Affinity and Avidity of the LFA-1 Integrin is Regulated by Phosphorylation

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals, and on unpublished results presented in the text.

I. Fagerholm, S.*, Hilden, T.J. * and Gahmberg C.G. (2002) Lck tyrosine kinase is important for activation of CD11a/CD18-integrins in human T lymphocytes. *Eur. J. Immunol*. **32**, 1670-1678

II. Valmu, L, Hilden, T.J., van Willigen, G. and Gahmberg, C.G. (1999) Characterization of β_2 (CD18) integrin phosphorylation in phorbol ester activated T lymphocytes. *Biochem. J*. **339**, 119-125

III. Hilden, T.J., Valmu, L., Kärkkäinen, S. and Gahmberg C.G. (2003) Threonine phosphorylation sites in the β_2 and β_7 leukocyte integrin polypeptides. *J. Immunol*. **170**, 4170-4177

IV. Fagerholm, S.C.*, Hilden, T.J. *, Nurmi S.M. and Gahmberg C.G. (2005) Specific integrin α and β chain phosphorylations regulate LFA-1 activation through affinity-dependent and –independent mechanisms. *J. Cell Biol.* **171**, 705-715

* These authors have contributed equally to the work.

ABBREVATIONS

ADMIDAS	adjacent to the MIDAS
AP-1	activator protein-1
APC	antigen-presenting cell
Arf-GAP	ADP-ribosylation factor GTPase-activating protein
βTD	β-tail domain
DAG	diacylglycerol
ECM	extracellular matrix
EGF	epidermal growth factor
FAK	focal adhesion kinase
GEF	guanine nucleotide exchange factor
FRET	fluorescence resonance energy transfer
ICAM	intercellular adhesion molecule
I-EGF	integrin- epidermal growth factor
IgSF	immunoglobulin superfamily
IP_3	inositol triphosphate
IS	immunological synapse
JAB-1	Jun activation domain-binding protein 1
JAM-1	junctional adhesion molecule
LAD-I	leukocyte adhesion deficiency-I
LFA-1	leukocyte function-associated antigen-1
MAP-kinase	mitogen-activated protein-kinase
MIDAS	metal ion-dependent adhesion site
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PSI	plexins, semaphorins, and integrin
PTB	phosphotyrosine-binding domain
RAPL	regulator of cell polarization and adhesion enriched in lymphoid tissues
RGD	Arg-Gly-Asp peptide
SH2/3-domain	Src-homology 2/3 - domain
SLP-76	Src homology 2 domain–containing leukocyte protein of 76 kDa
TCR	T cell receptor
WAIT-1	WD protein associated with integrin tails
ZAP-70	zeta-associated protein of 70 kDa

SUMMARY

Integrins are heterodimeric adhesion receptors at the cell membrane that transmit signals bidirectionally across the plasma membrane. LFA-1 is the major leukocyte integrin and it is of fundamental importance for the function of the immune system. LFA-1 mediates several different functions in the immune system, like adhesion between T cells and antigen presenting cells, emigration of leukocytes into the sites of inflammation, and costimulation of immune cells. The adhesive activity of leukocyte integrins must be tightly regulated, ensuring that adhesive interactions with ligands occur only after cell activation. This process, known as "inside-out" signalling, modulates integrin adhesiveness. This occurs through two modes; affinity modulation, where ligand-binding is altered by conformational changes, and avidity modulation, which modifies integrin diffusion and clustering in the membrane that is mediated by cytoskeletal interactions. Different modes of activation seem to use different mechanisms at the molecular level.

The short intracellular tails of integrins are devoid of catalytic activity, but are important for adhesion and signalling. Integrins probably work through the cytoplasmic proteins that modify or bind to the integrin cytoplasmic tails. Phosphorylation is a common mechanism for the regulation of the surface receptor functions and has also been suggested for integrin regulation. LFA-1 is phosphorylated on both the α and the β-chain, the α-chain being constitutively phosphorylated, while β-chain phosphorylation becomes detectable after inside-out stimulation of the integrin. Integrin phosphorylation may be a mechanism that regulates molecular interactions between integrins and cytoplasmic molecules.

