special inhibitors established the crucial role of PI3K in migration signalling that is manifested via its effectors. One of PI3K target proteins is the serine/threonine kinase PKB (protein kinase B), which takes part in regulation of the transcription factor NFκB (120).

The ability of Csk to inhibit the chemotactic response (118) further highlights the role of Src kinases. Fyn may have a regulatory role in migration via association with the tyrosine phosphatase SHP-2 and phosphorylation of the adaptor molecule Cbl. SHP-2 interacts with CXCR4 and forms a complex with SHIP (SH2-containing inisitol phosphatase), Cbl, PI3K and Fyn. While SHIP down-regulates migration, SHP-2 has a positive regulatory function. Cbl may have a dual function: as an adaptor protein it associates with SHP-2, Vav and PI3K thereby taking part in positive regulation. On the other hand, as a ubiquitin ligase it is involved in degradation of various receptors and tyrosine kinases (121). The fourth protein that negatively regulates migration is p62Dok-1 (downstream of tyrosine kinase). As Src kinases play role in phosphorylation and activation of this protein, it provides a feed back loop which down-regulates the migratory signals (126). CD45 is the second phosphatase (beside SHIP) involved in regulation. It activates Src kinases (via dephosphorylating their negative regulatory tyrosine) and the components of the focal adhesion complex (127).

Proteins of the focal adhesion complex (Pyk2, FAK, Crk, p130Cas, paxillin) are stimulated by SDF-1 (125, 126). Pyk2 (proline-rich tyrosine kinase 2) is critical for signalling through G protein coupled receptors. It associates with ZAP-70 and Vav and participates in the activation of the MAPK pathway (126). As chemokine receptors are G-protein coupled, G protein subunits also take part in the migratory response of lymphocytes. GTPases (e.g. Rac, Rho, Rap1) that play a role in actin cytoskeleton

reorganisation and cell polarisation are activated by the G protein subunits β and γ (120, 128).

There is evidence for interplay between the TCR and CXCR4 mediated signalling pathways. It has been shown that proteins that play an important role in proximal TCR signalling (e.g. Src kinases, ZAP-70, PLCγ (125)) are activated upon SDF-1 stimulation and are involved in chemokine receptor signalling (129). A recent paper (130) has identified with the help of FRET analysis and coimmunoprecipitation that there is a physical interaction between CXCR4 and the TCR. They also show that upon SDF-1 stimulation the CXCR4 mediated signalling pathway exploits the constitutively tyrosine phosphorylated pool of TCR-ZAP70 complexes leading to Ras activation and prolonged ERK activation. Earlier studies have revealed the existence of low amounts of constitutively associated TCR and ZAP-70 (131). Via depleting key TCR signalling proteins, SDF-1 may regulate the threshold of T-cell activation (129). Once this threshold was overcome, a stable contact forms between the APC and the T-cell, which would not occur without inhibiting the cells´ mobility. The calcium release that is induced via TCR stimulation leads to phosphorylation of the T-cell specific myosin MyH9 (nonmuscle myosin heavy chain IIA), which was shown to be the motor of T-cell crawling. Phosphorylation down-regulates its motor function and this leads to the “stop migration signal”, despite the high calcium concentration, that would otherwise activate calcium-dependent proteins, like myosin (132, 133). TCR stimulation also blocks SDF-1 induced chemotaxis via PKC-dependent internalisation of CXCR4 receptor (129).

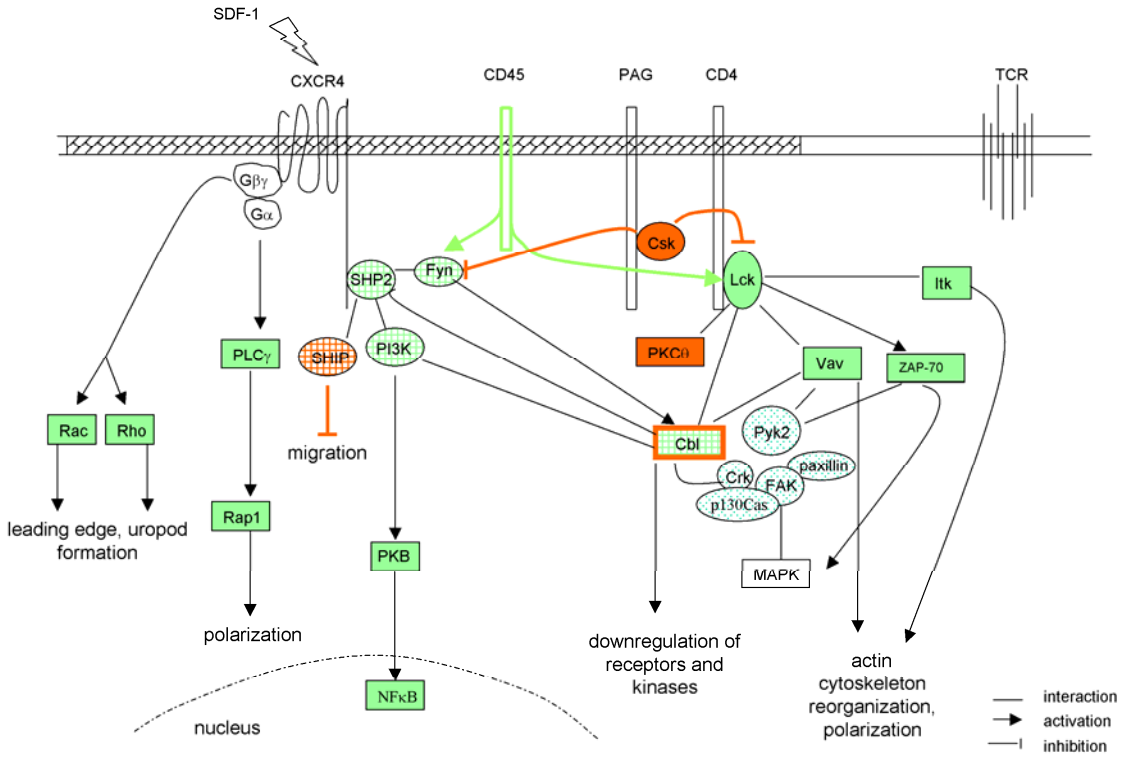


Figure V. Proteins involved in migration signalling

Some of the proteins and their interactions that take part in SDF-1 induced signalling pathways are shown in this figure. Actin cytoskeleton reorganisation and cell polarisation are mediated in a Src kinase (via Vav and Itk)- and G protein dependent manner (via GTPases). The Src kinases (Fyn and Lck) are both complex forming units and activators of other proteins. Members of the focal adhesion complex are distinguished with a fence pattern, while squared lines indicate a complex formed by both positive and negative regulators: Fyn, SHP-2, PI3K, SHIP, Cbl. Red colour indicates inhibitory role (Cbl has a dual function). The pattern of bricks represents lipid rafts.

RESULTS I

1. How much does palmitoylation contribute to the function of PAG?

Like all lipid raft associated transmembrane adaptor proteins, PAG also contains a dicysteine motif (CSSC) within its cytoplasmic domain that is located in a juxtamembrane position and is conserved among species (*Figure 1.1*.).

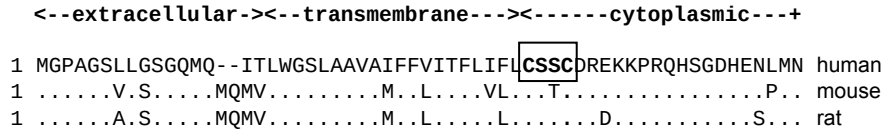


Figure 1.1. Alignment of the first 60 amino acids of PAG
Dots represent conserved amino acids, hyphens indicate gaps in the alignment.

This motif is a potential target for S-acylation and indeed, PAG has been shown to be S-acylated by ^{3}[H]palmitate-labeling. This lipid modification is thought to be responsible for the GEM targeting of the protein (4) based upon results obtained with LAT mutants (34), where palmitoylation was shown to be indispensable for targeting LAT to the GEMs. It has also been shown that lipid raft targeting of LAT is required for its function and for proper T-cell signalling (34, 35). However, the importance of GEM localisation was recently brought into question, as the non-raft associated LAX/LAT chimera was able to restore T-cell function in LAT deficient cells (134). LAX has been described as an adaptor protein which is excluded from the GEMs (135). The distribution within the plasma membrane of both, LAX and the LAX/LAT chimera was studied using sucrose gradient centrifugation. The apparent contradiction between the two studies (34, 134) dealing with the role of lipid rafts in LAT function may be solved with the newly emerging results from the lab of Vaclav Horejsi (Horejsi V. Signalling roles of lipid rafts and raft associated adapter proteins. 16th European Congress of Immunology, Paris, France, 2006, Book of Abstracts p. 41). Using a gel filtration method, they detected LAX and LAX/LAT in the same fractions as LAT, which explains why the chimera could restore the function of the wt LAT. The palmitoylation mutant of LAT was excluded from the raft fractions which is in line with the previous results (34). Horejsi proposed a new view of lipid rafts suggesting that two kinds of rafts exist: light and heavy rafts. The latter consist less of lipids and more of proteins than the light rafts and therefore do not float upon sucrose gradient centrifugation. The reason why LAX and the LAX/LAT chimera were detected in the non-

raft fractions may be that they are associated with the heavy rafts, possibly as part of a larger protein complex.

However, the question of whether the palmitoylation of PAG is essential for its localisation and function remained open. Therefore we have investigated both the subcellular distribution and the function of the PAG palmitoylation mutant.

1.1. Expression and localisation of the palmitoylation mutant

To study the effect of the CSSC → ASSA mutation (hereafter referred to as CxxC) it was necessary to incorporate an epitope tag into both the wild type and the mutant PAG construct to distinguish them from the endogenous protein upon transfection since PAG-deficient cell lines do not exist at present. Therefore an 8 amino acid peptide, Flag-tag (DYKDDDDK) was fused to the carboxyl terminus of PAG. Using Western blot and microscopic approaches the expression of the fusion proteins was found to be unaltered. Our aim was to generate stably transfected Jurkat T-cell lines, however we did not succeed. Therefore Jurkat E-6 T-cells (clone Kab14) were transiently transfected with the PAG constructs in all experiments performed. The expression levels of both the Flag-tagged wild type PAG (hereafter referred to as wt PAG) and the mutant protein (CxxC) were comparable (*Figure 1.2.A*). Lysates from human peripheral blood T-cells and Jurkat T-cells were probed for endogenous PAG. The expression pattern of endogenous PAG did not show any difference compared to the overexpressed PAG as both molecular weight forms (at 72 and 60 kDa) were detected and showed similar distribution to the overexpressed constructs (*Figure 1.2.B*). Since both the wild type and CxxC mutant proteins are expressed in two forms, the difference in size is not due to the post-translational lipid modification.

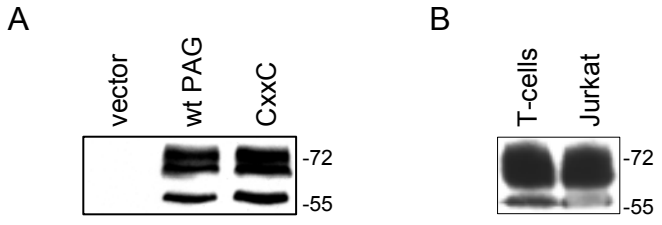


Figure 1.2. Expression of wt PAG and CxxC mutant
A) Whole cell lysates from Jurkat T-cells transfected with either vector alone, wt PAG or CxxC mutant. The expression was visualised with rabbit-anti-Flag antibody. **B)** Whole cell lysates from human peripheral T-cells and Jurkat T-cells stained with mouse-anti-PAG antibody.

The subcellular localisation of the mutant PAG was investigated by immunofluorescence microscopy. *Figure 1.3.A* shows that neither the inclusion of an epitope tag nor mutation of the palmitoylation motif affected PAG expression and its plasma membrane localisation. The specificity of anti-Flag staining is demonstrated by the negative control where cells transfected with vector alone show no staining. Flag-tagged TRIM served as a positive control, as a transmembrane adaptor protein having a staining pattern that is typical for a membrane associated protein.

Although the ring shaped staining observed under the fluorescence light microscope was identical for endogenous PAG, the Flag-tagged constructs and TRIM, and seemed to suggest plasma membrane localisation of the palmitoylation mutant, a confocal microscopy approach that enabled us to analyse the cells with higher resolution was used to strengthen these data. Jurkat T-cells transfected with either the wild type or mutant PAG were co-stained for the Flag-tag and a membrane associated protein which was either endogenous TRIM or Fyn (*Figure 1.3.B*). The staining pattern and the colocalisation with typical plasma membrane associated proteins is a clear proof of membrane localisation for the mutant PAG and excludes the possibility of a submembrane position. Anti-Flag staining also shows some intracellular staining that supports the idea of ER-association mentioned above.

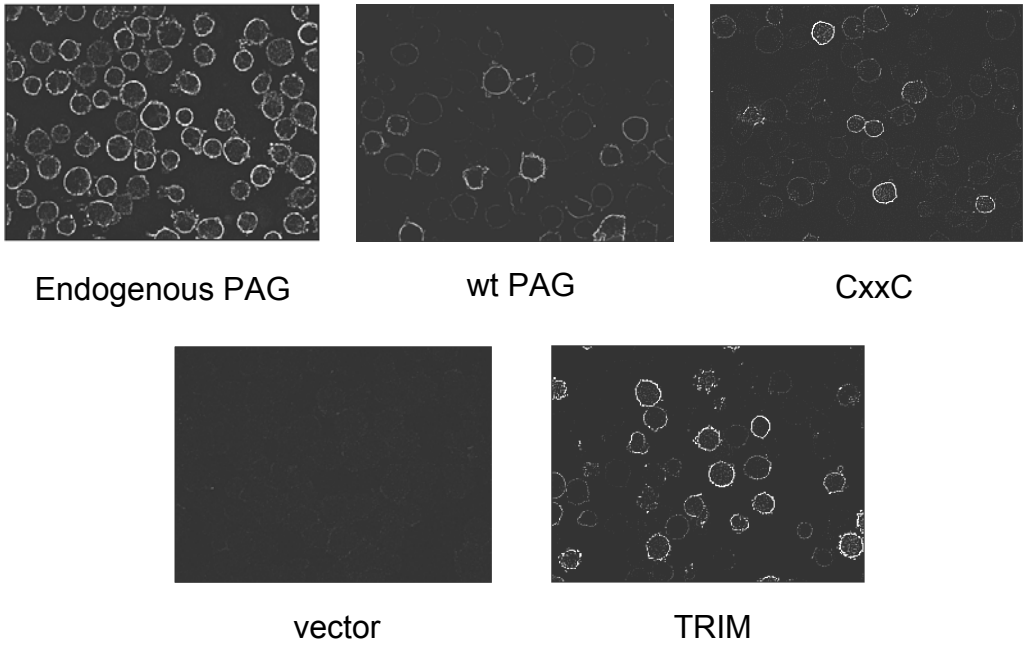


Figure 1.3.A Membrane localisation of wt PAG and the CxxC mutant
Jurkat T-cells transfected with the indicated constructs were stained for the Flag tag with mouse-anti-Flag antibody, and visualised with fluorescence microscope. Endogenous PAG was stained with mouse-anti-PAG antibody.

