3. Discussion

L-selectin is a leukocyte adhesion molecule crucial for blood cell extravasation in inflammatory responses and lymphocyte homing to peripheral lymph nodes. Additional to the extracellular adhesive function it also elicits signal transduction events inside the cell. This study aimed at a better understanding of the L-selectin mediated signalling by identification of novel cytoplasmic interaction partners for L-selectin and examining their involvement in L-selectin signalling.

3.1. Isolation of interaction partners for the cytoplasmic domain of L-selectin

At the start of this project, a suitable method for the identification of proteins that interact with the intracellular domain of L-selectin had to be chosen. In principle, isolation of protein binding partners can be approached by two ways:

a) screening of cDNA-encoded expression libraries, e.g. using yeast 2-hybrid or phage display techniques and b) affinity isolation from crude protein mixtures, usually cell lysates, followed by identification of the purified protein. Both isolation techniques offer distinct advantages, but also have inherent limitations.

The major advantage of expression library screening is the direct access to the cDNAs encoding the interacting proteins, which allows easy identification by DNA sequencing. This also facilitates further studies like construction of vectors for recombinant expression and deletion/mutation screening for the determination of interacting domains and motifs. Expression libraries contain cDNAs expressed in a certain cell type and have to be chosen carefully to match the expression pattern of the target protein. This libraries usually represent a large portion of cellular mRNAs, however, rare mRNAs may be lost during the construction process, and, depending on the priming method used, many cDNAs present in the library only contain part of the coding sequences, so that interaction domains may be missing or truncated. A significant restriction of expression library usage is the requirement of many proteins from higher eukaryotes for post-translational modifications. In many of the organism used for expression, the modification systems necessary for proper protein function are not present. Furthermore, during interaction screening proteins are displayed in an artificial environment, which not necessarily provides the correct conditions for interaction. For example, the yeast 2-hybrid system relies on interaction of the target proteins in the yeast cell nucleus, which is often considered an advantage since the interaction occurs *in vivo* (McAlister-Henn *et al*., 1999). However, for many proteins which normally exert their function in other cellular compartments, the conditions they encounter in the nucleus are strikingly different from the physiological situation (Fashena *et al*., 2000).

Biochemical affinity isolation of interaction partners from cell extracts provides proteins that are folded and modified in the correct way by native cellular systems. It also allows identification of activation-dependent interaction partners that require rapid modifications like phosphorylation by appropriate treatment of cells prior to lysis. It enables detection of complex interactions that

are dependent on additional protein or non-protein components, which are present in the cell lysates. On the other hand, proteins need to be highly purified to allow identification by sequencing or mass spectrometry. Analysis can be impaired by the presence of contaminating proteins, terminal blocking or low amounts of purified protein.

Because of the limitations of different methods, the conditions under which a certain target protein binds its interaction partner have to be considered carefully and a system that meets these requirements must be chosen.

3.1.1 Yeast Two-Hybrid screen

The yeast 2-hybrid method allows sensitive detection of protein-protein interactions inside living cells (Fields and Sternglanz, 1994). Known limitations of this technique concern the use of extracellular and transmembrane proteins, which are often not properly modified or expressed, and transcriptional activators that are likely to induce reporter gene expression without need for an interaction partner (Allen *et al*., 1995). Since only the cytoplasmic domain of the transmembrane receptor L-selectin was intended to be used in the interaction screen, these constraints did not apply. Therefore, a Gal4-based 2-hybrid system was chosen to isolate potential interaction partners from a leukocyte cDNA library.

The major problems to be encountered when conducting a yeast 2-hybrid screen are a) insufficient bait protein expression, b) autonomous activation of reporter genes by the bait protein used and c) toxicity of the expressed protein for the yeast cell. A fusion construct containing the yeast Gal4 transcription factor DNA-binding domain (BD) together with the cytoplasmic domain of L-selectin was generated. Yeast cells transformed with this construct were tested for expression of the fusion protein and reporter gene activation before the start of the library screen. Transformed yeast cells grew normally, indicating that protein expression did not interfere with cell viability. Expression of the fusion protein was confirmed with antibodies against both the cytoplasmic domain of L-selectin and the Gal4-BD. Autonomous activation of the lacZ gene, serving as a reporter in the system used, was not observed even after prolonged incubation.

Screening of the library with a fusion protein that contained a single copy of the L-selectin cytoplasmic domain yielded no positive colonies. A possible reason for this is the size of the cytoplasmic tail comprising only 17 amino acids, which might be to small to be displayed properly on the surface of the fusion protein. To enhance accessibility for binding partners, a fusion protein containing three consecutive copies of the cytoplasmic tail was used for further experiments. Using this bait protein, a small number of colonies expressing both reporter genes were isolated. Four of the five different cDNAs isolated coded for DNA- or RNA-binding proteins, one contained no detectable coding region in frame with the GAL4-AD fusion part.

Proteins with nucleic acid-binding domains have been reported to occur frequently in yeast 2-hybrid screens as false positive clones (Serebriiskii et al., 2000). False positive signals result from activation of reporter gene transcription by the library-encoded protein without the need for interaction with the bait protein, e.g. by binding to sequences in or near the promoter (Bartel *et al*., 1993). Alternatively, these proteins could activate transcription by weak and unspecific

interaction with the L-selectin cytoplasmic domain. This sequence contains a cluster of positively charged amino acids, whereas transcription factors often possess extended acidic stretches. Further indication against a specific interaction was the need of prolonged incubation necessary before a positive signal was observed.

Since the isolated proteins are unlikely to be physiological binding partners of L-selectin, the ability of this system to detect L-selectin interactions was tested by co-transformation of the bait protein with the cytoskeletal linker protein α-actinin. This protein has been reported to bind directly to L-selectin in *in vitro* assays (Pavalko *et al*., 1995). No positive signal was obtained in these experiments, although expression of both fusion proteins was detected in Western blots. This result was a strong indication that the yeast 2-hybrid method is not suitable for the identification of L-selectin interaction partners.

The failure to detect genuine L-selectin interaction partners can be explained in several ways (McAlister-Henn *et al*., 1999; Toby and Golemis, 2001). Due to the size of the L-selectin cytoplasmic tail, it is possible that this part of the bait protein is not displayed on the outside of the much larger globular GAL4-BD and therefore not accessible for potential interacting proteins. The L-selectin fusion part could also impair proper folding of the DNA-binding domain, resulting in a non-functional or unstable protein, or prevent transport to the nucleus (Golemis and Brent, 1992). Difficulties in identifying L-selectin cytoplasmic interaction partners can moreover be caused by the strong positive charge of the L-selectin cytoplasmic domain. This domain carries an unusual cluster of basic amino acids. Eight of the 17 residues are either lysine or arginine, resulting in a pI of 10,2. Since the fusion proteins used in 2-hybrid screening are transported to the yeast cells nucleus, where the interaction has to take place to be detected, this bait protein is surrounded by a great density of negative charges on nucleic acids and proteins containing acidic stretches. This may lead to a unspecific electrostatic adherence of these molecules to the bait protein, preventing specific interactions with potential protein partners.

Since the yeast 2-hybrid system proved unsuitable to identify potential L-selectin interaction partners, affinity isolation of binding proteins from cell lysates was chosen as an alternative approach.