In this study, different phosphorylation events regulating LFA-1 have been examined. Lck, a protein tyrosine kinase, has been identified as a novel component of the inside-out signalling involved in regulating LFA-1-dependent adhesion and cell aggregation. In addition, site-specific LFA-1 phosphorylation has been characterized. We have shown that phosphorylation of both cytoplasmic domain of LFA-1 mediates different modes of integrin activation. α-chain phosphorylation on Ser1140 is needed for the conformational changes in the extracellular domain of integrin. In contrast, the β-chain Thr758 phosphorylation mediates selective binding to the 14-3-3 proteins, resulting in cytoskeletal rearrangements and adhesion strengthening. These results indicate that the different phosphorylation events play distinctive roles in integrin activation. This gives the possibility for regulation of both fast and transient adhesive events by affinity changes and long-term adhesion strengthening by avidity changes.

The identification of components of the regulatory network and the investigation of the mechanism of integrin regulation is important, since leukocyte adhesion regulates most aspects of the immune system, and deregulation of LFA-1 function leads to autoimmune disease and fundamental defects of the immune system.

REVIEW OF THE LITERATURE

1. LEUKOCYTE FUNCTION-ASSOCIATED ANTIGEN-1

1.1. Introduction

Integrins are cell-surface receptors that mediate adhesion to cells and to the extracellular matrix (ECM). In addition, integrins make transmembrane connections to the cytoskeleton and activate many intracellular signalling pathways. Thus, the term "integrins" was coined to reflect the capacity of members of this family to integrate the extracellular and intracellular environment (Hynes 1987). Integrins are present in all metazoans, and the number of integrins in the genome generally increases with the complexity of the organism (reviewed in Bokal and Brown, 2002). They play central role in cell adhesion, cell migration and control of cell differentiation, proliferation and programmed cell death. Integrins are heterodimeric receptors formed by the non-covalent association of α and β subunits. Mammals contain 18 α and 8 β subunits that combine to produce at least 24 different heterodimers, each of which can bind to a specific repertoire of cell-surface, ECM or soluble protein ligands. Each subunit is a type I transmembrane glycoprotein that has a relative large (>700 residue) extracellular domain and, with the exception of the β4 subunit, a short cytoplasmic domain (reviewed in Hynes, 2002).

Leukocytes can express at least 12 of the 24 known integrin heterodimers, and the expression pattern depends on the subset and the maturation state of the cell (Hynes, 2002). They express four leukocyte-specific β2 integrins (αLβ2, αMβ2, αXβ2, αDβ2); αLβ2 or leukocyte function-associated antigen-1 (LFA-1) being the most abundant and widespread in expression (Gahmberg et al, 1997). Leukocytes also express the two β7 integrins (α4β7 and αEβ7) and, in common with many other cell types, the ECM-binding β1 integrins (α1-α6β1). LFA-1 has an essential role in various processes, including leukocyte attachment to endothelial cells and antigen-presenting cells (APC), cytotoxic killing, and migration across the endothelium both during normal recirculation through lymph nodes and in response to inflammatory signals (Scheeren et al, 1991, Davignon et al, 1981, van Epps et al, 1989, Kavanaugh et al, 1991). Furthermore, LFA-1 has a role in the "immunological synapse", which forms between the T cell and the APC during an immune response (Grakoui et al, 1999). Thus, the LFA-1 interaction with its ligands must be tightly regulated.

On leukocytes integrins are usually in an inactive state meaning that these receptors do not bind all the time to their ligands. Two mechanisms have been proposed to explain how leukocyte integrins become able to bind ligand (Figure 1) (reviewed in Dustin et al, 2004). Firstly, the integrin can undergo conformational changes that increase the affinity of individual integrins for their ligands. Secondly, stimulation of leukocytes causes clustering of integrins, resulting in increased avidity and providing stronger adhesion at sites of cell-cell contact. Importantly, because affinity regulation and avidity regulation are distinct processes, they are not mutually exclusive and can often occur at the same time.