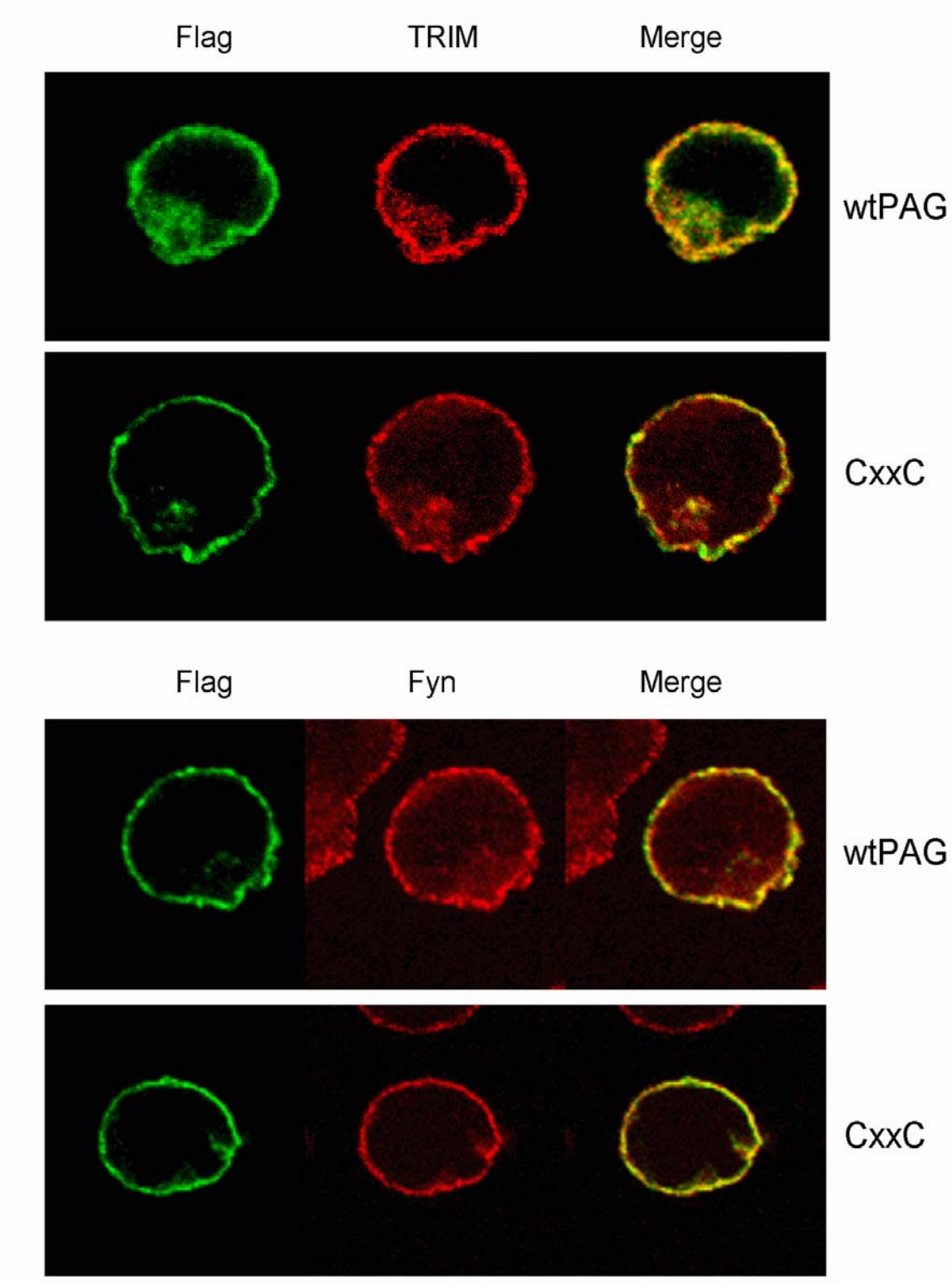


Figure 1.3.B Colocalisation of wt and CxxC mutant PAG with TRIM and Fyn
Jurkat T-cells transfected with wild type and mutant PAG were co-stained for Flag-tag (rabbit-anti-Flag/donkey-anti-rabbit-FITC) and either for the transmembrane adaptor protein TRIM (mouse-anti-TRIM/goat-anti-mouse-Texas red) or the plasma membrane anchored Fyn (mouse-anti-Fyn/goat-anti-mouse-Texas red) and visualised with confocal microscope.

1.2. The effect of palmitoylation mutation upon GEM localisation

Once the membrane localisation of the mutant PAG was established, the next step was to determine whether it was still capable of being targeted to the GEMs. Lysates from transfected Jurkat T-cells were fractionated upon sucrose density gradients and subsequently 9 fractions were collected. Since CD59 is a glycosylphosphatidylinositol-anchored protein it is considered to be a proper GEM marker (4, 50), therefore a dot blot with a sample from each fraction was stained with anti-CD59 antibody. Based upon the CD59 staining we conclude that fractions 2-4 represent the GEMs (*Figure 1.4.A*) and the heavy fractions 8-9 are considered as non-GEM fractions.

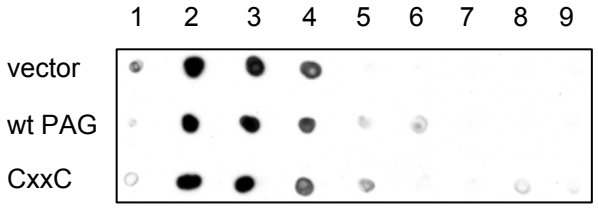


Figure 1.4.A Staining for GEM fractions upon sucrose density gradient fractionation
1µl from each fraction was dotted on a nitrocellulose filter and was stained with mouse-anti-CD59 antibody.

The distribution analysis of wt and the mutant PAG shows that while wt PAG preferentially localises to the GEMs, the CxxC mutant is more abundant in the non-GEM fractions. The 60 kDa form of PAG was always found in the heavy fractions (*Figure 1.4.B*).

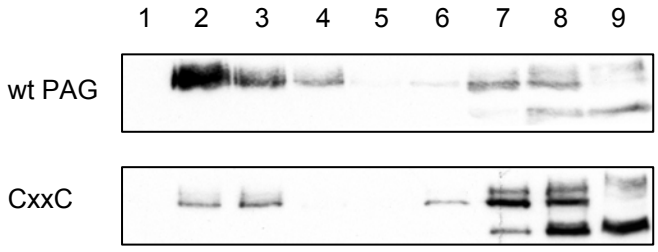


Figure 1.4.B Distribution of wt and CxxC PAG among the membrane fractions
Rabbit-anti-Flag staining.

Since a recent paper pointed out that acylation alone was not sufficient for the complete targeting of LAT to the lipid rafts (136), it is not surprising that the palmitoylated wt PAG is not exclusively raft localized. The idea of Shogomori et al. is that S-acylation alone is insufficient to overcome the large energetic cost of packing transmembrane helices into rafts. Constitutively raft associated proteins (e.g. GPI-anchored proteins or certain adaptor proteins) probably serve as anchors via protein-protein interactions for

those proteins which have less affinity for lipid raft binding (136). This could explain the remaining GEM localization of the mutant PAG. One candidate for such interaction could be EBP-50, which possesses two PDZ domains and could thereby potentially bind two PAG molecules. A dimer forming in this way, consisting of one endogenous and one mutant PAG could recruit the mutant into the lipid rafts. Another candidate protein that may support PAG recruitment to the lipid rafts is Fyn. Dual acylation (i.e. myristoylation, palmitoylation) of the Src family kinases also attributes them with an anchoring role. Based upon the observation that the enrichment of CD4 in the GEMs is not only palmitoylation-dependent, but also requires Lck association (57), Fyn could play a similar role in anchoring PAG as Lck does in recruiting CD4.

Pooling together the GEM- (2-4) and the non-GEM (8-9) fractions highlighted the differences and also made it possible to compare the samples on the same gel excluding the possibility that variations in electrophoresis or blotting were responsible for the differences observed. Staining for LAT, another lipid raft associated protein, shows that the integrity of GEMs remained intact and that the overexpression of the PAG constructs did not alter the distribution of other GEM localised proteins. Whole cell lysates prepared from the same transfectants prior to GEM preparation were included as controls for protein expression. (*Figure 1.4.C*)

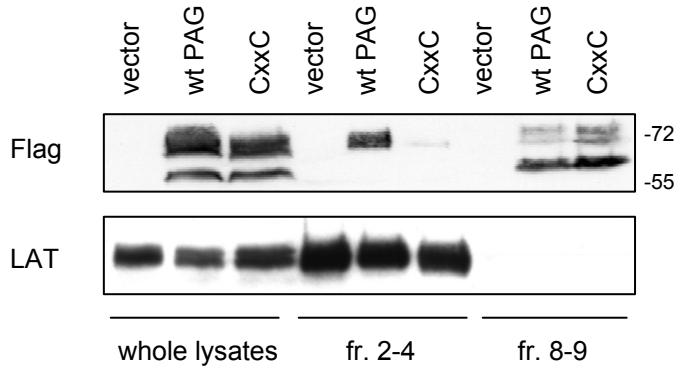


Figure 1.4.C GEM localisation of wt and CxxC PAG
Pooled fractions 2-4 and 8-9 represent GEM- and non-GEM fractions, respectively. Rabbit-anti-Flag staining shows the localisation of the PAG constructs, rabbit-anti-LAT staining indicates intact GEM architecture. As a control for expression levels $1x10^6$ cells from each transfection were lysed and loaded together with the fractions.

1.3. Is the palmitoylation mutant phosphorylated?

PAG is one of the most abundant phosphoproteins in unstimulated T-cells (4). Therefore, we looked to see whether the non-raft associated PAG mutant becomes tyrosine phosphorylated. PAG was immunoprecipitated using Flag M2 affinity gel. Since the presence of the antibody chains in immunoprecipitates (IPs), particularly the heavy chain may interfere with detecting proteins of similar size (~55 kDa) on Western blot the captured constructs were eluted from the Flag-agarose with Flag peptide. *Figure 1.5.* clearly shows that the equally expressed wt and CxxC PAG were phosphorylated to the same extent. One can also observe that the lower molecular weight form of PAG is less phosphorylated, suggesting that the 60 kDa form of PAG differs from the 72 kDa form in the extent of phosphorylation.

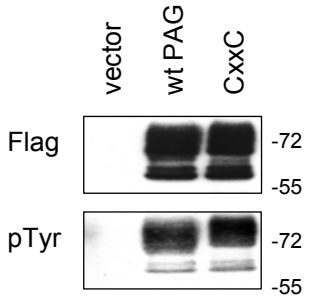


Figure 1.5. Both wt and CxxC mutant PAG become phosphorylated
Flag immunoprecipitates from transfected Jurkat T-cells were immunoblotted with rabbit-anti-Flag and mouse-anti-phosphotyrosine-HRP [pY20] antibodies.

1.4. Which kinase phosphorylates PAG?

Studies on peripheral T-cells and thymocytes from $Fyn^{-/-}$ mice have clearly demonstrated that Fyn is predominantly responsible for the phosphorylation of PAG in resting T-cells (98). However, PAG phosphorylation was not completely inhibited in Fyn-deficient cells indicating that other kinases may be involved in PAG phosphorylation (98). The kinase activity of Fyn and Lck show different localisation within the plasma membrane of both resting and stimulated T-cells. Lck kinase activity was mainly detected outside of the lipid rafts, while Fyn showed a much higher activity in the rafts than in non-raft fractions, but the amount of Fyn is also much higher in GEMs (14, 98). This means that the relative kinase activity of individual Fyn molecules may be equal throughout the plasma membrane or even higher in the non-raft fractions and the reason why less Fyn activity is detected outside of the rafts might be that less Fyn localises to this membrane fraction. From this point of view, it is worth to speculate that the few Fyn molecules residing in the non-raft fraction may have a comparable enzymatic activity to the raft-associated Fyn

population and could be responsible for the phosphorylation of the PAG palmitoylation mutant. On the other hand, one has to take into consideration that the low amount of Fyn may be inefficient in phosphorylating PAG and that another kinase might also be involved. Indeed, Brdicka et al. showed that both Fyn and Lck are capable of phosphorylating PAG (4) and in mast cells Lyn plays this role (137).

To determine which kinase is responsible for the phosphorylation of the palmitoylation mutant, Jurkat T-cells transfected with vector, wt PAG and CxxC PAG were treated with the Src kinase inhibitor PP2 (pyrazolopyrimidin) prior to lysis. PP2 was also included in the lysis buffer to prevent post-lysis phosphorylation. Anti-Flag IPs were probed with anti-Flag antibody to ensure that equal amounts of the PAG constructs were immunoprecipitated and subsequently the blots were stained for phosphotyrosine. *Figure 1.6*. shows that despite the higher protein amounts in the PP2 treated immunoprecipitates, the tyrosine phosphorylation was markedly reduced, indicating a Src kinase dependency for the phosphorylation of both wt and the CxxC mutant of PAG.

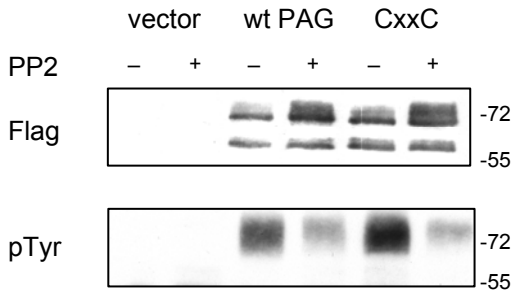


Figure 1.6. Phosphorylation of wt and CxxC PAG is PP2 sensitive
Flag immunoprecipitates from transfected Jurkat T-cells treated with PP2 (+) or left untreated (-) were immunoblotted with rabbit-anti-Flag and mouse-anti-phosphotyrosine [4G10] antibodies.

To get further insight into the mechanism of CxxC PAG phosphorylation, anti-Flag immunoprecipitates were tested for Fyn binding. Elution of the PAG constructs from the agarose with a Flag peptide was especially useful in these studies since the heavy chain of immunoprecipitating antibody would interfere with the Fyn staining because of the similarity of the molecular weights of these two proteins (55 and 59 kDa, respectively). To avoid that the lower molecular weight band of the overexpressed PAG construct overlaps with the band representing Fyn, the immunoblots were first probed for Fyn. Whole cell lysates were also included to show the expression level of the proteins of interest. *Figure 1.7.* shows that in addition to comparable levels of expression, Fyn was found to associate with both wt PAG and the CxxC mutants.

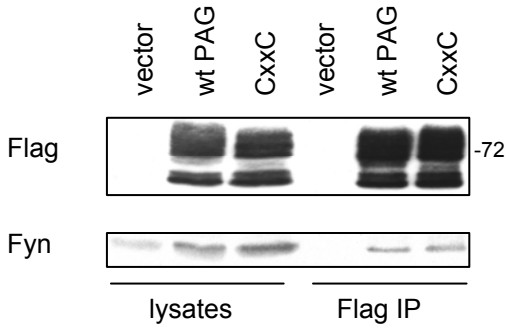


Figure 1.7. Both wt and CxxC PAG associate with Fyn
Jurkat T-cells were transfected with the indicated constructs and the immunoblot was stained with rabbit-anti-Flag and mouse-anti-Fyn antibodies.

Since Lck was also shown to be capable of phosphorylating PAG (4), the possibility exists that it could also contribute to the phosphorylation of the mutant PAG, which mainly localises outside of the lipid rafts where Lck is much more abundant than Fyn. To test this, Lck deficient Jurkat T-cells (JCam 1.6) were transfected either with one of the PAG constructs or the vector alone. One set of transfectants was cotransfected with Lck as a control to show that a possible difference in phosphorylation is due to lack of Lck and does not originate from the use of a different cell line. The expression level of Lck was checked in whole cell lysates. The lowest panel on *Figure 1.8*. shows three Lck species: the band at 45 kDa represents the defective Lck (Δexon7) from the mutagenesis, which does not have kinase activity. The upper bands (at 70-75 kDa) represent the kinase active Lck transcribed from the transfected CFP containing construct (the CFP that is inserted between the SH4 and SH3 domains causes the increase in size of Lck). The appearance as a double band is probably due to phosphorylation. Flag IPs were probed for Lck association and with anti-phosphotyrosine. Although the phosphorylation of both PAG constructs required the presence of Lck no direct association of Lck to PAG was detected (*Figure 1.8.*). However, the lack of association does not exclude the possibility that the phosphorylation was mediated by Lck. Although it is more likely that the two pools of Fyn, the lipid raft associated and the non-raft pool, are responsible for phosphorylation of PAG wherever it localizes. The indispensable role of Lck is most likely in the activation of Fyn (14, 73) that in turn phosphorylates PAG. Experiments employing siRNA against Fyn in wt Jurkat cells would be required to clarify this issue.

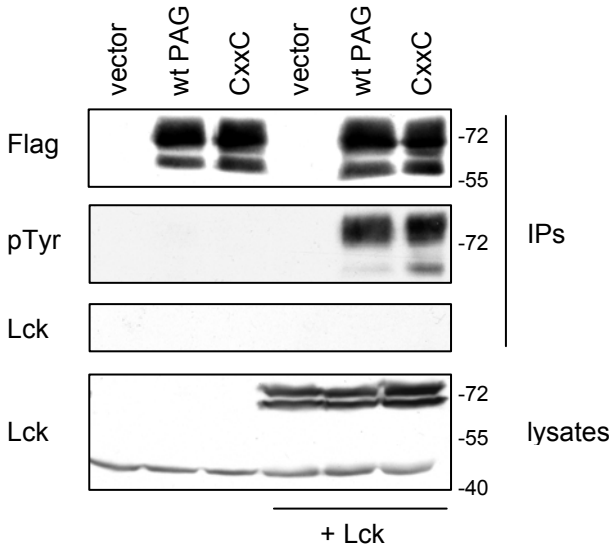


Figure 1.8. Lck plays a role in phosphorylation of PAG
JCam 1.6 cells were transfected with the indicated constructs. The immunoblot with Flag IPs was probed with rabbit-anti-Flag, mouse-anti-phosphotyrosine [4G10] and mouse-anti-Lck antibodies. Whole cell lysates were probed with mouse-anti-Lck to control the expression level of Lck.