3.1.2 Affinity-isolation of binding partners from cell lysates

As a tool for isolation of intracellular binding proteins, a recombinant protein containing the cytoplasmic domain of L-selectin fused to glutathione S-transferase (GST-Lscyto) was expressed in *E. coli* and purified by glutathione-Sepharose affinity chromatography. Pull-down experiments from metabolically labelled cell lysates using this fusion protein bound to a glutathione-Sepharose matrix revealed a number of cellular proteins specifically associating with the fusion protein. Analysis of the isolated proteins by mass spectrometry revealed the identity of six proteins: nucleolin, nucleophosmin, RS4, RS6, RL5 and PHAPII.

Nucleolin, nucleophosmin, RS4, RS6, RL5

Three of the identified proteins (RS4, RS6 and RL5) are ribosomal proteins of the small and large subunits, two others, nucleolin and nucleophosmin, are known to be major constituents of pre-ribosomal ribonucleoprotein (RNP) complexes (Ginisty *et al*., 1999; Pinol-Roma, 1999; Srivastava and Pollard, 1999). These complexes are established during transcription of ribosomal precursor RNA in the nucleolus, where the emerging rRNA is bound throughout their length by nucleolar RNA-binding proteins, and persist during rRNA processing, ribosomal maturation and export into the cytoplasm. RNP complexes have been shown to contain nucleolin, nucleophosmin (B23) and other non-ribosomal proteins involved in packaging, splicing and transport, as well as integral protein subunits of the mature ribosome.

This complex as a whole is not likely to be associated with L-selectin in a physiological context, but some of the members have been shown to fulfil unrelated cellular functions, especially nucleolin, which interacts with CD3 ζ-chain, cytoskeletal proteins and PI3-kinase in the cytoplasm (Hollander *et al*., 1999; Barel *et al*., 2001; Gil *et al*., 2001). In this light, it was conceivable that one or several of the proteins in the complex could bind specifically to L-selectin, thereby transporting the other members into the pull down eluates. The RNP particles are held together mainly by association of the proteins with the ribosomal RNAs rather than by protein-protein interactions and can be dissociated by treatment with nucleases (Pinol-Roma, 1999). To test whether one of the proteins was able to bind directly to L-selectin, the complexes were disrupted by treatment with RNase A, which digests the RNA molecules that bridge the proteins. Surprisingly, in pull-downs from RNase-pretreated cell lysates, none of the proteins belonging to RNP particles could be detected associated with the Lscyto fusion protein. The most likely explanation for this is that association of the RNP complex with Lscyto was mediated by electrostatic adherence of the rRNAs rather than by protein-protein interactions. The cytoplasmic tail of L-selectin contains a large portion of positively charged residues, whereas RNA is highly basic due to the phosphate groups in the nucleic acid backbone.

The RNP complexes are mainly localised in the nucleolus, where rRNA transcription and processing takes place. The presence of these particles in the membranous fraction used as a starting material for isolation of Lscyto binding partners was puzzling, since cell nuclei should remain largely intact during mechanical cell disruption and are removed by low-speed centrifugation at the start of the fractionation process. However, it has been shown that nucleolin function extends to the export of pre-ribosomal particles from the nucleus to the cytoplasm, where they persist for a certain time before the mature ribosome is released (Borer *et al*., 1989). Moreover, during mitosis, nucleolar components are concentrated in large aggregates, the nucleolus-derived foci (NDF), which disperse throughout the cytosol (Dundr and Olsen, 1998). Since the cellular fractions used as a starting material for pull-down experiments originated from logarithmically growing cell lines that show a high degree of ribosome biogenesis and cell division, both the newly exported ribosomal precursors and NDFs from dividing cells could contribute to the presence of RPNs in the nuclei-free cell fraction. The distribution of these complexes to the membranous fraction obtained by differential centrifugation can be explained by their large size, which causes

sedimentation during ultracentrifugation, rather than by direct association of these proteins with membranous structures.

Nucleolin was also found as a Lscyto binding partner from cytoplasmic fractions, where little of the other components of RNP particles were detectable. This initially made it a promising candidate for a direct interaction partner. However, nucleolin association with L-selectin was as well abolished by RNase-treatment, indicating that nucleolin from cytosolic preparations is also associated with RNA. This could be due to the presence of smaller aggregates derived from RNPs during processing, that do not contain all of the proteins present in the larger complexes. In addition, nucleolin has also been shown to be involved in stabilising several mRNAs and is found tightly associated with them in the cytosol (Zaidi and Malter, 1995; Chen *et al*., 2000).

According to the results obtained by the characterisation of the nature of interaction of these proteins with Lscyto, they are unlikely to represent physiological binding partners for L-selectin.

PHAPII (SET)

In addition to nucleolin, a 41 kDa protein was isolated from cytosolic fractions, whose binding to Lscyto was not disrupted by RNase treatment. This protein was identified as PHAPII (putative HLA-associated protein II), also known as SET. In contrast to the RNA-dependent association of the other proteins, binding of PHAPII was enhanced by RNase digestion, probably due to unmasking of the binding site which was occupied by RNA unspecific adhering to the fusion protein in untreated samples.

Little is known about the physiological functions of PHAPII in cells, although the protein has been isolated by several research groups in different contexts. Initially described as a breakpoint protein found in acute undifferentiated leukaemia (AUL), where it is fused to the putative oncogene CAN that is thought be activated by this fusion (von Lindern *et al*., 1992), it was characterised as a widely expressed phosphoprotein which is phosphorylated on serines in PKC consensus sites (Adachi *et al*., 1994a and 1994b).

PHAPII possesses an extended, highly acidic sequence in the C-terminal region, which is thought to be responsible for the discrepancy between the deduced molecular weight of 32 kDa and the observed migration behaviour on SDS-PAGE. Most of the cellular PHAPII is located in the cell nucleus, where it may serve as a nucleosome organising factor (Nagata *et al*., 1995). Nevertheless, it has also been detected in the cytosol and was isolated from cytosolic preparations in several instances (Vaesen *et al*., 1994, Beresford *et al*., 1997)

It was designated PHAPII by investigators identifying proteins that interacted with the cytoplasmic domain of HLA class II DR2α chain from B-cells in a search for interaction partners that are involved in HLA signal transduction (Vaesen *et al*., 1994). HLA class II molecules are heterodimeric glycoproteins on antigen-presenting cells that display processed antigenic peptides for the activation of T-helper cells. Crosslinking of these molecules on B-lymphocytes causes signalling events inside the cell, including tyrosine phosphorylation, phosphatidylinositol turnover, increase of intracellular free calcium and activation of PKC (Mooney *et al*., 1990, Brick-Ghannan *et al*., 1991). Affinity isolation of binding partners from cytosolic extracts with peptides spanning the short cytoplasmic domain revealed

specific binding of PHAPII to this protein, although physiological significance of this association was not further investigated.