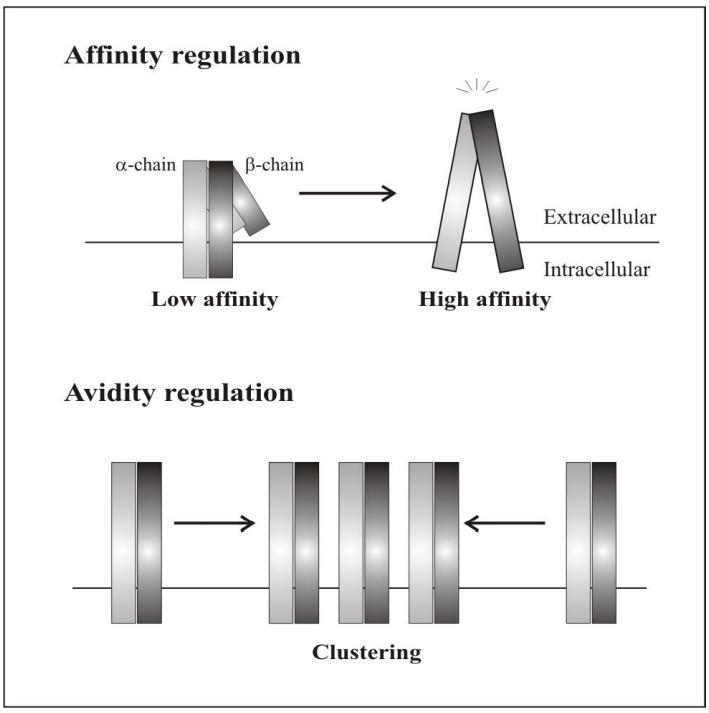

Figure 1. Models of integrin affinity and avidity. The upper panel shows different conformations of the integrins: the bent (low affinity) and the extended conformation (high affinity). The lower panel shows clustering of integrins on the surface of a cell.

1.2. LFA-1 binds to ICAMs

LFA-1 mediates leukocyte adhesion to cells by binding to any of five intercellular adhesion molecules (ICAM-1-5), which play important roles in inflammation, immune responses, and also in tumour progression (reviewed in Gahmberg, 1997, Cavallaro and Christofori, 2004). The ICAM molecules belong to the immunoglobulin superfamily (Ig-SF), consisting of two to nine tandem Ig domains. These Ig domains form the extracellular portion of the protein. Despite their structural similarity and integrin-binding capacity, the ICAMs have distinct patterns of expression, functions and cell signalling capacities (reviewed by Hayflick et al, 1998).

ICAM-1, the most widely distributed ICAM, is expressed constitutively only at low levels on a variety of both haematopoietic and non-haematopoietic cells, including leukocytes and endothelial cells (Rothlein et al, 1986, Patarroyo, et al, 1987), and expression levels can be upregulated by a number of inflammatory mediators such as interleukin-1, tumour necrosis factor α and interferon γ (Dustin et al, 1986, Pober et al, 1986a, 1986b). It is composed of five Ig domains, and because of a 90° bend between domains three and four, ICAM-1 monomers are L-shaped (Kirchhausen et al, 1993). ICAM-1 probably organizes into non-covalent homodimers on the cell surface (Miller et al, 1995, Reilly et al, 1995). The link is formed via Ig domain 4 interactions, which can then bring together domains 1, resulting in W-shaped tetramers (Casasnovas et al, 1998). ICAM-1 plays an important role in immune and inflammatory responses, in the development of the nervous system and in embryonic development (Springer, 1990, Tessier-Lavigne and Goodman, 1996). Additionally, ICAM-1 has been shown to act as a co-stimulatory ligand that binds to LFA-1, thereby promoting the T cells activation, and it can initiate several intracellular signalling pathways via dimerization (Lebedeva et al, 2005).

ICAM-2, a second LFA-1 ligand, is expressed constitutively at low levels on resting lymphocytes and monocytes and at higher levels on vascular endothelial cells, and expression is refractory to inflammatory

mediators (de Fougerolles et al, 1991, Nortamo et al, 1991). ICAM-2 mediates leukocyte trafficking (Staunton et al, 1989, Gerwin et al, 1999), and recent data indicate that ICAM-2 also play a role in angiogenesis (Huang et al, 2005). Furthermore, it acts as a stimulatory molecule of LFA-1 mediated adhesion (Li et al, 1993, Kotovuori et al, 1999).