1.5. Does the palmitoylation mutant recruit Csk and EBP50?

Having shown that the palmitoylation mutant of PAG is capable of binding Fyn and is phosphorylated to the same extent as the wt protein, we next asked whether it is also capable of recruiting Csk to the plasma membrane, since the interaction between PAG and Csk is phospho-dependent (4, 59, 138). Flag immunoprecipitates probed for Csk clearly show that the mutant is indeed capable of binding Csk (*Figure 1.9*.).

The third binding partner of PAG is EBP-50, a cytoplasmic adaptor protein that binds to the C-terminal TRL motif of PAG via one of its PDZ domains in resting T-cells. Upon TCR stimulation this association is lost (62). We wished to test whether this interaction occurs between the mutant PAG and EBP-50 as well, but as we could not detect EBP-50 association with the wt PAG we suspected that the C-terminal Flag-tag may interfere with EBP-50 binding. Therefore, we took the advantage of the N-terminally tagged PAG construct (N-PAG) that was newly designed during the course of my other project (generation of this construct is described in *Results II.*). First, we checked whether this construct localises to the plasma membrane (see in *ResultsII/2.4.*) and associates with its binding partners in a similar manner to the C-terminally tagged PAG (*Figure 1.10.A*), then we generated the N-terminally tagged palmitoylation mutant by site directed mutagenesis ($CxxC_N$). The N-terminally tagged PAG constructs were not as

efficiently expressed as the C-terminally tagged ones, therefore the lower molecular weight form (~60kDa) of PAG was detected only in experiments where higher cell numbers were used (e.g. GEM preparation, *Results II/Figure 2.8.*). Despite the lower expression level and altered lipid raft localisation (Results II/4.) we found these constructs suitable for EBP-50 interaction studies. As *Figure 1.10.B* demonstrates, EBP-50 was present not only in Flag-IPs from wt N-PAG (as expected), but it was also associated with the palmitoylation mutant. Because of the high amount of EBP-50 expressed in Jurkat T-cells and the sensitivity of the antibody, it was necessary to cut the blot and probe the lysates and Flag-IPs with different antibody dilutions (1:10.000 vs. 1:1000, respectively).

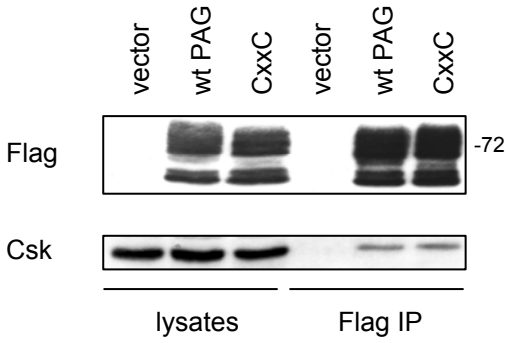


Figure 1.9. Wt PAG and the CxxC mutant equally bind Csk
Jurkat T-cells were transfected with the indicated constructs and the immunoblot was stained with rabbit-anti-Flag and rabbit-anti-Csk antibodies.

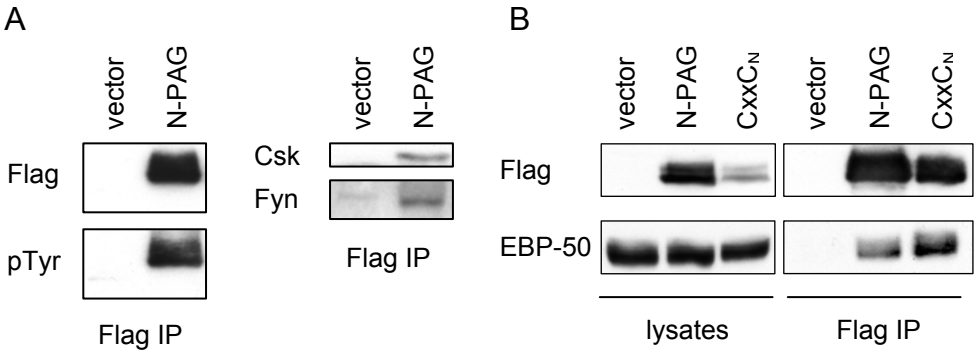


Figure 1.10. Interaction with EBP-50 is not altered
Jurkat T-cells transfected with the indicated constructs were lysed and immunoprecipitated with anti-Flag antibody. **A)** Expression, phosphorylation level, Csk and Fyn binding ability of N-PAG is demonstrated by rabbit-anti-Flag, anti-phosphotyrosine [pY20-HRP], rabbit-anti-Csk and rabbit-anti-Fyn staining of Flag immunoprecipitates. **B)** Lysates and IPs were probed for Flag expression with rabbit antibody. Rabbit-anti-EBP-50 staining was carried out on cut blots with 1:10.000 and 1:1000 antibody dilutions for lysates and IPs, respectively.

1.6. The CxxC mutant enhances specific TCR proximal events

PAG is a negative regulator of T-cell signalling as it down-regulates Src kinase activity by recruiting Csk to the lipid rafts. Since Src kinase activation is one of the first events upon TCR stimulation, we carried out a detailed analysis of proximal signalling. To monitor changes in the activity of the Src kinases, we stained the lysates obtained from vector, wt PAG or CxxC PAG transfected Jurkat T-cells, with Lck phospho-specific antibodies (*Figure 1.11*.A). The phosphorylation of the Lck activatory tyrosine (Y-394) is increased both in the basal state and upon stimulation due to CxxC mutant overexpression while no significant differences were observed in the phosphorylation of

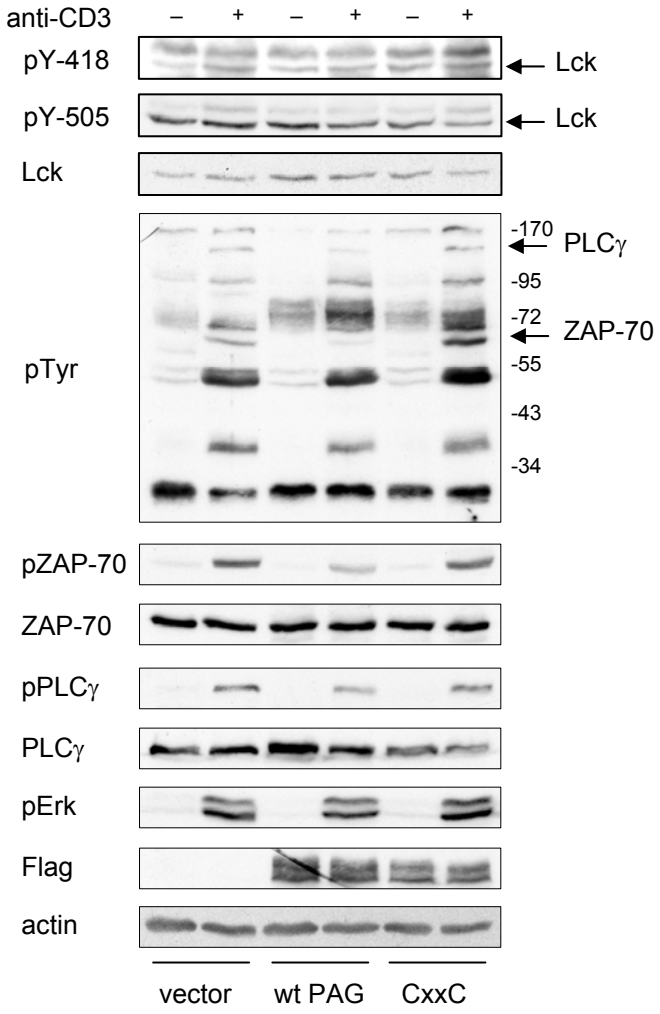


Figure 1.11.A In contrast with the inhibitory role of wt PAG, overexpression of the CxxC mutant has a positive effect on activation of several proximal signalling elements
Jurkat T-cells transfected with the indicated constructs were either left unstimulated (-) or were stimulated (+) via CD3 with OKT3 antibody for 2min. Lysates were stained against Lck, ZAP-70, PLCγ and Erk with total or phospho-specific rabbit antibodies (note, the anti-pY-418 antibody recognises the activatory tyrosine of Src, as well as Fyn and Lck – see *Figure 1.12*.).The blot was probed for total phosphotyrosine with

mouse-anti-pY [4G10]. Rabbit-anti-Flag staining monitors the expression level of PAG constructs. Mouse-anti-actin staining serves as loading control. Cross-reactivity was avoided by stripping the blot.

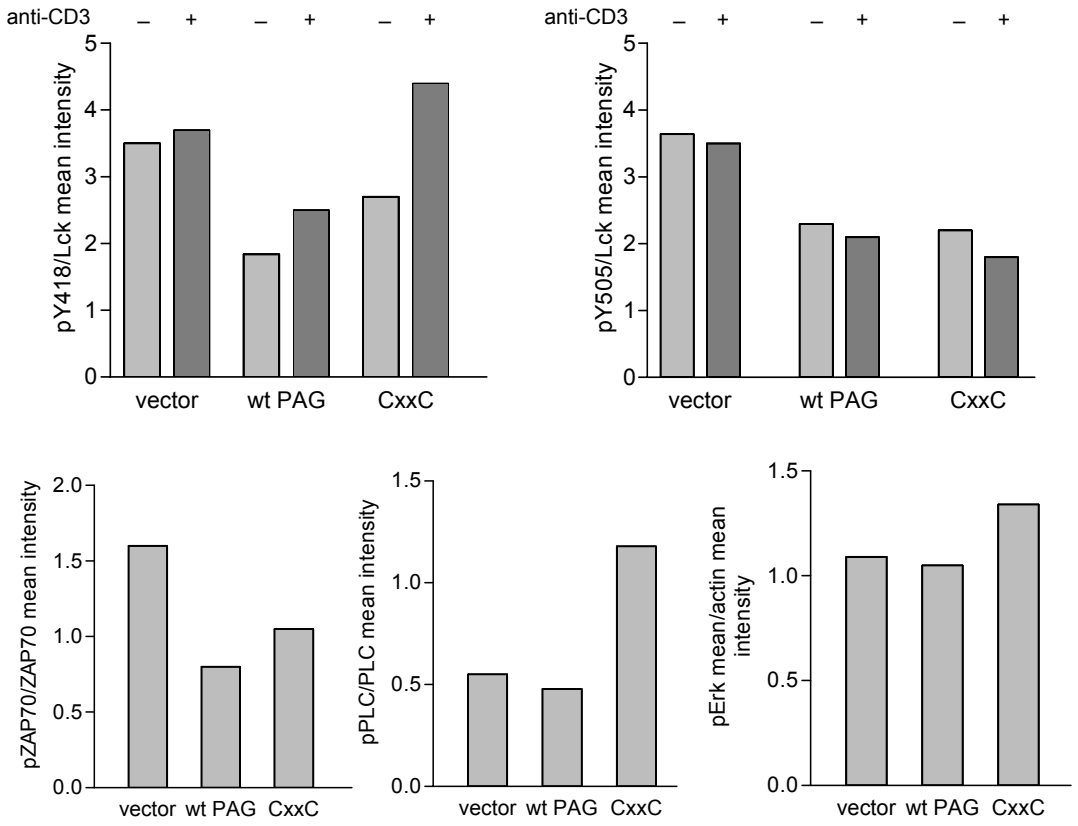


Figure 1.11.B CxxC PAG overexpression increases phosphorylation of Lck, PLCγ and Erk
The graphs represent the ratio of phospho-specific staining to total protein amounts for Lck, ZAP-70 and PLCγ. Phospho-Erk staining is quantified to actin. Note, that the increase in phosphorylation of Y-418 is 46% and 76% in CxxC overexpressing cells compared to the wt PAG in unstimulated and stimulated samples, respectively. The mean intensity of phospho-Erk staining shows 30% increase as an effect of the palmitoylation mutant. To calculate the mean values for Erk phosphorylation the values of Erk1 and Erk2 were added, divided with 2 and the result of this operation was divided with the values of actin signal.

the inhibitory (pY-505) tyrosine (*Figure 1.11.B*). Phospho-tyrosine staining revealed differences in phosphorylation of four proteins migrating at 170kDa, 150kDa, 70kDa and 36kDa. Phosphorylation of these proteins was inhibited by wt PAG and was enhanced upon overexpression of the CxxC mutant. Based upon the size of these proteins, we probed the blots further for two possible candidates, ZAP-70 (70kDa) and PLCγ (150kDa). Quantification of the signal intensities presented as the ratio of phospho-specific staining to total protein further highlight the observed differences for PLCγ (*Figure 1.11.B*). Using a phospho-specific antibody for ZAP-70, the increase in phosphorylation caused by the CxxC PAG compared to the vector control that was seen in the total pTyr blot disappeared. One explanation for this is the presence of multiple tyrosine phosphorylation sites within ZAP-70, which would be measured by anti-pY

probing but not by a single site-specific antibody. Phospho-Erk staining is a widely accepted indicator of more distal signalling. Quantification of pErk amounts to actin, showed 30% increase in CxxC mutant compared to the vector control and the wt PAG overexpressing cells (*Figure 1.11.B*).

```
409 GLARLIEDNEYTARQGAKFPI   Src
407 ..........Y.........   Fyn
384 ..........Y...E......   Lck
```

Figure 1.12. Sequence alignment of human Src, Fyn and Lck
The pY-418 Src antibody was raised against pY-419 in Src. Due to the obvious similarities in the amino acid sequence of the Src kinases the antibody recognizes the activatory tyrosine of Fyn (Y-417) and Lck (Y-394) as well. The target tyrosine is highlighted with underlining. Dots represent conserved amino acids.

Seeing the enhanced PLCγ phosphorylation induced by the overexpression of the palmitoylation mutant one might expect to detect an increase in intracellular Ca^{2+} flux. However, as *Figure 1.13.* shows we could not detect enhancement in CxxC PAG overexpressing cells, while wt PAG caused a moderate decrease. One can speculate that the signal measured in vector transfected cells is the maximum response and overexpression of the palmitoylation mutant could not increase it more. As a positive control the maximal level of calcium mobilisation was induced with ionomycin, an ionophore that is capable of elevating the intracellular calcium concentration.

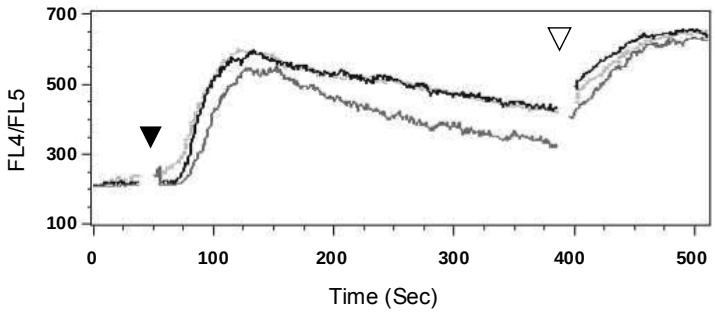


Figure 1.13. CxxC PAG does not increase Ca^{2+} flux
After recording the baseline activity, anti-CD3 [c305] was added (filled triangle) to the Jurkat T-cells transfected with either vector alone ( ), wt PAG ( ) or CxxC PAG ( ). Addition of ionomycin is indicated by the white triangle.

1.7. The effect on SDF-1 induced migration

Since PAG regulates Src kinase activity, processes mediated by the Src kinases appear to be the best read-outs for the functional consequences of depleting PAG from the lipid rafts. In addition to TCR signalling, Src kinases play an essential role in T-cell migration

(118, 122) therefore we extended our functional analysis with migration assays. We have chosen SDF-1 for stimulation because it is known that its receptor, CXCR4 is lipid raft localised and the signalling pathway generated upon SDF-1 stimulation involves exclusively the raft fraction of the plasma membrane (51, 116, 117).

Jurkat T-cells transfected either with wt PAG or the CxxC mutant were allowed to migrate through a polycarbonate membrane (5μm pore size) for four hours following the addition of the chemoattractant SDF-1 to the lower chamber of the transwells. As a negative control, cells from each transfection were also left unstimulated. This way we were able to take into account the spontaneous migratory activity of the cells that occurs without chemokine stimulation. Data obtained from six independent experiments demonstrate that while wt PAG mildly suppresses the migratory response, the palmitoylation mutant enhances it (*Figure 1.14*.). To exclude the possibility that the observed difference was due to altered cell surface expression of the chemokine receptor, the expression levels were measured by FACS. The profiles presented in *Figure 1.15.* demonstrate that the expression levels were comparable on the surface of vector and CxxC PAG transfected Jurkat T-cells. The wt PAG overexpressing cells show a somewhat higher expression level which further strengthens the phenotype in the sense that these cells showed a decrease in migratory response even though they were potentially more receptive to the stimulus.