An involvement of PHAPII in cellular signalling processes has been shown by Al-Murrani *et al*. (1999). Overexpression of PHAPII in cells increased concentration, phosphorylation and DNA-binding of the transcription factors c-Jun and enhanced transcriptional activity of the c-Jun containing AP-1 transcription factor complex. Interestingly, PHAPII was purified by a different group from bovine kidney as I_2PP2A, a potent, noncompetitive and specific inhibitor of protein phosphatase 2A, which is one of the major mammalian serine/thronine phosphatases in the cell (Li *et al*., 1995 and 1996). PP2A has a central role in the regulation of diverse cellular functions like proliferation, gene expression and signalling by counteracting the effects of numerous kinases involved in these processes (Janssens and Goris, 2001). The stimulatory effect of PHAPII on c-Jun was reversed by co-expression of the catalytic subunit of PP2A. This phosphatase has been previously shown to negatively regulate c-Jun activity by dephosphorylation of serine residues which are phosphorylated in the active transcription factor (Black *et al*., 1991). The results obtained by PHAPII overexpression correspond well with the effect obtained by treatment of cells with okadaic acid, a phosphatase inhibitor preferentially blocking PP2A. (Lee *et al*., 1994). Therefore, PHAPII elicits effects that are consistent with it acting as an inhibitor of PP2A in intact cells. A physical association of PHAPII and PP2A has been shown in a heterocomplex also containing the HRX leukemic fusion protein and this interaction has been discussed to have a role in the HRX induced deregulation of cell growth (Adler *et al*., 1997).

These results taken together provide growing evidence that PHAPII is involved in cellular signal transduction processes by acting as a negative regulator of protein phosphatase 2A, although nothing is known about the mode of action of this inhibitor and the regulation of this protein itself.

PHAPII can associate with the cytoplasmic tail of L-selectin in *in vitro* experiments and is found in L-selectin immunoprecipitates from the Jurkat T-cell line. This hints towards an involvement of the protein in L-selectin-mediated signal transduction. Interestingly, L-selectin was shown to be a target for PKC phosphorylation in this study, a kinase that itself is under regulation of PP2A and whose effects are often counteracted by this phosphatase. Therefore, a signal-modulatory complex containing PKC, PP2A and PHAPII could be involved in the regulation of the phosphorylation status of L-selectin (for further discussion see chapter 3.3.1) .

3.2. L-selectin interacts with PKC in an isotype-specific manner

The work of Haribabu *et al*. (1997) showed that L-selectin becomes phosphorylated on serine residues after stimulation of intact cells with phorbol esters and chemokines. The kinase(s) responsible for this modification have not been identified, however, the involvement of protein kinase C (PKC) in the process was suggested by its induction with PMA that can act as a direct activator of PKC, and the inhibitory effect of staurosporine which inhibits this kinase. Both agents have been widely used to dissect PKC functions, although it is clear now

that they do not act exclusively on PKC (Marks and Gschwendt, 1996). Moreover, it was not known whether these agents influence the L-selectin kinase directly or rather act on an upstream activator in a signalling cascade leading to L-selectin phosphorylation. This study set out to investigate the mechanisms leading to L-selectin serine phosphorylation.

3.2.1 PKC phosphorylates L-selectin in vitro

Staurosporine, which blocks L-selectin phosphorylation, strongly inhibits PKC, but also acts on a number of other kinases, including PKA and PKG. These kinases, similar to PKC, prefer target sites surrounded by basic amino acid residues (Pearson and Kemp, 1991; Tegge *et al*., 1995). The two serine residues that can serve as potential phosphoryl acceptors in the cytoplasmic tail of L-selectin are in the context of a highly basic sequence. To obtain information whether these kinases are able to phosphorylate L-selectin, *in vitro* kinase assays were performed with purified enzymes, using the GST-Lscyto fusion protein as a substrate. These experiments showed that PKC and PKG can serve as L-selectin kinases *in vitro*, whereas PKA shows very little activity towards this protein. PKC and PKG are able to use both serines as acceptor sites, but have different preferences: PKC has a higher affinity to serine 377, whereas PKG preferentially phosphorylates serine 380. These *in vitro* assays identified PKC and PKG as candidates for the L-selectin serine kinase.

3.2.2 The cytoplasmic tail of L-selectin precipitates a PKC-like kinase activity from cell lysates

With the aim of isolating the kinase that phosphorylates L-selectin in intact cells, the GST fusion protein containing the cytoplasmic domain of L-selectin was used to test for an associating kinase. When pull-down precipitates from T-cell extracts were subjected to *in vitro* kinase assays, phosphorylation of the fusion protein was observed. This phosphorylation occurred exclusively on serine residues, indicating that a serine/threonine kinase from cell lysates associates specifically with the cytoplasmic domain of L-selectin This was supported by the ability of this kinase to phosphorylate the typical serine/threonine kinase substrates myelin basic protein (MBP) and Histon H1. Phosphorylation of Lscyto occurred preferentially on serine 377, in accordance with the phosphorylation pattern observed with purified PKC. The association was stable and not impaired by high salt conditions. Closer characterisation of this kinase activity was achieved by inhibitor/activator studies. Use of different inhibitors demonstrated, that the kinase was sensitive to both the broad range serine/threonine kinase inhibitor staurosporine and the specific PKC inhibitor bisindolylmalimide, whereas the phosphorylation of Lscyto was not affected by inhibitor of tyrosine kinases and Protein Kinase G. Treatment of cells with PMA prior to cell lysis induced a strong increase in the activity of the associated kinase. These results strongly indicated that the kinase activity purified from T-cell lysates by binding to the cytoplasmic domain of L-selectin was PKC and that this kinase is involved in cellular L-selectin phosphorylation.

3.2.3 Association of PKC with L-selectin is isotype-specific

PKC constitutes a family of serine/threonine kinases under complex regulation by phosphorylation events, Ca^{2+} and interaction with lipid mediators (Liu and Heckman, 1998; Parekh *et al*., 2000). The eleven PKC isozymes known to date are grouped into three subfamilies according to their susceptibility for activation by the second messengers Ca^{2+} and diacylglycerol (DAG): (1) conventional cPKCs (α, β_{II}, β_I, γ) are activated by both Ca^{2+} and diacylglycerol, (2) novel nPKCs (δ, ε, η, θ, μ) are isotype-specifically activated by diacylglycerol and/or other lipid mediators, but independent of Ca^{2+}, (3) atypical aPKCs (ζ, ι/λ) are independent of both diacylglycerol and Ca^{2+}. The activation mechanisms of the atypical isozymes are largely unknown but might involve lipid compounds like phosphatidylinosites and unsaturated fatty acids (Toker, 1998).

Despite the extensive sequence and structural homology between the PKC isozymes and the fact that PKCs show little isozyme-specific discrimination towards substrates *in vitro*, individual cellular functions have been determined for the different isozymes *in vivo* (Dempsey *et al*., 2000; Way *et al*., 2000). This specificity is thought to be achieved by a combination of cell type-specific expression, differential regulation by upstream signals and intracellular localisation. PKCs have been shown to translocate to distinct cellular sites upon activation, indicating that specificity is obtained by selective confinement of the enzymes in proximity of upstream regulators and substrates.