Many evidences suggest that the third ligand of LFA-1, ICAM-3, may be involved in the initial steps of the immune response. It shows a high constitutive expression in resting T lymphocytes (de Fougerolles and Springer, 1992), co-stimulatory activity in T cells (Hernandez-Caselles et al, 1993, Juan et al, 1994, Bleijs et al, 1999, Montoya et al, 2002), and a capacity to induce LFA-1-mediated adhesion (Campanero et al, 1993). Moreover, ICAM-3 enhances the T lymphocyte adhesion to endothelial cells and ECM, probably by inducing the activation of β1 and β2 integrins (Bleijs et al, 2000). ICAM-3 has also been suggested to contribute to angiogenesis and cancer cell proliferation (van Buul et al, 2004, Kim et al, 2005).

The two other ICAM molecules also have been shown to bind to LFA-1: ICAM-4 (or LW blood group antigen) and ICAM-5 (or telencephalin) (Bailly et al, 1995, Mizuno et al, 1997, Tian et al, 1997). Their expressions are more restricted; ICAM-4 is expressed only on the red cell lineage of haematopoietic cells (Bailly et al, 1994) and ICAM-5 in the brain (Yoshihara et al, 1994). The physiological functions of ICAM-4 and ICAM-5 remain to be determined; however, ICAM-4 might play some role in red cell turnover and ICAM-5 seems to be involved in neurite outgrowth by homophilic interactions (Gahmberg et al, 1997, Tian et al, 2000).

Additionally, LFA-1 may bind to a few other proteins. Interactions have been shown with E-selectin (Kotovuori et al, 1993), type I collagen (Garnotel et al, 1995) and junctional adhesion molecule 1 (JAM-1) (Ostermann et al, 2002).

1.3. Biology of LFA-1

LFA-1 is involved in several fundamental biological processes

Many lymphocyte functions are dependent on adhesive interactions. T lymphocytes use integrins to migrate in and out of lymph nodes during normal recirculation (“homing”) and, following infection, to move into infected tissues. LFA-1 also participates in the immunological synapse (IS) formed between T lymphocyte and APC that is needed for T cell cytotoxicity and the antibody production process. In addition, LFA-1 also plays a key role in signalling although it has no enzymatic activity (reviewed in Hogg et al, 2003).

Leukocyte migration into target tissue is a sequential process that involves tethering and rolling of leukocytes on the blood-vessel wall, firm adhesion and crossing through the endothelial barrier (Figure 2A) (reviewed in Worthylake and Burridge, 2001). Activated endothelial cells participate in the process by expressing an array of important molecules, such as selectins, chemokines and integrin ligands on their surface. Initial attachment (tethering and rolling) of leukocytes to the endothelium is mainly mediated by the interaction of selectins with their ligands. L-selectin on T cells binds to its glycosylated ligands on the endothelium or, alternatively, E- or P-selectin expressed on stimulated endothelium bind to ligands on the T cell surface