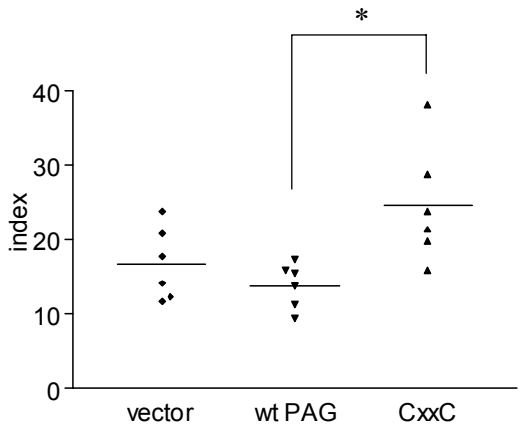


Figure 1.14. CxxC PAG enhances SDF-1 induced migratory response
SDF-1 induced migration of Jurkat T-cells transfected with the indicated constructs. The migration index shows the relative number of migrating cells compared to the unstimulated control. Statistical significance is defined as $P < 0,05$. The $*$ indicates P=0,01.

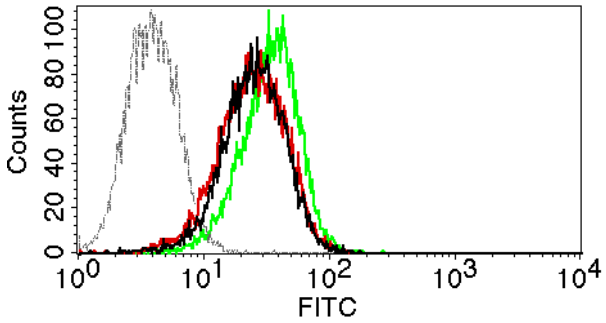


Figure 1.15. CXCR4 expression is not altered upon transfection
Vector (━), wt PAG (━) and CxxC PAG (━) transfected cells were extracellularly stained with mouse-anti-CXCR4/donkey-anti-mouse-FITC antibodies. Mean fluorescence intensity values (MFI) are: 30,3 for vector, 36,5 for wt PAG, and 31 for CxxC PAG. The grey curve (━) demonstrates that the aspecific background fluorescence from the secondary antibody was very low (MFI: 4).

1.7.1. SDF-1 induced calcium mobilisation

Although there are no available studies on calcium mobilisation upon CXCR4 receptor stimulation in T-cells, there are data which indicate that in different lymphocytic cell lines SDF-1 induces a transient increase in the intracellular Ca^{2+} concentration (139). Therefore, we performed experiments with our PAG mutants where we measured Ca^{2+} flux upon SDF stimulation. Our results indicate that Jurkat T-cells mobilize only moderate calcium amounts compared to other cell lines, such as SupT1 or MOLT-4 (139). However there is a clear difference between the Ca^{2+} flux generated by the CxxC PAG and wt PAG overexpressing cells. As a positive control the maximal level of calcium mobilisation was induced by ionomycin. (*Figure 1.16.*)

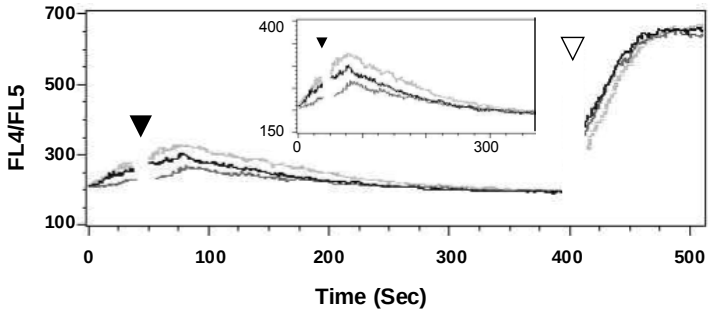


Figure 1.16. CxxC PAG slightly increases intracellular calcium flux upon SDF-1 stimulation compared to wt PAG
After recording the baseline activity, SDF-1 was added (filled triangle) to the Jurkat T-cells transfected with either vector alone (━), wt PAG (━) or CxxC PAG (━). Addition of ionomycin is indicated by the white triangle. The inset box is enlargement of the Y-axis.

1.7.2. Proximal signalling upon SDF-1 stimulation

As migration is a complex phenotype we also analysed the proximal signalling events upon SDF-1 stimulation. Src kinase activation was monitored by phospho-specific staining of the lysates acquired from SDF-1 stimulated Jurkat T-cells transfected with either of the PAG constructs or the vector alone. Overexpression of wt PAG strongly reduced the phosphorylation of the activatory tyrosine of Src kinases (note, the anti-pY-418 antibody recognizes the activatory tyrosine of Src, as well as Fyn and Lck – see *Figure 1.12*.). The palmitoylation mutant overexpressing cells showed not only an increased and more sustained activity of the Src kinases upon stimulation, but also an enhancement of their basal activity (compare time 0 in *Figure 1.17*.A). Erk phosphorylation indicated that the cells were indeed stimulated. Quantification of pErk

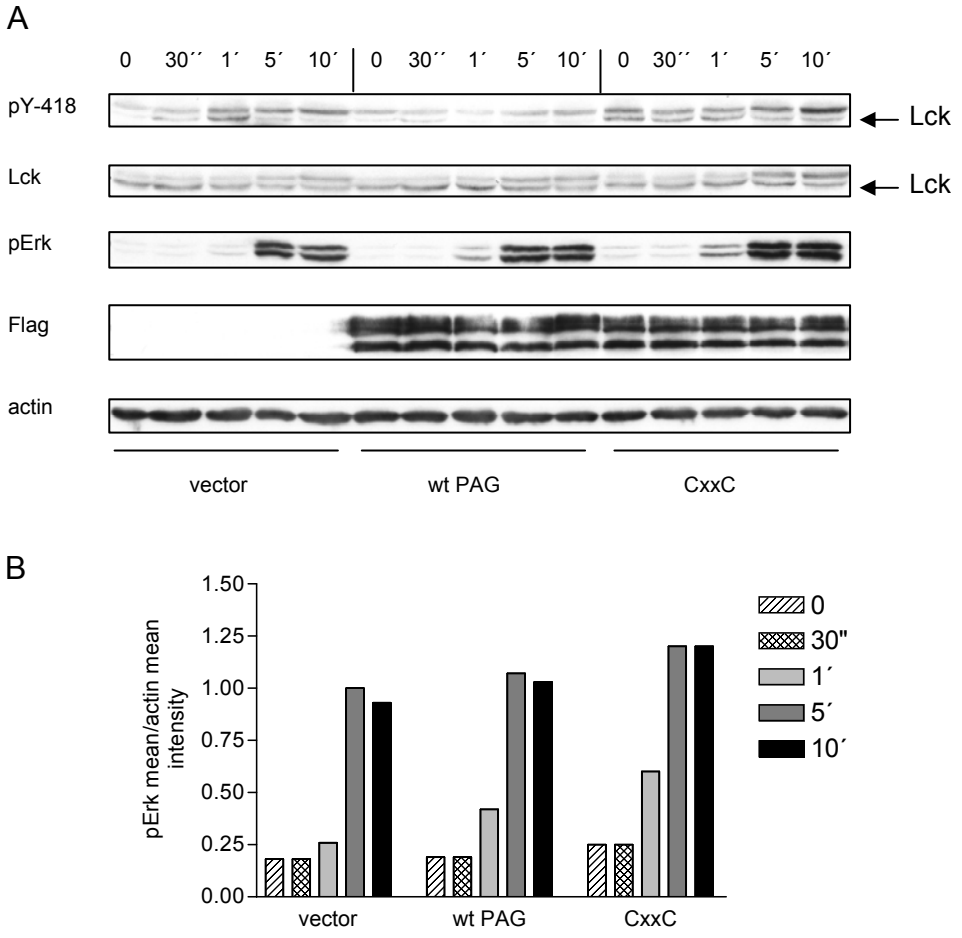


Figure 1.17. Src kinase activation is enhanced by the CxxC mutant upon SDF-1 stimulation **A)** Jurkat T-cells transfected with the indicated constructs were stimulated with SDF-1 for the indicated times. Lysates were probed with rabbit-anti-pErk, rabbit-anti-Flag and mouse-anti-actin antibodies. The phosphorylation level of the Src kinases was visualized by rabbit-anti-pY-418 staining (the lower band is Lck, the upper band represents Fyn). Mouse-anti-Lck staining shows total Lck amounts (lower band). **B)** Quantification of total pErk staining to actin counted from the mean values.

indicates a small (20%) increase in phosphorylation of total Erk in the cells overexpressing the palmitoylation mutant (see the graphs in Figure 1.17.B). Flag staining demonstrates comparable expression levels of the constructs while actin staining serves as loading control. These results further support those from the migration assays.

1.7.3. The effect of PAG downregulation on migration

In order to further investigate the role of PAG in SDF-1 induced migration, we examined the effect of small interfering RNA (siRNA) of PAG on the migratory response. As *Figure 1.18.A* shows, PAG gene expression is strongly downregulated by the siRNA in Jurkat T-cells. Reduction of PAG expression through siRNA clearly enhances the migratory response upon SDF-1 stimulation. It is noteworthy that the spontaneous migratory activity is also increased to a curtain extent by siRNA inhibition of PAG, however the difference is not significant (*Figure 1.18.B*).

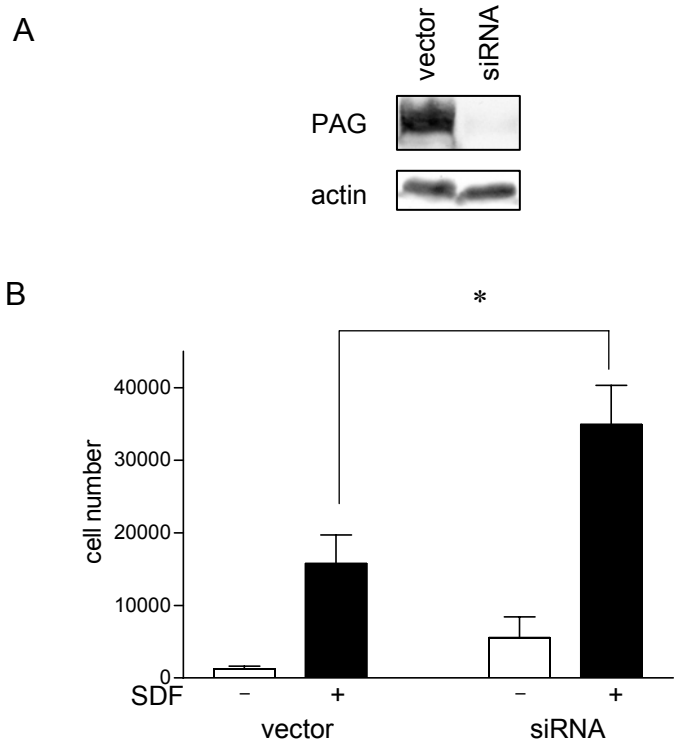


Figure 1.18. PAG downregulation results in increased migratory response
A) Lysates from Jurkat T-cells transfected either with vector control or PAG specific siRNA were probed with mouse-anti-PAG (MEM255) and mouse-anti-β-actin antibodies. **B)** Vector or siRNA transfected cells were loaded into transwells and either left without stimulation (-) or stimulated with SDF-1 (+). The number of migrated cells was counted in the lower chamber of the transwells after 4 hours of incubation by flow cytometer for 1min. Statistical significance is counted from three independent experiments and is defined as $P < 0,05$. The $*$ indicates P=0,01.

The stimulatory effect of PAG specific siRNA on T-cell migration further highlights the negative regulatory role of PAG in SDF-1 mediated signalling pathways. The observation that downregulation of the endogenous PAG results in the same functional disorder as dislocation of PAG from the lipid rafts points on the importance of localisation in the regulation of proteins and cellular processes.

1.8. Altered Csk distribution and its effects

Although the palmitoylation mutant of PAG is still capable of recruiting Csk to the plasma membrane, it localises it to a different membrane fraction than wt PAG. In this way, the inhibition imposed by Csk over the Src kinases within the lipid rafts is released and should lead to an increased and more sustained activation of Fyn and Lck.

To test this hypothesis, we reprobed the GEM fractions for Csk. While probing of whole lysates indicated that the levels of total Csk were equal between the transfectants, we found that the level of Csk within the GEMs was reduced to the level of vector transfected cells by over-expression of the CxxC mutant. Additionally, one can see that over-expression of the wild-type PAG increased Csk amounts within the GEM fractions (*Figure 1.19.*). Together these results suggest that the mechanism of action involves the redistribution of Csk within the cell. However, as PAG is capable of binding 20-30% of the total cellular Csk amount (14), the differences in PAG mediated Csk distribution are not detectable within non-GEM fractions where the bulk of Csk resides.

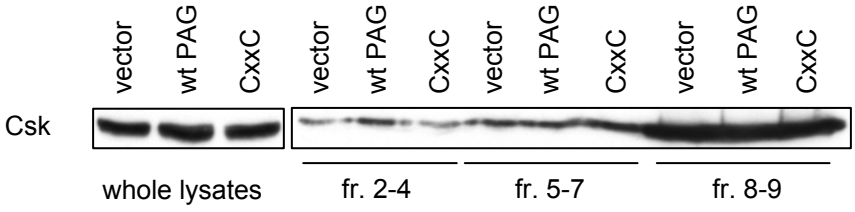


Figure 1.19. Distribution of Csk throughout the membrane fractions
Sucrose density gradient fractions 2-4 (GEM), 5-7 and 8-9 (non-GEM) were pooled and immunoblotted with rabbit-anti-Csk antibody. Whole cell lysates from $1x10^6$ cells, not exposed to the fractionation process, are showing equal Csk expression in all three transfectants.

If our proposed mechanism of CxxC mutant PAG function is correct, then the altered distribution of Csk should also be reflected by changes in the phosphorylation of the activatory and inhibitory tyrosines of Src kinases within the GEM and non-GEM compartments. Therefore we reprobed the pooled fractions using phospho-specific antisera for the activatory tyrosine of Fyn (pY-418). *Figure 1.20.A* shows that there is

indeed an increase in the activatory tyrosine phosphorylation within the GEM fractions of cells expressing the palmitoylation mutant. To demonstrate this more clearly, graphs with the quantified data normalised to total Fyn amounts are presented in *Figure 1.20.B*.

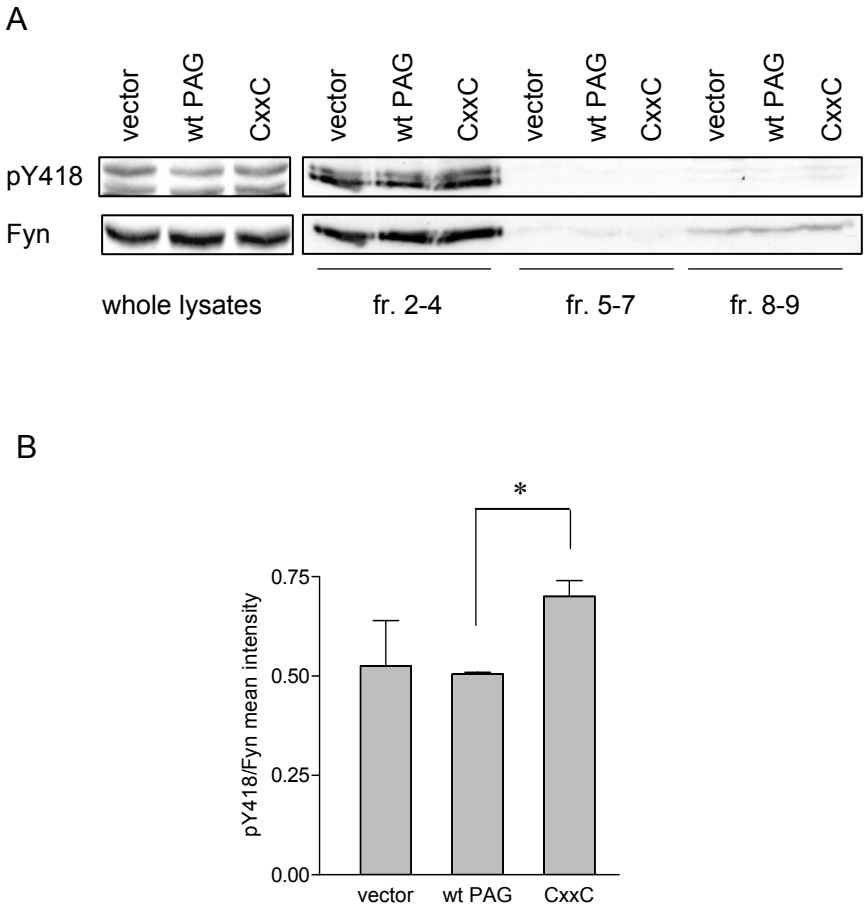


Figure 1.20. Fyn Y-418 phosphorylation is enhanced within the GEMs of CxxC PAG overexpressing cells

A) Rabbit-anti-pY-418 and mouse-anti-Fyn stainings of the pooled GEM fractions mirror the altered phosphorylation pattern of Fyn. The antibody against pY-418 recognises both Fyn and Lck. The upper band corresponds to Fyn. **B)** Quantification of pY-418 staining from two independent experiments. The bars represent the ratio of pTyr staining to total Fyn amount within GEM fractions. Statistical significance is defined as $P < 0{,}05$. The $*$ indicates P=0,04.