To determine if PKC association with the cytoplasmic tail of L-selectin is isozyme-specific, Western blotting experiments with antibodies directed against several PKCs were performed. Of the isozymes previously shown to be expressed in Jurkat T-cells that were used for the isolation of the PKC kinase activity, exclusively nPKC theta (θ) and aPKC iota/lambda (ι/λ) were found in pull-down eluates, whereas the other isozymes tested (PKCα, β, δ, and ε) were not present in these samples. This is in good agreement with the results from experiments testing the cofactor requirements for the L-selectin-associated kinase activity. This activity was not enhanced by either Ca^{2+} or the lipid activators phosphatidylserine and diacylglycerol (DAG). Atypical PKC isozymes like ι/λ are independent of these factors. nPKCθ, which, like all novel PKC isozymes, possesses the conserved region involved in DAG-binding, has been shown not to be responsive to this lipid (Pietromonaco *et al*., 1998). These authors also reported, that PKCθ showed little activity when Mg^{2+} was provided as a divalent cation, but rather depends on Mn^{2+}, as was shown here for the L-selectin kinase.

Taken together, the results reported in this study show that a kinase activity from T-cell lysates associates with and phosphorylates L-selectin. This kinase shows characteristics reported for novel and atypical PKC isozymes. The PKC isozymes nPKCθ and a PKCι/λ are present in pull–down eluates obtained under the same conditions that isolated the L-selectin kinase. Although all recombinant PKC isozymes tested (α, βI, βII, γ, δ, ε, η, θ) were able to phosphorylate the cytoplasmic tail of L-selectin in *in vitro* experiments (data not shown), the association of this protein with PKC occurs in a isozyme-specific manner. An investigation if one or both of these isozymes are the cellular L-selectin kinase requires reagents that allow isozyme-specific inhibition of the kinases, which are not available at the moment.

3.2.4 PKC association is enhanced when L-selectin is phosphorylated on serine residues

Phosphorylation of proteins involved in signal transduction events frequently creates binding sites necessary for protein-protein interactions that trigger further advances of the signalling cascade. With the knowledge that L-selectin can be phosphorylated by PKC it was compelling to investigate whether serine phosphorylation of L-selectin cytoplasmic tail leads to a specific association of protein binding partners. To test this, *in vitro* phosphorylated Lscyto fusion protein was used to purify binding partners from T-cell lysates. Comparison of eluates from pull-downs with phosphorylated and unphosphorylated Lscyto revealed the presence of two protein bands of approx. 79 and 81 kDa specifically associating with the phosphorylated protein. Mass-spectrometric analysis of these proteins identified them as PKC isozymes alpha and theta. Carryover of PKCα as a contamination from the previously performed *in vitro* phosphorylation reaction, which used a PKC preparation from rat brain containing mainly PKCα, β, and γ, could be excluded by control experiments where incubation with cell lysates was omitted. In these control samples, no PKCα was detectable. Moreover, peptide sequencing by electrospray ionisation (ESI) mass spectrometry revealed that the amino acid composition of the Lscyto-associated PKCα was identical to that of the human kinase, which differs from the sequence of rat PKCα in two residues in the N-terminal peptide. PKCθ was not detected in the preparation used for *in vitro* phosphorylation. Hence, PKCθ associated with Lscyto was of T-cell origin. The amount of PKCθ associated with the phosphorylated cytoplasmic domain was much higher than with the unphophorylated protein as judged from silver stained gels. Also, immunological detection of PKCθ in precipitates with unphosphorylated Lscyto produced weaker signals compared to samples using phosphorylated protein. The binding of both PKCα and PKCθ was stable and resistant to elution by high salt washes, indicating that the association did depend on other than electrostatic forces. No other PKC isozymes could be detected associated with phosphorylated Lscyto.

The fact that the cytoplasmic of L-selectin tightly associates with PKCα from cell lysates, but not with the enzyme used for *in vitro* phosphorylation, indicates that binding of the kinase requires additional cellular components that are not present in *in vitro* phosphorylation assays. This tight binding could involve additional protein(s), although no other phosphorylation-specific binding partners than the 79 and 82 kDa PKC isozymes showed on silver stained gels. However, this possibility cannot be excluded, since some proteins do not stain well with silver or may be obscured by strong signal like the one obtained from Lscyto eluted from the GSH-matrix. It is also possible that the binding is dependent on the presence of phospholipids, which have been shown to have a large role in PKC activation and interaction with other proteins (Toker *et al*., 1994; Liu and Heckman, 1998).

3.2.5 L-selectin associates with PKC in vivo

Having found that the recombinant cytoplasmic domain of L-selectin associates with protein kinase C from cell lysates in an isotype-specific manner *in vitro,* it was a compelling question whether this association can also be detected in intact cells under conditions previously shown to induce serine phosphorylation of the receptor. For this, Jurkat T-cells were stimulated with the phorbol ester PMA, which has been reported to induce L-selectin phosphorylation on serine residues in this cell line. Immunoprecipitations were performed with antibodies directed against the extracellular part of L-selectin and the precipitates tested for the presence of PKC isozymes. Both PKCα and θ were detected in cells treated with PMA, but not in unstimulated cells. The same effect was observed in cells treated with okadaic acid, which inhibits serine/threonine phosphatases, especially PP2A, that reverses many of the PKC mediated phosphorylations. Inhibition of this phosphatase is thought to lead to an activation of signalling cascades. This is due to a basic level of phosphorylation events taking place in resting cells, which are mediated by partially active kinases. These phosphorylations are usually reversed immediately by a corresponding phosphatase, preventing further signalling events. Signalling is induced when phosphorylation exceeds a certain threshold, either by activation of the kinase, inhibition of the phosphatase or often both. Therefore, it is likely that treatment with okadaic acid leads to an accumulation of serine-phosphorylated L-selectin by deregulation of the corresponding signalling pathway. Pervanadate, an inhibitor for tyrosine phosphatases, did not induce association of PKC with L-selectin, indicating that upstream regulation of this event by tyrosine kinases does not occur or is not sufficient for PKC activation. Association of PKC isozymes with L-selectin in unstimulated cells was not detected, whereas *in vitro* experiments showed, that PKCθ and ι/λ are able to bind to the unphosphorylated cytoplasmic tail. The amount of kinase co-precipitating with unphosphorylated L-selectin under *in vivo* conditions may be below the detection limit, since, in the case of PKCθ, binding has been shown to be strongly enhanced with phosphorylated protein. More likely, though, is the possibility that binding of PKC in resting cells is prevented by interaction of L-selectin with alternative binding partners, e.g. calmodulin (see below), that block the PKC binding site.

The results of this study show that specific PKC isozymes have the ability to associate with the cytoplasmic tail of L-selectin both *in vitro* and *in vivo*. Phosphorylation of serine residues in the cytoplasmic sequence greatly enhances the binding of the novel PKC isozyme theta. The atypical PKC lambda/iota exclusively interacts with unphosphorylated Lscyto, whereas phosphorylation induces a strong association with PKC alpha, which is not found together with the unphosphorylated protein.