(McEver, 2002). Also α4 integrins (α4β1 and α4β7) are able to support rolling (Alon et al, 1995, Berlin et al, 1995). LFA-1 molecules cannot on their own mediate rolling, but importantly, they can contribute to the rolling process together with selectins or α4 integrins (Perry and Granger, 1991, Henderson et al, 2001). The next step of leukocyte migration is the firm attachment of leukocytes to the endothelium. The most important molecules in this process are LFA-1, α4β1 and α4β7 that bind to their ligands expressed on chemokine activated endothelium cells (Andrew et al, 1998, Berlin-Rufenach et al, 1999). Before cell migration leukocytes undergo polarization, with the formation of a lamellipodium at the leading edge and a uropod at the trailing edge (reviewed in Vicente-Manzanares and Sanchez-Madrid, 2004). Although the response of transendothelial cell migration is believed to occur largely as a result of leukocyte migration through junctions between adjacent endothelial cells (paracellular route), there is now a renewed interest in the leukocyte migration through the body of the endothelial cells (transcellular route) (Engelhardt and Wolburg, 2004, Carman and Springer, 2004). ICAM-1 plays a key role in regulating leukocyte transmigration by both pathways (Yang et al, 2005). ICAM-1 and vascular cell adhesion molecule-1 concentrate at the leukocyte-endothelial cell contacts, forming cuplike structures surrounding migrating cells which provides directional guidance to leukocyte (Carman and Springer, 2004). The formation of the transmigration cup structure is essential for both para- and transcellular migration events by monocytes, neutrophils and lymphocytes, indicating that the structure represents a general feature of transendothelial cell migration. JAM-1, a molecule which normally regulates endothelial cell-cell junctions, is also involved in transmigration (Ostermann et al, 2002). It has been speculated that LFA-1 swaps ligands from the ICAMs to JAM-1 during the migration. In addition, chemokines have been shown to be key regulators of lymphocyte transmigration (Cinamon et al, 2001). Some β2 integrins have also been shown to be associated with matrix metalloproteases and this interaction seems to be important for migration (Stefanidakis et al, 2003, 2004). However, interaction with LFA-1 and matrix metalloproteases has not reported.

LFA-1 also has an essential role in T cell – APC interactions. Antigen presentation occurs when a T cell interacts with an APC that expresses its specific antigen. The initial interaction involves exploratory contacts that are mediated by chemokine activated LFA-1 and its ligands, which culminates in IS formation. The IS is a specialized cell-cell junction between a T cell and APC which is required for T cell signalling (Grakoui et al, 1999). The IS can be stable over many hours, and it naturally delivers a stop signal for T cell migration that is an essential event in T cell activation (Dustin et al, 1997). Like migration, IS formation is a multistep process with two major morphologically defined stages: immature IS and mature IS (reviewed in Sims and Dustin, 2002). In the immature IS the integrins are initially engaged in the center of the contact area, and T cell receptors (TCRs) are engaged in the periphery of the contact (Figure 2B). A major feature of the mature IS is the concentration of TCRs at the center surrounded by a ring of LFA-1 (Monks et al, 1998). Also additional molecules involving in T cell activation clustered at the center of the contact, such as CD2, CD28, PKC-θ, Lck, Fyn, CD4 and CD8 (reviewed in Huppa and Davis, 2003). In contrast, only LFA-1 and talin are known to reside in the peripheral area. Some molecules such as CD43 and CD45 are excluded from the contact area entirely probably for reasons of large size. This lateral movement induces the formation

of a second protein complex distal to the site of TCR engagement, which has been shown to be important for appropriate T cell activation (Cullinan et al, 2002). The precise mechanism by which the mature IS is formed is unknown, but it is believed to be dependent on signals generated by the TCR and the actin cytoskeleton (reviewed in Lin et al, 2005).