DISCUSSION I

The aim of this project was to clarify the role of palmitoylation in the localisation and function of PAG. The questions we have propounded were similar to those addressed in studies of LAT, which have recently brought controversial results (34, 35, 134). LAT is one of the lipid raft associated transmembrane adaptor proteins and, as such, it possesses a dicysteine motif that was shown to be palmitoylated and play a role in targeting of the protein to the GEMs (34). As lipid raft localisation of LAT was shown to be required for its phosphorylation and the initiation of signalling pathways downstream of the TCR (35, 95) one could conclude that the palmitoylation of LAT is crucial for proper T-cell function. However, the newly published data on the LAX/LAT chimera (134) seemed to scramble the picture formed about LAT over the past decade. The attractive hypothesis concerning two types of lipid rafts (described in *Results I.*), i.e. light and heavy rafts (V. Horejsi) offers an explanation to the contradiction regarding the necessity of lipid raft association for the function of LAT.

Both the endogenous PAG and the overexpressed PAG constructs are expressed in two molecular weight forms: 72 and 60 kDa (*Figure 1.2.*). The 60 kDa form is characteristic also of the palmitoylation mutant meaning that the difference in size is not due to post-translational lipid modification. It is more likely that they differ in the extent of phosphorylation, as the 60 kDa form is less phosphorylated (*Figure 1.5.*). Based upon microscopy stainings (*Figure 1.3.B*) we propose that the smaller form does not localise at the plasma membrane but in the ER and/or cytoplasmic vesicles. GEM preparation strengthens this idea as the low molecular weight form does not associate with the lipid rafts (*Figure 1.4.B*).

Similarly to LAT, mutation at the palmitoylation site in PAG excluded the majority of the protein from the lipid rafts (*Figure 1.4.B,C*). Despite its dislocation, the mutant PAG was properly phosphorylated (*Figure 1.5*.), recruited Csk to the plasma membrane (*Figure 1.9.*) and bound EBP-50 (*Figure 1.10.*) to the same extent as wt PAG. The association with EBP-50 occurs via the C-terminal TRL motif and one of the PDZ domains of EBP-50 (61, 62). Due to the presence of a second PDZ domain, EBP-50 has the potential to support the formation of PAG dimers. Dimerisation between endogenous PAG and the overexpressed palmitoylation mutant could explain the presence of the small population detected within the GEMs in the absence of S-acylation. To test this idea, we have generated a mutation (T430A) in wt and CxxC PAG that was reported to

be unable to bind EBP-50. (62). The experiments with these mutants are currently ongoing in our laboratory.

Although we did not observe a difference in the level of phosphorylation or in the binding of the associated proteins, we set out to test whether there is an effect upon signalling processes caused by the dislocation of the palmitoylation mutant from the lipid rafts. Following short term (2min) stimulation via CD3, we observed 46% enhancement in Lck basal activity that was further induced (up to 76%) by the palmitoylation mutant upon stimulation compared to the wt PAG overexpressing cells. Overexpression of the wild type protein results in an irreversible inhibition (4), which abrogates Lck activation even upon stimulation. This inhibitory effect is diminished when the palmitoylation mutant is overexpressed and a marked increase is detected in Lck autophosphorylation following CD3 stimulation. The strong decrease in tyrosine phosphorylation of ZAP-70 and a milder inhibition in PLCγ activation in cells overexpressing wt PAG is in line with the literature (4, 100). The enhanced phosphorylation level of these proteins due to the CxxC mutant was also propagated to distal signalling events, like Erk phosphorylation (*Figure 1.11.A,B*). The marked effect upon PLCγ activation called for the next set of experiments, which measured intracellular Ca^{2+} flux in Jurkat T-cells overexpressing the PAG constructs. Wt PAG partially suppressed the release of calcium, which was expected from the proximal signalling data however, the palmitoylation mutant did not cause any change compared to the vector control *(Figure 1.13.*). Looking at the ionomycin induced calcium signal it is tempting to assume that the calcium response is already saturated upon stimulation and a potential positive effect (i.e. removal of a negative regulator) can not increase it further.

As functional read-out we have applied migration assays since migration is a Src kinase mediated response (118, 122) and PAG is involved in the regulation of Src kinases via recruiting Csk. Our results clearly demonstrate that removal of PAG from the lipid rafts leads to an enhanced migratory response (*Figure 1.14.*). Whilst we have seen striking differences in Src kinase activation between wt PAG and the CxxC mutant overexpressing cells (*Figure 1.17.A*), the Ca^{2+} flux does not seem to be as much affected (*Figure 1.16.*). The Ca2+ response is initiated by IP3 that is generated upon PLC activation. It is still unclear whether the PLCs become activated via a Gα (140) or Gβγ (141) mediated pathway. Tyrosine phosphorylation of PLCγ is increased in a rapid and transient manner upon SDF-1 stimulation in a myeloid cell line (125). In addition to PLCγ, PLCβ was also found to be involved in the chemokine-induced responses. However,

data from neutrophils show that although PLCβ isoforms were activated by chemokines, they were not required for chemotaxis (142). The difference in the two read-outs, i.e. proximal signalling and Ca-flux measurement, may be that the pathways leading to Src kinase activation and to calcium mobilisation diverge. The slight increase in intracellular Ca^{2+} seen in the palmitoylation mutant overexpressing cells may be due to a cross-talk between these two pathways. This idea is supported also by the observed 20% increase in Erk phosphorylation.

Altered distribution of Csk caused by the changes in lipid raft localisation of the mutant PAG was shown by staining for Csk in lipid raft fractions isolated from transfected Jurkat T-cells (*Figure 1.19.*). The consequences of Csk dislocation from the lipid rafts were reflected in the phosphorylation of the activatory tyrosine of Fyn (*Figure 1.20.*). PAG dislocation from the lipid rafts resulted in increased Src kinase activity within the rafts what is in concert with the SDF-1 induced proximal signalling data (*Figure 1.17.A*).

The results obtained with the siRNA of PAG (*Figure 1.18.*), namely that downregulation of PAG expression increased the migratory response of Jurkat T-cells, not only supported our proximal signalling and functional data generated by SDF-1 stimulation but also further highlighted the importance of localisation in regulation of the signalling machinery and cellular functions. Dislocation from the lipid microdomains leads to the same phenotype as downregulation of PAG what clearly shows that lipid raft association is indispensable for PAG to fulfil its negative regulatory function.

RESULTS II

2. Localisation of the Fyn binding site

In addition to studying the role of palmitoylation in PAG function, we have been working on localising the Fyn binding motif within PAG. Although it has been previously shown that Fyn is the kinase responsible for PAG phosphorylation (4, 98), the site of interaction remained unknown. By identifying the Fyn binding site we hope to better understand how the kinase interacts with its target protein, and thereby study the functional consequences of Fyn-PAG association.

Our strategy was to generate truncation mutants to localise the region of interaction, thus arose the need for an N-terminally tagged PAG construct (here after referred to as N-PAG). We chose Flag as an epitope tag since we had good experience with it in our laboratory.

2.1. Generation of N-terminally Flag-tagged PAG

To ensure membrane targeting of the construct, we synthesized an oligonucleotide where the CD8 leader sequence was added to the Flag epitope and which had *Aat* II and *Nco* I restriction sites on its 5´ and 3´ ends, respectively (see the sequence in the *Methods*). The CD8-Flag sequence was ligated into the pGEM-5Zf (+) vector, which was previously linearised by *Aat* II/*Nco* I double digestion (see the strategy presented in *Figure 2.1*.). We have chosen this vector because of its relatively small size (3 kb) that unburdens the insertion of a small (98bp) oligonucleotide and because it had a multicloning site with *Nco* I and *Not* I restriction sites which were necessary to preserve the full length and original sequence of PAG during the ligation. Also, this vector is suitable for blue/white screening of the colonies that makes the cloning process more convenient. To prevent recircularisation of the plasmid without the insert, we alkaline-phosphatase treated the linearised vector and phosphorylated the oligos to provide the phosphates on both ends of the insert which are indispensable to achieve ligation. Following restriction digest and alkaline phosphatase treatment, the vector was purified on a 1% agarose gel and extracted with the Nucleo Spin Extract Kit. The dimerised oligos were phosphorylated by polynucleotide kinase treatment and extracted from the protein contents of the reaction by phenol/chloroform/isoamylalcohol. The success of the ligation was tested by *Nhe* I digestion. Since the vector does not carry this restriction

site, linearisation can only be achieved by the presence of the insert (*Figure 2.2.A*). *Aat* II and *Nco* I digestions were necessary to check the integrity of the restriction sites that were required for further ligations (*Figure 2.2.B*).

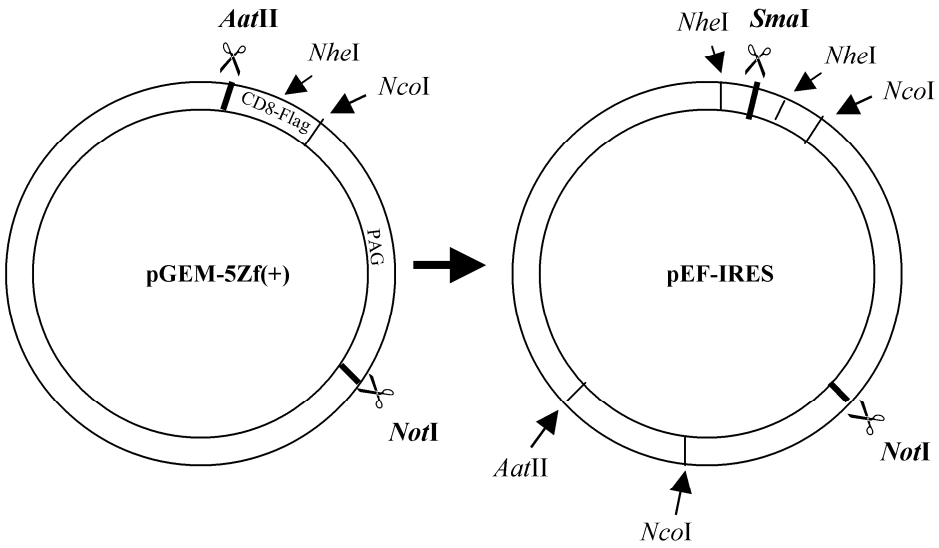


Figure 2.1. Strategy for N-terminally Flag-tagged PAG generation
As a first step CD8-Flag was ligated into pGEM-5Zf vector using the *Aat* II-*Nco* I sites. PAG was then inserted at the *Nco* I-*Not* I site. The Flag-tagged PAG was excised from the pGEM vector and inserted into pEF-IRES using *Sma* I-*Not* I ligation. The *Nhe* I site within the CD8-Flag tag was used for screening the colonies.

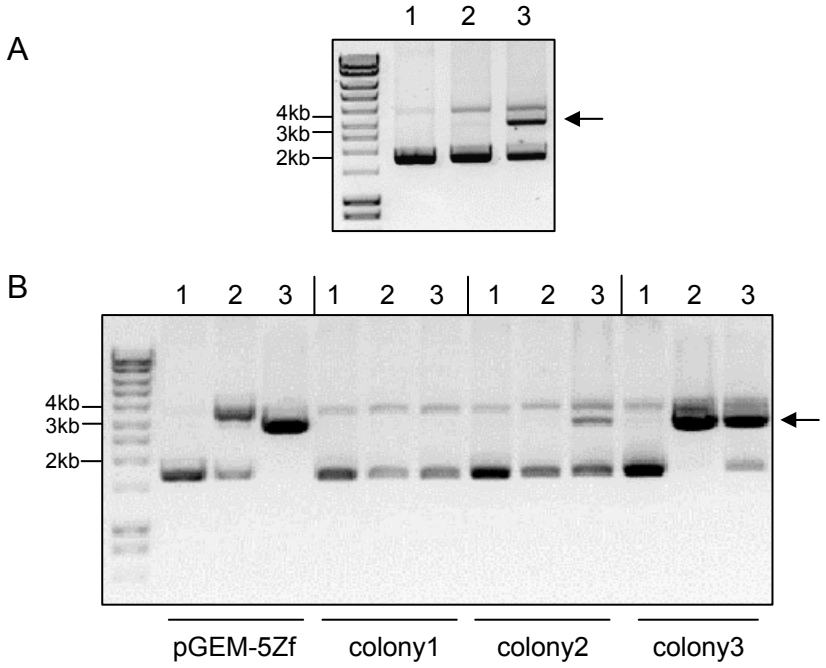


Figure 2.2. Screening for CD8-Flag-pGEM positive colonies
A) *Nhe* I digestion of one colony: 1. Intact pGEM-5Zf vector, 2. Undigested plasmid isolated from a single colony, 3. *Nhe* I digestion. The arrow indicates the linearised plasmid. **B)** *Aat* II and *Nco* I digestion of the vector and three colonies. The restriction sites of colony 1 and 2 are damaged. 1. Intact vector, 2. *Aat* II digestion, 3. *Nco* I digestion. The marker is Hyperladder I.

Once we had the CD8-Flag oligonucleotide in the vector the next step was to ligate *Nco* I/*Not* I digested PAG to the 3´end of the CD8-Flag sequence (*Nco* I/*Not* I digested, alkaline phosphatase treated CD8-Flag/pGEM-5Zf). To screen positive colonies, a PCR reaction was designed in a way such that two PCR products (217bp and 371bp) would indicate a positive colony (*Figure 2.3.*).

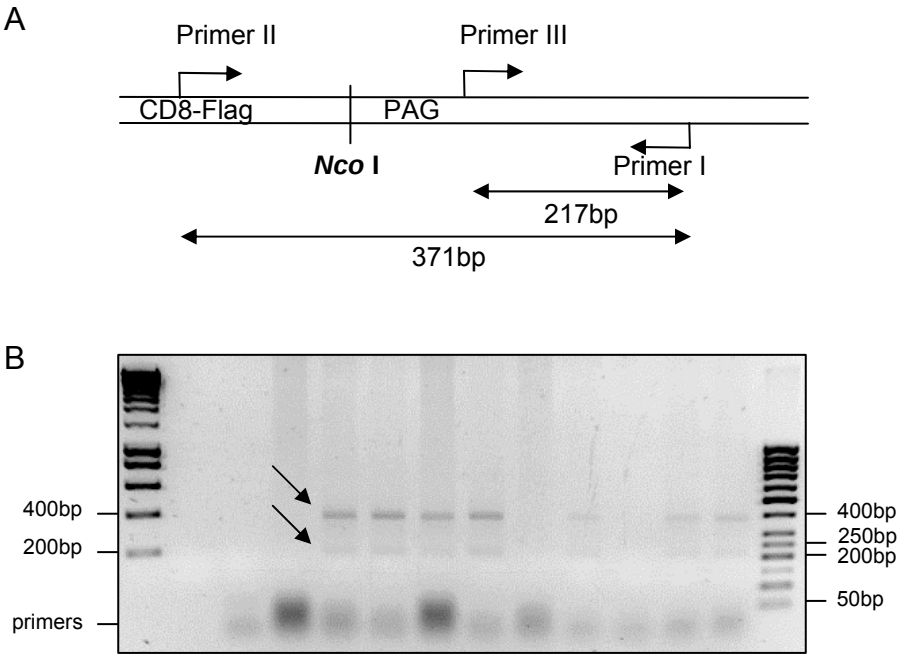


Figure 2.3. PCR reaction for screening CD8-Flag-PAG positive colonies
A) Schematic depiction of the primers and the expected products on CD8-Flag-PAG sequence. **B)** The result of the PCR reaction. The arrows point on the PCR products of expected size. The marker on the left side is Hyperladder I, on the right side Gene Ruler 50bp Ladder.