3.3. PKC-a central mediator of L-selectin function?

3.3.1 Regulation of L-selectin function by PKC and Calmodulin

The cytoplasmic domain of L-selectin has been reported to associate with the Ca^{2+}-sensing protein calmodulin (CaM) (Kahn *et al*., 1998). The study presented here now shows that PKC-dependent phosphorylation and tight binding of the

kinase occurs after activation of this enzyme or inhibition of the phosphatase that can reverse its action. Orchestrated regulation by PKC and CaM has been reported for a number of proteins. In all of these cases, the binding site for calmodulin overlaps with a phosphorylation site for PKC. Typical calmodulin binding sites are, like target sites for PKC, highly basic sequences, that have been shown for several proteins to form amphipathic helices. These sites are regulated in an opposing manner, i.e. calmodulin blocks PKC access to the phosphorylation site when bound to the target and, in reverse, the presence of phospho-serines in the calmodulin binding site strongly decreases its affinity for CaM (Rhoads and Friedberg, 1997).

The regulation of cellular functions by proteins that integrate CaM and PKC signals is best understood in the case of synaptic neurotransmitter release, involving the proteins neuromodulin (GAP-43) and neurogranin, as well as the metabotrophic glutamate receptor (mGluR) and NMDA receptor (Skene, 1990; Nakamura *et al*., 1999). Neurogranin and neuromodulin both bind CaM in the absence of elevated Ca^{2+} levels and serve as calmodulin reservoirs at synaptic sites. The opening of voltage-gated Ca^{2+} channels in the presynaptic membrane leads to binding of Ca^{2+} to CaM and the release of Ca^{2+}-CaM from neuromodulin. The concurrent activation of PKC induces phosphorylation of neuromodulin in the CaM binding site, preventing rebinding of CaM to this protein. This leads to a sustained high level of Ca^{2+}-CaM in the axon terminals, inducing release of the neurotransmitter glutamate from synaptic vesicles. On the postsynaptic side, a similar mechanism is used to regulate release of Ca^{2+}-CaM from neurogranin following increase of intracellular Ca^{2+} levels mediated by glutamate-activated NMDA-receptors and metabotrophic glutamate receptors (mGluR).

For L-selectin, binding of calmodulin has been shown in neutrophils and is constitutive in resting cells. Disruption of the L-selectin-CaM association by calmodulin inhibitors in the absence of activating stimuli induces shedding of the surface receptor (Kahn *et al*., 1998). This has been attributed to a conformational change in the L-selectin molecule after dissociation of calmodulin, leading to accessibility of the extracellular cleavage site for the protease. Regulation of L-selectin shedding by cellular signals can occur via different pathways. Induction of shedding by PMA, fMLP and TCR crosslinking is staurosporine-sensitive and therefore regulated by PKC, whereas shedding caused by crosslinking of L-selectin itself or the Leu13 molecule, or by growth factor stimulation is not blocked by staurosporine, but tyrosine kinase inhibitors (Frey *et al*., 1997; Fan *et al*., 1999; Alexander *et al*., 2000). The occurrence of PKC-independent shedding events indicates that serine phosphorylation of the L-selectin cytoplasmic domain is not a critical step for this process. This was supported by the finding that L-selectin variants lacking the serine residues in the cytoplasmic tail are shed at a normal rate (Haribabu *et al*., 1997). CaM-dependent shedding was observed by direct disruption of the binding by inhibitors, which is an isolated event. In a cellular context, however, the signal transduction pathway that leads to dissociation of CaM from L-selectin is likely to produce additional signals that act on the intracellular domain and regulate L-selectin function. In T-cells, proteolytic cleavage of the receptor is preceded by an increased binding activity of L-selectin when cells were activated by either PMA or T-cell receptor crosslinking (Spertini *et al*., 1991). Both modes of cellular activation are known to act on PKC and, at

least in the case of PMA, have been shown to induce serine phosphorylation of L-selectin. It is therefore imaginable, that Ca^{2+}-induced release of calmodulin from the cytoplasmic domain of L-selectin allows activated PKC to occupy the vacant binding site after phosphorylation of the serine residues, thereby preventing the conformational change that leads to immediate proteolytic cleavage. Shedding might then only occur after termination of the PKC signal.

A possible mechanism for the coordinated action of calmodulin and PKC on the cytoplasmic domain of L-selectin is shown in Fig. 40. Calmodulin is bound to the unphosphorylated L-selectin cytoplasmic tail in resting cells. Activating signals that cause Ca^{2+}-influx induce the dissociation of CaM from the cytoplasmic domain. This allows phosphorylation of the unoccupied L-selectin cytoplasmatic domain by PKC, which is activated by parallel signalling events.

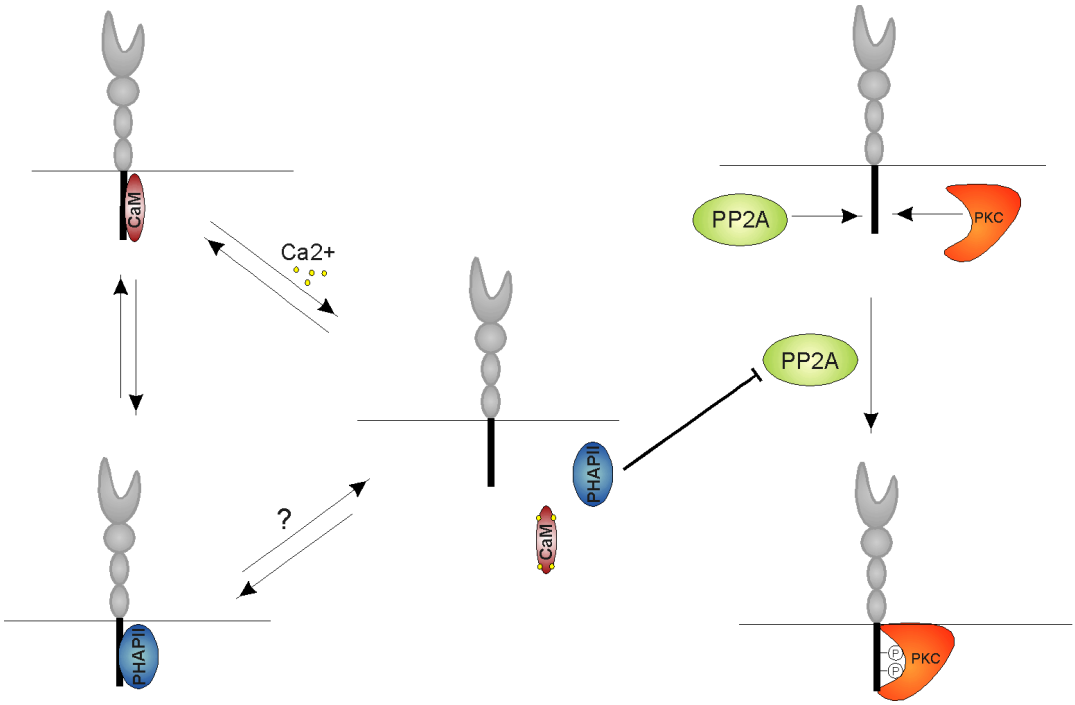

Fig. 40: Coordinated regulation of L-selectin by calmodulin and PKC.
This picture presents a model for the orchestrated action of calmodulin (CaM), PHAPII, protein phosphatase 2A (PP2A) and protein kinase C (PKC) on the cytoplasmic tail of L-selectin. For detailed explanation see text of this chapter.