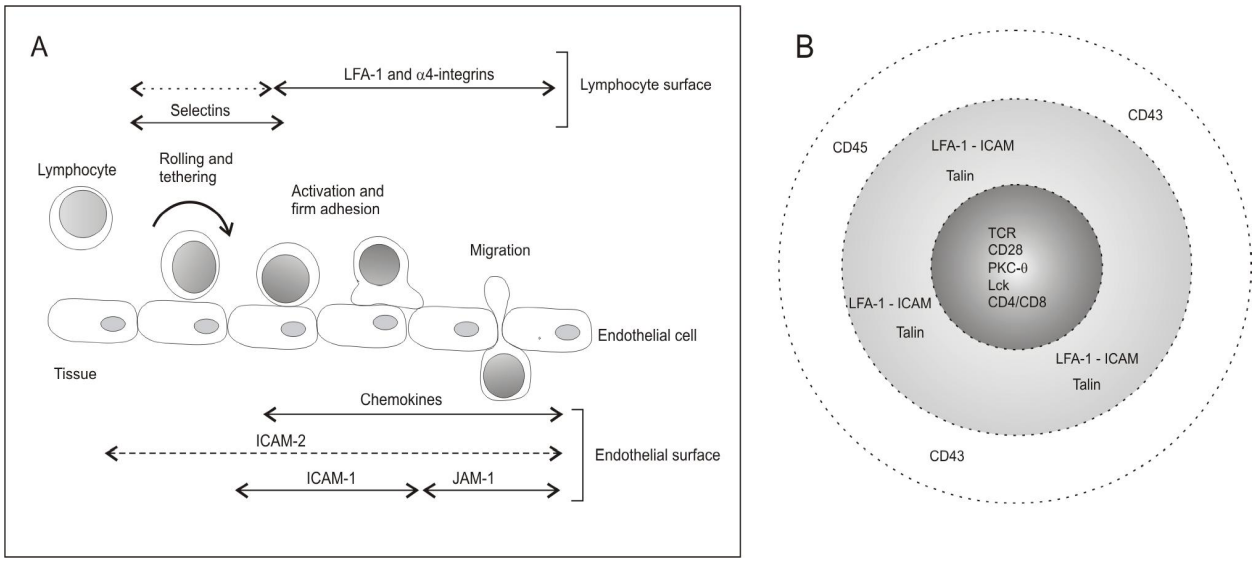

Figure 2. T cell adhesion events. A. The multistep model of leukocyte interactions with the endothelium. Adapted from Kinashi, 2005. B. The face of the IS, including the central region (dark gray), the peripheral ring surrounding the central region (light gray) and the region distal to the synapse (white), and the molecules that are found enriched within each region. Adapted from Huppa and Davis, 2003.

Leukocyte adhesion deficiency and LFA-1-deficient mice

The physiological importance of LFA-1 in leukocyte function has also been verified by the study of a naturally occurring human disease, leukocyte adhesion deficiency-I (LAD-I) (reviewed in Hogg and Bates, 2000). A range of mutations in the β2 gene have been identified including deletions, truncations, substitutions, and frame shifts that result in abnormalities of the β2 polypeptides and β2 integrin expression levels. The lack of β2 integrin results in elevated numbers of circulating neutrophils because these cells fail to migrate across the endothelium. Patients typically have recurrent bacterial or fungal infections of the skin and mucous membranes, impaired mobilization of leukocytes to infected area, severe gingivitis, and impaired tissue remodeling and wound healing. Severely affected people often die of infections in early childhood or adulthood unless bone marrow transplantation is successfully accomplished. A few variant LAD-1 syndromes have also been identified (Kuijpers et al, 1997, Hogg et al, 1999, McDowall, et al, 2003). In these syndromes the expression levels of LFA-1 were normal or only marginally reduced, but integrins were nonfunctional, indicating a defect in inside-out signalling. A new interesting LAD-1 variant has been identified recently, which has a major truncation of the β2 cytoplasmic domain (Hixson et al, 2004). This is the first report describing an LAD-1 that specifically involves the cytoplasmic domain. This truncation impaired binding function of LFA-1, but surprisingly not Mac-1, another β2 integrin.

Both $\alpha L^{-/-}$ and $\beta 2^{-/-}$ mice display similar phenotypic features as humans with LAD-I (Schmits et al, 1996, Mizgerd et al, 1997, Scharffetter-Kochanek et al, 1998). Animals deficient in LFA-1 demonstrate defects of T cell function in vivo, including leukocytosis, spontaneous skin infections, neutrophilia and failure in immunogenic tumor rejection. Mice also have impaired T lymphocyte and neutrophil transendothelial migration (Andrew et al, 1998, Berlin-Rufenach et al, 1999). Interestingly, LFA-1 mutant mice having a deletion of the conserved GFFKR motif in the αL cytoplasmic domain, which makes LFA-1 constitutively active, also have impaired immune responses (Semmrich et al, 2005). The phenotype was quite similar to that of LFA-1-deficient mice, including enlarged spleens and impaired lymphocyte responses, but in addition, T cell activation, cytotoxic T cell activity and T cell-dependent humoral immune responses were impaired. Thus, also deactivation of LFA-1 and disassembly of LFA-1-mediated cell contacts seem to be vital for the generation of normal immune responses.