Since the pGEM-5Zf vector is not compatible with mammalian cells, we had to transfer the CD8-Flag tagged PAG into an appropriate expression vector. Our choice was pEF-IRES, a mammalian expression vector which possessed the *Not* I restriction site. Since it lacked *Aat* II within the multicloning site we took the advantage of *Sma* I that enabled us to insert our DNA using a blunt-end ligation. The sticky ends of the CD8-Flag-PAG insert and the vector (*Sma* I/*Not* I digested) were filled in by Klenow enzyme (DNA Polymerase I Large fragment). Following this reaction phosphorylation of the insert was not necessary, however alkaline phosphatase treatment of the vector was required. The success of ligation was checked by PCR. *Not* I digestion served to visualise the linearised plasmid. We used *Nhe* I digestion to check the orientation of the insert as blunt end ligation gives the possibility for ligation in two orientations. If the insert is in the

right orientation, digestion results in a small fragment that we cannot detect on a 1% agarose gel and a long fragment of 7,2 kb. The wrong orientation would lead to detection of a fragment of 1,5 kb size. The colony presented on *Figure 2.4.* carries the plasmid with the insert in the right orientation. The positivity of the colonies was verified by sequencing (carried out by GATC Biotech).

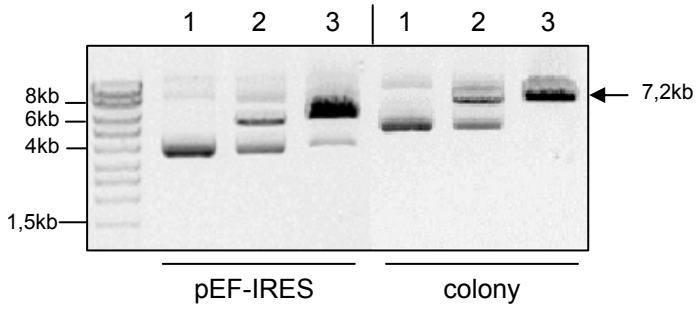


Figure 2.4. Testing of the CD8-Flag-PAG-pEF-IRES positive colonies
1. intact plasmid, 2. *Nhe* I digestion, 3. *Not* I digestion. The linearised pEF-IRES is 5.6kb, following ligation of the Flag-tagged PAG it is 7.2kb The marker is Hyperladder I.

2.2. Generation of truncation mutants

Once we had generated the N-terminally tagged PAG, we could then proceed with the generation of the truncation mutants. As Fyn association with PAG was shown to be phospho-independent (4), we therefore focused on the proline-rich regions within PAG as potential binding sites for the SH3 domain of Fyn. Like all Src kinases, Fyn possesses an SH2 and an SH3 domain, which are potentiated for protein-protein interactions. SH2 domains recognise phosphoproteins (143), while SH3 domains are specialized for proline-rich sequences containing a core PxxP motif (144). The literature provides examples for interactions via both Src homology domains of Fyn (145, 146). Additionally we have taken into consideration the RxxK motif juxtaposed to the palmitoylation motif, since Fyn has been shown to associate with the adaptor molecule SAP (SLAM-associated protein) via its SH3 domain and an RxxK motif in SAP (146).

The truncation mutants were generated by inserting STOP codons at the sites indicated in *Figure 2.5*. STOP4 lacks an unconventional proline-rich region (PRR) (PxxxP), STOP3 is missing the membrane distal PRR, STOP2 loses the membrane proximal PRR. STOP1 contains only the palmitoylation and the RxxK motifs with no tyrosines remaining in this sequence. The integrity of each construct was checked by sequencing.

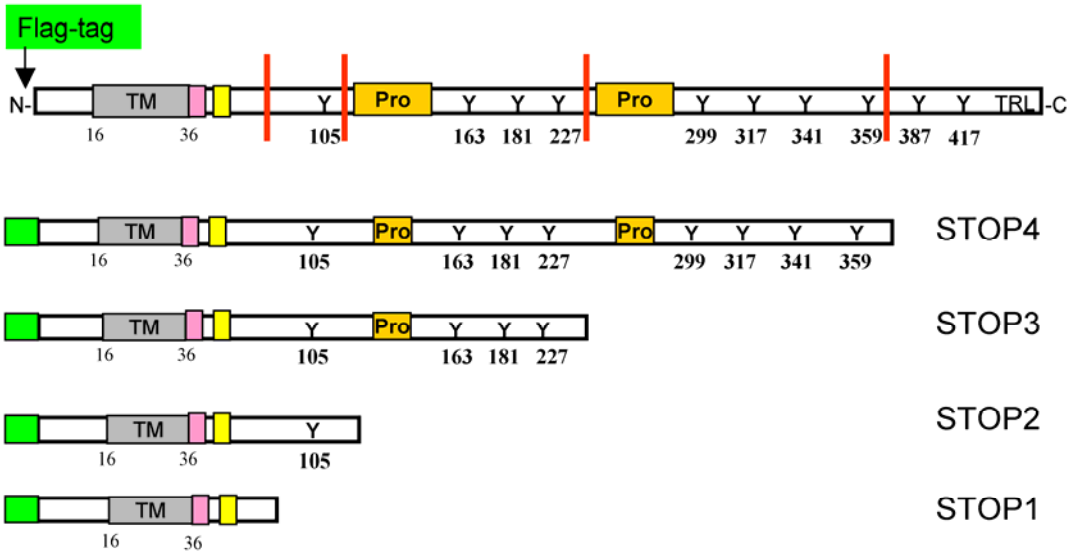
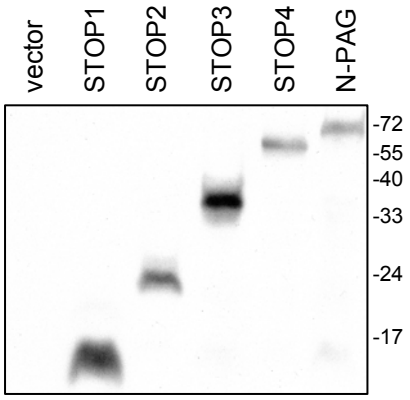


Figure 2.5. Truncation mutants generated from N-PAG
TM stands for transmembrane region. The green boxes label the Flag-tag, red stripes show the sites of STOP codon insertion, pink boxes label the palmitoylation site, and yellow boxes represent the RxxK motif.

2.3. Expression of the truncation mutants

The truncation mutants were tested for expression in HEK 293T cells (an embryonic kidney cell line) and in Jurkat T-cells. Lysates were prepared and loaded onto 12% ProSieve gels, which provide better resolution than the standard acrylamide:bis gel. To avoid loss of the smallest truncated protein by blotting through the membrane and to increase the efficiency of transfer, we chose PVDF (polyvinylidene difluoride) membranes for electroblotting. *Figure 2.6*. shows the expression pattern in HEK 293T cells, from which we could estimate the size of the truncated proteins. The predicted sizes were as much different as in the case of the full length PAG (4). The reason for this discrepancy may be due to phosphorylation (in unstimulated cells PAG is phosphorylated) and the acidic nature of the protein that together result in an anomalous binding of SDS and thus retarded migration during electrophoresis (4). The latter explanation is especially true for STOP1 that lacks all tyrosines, which are potential sites for phosphorylation.



	Size [kDa]	
protein	predicted	apparent
STOP1	5,7	15
STOP2	12,6	25
STOP3	26	37
STOP4	39,5	60
N-PAG	47	72

Figure 2.6. Expression of the truncated proteins in HEK 293T cells
Rabbit-anti-Flag staining of cell lysates transfected with the indicated proteins. Protein size was predicted with the help of Compute pI/MW tool at http//:www.expasy.ch web site.

2.4. Membrane localisation of the truncation mutants

Jurkat T-cells transfected with the truncation mutants were stained for Flag and analysed by confocal microscopy. We chose the membrane localised CD29 (integrin β1 chain, VLA-4 β chain) protein as a membrane marker. The pattern of the individual stainings indicates that the occurrence in the plasma membrane is comparable for the mutants and CD29. Each mutant was capable of localising to the plasma membrane to similar extent as the full length N-terminally tagged PAG with the exception of STOP1. In the case of STOP1 and partially also in STOP2 we could observe vesicular localisation. *Figure 2.7.* demonstrates representative cells from each transfection. In some cells STOP1 showed membrane localisation, in others vesicular and submembrane position. This can be due to the lack of association with a possible membrane anchoring protein, failure in processing the truncated protein, unstable membrane localisation or fast degradation. As the number of cells which showed membrane localisation of STOP1 was variable among the experiments and in some cases membrane association was the typical appearance, we assumed that the physical condition of cells prior to transfection may affect the capability of processing this mutant. Considering the staining results from several experiments and observed cells we decided to continue our studies also with the shortest construct.

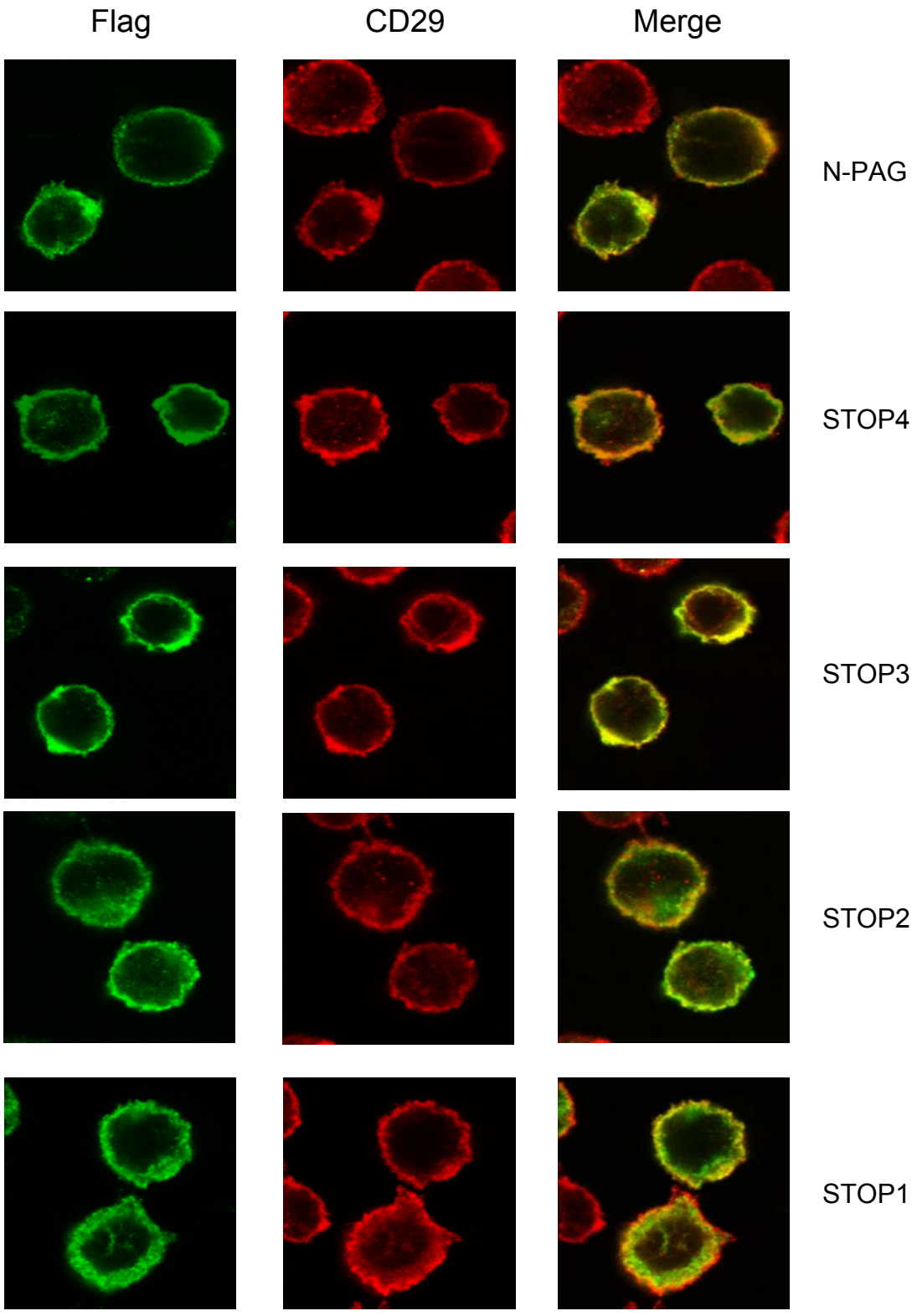


Figure 2.7. The truncated proteins localise to the plasma membrane
Jurkat T- cells transfected with the indicated constructs were stained with rabbit-anti-Flag/donkey-anti-rabbit-FITC, mouse-anti-CD29/donkey-anti-mouse-Cy5 antibodies.

2.6. Lipid raft association of the truncated mutants

The importance of protein-protein interactions in the localisation of proteins to certain membrane fractions is more and more evident in the literature (28, 53). Filipp et al. has recently reported that the C-terminus of Lck is indispensable for the lipid raft association and thereby for its function as the Fyn activating kinase (Filipp et al., 16th European Congress of Immunology, Paris, France, 2006, Book of Abstracts p. 115, PB-1434). These observations drove us to check lipid raft association of our truncation mutants since they would eventually lose contact with possible anchoring proteins.

Lipid raft localisation was determined by fractionation upon sucrose density gradient. The distribution of the N-terminally tagged constructs is different from the C-terminally tagged wtPAG (*Figure 2.8*.). The reason why the full length N-PAG is more abundant in the non-raft fractions is not clarified yet. The N-terminal Flag-tag may interfere with palmitoylation and therefore these constructs may be losing the GEM targeting signal. It has been suggested earlier in connection with the PUFA treatment that interferes with lipid raft localisation of LAT and Lck, but does not displace PAG, that the extracellular and/or transmembrane domain of PAG may have a particular structure that attributes it with PUFA-resistance (92, 95). This special structure might be modified by the addition of the Flag-tag and could lead to steric inhibition of palmitoyl acyltransferase and inefficient palmitoylation.

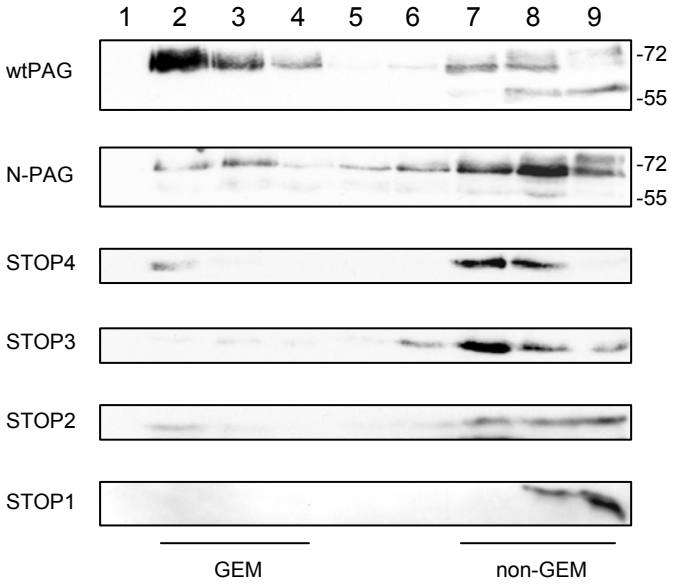


Figure 2.8. Membrane distribution of the truncated proteins
Rabbit-anti-Flag staining of sucrose density gradient fractions from the cells transfected with the indicated constructs.

Looking at the distribution pattern of the truncated mutants (*Figure 2.8*.) one can see that they are more and more excluded from the GEM fractions which reinforces the idea about an anchoring protein that plays a role in recruitment of PAG into the lipid rafts. Via shortening the cytoplasmic tail of PAG we seem to interfere more and more with the interaction(s) with a possible anchoring partner(s).

Seeing the membrane localization of N-PAG and the truncated mutants one could question their competence for Fyn binding site studies. Here I would like to note that we have shown that the palmitoylation mutant of PAG resides mainly outside of the lipid rafts but it is still capable of binding Fyn and the association with the non-raft pool of Fyn is sufficient to phosphorylate PAG (*Results I/1.4*.). Therefore the localisation of PAG does not seem to be relevant in structural studies. However, following the preliminary experiments where we have localised the possible Fyn binding region with the help of truncations, we turned back to the C-terminally Flag-tagged PAG and used it for the more sophisticated, site directed mutagenesis.

2.5. *In vitro* kinase assays

Since HEK 293T cells express high levels of the transfected truncation mutants, the experiments aimed at structural analysis of the Fyn binding motif were carried out in this cell line. As these cells express low levels of Fyn, we therefore co-transfected it and in this way avoided that limited Fyn results in lower phosphorylation of the PAG constructs. The truncated proteins as well as the full length N-PAG were immunoprecipitated from transfected cells and the associated Src kinase activity was measured in an *in vitro* kinase assay. The IPs were divided into two equal parts. One half was used for the IVK, the other served to control the amount of immunoprecipitated protein and the association of the binding partners. *In vitro* kinase assays clearly showed that while lack of the unconventional (STOP4) and membrane distal (STOP3) Pro-rich regions did not affect the phosphorylation of PAG, in the absence of the membrane proximal PRR (STOP2) it was completely abolished (*Figure 2.9.A*). Fyn IPs demonstrated on *Figure 2.9.B*, somewhat surprisingly showed association of STOP2 with Fyn. One reason why we could not detect kinase activity associated to the two shorter constructs may be that in STOP2 eight out of nine tyrosines, which are potential sites of phosphorylation, are deleted and STOP1 does not contain any tyrosines.