An additional regulatory step could occur on the level of the L-selectin phosphatase. In this context, the finding that PHAPII associates with the intracellular tail of L-selectin is of special interest. This protein has been shown to be a specific inhibitor for serine/threonine protein phosphatase 2A which is involved in dephosphorylation of numerous PKC substrates and the kinase itself (Sim and Scott, 1999; Janssens and Goris, 2001). The binding of PHAPII to L-selectin, as shown previously for calmodulin (Ivetic *et al*., 2002), is sensitive to high salt conditions and therefore dependent on electrostatic interactions. It was also observed in pull-down assays that PHAPII binds phosphorylated L-selectin to a lesser extent than the unmodified protein (data not shown). Therefore,

phosphorylation by PKC is likely to prevent association of PHAPII with L-selectin, allowing interaction of the free inhibitor to interact with PP2A molecules in the vicinity of L-selectin. This inhibition of the PKC antagonist might further enhance accumulation of phosphorylated and PKC-associated L-selectin that initiates further downstream signalling events.

3.3.2 L-selectin-a PKC anchoring protein?

PKC functions in intact cells have been shown to be strictly isozyme-specific and in many cases different PKC isozymes mediate opposite effects. This is seemingly in contrast to the finding that the optimal phosphorylation sites of different isozymes show only subtle differences (Nishikawa *et al*., 1997). *In vitro*, many PKC substrates, like MARCKS (myrostylated alanine-rich C kinase substrate), can be phosphorylated by all isoforms (Brumell *et al*., 1997; Ueberall *et al*., 1997). This suggests that other mechanisms than substrate specificity must control the involvement of PKC isozymes in distinct signalling pathway. An emerging concept for tight regulation of PKC function is the restricted, activation-dependent subcellular localisation of PKC by association with isozyme-specific anchoring proteins. Several of these proteins have been identified to date. RACKs (receptors for activated C-kinase) serve as anchoring proteins by binding to the regulatory domain of active PKC and this association further increases kinase activity (Robles-Flores and Garcia-Sainz, 1994). RACKs, apart from positioning PKCs at appropriate sites, also bind other signalling molecules like phosphatases and phospholipases. These adaptor molecules thereby serve as organisers of functional multienzyme complexes that mediate signalling events (Schechtman and Mochly-Rosen, 2001). PKCs have also been shown to undergo tight association with their own substrate proteins, which were collectively termed STICKs (substrates that interact with C-kinase). The PKC binding to STICKs occurs via the regulatory domain of the kinase, thereby allowing PKC phosphorylation of other substrates closeby (Jaken and Parker, 2000). In the case of Syndecan-4, a proteoglycan adhesion molecule acting in concert with integrins in focal adhesions, binding of PKC to the cytoplasmic tail of the receptor has been demonstrated to stimulate the phosphorylation activity of the kinase (Oh *et al*., 1998; Simons and Horowitz, 2001). This indicates, that STICKs, like RACKs, are important for both localisation and regulation of PKC.

L-selectin function has been reported to be influenced by various cellular signals and ligation of the receptor elicits intracellular signalling events. The mechanisms how these signals act on the cytosolic part of L-selectin is not known, but the finding that specific PKC isozymes can interact with the receptor poses an exciting perspective for integration of signalling activities, since this kinase has been shown to have an important role in many of the cellular events that are triggered by L-selectin ligand-binding. In this study, association of PKC with the cytoplasmic domain of L-selectin has been shown with both unphosphorylated and serine-phosphorylated protein. Yet, the amount of kinase associated with phosphorylated L-selectin is significantly higher in pull-down assays and immunoprecipitations. This indicates tight association between the proteins going beyond transient kinase-substrate contacts. L-selectin could therefore serve as a PKC anchoring protein by locating the kinases in the vicinity of further substrates and promoting their phosphorylation. Association with the cytoplasmic domain of

L-selectin would also bring the kinase in close contact with membrane lipids that are crucial for PKC activation.

Although the ability of PKC to be activated and phosphorylate other proteins when bound to the cytoplasmic tail of L-selectin remains to be investigated, this could explain many of the signalling actions of the receptor. The possible implications of PKC on L-selectin signalling events are discussed in detail below.

3.3.3 Cytoskeleton-association and inside-out activation

The mechanism how serine phosphorylation influences L-selectin function is not well understood at the present time. However, this event has been implicated in the reported upregulation of L-selectin binding activity on T-cells by PMA and TCR-crosslinking, since induced binding to HEV sections was inhibited by pretreatment with PKC-inhibitors (Spertini *et al*., 1991b; Haribabu *et al*., 1997). Recent reports have correlated the increase in binding activity to an inducible association of L-selectin with detergent insoluble cell fractions (Evans *et al*., 1999). The authors concluded that L-selectin connects to the cortical cytoskeleton following receptor ligation. Increased binding to HEV and translocation to the cytoskeletal fraction was also observed after activation of intracellular signalling events by exposure of cells to fever-range hyperthermia, which was shown previously to influence PKC activity (Di *et al*., 1997; Wang *et al*., 1998). Crosslinking of L-selectin also enhances actin polymerisation and capping of L-selectin, which is sensitive to staurosporine (Brenner *et al*., 1997a; Junge *et al*., 1999).

The finding that L-selectin interacts with PKC directly is of special interest for these events, since PKC has been identified as an important regulator of cytoskeletal functions in leukocytes (Kelleher *et al*., 1995; Keenan and Kelleher, 1998). PKC co-localises with numerous cytoskeletal components and phosphorylates many of them, including talin, vinculin and moesin (reviewed in Keenan and Kelleher, 1998). Inhibition of PKC in T-cells severely impairs actin-dependent lymphocyte migration (Southern *et al*., 1995), whereas activation of PKC leads to capping of numerous surface receptors and rearrangement of the actin cytoskeleton (Haverstick *et al*., 1992; Parsey and Lewis, 1993). Moreover, the major cellular PKC substrate MARCKS is an actin crosslinking protein, which has a role in actin reorganisation following leukocyte activation (Aderem, 1992).

L-selectin has been found associated with the cytoskeletal linker proteins α-actinin and moesin, but the exact mechanism of the cytoskeletal connection is unknown. PKC could have a central role in this event. L-selectin-associated α-actinin is complexed with vinculin and talin, which are both PKC targets (Pavalko *et al*., 1995; Hyatt *et al*., 1994). Interestingly, PKCθ has been reported to phosphorylate moesin in the actin binding sequence (Pietromonaco *et al*., 1998). This phoshorylation is thought to be involved in the induction of actin-mediated morphological shape changes (Nakamura *et al*., 1995). The recruitment of the kinase to the L-selectin cytoplasmic domain may therefore locate PKCθ in the optimal position to phosphorylate moesin or other cytoskeletal linker proteins and thereby induce the connection of the receptor to the actin cytoskeleton.

3.3.4 Influence of PKC on intracellular signalling pathways

L-selectin has been reported to activate several intracellular signalling pathways following receptor crosslinking (see also chapter 1.5.4). Several of the L-selectin mediated signalling events inside the cell are dependent on the src-family kinase p56lck and are therefore acting downstream of this kinase. These include activation of ERK and JNK kinase cascades, activation of the transcription factor NF-AT and the ability to roll on fucoidan-coated surfaces (Brenner *et al*., 1996; Brenner *et al*., 1997a and 1997b). Direct association of lck with the cytoplasmic tail of L-selectin has not been detected and appears unlikely, since binding of lck to associated signalling molecules occurs via the SH2 and SH3 domains of the kinase, whereas L-selectin does not possess any sequences resembling binding sites for these domains. Interestingly, a recent report showed that the L-selectin associated PKC isozyme theta closely cooperates with lck in T-cell receptor signalling (Liu *et al*., 2000).