1.4 LFA-1 as a signalling receptor

Two-way signalling through LFA-1

Integrins possess the unique ability to regulate dynamically their adhesiveness by bidirectional signalling across the plasma membrane (reviewed in Dustin et al, 2004, Travis et al, 2004). Ligation of various cell-surface receptors generates intracellular signals that increase LFA-1-mediated cell adhesion; this is termed *inside-out activation*. This pathway can modulate both the affinity of LFA-1 for its ligands and clustering of integrins in the cell membrane. LFA-1 is also capable of transducing signals into the cell after ligand binding in a process known as *outside-in signalling*. This outside-in signalling occurs particularly in the context of the IS, but probably also during endothelial attachment and migration (Pardi et al, 1989, van Seventer et al, 1990). Signalling events that follow direct LFA-ligation include tyrosine phosphorylation of phospholipase Cγ1 (PLCγ1) (Kanner et al, 1993), and activation of the tyrosine kinases ZAP-70 (zeta-associated protein of 70 kDa) (Soede et al, 1999), Pyk-2 and focal adhesion kinase (FAK) (Rodriguez-Fernandez et al, 1999). The functions of protein kinase Cδ (PKCδ), cytohesin-1 and Jun activation domain-binding protein 1 (JAB1) have also been compared directly in outside-in signalling (Perez et al, 2003). These signals may influence whether a T cell is differentiated to T helper type 1 or type 2 fates.

The initial activating signal in inside-out signalling can be triggered by a number of different cell-surface receptors. Several stimulating receptors are involved in the initial contact between T lymphocytes and APC, including CD3, CD2 (Dusting and Springer, 1989, van Kooyk et al, 1989), CD44 (Koopman et al, 1990) and CD45 (Spertini et al, 1994). Furthermore, multiple inflammatory mediators, such as chemokines, have been shown to induce LFA-1-mediated adhesion through inside-out signalling (Campbell et al, 1998, Constantin, et al, 2000).

In addition, LFA-1 can also be activated by extracellular treatment with divalent cations (Dransfield et al, 1992), ligands (Cabanas and Hogg, 1992, Li et al, 1993, Kotovuori et al, 1999, Bleijs et al, 2000) or certain monoclonal antibodies to the integrin extracellular domain (reviewed by Humphries, 2000).

Intracellular signalling controlling LFA-1 activation

Multiple intracellular signalling events contribute to LFA-1 activation (Figure 3). Ligation of the TCR through CD3 cross-linking activates a network of downstream signalling including activation of protein tyrosine kinases like Lck (reviewed in Dustin and Chan, 2000). Lck has been shown to be responsible for the early events in T cell activation due to tyrosine phosphorylation events, and the induction of calcium signals (Straus and Weiss, 1992). Relative little is known about the proximal signalling events that regulate TCR-activated LFA-1 adhesion. However, Lck has been shown to play a critical role in superantigen-induced LFA-1-mediated T cell - B cell conjugation independent of its ability to activate ZAP-70 (Morgan et al, 2001). Adhesion is dependent on the kinase activity of Lck and a functional Lck Src-homology 3 (SH3)-domain, indicating that one or more proteins that interact with the SH3 domain of Lck are required for the inside-out signalling from the TCR. In addition, overexpression of Lck has been shown to upregulate LFA-1 surface expression in a cytolytic T cell clone, and that way to increase adhesion to ICAM-1 (Torigoe et al, 1994). Lck has been shown to regulate the affinity of β1-integrins in T cells (Feigelson et al, 2001), but the involvement of Lck in integrin activation remains poorly understood.

SLP-76 (Src homology 2 domain – containing leukocyte protein of 76 kDa) is a scaffold molecule that is required for TCR signalling (Yablonski et al, 1998). This protein lacks enzymatic domains and instead carries binding domains that are needed for the assembly of complexes. SLP-76 associates in a TCR-inducible fashion with SLAP-130/Fyb, another hamatopoietic-specific adaptor. T cells from SLAP-130/Fyb-deficient mice fail to enhance TCR-induced LFA-1-dependent adhesion and clustering of the LFA-1 (Peterson et al, 2001). The other SLP-76-binding protein, ADAP (adaptor adhesion and degranulation promoting adaptor protein), has also been shown to be needed for TCR-induced LFA-1 adhesion and T cell –APC conjugation (Wang et al, 2004), indicating that intermolecular interaction might constitute a link in TCR-mediated activation of LFA-1.