Since the interaction between PAG and Fyn has been shown to be phospho-independent (4) we speculated that the association of Fyn with the truncation mutant that still possesses a single tyrosine and no PRR (STOP2), may occur via the SH2 domain of

Fyn and probably has stabilizing functions. We based our hypothesis on the Fyn-FAK association where closely spaced proline and phosphotyrosine motifs in FAK bind simultaneously to both the SH3 and SH2 domains of Fyn (147). Binding of the Fyn SH2 domain to PAG was previously shown (4, 99). Results from our laboratory with Fyn-SH2-GST pulldowns further strengthen our idea, as they indicated Y-105 as Fyn binding site (Lindquist, unpublished observations). The latest publication from Davidson et al. also highlight that a tyrosine(s) of PAG, other than Y-314, is implicated in Fyn binding (99).

Although we have included enolase as an exogenous substrate (74), in contrast to Fyn immunoprecipitates, we could not detect its phosphorylation in IVKs from Flag IPs (*Figure 2.9.A*). This could mean that PAG bound Fyn has different specificity than the non-bound Fyn population that is present in the Fyn IPs. To test the kinase function of Fyn we are planning to generate a better substrate, possibly a PAG peptide for *in vitro* kinase assays. If we assume that Fyn binds to PAG via its SH2 domain than the interaction should lead to an increase in kinase activity as binding via the SH2 domain leads to an open and thus active conformation of Src kinases (75). Finding the proper substrate is of importance because it would help to better understand the activatory mechanism and the requirement for kinase function of Fyn.

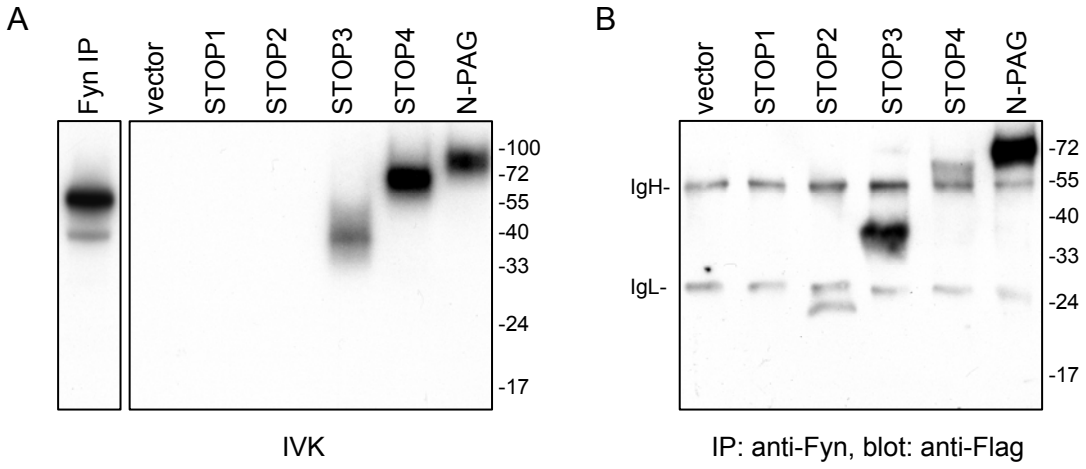


Figure 2.9. Fyn association with PAG truncation mutants
A) In vitro kinase assay with Flag IPs from HEK 293T cells transfected with the indicated constructs. The panel on the left side presents kinase activity in Fyn overexpressing cells. The upper band correspond to Fyn the lower band to enolase. **B)** Fyn IPs stained for Flag with rabbit antibody. The bands of 55 and 25kDa size are the heavy and light chains of the IP-ing antibody.

To demonstrate that the kinase activity observed is due to Src kinases, we repeated the experiment in the presence of the Src kinase specific inhibitor, PP2. *Figure 2.10.* demonstrates that the phosphorylation of STOP3 and STOP4 was indeed abolished in the presence of Src inhibitor.

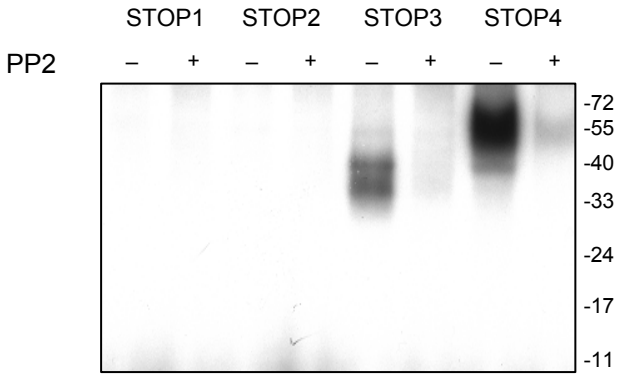


Figure 2.10. Phosphorylation of STOP3 and STOP4 are PP2 sensitive
In vitro kinase assay carried out on Flag IPs from PP2 treated HEK 293T cells that were previously transfected with the indicated constructs.

To get a better view of the Fyn binding site we have generated mutants using the C-terminally tagged PAG as a template to avoid any discrepancies caused by the dislocation of the N-terminally tagged PAG from the lipid rafts. Single mutants where the prolines of the membrane proximal PRR were mutated to alanine (here after referred to as PxxP) or where Y-105 was mutated to phenylalanine (here after referred to as Y105F), or the double mutant possessing both mutations (here after referred to as Y105F-PxxP) were generated and tested using *in vitro* kinase assays. In contrast to our expectations, kinase activity was also detected in association with the double mutant. Stainings of the control blots demonstrate that neither the association with Fyn, tyrosine-phosphorylation, nor Csk binding were altered (*Figure 2.11*.).

The kinase assay gave us information about the associated Src kinase activity (which was inhibited by the Src kinase specific inhibitor PP2), therefore the experiment was repeated in the JCam1.6 cell line, which is Lck deficient, to exclude Lck as the kinase responsible for the detected kinase activity. Since Lck is indispensable for Fyn activation, constitutively active Fyn was cotransfected together with the PAG constructs. The results obtained from JCam 1.6 cells (and also in Jurkat T-cells) were identical with the results from HEK 293T cells. This means that although the experiments with the truncated mutants identified the membrane proximal proline-rich region and Y105 as potential binding sites for the SH3 and SH2 domains of Fyn, respectively, the single and double mutants of the full length PAG still bind Fyn and PAG phosphorylation is not completely altered. Given the inconsistencies between the results obtained with the truncation mutants and the site directed mutants, we suspect that EBP-50-induced PAG

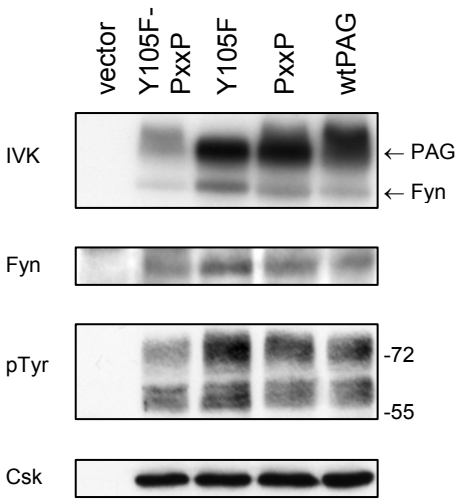


Figure 2.11. Fyn associates with the Y105F PxxP double mutant
Flag IPs from HEK 293T cells transfected with the indicated constructs were prepared and divided into two aliquots. One aliquot was used for *in vitro* kinase assay (first panel). Western blot with the second aliquot of the Flag IPs was stained for Fyn (rabbit-anti-Fyn), phosphorylation (anti-phosphotyrosine-HRP) and Csk (rabbit-anti-Csk). Note, that these mutants were generated from the C-terminally tagged PAG construct.

dimerisation, as previously mentioned (*Results I/1.2*. and *Discussion I*) may occur between the endogenous PAG and the overexpressed full length PAG constructs, which prevents us from detecting altered levels of Fyn association and PAG phosphorylation. Itoh et al. (62) published that the substitution mutant T430A is unable to bind EBP-50, therefore we mutated this site of the double mutant (*Figure 2.12.*) and plan to perform *in vitro* kinase assays with the triple mutant. If this mutation is success in abolishing Fyn association and the residual kinase activity detected, then we will have identified a novel mechanism for PAG dimerisation in addition to heaving identified the Fyn binding site.

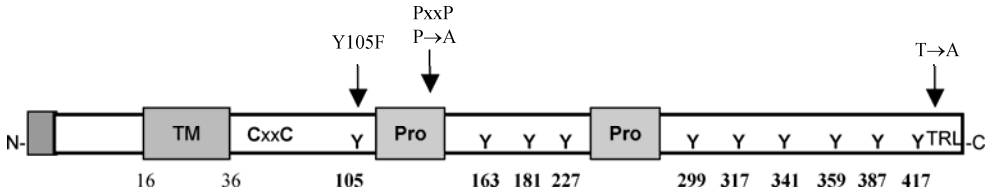


Figure 2.12. The Y105F PxxP T430A triple mutant

DISCUSSION II

PAG has three published binding partners, Fyn, Csk and EBP-50. While the binding sites of Csk and EBP-50 have been identified (4, 60-62, 98), the site of Fyn association remains unknown despite the importance of Fyn for PAG phosphorylation. Therefore to further our understanding of PAG we decided to address this question. We tagged PAG on its N-terminus and generated a number of C-terminal truncation constructs with the hope of identifying the region of Fyn binding. The N-terminally tagged wild type PAG localised to the plasma membrane (*Figure 2.7.*) and was capable of binding Fyn, Csk and EBP-50 (see *Figure 1.11.* in Results I). The truncation mutants also resided in the plasma membrane (*Figure 2.7.*), however the ability of the cells to express the shortest construct within the plasma membrane varied among experiments and cells. This can be due to the lack of association with a possible membrane anchoring protein thereby instable membrane localisation, short half-life of the protein or failure in the posttranslational vesicular transport. STOP4, the mutant which still contains Y-317 associated with Csk, as expected.

Once we had localised the potential Fyn binding site to the region of the membrane proximal PRR with the help of the truncation mutants, the next step was to mutate the potential binding sites on the full length protein by site directed mutagenesis. The results obtained from *in vitro* kinase assays were surprising at first. Seeing that there was no kinase activity associated to the shorter truncated mutants lacking the membrane proximal proline-rich region, one would expect that the point mutations at this region would bring similar results. Instead, we found that neither the proline-rich region, nor Y-105 mutations interfered with PAG phosphorylation. The contradiction between the results from these two approaches i.e. truncation and site directed mutagenesis may result from the differently tagged PAG constructs that were used for the mutation studies. For the first approach N-terminal tagging of PAG was indispensable. Based upon our results with the palmitoylation mutant of PAG (i.e. that it binds Fyn to the same extent as wt PAG) we assumed that displacement from the lipid rafts does not interfere with Fyn binding and the non-raft associated truncated mutants are suitable for preliminary studies. It is obvious from our results with the palmitoylation mutant of PAG, that there is enough Fyn in the non-raft membrane fractions to phosphorylate PAG.

In connection with the interesting observation that unlike LAT and Lck, PAG is not displaced from the lipid rafts upon PUFA treatment (95), it has been proposed that either

a special structure of the extracellular and/or transmembrane domain of PAG or a lipid raft associated anchoring partner attributes PAG with PUFA resistance (92). The unique structure of the amino-terminal domains might, in this case, have been disturbed by the N-terminal tagging. It could also lead to altered palmitoylation, which would explain the reduced raft association of the N-terminally tagged PAG constructs. However the fact that the truncated mutants are less and less detected in the lipid rafts points to the second explanation of PUFA resistance, namely to a possible interacting partner that supports lipid raft association of PAG. This may be a loose association that plays a minor role when PAG is palmitoylated, but if there is indeed a palmitoylation defect in the case of the N-terminally tagged PAG molecules the role of an anchoring protein would become more important. Truncation mutations could interfere with this association and therefore the smaller constructs would lose their ability to localise in rafts. Experiments with ^{3}H palmitate would be useful to determine whether the N-terminally labelled constructs are palmitoylated.

The idea of Vaclav Horejsi concerning light and heavy rafts (described in *Results I*) offers a possible explanation for the detection of the STOP mutants in the non-raft membrane fractions. With the help of a gel filtration method, his group has shown that the adaptor molecule LAX localises to the lipid rafts just like LAT. The earlier view that LAX is a non-raft protein was based upon the sucrose gradient method that we also used to determine the distribution of the PAG constructs. To clarify whether the N-terminally tagged PAG constructs are indeed excluded from the lipid rafts or they reside in the heavy rafts we should apply this method.

One possible explanation for the observed inconsistency between the results obtained with the truncation mutants and point mutations is that dimerisation occurs between the endogenous PAG and the full length mutants via EBP-50, which prevented us from detecting the effect of the site directed mutations. To address this possibility we have generated an additional T430A mutation in the EBP-50 binding site of the Y105F, PxxP and Y105F PXXP mutants and are planning to test these new constructs for associated kinase activity.

Let us suppose that the EBP-50 binding site mutant reveals that dimerisation is not the reason for the remaining kinase activity associated with the Y105F, PxxP and Y105F-PxxP mutants. What mechanisms of interaction then remain? Fyn has been published to bind other proteins via its SH2 and SH3 domains (145, 147). However, there is one more mode of recognition that was first described for the SH3 domain of GADS, namely that it binds to the RxxK motif on SLP-76 (148), and also applies to the

SH3 domain of Fyn that binds via the same motif to the adaptor molecule SAP (146). Based upon these results and knowing that there is an RxxK motif within the PAG sequence (following the palmitoylation site), we think that the unconventional SH3 binding site may also be a good candidate to mediate Fyn binding to PAG. The next step would be to generate the R42A mutant and test whether this is still capable of Fyn binding. This approach is reasonable given that substitution of arginine with alanine at the RxxK motif of SAP and SLP-76 abrogated Fyn and GADS binding, respectively (146, 148).

Seeing the inability of Fyn to phosphorylate enolase, the exogenous substrate in the kinase reaction that was previously shown to be the proper substrate for Fyn (74), in Flag IPs (*Figure 2.9.A*), it is tempting to speculate that binding to PAG results in an alteration of Fyn kinase activity. Further developing the idea the following hypothesis emerges: Fyn may phosphorylate PAG without direct interaction, like Lck phosphorylates LIME (75) or ZAP-70 (149) and thus induces binding via its SH2 domain in a phospho-dependent manner. Binding of Fyn to either pY-105 or the membrane proximal PRR might be of sufficient strength that Fyn cannot dissociate to bind and phosphorylate other substrates and in this way PAG exerts an additional negative regulatory function namely by sequestering Fyn within the GEMs, and thus restricting the amount of available kinase. This is in agreement with our observations indicating that autophosphorylation of Fyn is altered in wt PAG overexpressing cells and is not inducible upon CD3 or SDF-1 stimulation.

SUMMARY

The phosphoprotein associated with GEMs (PAG) was simultaneously identified by two groups. One described it as a member of the Fyn complex, the other found it as a Csk binding protein (Cbp). According to the current model of PAG function, both of these associations play a crucial role in the regulation of T-cell responses.

PAG is ubiquitously expressed, which is unique among the transmembrane adaptor proteins, suggesting that PAG has a more general role in regulating cellular functions throughout the organism. PAG belongs to the lipid raft associated group of transmembrane adaptor proteins. In resting T-cells, PAG is phosphorylated and binds Csk. Following stimulation via the TCR, a yet unknown phosphatase dephosphorylates PAG and thereby releases Csk, allowing Src kinases to then become activated. Lck translocates to the lipid rafts where it mediates Fyn activation. By phosphorylating their substrates the Src kinases initiate the signalling cascade, which leads to cytokine production and to the differentiation from resting into effector cells. One substrate of Fyn is PAG, so the activated kinase in turn rephosphorylates PAG enabling it to bring Csk back to the membrane. In this way the negative regulatory feed back loop closes, and the signalling cascade is silenced. In addition to binding Csk, PAG also serves as a link between lipid rafts and the cytoskeleton via its association with EBP-50 (ezrin-radixin-moezin binding phosphoprotein of 50kDa). This interaction occurs between the C-terminal TRL motif of PAG and a PDZ domain of EBP-50. EBP-50 interacts with ERM (ezrin, radixin, moezin) proteins, which bind F-actin. Anchoring of lipid rafts through the PAG-EBP50-ERM complex regulates lipid raft mobility. Following T-cell activation this interaction is lost allowing lipid raft aggregation and immune synapse formation.