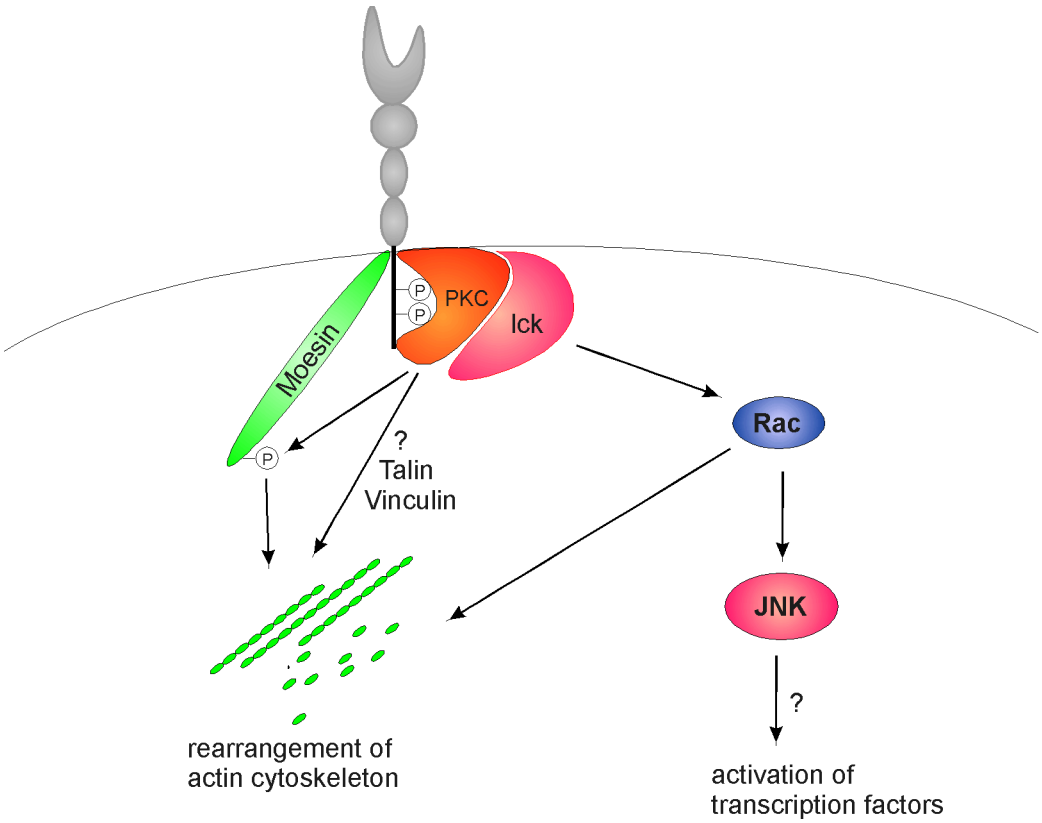

Fig. 41: Possible involvement of PKC in L-selectin mediated signalling events.
Several signalling pathways that are activated by L-selectin have also been shown to be influenced by PKC.

Expression of PKCθ is confined to haematopoietic cells and skeletal muscle. In the haematopoietic lineage, expression is most prominent in T-cells and platelets, whereas B-cells, neutrophils and monocytes possess little or none PKC theta (Baier *et al*., 1994; Oshevski *et al*., 1999). This isozyme has been shown to be involved in T-cell specific signalling events (reviewed in Meller *et al*., 1998; Altman *et al*., 2000). PKC theta translocates to the membrane and co-localises with the T-cell receptor (TCR) complex in T lymphocytes upon antigenic

stimulation (Monks *et al*., 1997). TCR engagement also resulted in an increase in the enzymatic activity of PKC theta. This activation has been shown to be due to tyrosine phosphorylation of the kinase by lck (Liu *et al*., 2000). lck is closely associated with the TCR complex after antigenic stimulation and phosphorylates several compounds of this complex (Wange, 2000). Phosphorylation by lck also has a important regulatory role for PKCθ function, since inactivating mutations of this phosphorylation site lead to a severe impairment of the ability of the kinase to induce T-cell proliferation and NF-kB activation. PKCθ and lck are constitutively associated via the PKC regulatory domain and association of the proteins is enhanced by TCR stimulation. PKC, in reverse, is also able to phosphorylate lck, an event that as well has been implicated in the regulation of signalling events downstream of both kinases (Hurley *et al*., 1989). Association of lck with PKCθ bound to the cytoplasmic domain of L-selectin could provide a mechanism to position lck close to the receptor, where it can exert its central role in L-selectin induced signalling events.

Several of the signal transduction components activated by L-selectin also have been shown to be controlled by PKC. One downstream target of lck-mediated L-selectin signalling is the Jun N-terminal kinase (JNK) (Brenner *et al*., 1997c). Activation of this kinase is dependent on the small G-protein Rac, which also regulates enhanced actin polymerisation triggered by L-selectin ligation (Brenner *et al*., 1997a). In T-cells the JNK signalling cascade is triggered by TCR/CD28 ligation, which leads to the activation of the transcription factors NF-κB and AP-1 and IL-2 expression (Ghaffari-Tabrizi *et al*., 1999; Su *et al*., 2000). This event is dependent on both PKCθ and Rac (Werlen *et al*., 1998).

Another signalling pathway induced by L-selectin is the mitogen-activated protein kinase (MAPK) cascade (Brenner *et al*., 1996). PKCα, which was also found associated with L-selectin, is known to be one of several upstream activators of the MAP kinases Erk-1 and Erk-2. This activation is thought to occur via the Raf-1 kinase, which is a direct target for PKCα phosphorylation (Siegel *et al*., 1990; Kolch *et al*., 1993). Raf-1-dependent activation of the MAP kinases and subsequent IL-2 expression has been shown to occur in T-lymphocytes after crosslinking of the T-cell receptor (Genot *et al*., 1995).

3.3.5 Crosstalk with other transmembrane receptors

L-selectin is co-localised with the TCR complex and β2 integrins (CD18) in the plasma membrane (Murakawa *et al*., 1992; Simon *et al*., 1999). These receptors also modulate each others functions by signalling crosstalk (see below). The mechanism how these transmembrane molecules are brought into close contact is not understood. However, it is well known, that both β2-integrins and the T-cell receptor complex are localised in specialised membrane microdomains after ligand binding. These membrane domains, designated as membrane rafts, detergent insoluble domains (DIGs) or glycolipid enriched microdomains (GEMs), are rich in cholesterol, glycosphingolipids and GPI-linked membrane proteins, and exist in a highly ordered liquid phase compared to the bulk of the plasma membrane. These domains also contain a number of intracellular signalling molecules, that are constitutively or inducibely associated with the membrane by lipid modifications. The abundance of these molecules in

membrane rafts has raised the hypothesis, that GEMs have a important role in the assembly of multi-enzyme signalling complexes by positioning the compounds of certain signalling pathways in close spatial proximity.