Based upon observations that Csk plays a role in the prevention and/or suppression of tumour formation and metastasis, one can infer that the PAG-Csk complex functions as a type of tumour suppressor that helps to inhibit the potential oncogenic effect of unregulated Src kinase activity.

The aim of my thesis is to gain a better understanding of the regulatory function of PAG by studying the role of various motifs in localisation and Fyn binding. We demonstrate the importance of the palmitoylation motif in targeting PAG into the lipid rafts and the functional consequences of PAG dislocation. Fyn is the kinase responsible for phosphorylation of PAG, but unlike Csk and EBP-50, the site of interaction is still unknown. Via phosphorylating PAG, Fyn enables Csk recruitment to the proximity of Src

kinases. Identification of the Fyn binding site should help us to better understand how the kinase interacts with its target protein and by mutating the site of interaction, we can study whether direct association is necessary for phosphorylation and/or Fyn has an additional adaptor function.

For LAT (linker for activation of T cells), which like PAG, is a lipid raft associated transmembrane adaptor molecule, it was shown that lipid modification (palmitoylation) at its membrane proximal cysteines is responsible for its lipid raft association. Both LAT and PAG possess a CxxC motif in their cytoplasmic tails in a juxtamembrane position, which was shown to be palmitoylated. Knowing the importance of palmitoylation for the lipid raft localization of LAT, we began investigating the palmitoylation mutant of PAG (CxxC→AxxA). To determine whether the mutated protein was still capable of being targeted to the GEMs, cell lysates from transfected Jurkat T-cells were fractionated upon sucrose density gradient. While the wild type protein does localise to the GEM fractions, mutation of the palmitoylation motif clearly reduces the targeting of PAG into these microdomains. Therefore, we can conclude that palmitoylation of the CxxC motif is required for the targeting of PAG into the GEMs. Next we looked to see whether exclusion from the GEMs had an effect upon the phosphorylation of PAG or upon its interactions with the known binding partners. By immunoblotting the PAG immunoprecipitates we could see equal tyrosine phosphorylation of both wt PAG and the palmitoylation mutant as well as equal association of Fyn, Csk and EBP-50.

PAG is believed to exert its negative regulatory function by recruiting Csk to the GEMs. Since the CxxC mutant could also recruit Csk to the membrane, the question we next addressed was whether this mutant is capable of suppressing cellular activation, as cells over-expressing wt PAG can not be activated via CD3 stimulation. Mutation of the CxxC motif and thereby dislocation from the lipid rafts led to increased phosphorylation of several signalling molecules.

Since Src kinases are also important in migration, we next tested our mutant in an SDF1-based migration assay, as SDF-1 is known to preferentially signal from within the GEMs. Our results indicate that while wt PAG is capable of mildly suppressing SDF1-induced T-cell migration, the CxxC mutant enhanced the migratory response. The role of PAG in regulation of T-cell migration was further proved with the help of PAG specific siRNA. Suppression of PAG expression in Jurkat T-cells led to increased migratory response, similarly to the effect of PAG dislocation from the lipid rafts.

To explain the effects of the CxxC mutant, we hypothesised that over-expression of wt PAG increases the level of Csk within the GEMs, thereby creating an irreversible

block in activation. The CxxC mutant on the other hand could recruit Csk out of the GEMs and thereby relocate the block in Src kinase activation to the non-GEM compartment. For events occurring within the GEMs, the relocation of Csk outside of the GEMs has a profound effect, since it allows for an enhanced activation of Src kinases within the GEMs.

To test this hypothesis, we reprobed the GEM fractions for Csk content. Indeed, we found that the level of Csk within the GEMs was reduced by overexpression of the CxxC mutant. Together these results indicate that the mechanism of action appears to be effected by a redistribution of Csk within the cell. If this mechanism is correct, then this should also be reflected by changes in the phosphorylation of activatory and inhibitory tyrosines of Src kinases within the GEM and non-GEM compartments. By reprobing the fractions with phospho-specific antisera for the activatory tyrosine of Fyn (pY-418) we could show that there is indeed an increase in activatory tyrosine phosphorylation within the GEM fractions of cells expressing the PAG CxxC mutant.

Using Src kinase specific inhibitors the phosphorylation level of PAG was greatly reduced, however Fyn association was unaffected, indicating that the interaction occurs in a phospho-independent manner. There are several motifs apart from the phosphotyrosines within the PAG sequence that could support Fyn binding: PAG has two conventional (PxxP) and one non-conventional (PxxxP) Pro-rich regions, which are potential binding sites for SH3 domains, additionally an RxxK motif is located in a submembrane position in PAG that may also be a target for Fyn SH3 binding. As both Fyn and PAG are S-acylated (myristoylated and palmitoylated, respectively) one can also speculate that hydrophobic (lipid-lipid) interactions in or at the membrane might also play a role in Fyn association.

To study the mechanism of Fyn binding, several truncation and site directed mutants needed to be generated. We designed the truncation mutants that would eliminate the potential Fyn binding sites one by one. Once we had localised the region of the interaction, we analysed the mechanism of binding via single mutations. Our results indicated that even though Fyn binding was reported to be phospho-independent, both a proline-rich region and the phospho-Y-105 might be involved in forming the PAG-Fyn complex. Such a bipartite interaction has been already published for Fyn and its binding partner FAK. The SH2 domain of Fyn seemingly plays a role in stabilising the interaction formed between the SH3 domain and the membrane proximal proline-rich region of PAG. By mutating the Fyn binding site we will be able to determine the functional consequences of Fyn-PAG association.

During our studies several exciting questions opened up. One of them is whether EBP-50 is capable to assist dimerisation of PAG molecules. Some of our observations, like residual lipid raft localisation of the palmitoylation mutant and the contradictory results with the substitution mutants at the potential Fyn binding sites, all point to EBP-50 as a possible causative factor. Via demonstrating the role of EBP-50 in these observations, we would identify a novel mechanism for PAG dimerisation.

The next interesting question of our experiments is whether application of the new lipid raft isolating method from Vaclav Horejsi's lab, namely the gel filtration would bring different results regarding raft localisation of the PAG truncation mutants and whether the enigma of these constructs can be explained by the existence of the heavy rafts.

The third question that we would like to clarify in the near future is the mechanism of Fyn binding to PAG. We hope that the T430A mutants of the EBP-50 binding will bring us closer to the solution. If the experiments with these mutants reveal that dimerisation does not play a role, we will continue our studies with the R42A mutant of PAG that aims to clarify the involvement of a non-conventional SH3 binding motif in Fyn association to PAG. Once we identify the binding site, the next step is to study the effect of mutations that disturb Fyn association on the fate of T-cells. Identifying the site and mechanism of binding will also bring us closer to understanding the nature of PAG phosphorylation by Fyn and a new view of negative regulation of Src kinases by PAG may also emerge from these studies.

ABBREVIATIONS

ADAP	adhesion and degranulation promoting adaptor protein
AP-1	activator protein 1
APC	antigen presenting cell
BCR	B-cell receptor
BSA	bovine serum albumine
Cbp	Csk binding protein
Cbl	Casitas B-lineage lymphoma
CD	clusters of differentiation
Csk	C-terminal Src kinase
DAG	diacylglycerol
DMSO	dimethylsulfoxide
DRM	detergent-resistant membrane
EBP-50	ezrin-radixin-moezin binding phosphoprotein of 50kDa
EDTA	ethylene-diamine tetra-acetic acid
ER	endoplasmatic reticulum
Erk	extracellular signal-regulated protein kinase
FAK	focal adhesion kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLIM	fluorescent life time imaging
FRET	fluorescence resonance energy transfer
G3BP	GAP SH3-domain binding protein
GADS	Grb2 related adaptor protein
GEM	glycosphyngolipid-enriched microdomain
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor bound protein 2
Hck	haematopoietic-cell kinase
Ig	immunoglobulin
IP	immunoprecipitate
IP3	inositol-1,4,5-triphosphate
IPTG	isopropylthiogalactoside
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
Itk	interleukin-2 inducible T-cell kinase
LAT	linker for activation of T-cells
LAX	linker for activation of X-cells
Lck	lymphocyte-specific cytoplasmic protein tyrosine kinase
ld	liquid-disordered
LIME	Lck-interacting membrane protein
lo	liquid-ordered
MAPK	mitogen-activated protein kinase
MyH9	nonmuscle myosin heavy chain IIA
Nck	non-catalytic region of tyrosine kinase
NFκB	nuclear factor κB
NFAT	nuclear factor of activated T-cells
NTAL	non-T-cell activation linker
p62Dok	downstream of tyrosine kinase

PAG	phosphoprotein associated with glycosphingolipid enriched microdomains
PAK	p21 activating kinase
PAT	palmitoyl acyltransferase
PBL	peripheral blood lymphocyte
PBS	phosphate-buffered saline
PDZ	postsynaptic density/disc-large/ZO1
pEF-IRES	polypeptide chain elongation factor 1α - internal ribosomal entry sites
PEP	PEST enriched PTP
PH	pleckstrin homology domain
PI3K	phosphatidylinositol-3 kinase
PIP	phosphatidylinositol phosphate
PIP2	phosphatidylinositol-4,5-biphosphate
PIP3	phosphatidylinositol-1,4,5-triphosphate
PKB	protein kinase B
PKCθ	protein kinase C
PLCγ1	phospholipase C-γ1
PP2	pyrazolopyrimidin
PRR	proline-rich region
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTPα	protein tyrosine phosphatase α
PTP-PEST	PTP with PEST sequence
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene difluoride
Pyk2	proline-rich tyrosine kinase 2
RasGRP	Ras guanyl-releasing protein
SAP	SLAM-associated protein
SDF	stromal cell-derived factor
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFKs	Src family kinases
SH2	Src homology 2 domain
SH3	Src homology 3 domain
SHIP	SH2-containing inositol phosphatase
SHP-1	SH2 domain containing PTP
siRNA	small interfering RNA
SIT	SHP-2 interacting transmembrane adaptor protein
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
SOS	son of sevenless homologue
TCR	T-cell receptor
TRAP	transmembrane adaptor protein
TRIM	T-cell receptor interacting molecule
VAV	guanine nucleotide exchange factor
WASP	Wiskott-Aldrich syndrome protein
ZAP-70	Zeta-associated protein of 70 kDa

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APPENDIX-Antibodies

Antibody	Species	Isotype	Clone	Usage	Dilution	Source
beta-actin	mouse, monocl.	IgG1	AC-15	WB	10000	Sigma
CD3	mouse		OKT3	stimul.	supernat.	our institute
CD3	mouse		c305	Ca-flux	supernat.	our institute
CD3-FITC	mouse	IgG1	UCHT1	FACS	100	BD Bioscience
CD29	mouse		MEM101A	M	1000	Dr. Vaclav Horejsi
CD59	mouse		MEM43/5	WB	250	Dr. Vaclav Horejsi
Csk	rabbit, polycl.	IgG	C-20	WB	200	Santa Cruz
Csk	mouse	IgG1		WB	500	BD Bioscience
CXCR4	rabbit, polycl.	IgG		WB	1000	abcam
CXCR4	mouse			FACS	100	PharMingen
EBP-50	rabbit, polycl.	IgG		WB-IP	10000	abcam
EBP-50	rabbit, polycl.	IgG		WB-lysate	25000	abcam
ERK1/2	rabbit, polycl.			WB	5000	Promega
Flag	rabbit, polycl.	IgG		M ,WB	400	Sigma
Flag M2	mouse, monocl.	IgG1		WB	350	Sigma
Flag M2	mouse, monocl.	IgG1		FACS, M	10000	Sigma
Fyn	mouse			WB, IP	1000	Dr. Vaclav Horejsi
Fyn	mouse	IgG1		M	50	Biosource
Fyn	rabbit, polycl.			WB	2000	Biosource
Fyn	rabbit			M	50	Dr. Paul Burn
LAT	rabbit			WB	1000	Dr. Vaclav Horejsi
Lck	rabbit			WB	1000	Dr. Anthony Magee
MHCII	mouse		W6/32	FACS	100µl supernat.	our institute
PAG	mouse		MEM255	M	50	Dr. Vaclav Horejsi
PAG	mouse		MEM255	WB	100	Dr. Vaclav Horejsi
PAG	rabbit			FACS	50	Dr. Vaclav Horejsi
PAG	rabbit			M	250	Dr. Vaclav Horejsi
PLC gamma	rabbit			WB	1000	Santa Cruz
TRIM	mouse			M	300	Dr. Vaclav Horejsi
ZAP-70	mouse			WB	1000	BD Bioscience
phosphospecific antibodies						
pERK1/2	rabbit, monocl.			WB	1000	Cell Signaling
pPLCgamma (Y-783)	rabbit			WB	1000	Santa Cruz
pY-317 (PAG)	rabbit			WB	10 000	our intitute
pY-418 (Src)	rabbit, polycl.			WB	1000	Biosource
pY-505 (Lck)	rabbit, polycl.			WB	1000	Biosource
pY-529 (Src, Fyn)	rabbit, polycl.			WB	1000	Biosource
pY	mouse		4G10	WB	100	our intitute
pY-HRP	mouse	IgG2b	pY20	WB	1000	Southern Biotech
pZAP-70 (Y-319)	rabbit			WB	1000	Cell Signaling
secondary antibodies						
anti-mouse-HRP	goat	IgG		WB	10 000	Dianova
anti-rabbit-HRP	goat	IgG		WB	10 000	Dianova
anti-mouse-FITC	donkey	IgG		FACS	250	Dianova
anti-mouse-FITC	donkey	IgG		M	500	Dianova
anti-rabbit-FITC	donkey	IgG		FACS	100	Dianova
anti-rabbit-FITC	donkey	IgG		M	500	Dianova
anti-mouse-Texas red	goat	IgG		M	200	Dianova
anti-mouse-Cy5	donkey	IgG		M	100	Dianova

WB - Western blot M - microscopy staining IP - immunoprecipitation

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1994-1998	Grammar School Svetozar Markovic, Subotica, Yugoslavia

Grants and scholarships

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2002-2003 Scholarship from the Republic of Hungary
2002-2003 Scholarship from the local government of Szeged, Hungary

1998-2003 Grant from the Hungarian Ministry for Education

Awards and prizes

Signal Transduction Society (STS)-member since 2005
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2002- Young Researcher's Competition, local round, Szeged, Hungary (29.November) : 3rd prize

Publication

Posevitz-Fejfár, A., *Smida,M., Kliche, S., Hartig, R., Schraven, B., Lindquist, J.A.*: A displaced PAG enhances proximal signaling and SDF-1 induced T-cell migration. *Accepted in Eur.J.Immunol.*

Smida,M., ***Posevitz-Fejfár, A.****, Horejsi, V., Schraven, B., Lindquist, J.A*.: A novel negative regulatory function of the phosphoprotein associated with glycosphingolipid-enriched microdomains: blocking Ras activation. *Blood, 2007, 110(2): 596-615*

Poster

Posevitz Fejfar,A*., Kliche,S., Schraven,B., Lindquist,J.:* A displaced PAG enhances SDF-1 induced T-cell migration. International Symposium: Inflammation, Degeneration and Regeneration from Basic Mechanisms to Clinical Manifestations (30. November-01. December 2006, Magdeburg, Germany)

Posevitz Fejfar,A*., Smida,M., Kliche,S., Schraven,B., Lindquist,J*.: Is GEM localization required for PAG function? 16th European Congress of Immunology (6-9. September 2006, Paris, France)

Posevitz Fejfar,A*., Kliche,S., Schraven,B., Lindquist,J.:* Is GEM localization required for PAG function? Cell Signaling, 3rd annual symposium (11-14. June, 2006, Dundee, United Kingdom)

Posevitz Fejfar,A*., Smida,M., Kliche,S., Schraven,B., Lindquist,J.:* Is GEM localization required for PAG function? 9th STS meeting, Signal transduction: receptors, mediators and genes (10-12. November 2005, Weimar, Germany)

Posevitz Fejfar,A.*, Smida,M., Kliche,S., Schraven,B., Lindquist,J.:* Is GEM localization required for PAG function? 36th Annual Meeting of the German and Scandinavian Societies of Immunology (20-24. September 2005, Kiel, Germany)

Fejfár,A*., Kucsera,J., Golubev,W.I.:* Extrachromosomal DNAs in yeasts isolated from spring sapwood fluxes. 1st FEMS Congr European Microbiologists (29. June –03. July 2003, Ljubljana, Slovenia)

Pfeiffer,I., ***Fejfár,A****., Kucsera,J., Golubev,W.I.*: Occurrence of DNA plasmids in psychrophile yeast. Jubilee Conference of the Hungarian Microbiological Society, Balatonfüred, Hungary (2001)