The association of L-selectin with membrane microdomains has not been demonstrated yet. Translocation of L-selectin to the detergent-resistant cellular fraction following crosslinking has been attributed to cytoskeletal connection, as described above (Evans *et al*., 1999), which is plausible considering the association of L-selectin with the cytoskeleton linker proteins α-actinin and moesin (Pavalko *et al*., 1995; Ivetic *et al*., 2002). Nevertheless, localisation of receptors in membrane rafts also leads to detergent-insolubility and often occurs together with cytoskeleton association, which might be necessary for the translocation of the protein to this specialized membrane regions.

In this respect, it is interesting that in the study presented here, two L-selectin-associated proteins were identified that have previously been shown to be preferentially localised in membrane rafts. PKCθ translocates to GEMs after TCR stimulation by association with lck kinase, which is constitutively found in these membrane domains (Zhang *et al*., 1998; Bi *et al*., 2000). This localisation of PKCθ has been shown to be essential for the TCR-induced activation of the transcription factor NF-κB.

Furthermore, analysis of L-selectin-associated membrane complexes revealed the presence of the transmembrane linker protein LAT (Linker for Activation of T-cells) as well as two unidentified tyrosine-phosphorylated proteins in these assemblies. LAT is constitutively localised in GEMs and serves as a binding platform for a number of proteins involved in TCR signalling, including phospholipase C, PI-3 kinase and the adaptor proteins Grb2 and Cbl. Close association of L-selectin with LAT is only observed after receptor crosslinking, pointing towards an inducible translocation of L-selectin into membrane rafts, which might be essential for contact with other signalling molecules. A close association with β_2-integrins and the TCR in membrane rafts might facilitate receptor crosstalk and coordinate the shared signalling pathways.

3.3.6 L-selectin-mediated integrin activation

Ligation of L-selectin activates the leukocyte-specific β2-integrins in both neutrophils and lymphocytes (Sikorski *et al*., 1996; Tsang *et al*., 1997) and the L-selectin induced increase in β2-integrin binding is synergistic to chemoattractant stimulation (Simon *et al*., 1995). Co-localisation of L-selectin with the CD18 integrin subunit is observed after L-selectin ligation (Simon *et al*., 1999). Recently it was shown that PKCα phosphorylates the CD18 integrin chain in a threonine-rich motif in the cytoplasmic tail (Fagerholm *et al*., 2002). Phosphorylation of this motif is assumed to be involved in upregulation of integrin binding activity (Hibbs *et al*., 1991) and association with the actin cytoskeleton (Peter and O'Toole, 1995; Valmu *et al*., 1999), and occurs in intact cells after stimulation with phorbol ester or T-cell receptor engagement (Valmu *et al*., 1995). Since PKCα associates with L-selectin, this could constitute the mechanism to bring the active kinase into proximity to the CD18 chain, allowing phosphorylation and activation of the integrin.

3.3.7 Intersection with TCR signal transduction

L-selectin has been shown by fluorescence energy transfer measurements to localise in close proximity to the TCR complex (Szabo *et al*., 1994), to precipitate together with the CD3 ζ-chain and to produce a co-stimulatory signal for mitogenic stimuli via the TCR (Murakawa *et al*., 1992). Triggering of TCR signalling by crosslinking of the CD3 complex also influences L-selectin function. Crosslinking of the CD3-complex enhances L-selectin binding activity, followed by proteolytic cleavage of the receptor (Spertini *et al*., 1991b). Both events have been shown to be inhibited by staurosporine and are therefore PKC dependent (Haribabu *et al*., 1997). Additionally, L-selectin and the TCR have been shown to share many of the signalling compounds involved in mediating downstream events, as described above. It is therefore likely, that signalling converges at a point close to the receptors.

Activation of PKCθ is an early event in TCR signalling. PKCθ is recruited to the membrane and co-precipitates with both the TCR and lck after stimulation of the receptor. Since PKCθ has been shown here also to associate tightly with the cytoplasmic domain of L-selectin, it appears likely, that this molecule mediates crosstalk between the two receptors that are located in close proximity. Activation and recruitment of the kinase by the TCR might lead to serine-phosphorylation of L-selectin, which has been implicated in the upregulation of receptor binding activity. *Vice versa*, additional recruitment of active PKCθ by L-selectin to the vicinity of the TCR complex could lead to the observed enhancement in TCR signalling by increased phosphorylation of downstream targets.

3.4. Conclusion and future prospects

The study presented here shows that L-selectin interacts via its cytoplasmic domain with Protein Kinase C in an isotype-specific manner both *in vitro* and *in vivo*. Many of the cellular signalling events regulated by the PKC isozymes alpha and theta also have been shown to be targets for L-selectin signalling. This suggests a central role of this kinases in L-selectin mediated signal transduction. The association of L-selectin with PKC opens a wide field for further investigations. Points that have to be addressed in the future include:

Determination of the nature of the L-selectin-PKC interaction.

The binding conditions, under which L-selectin associated with PKC suggested that additional components are necessary for interaction. Many PKC interactions have been shown to depend on lipid compounds like phosphatidylserine or phosphatidylinosites. Further work will investigate the ability of L-selectin to interact with membrane lipids that might assist in PKC binding. Also, the question which PKC domains are involved in L-selectin binding will be addressed. The tight adhesion observed in the experiments presented here exceeds transient substrate-kinase contacts and indicates a association via the PKC regulatory domain, as described for numerous other PKC substrates. This interaction mechanism would allow the L-selectin-bound kinase to phosphorylate further substrates. In this context, it is also interesting to test,

whether the cytoplasmic tail of L-selectin can have a modulatory function on PKC activity, similar to other PKC-anchoring substrate proteins.

Regulation of L-selectin serine phosphorylation and PKC association by cellular signals.

Phosphorylation of L-selectin and association of PKC is induced by stimulation with phorbol ester. However, physiological stimuli that lead to this events have not been identified. L-selectin serine phosphorylation has been shown in a basophilic cell line after stimulation via co-transfected chemoattractant receptors. The involvement of chemokines in the extravasation of T-cells, however, is still controversial. In general, T-cells are less responsive to stimulation by these agents than granulocytes and receptor numbers are significantly lower. Stimulation of Jurkat cells with T-cell specific chemoattractants have failed to induce PKC association with L-selectin (data not shown). However, that might be due to downregulation of these receptors in cell lines. Experiments using primary lymphocytes isolated from peripheral blood will show whether stimulation via chemokine-receptors can lead to L-selectin phosphorylation in a physiological context. Other stimuli that might cause these signalling events are ligation of the T-cell receptor and crosslinking of L-selectin itself.

Dissection of isotype-specific PKC effects.

The investigation of specific effects of single PKC isozymes in intact cells is difficult, since no strictly isozyme-selective pharmacolocical inhibitors are available yet. Due to the lack of these antagonists, it was not possible in the scope of this study to determine, whether one of the isozymes found associated with L-selectin is the kinase that phosphorylates the receptor in intact cells. Inhibition of these kinases requires the construction of dominant-negative variants that are transfected into cells to block single isozymes. This mutants are also a prerequisite for the investigation of the involvement of PKC in L-selectin induced downstream signalling events.