TCR-ligation and chemokine triggering also lead to activation of PLC, which generates the second messengers inositol triphospate (IP_3) and diacylglycerol (DAG). IP_3 is required for Ca^{2+} flux that has been shown to be crucial for LFA-1-mediated adhesion (Rothlein and Springer, 1986, Stewart et al, 1996, 1998). The effects of Ca^{2+} are mediated through calcium-binding proteins, like calmodulin and calpain (Pettit and Fay, 1998). DAG, in turn, activates PKCs which are candidates for activation signals for LFA-1, since phorbol esters, cell permeable analogs of DAG, have been shown to stimulate LFA-1-mediated adhesion (Patarroyo et al, 1985, Rothlein and Springer, 1986). In addition, involvement of PKC in LFA-1 activation was demonstrated using a PKC inhibitor (Dustin and Springer, 1989, Hauss, et al, 1993). The PKC family includes a growing number of isoforms that are classified into three major subgroups based on their structure and cofactor requirements for activation. Overexpression of active conventional PKCs and novel PKCs, but not atypical PKC isoforms induces LFA-1-mediated adhesion to ICAM-1 (Katagiri et al, 2000). On the other hand, the atypical ζ PKC has been shown to be involved in chemokine-induced LFA-1 lateral mobility (Giagulli et al, 2004). Moreover, PKCs have been shown to phosphorylate several residues in the LFA-1 β subunit (Fagerholm et al, 2002).

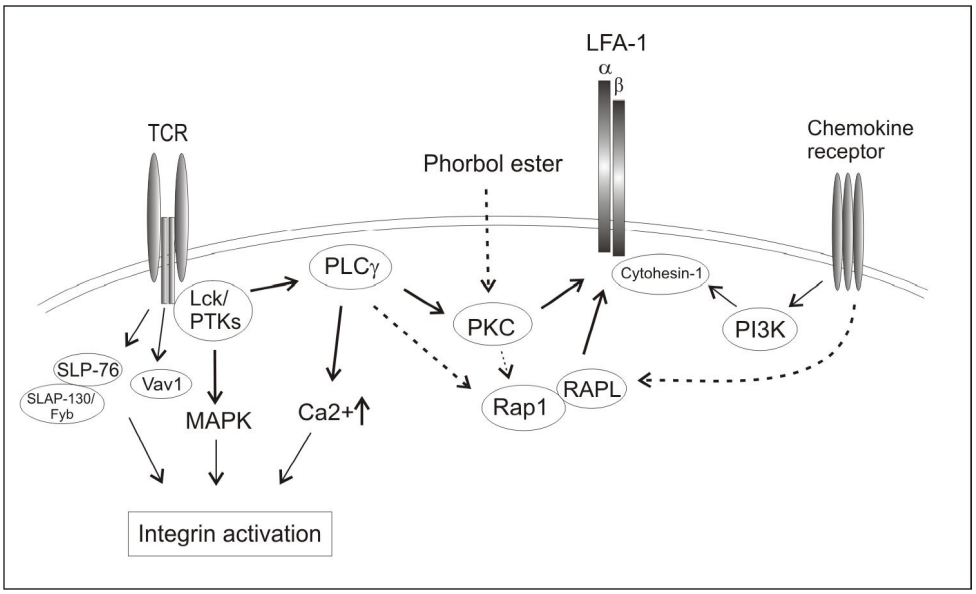

Figure 3. Schematic figure of intracellular signalling pathways controlling LFA-1 activation. T cells are activated through TCR or chemokine receptors. TCR-ligation leads to protein tyrosine kinase (PTK) activity resulting in the activation of multiple downstream pathways. The Ras-MAPK pathway is activated downstream of PTK-signalling. PTK activity also results in PLCγ activation that leads to PKC activation and Ca2+-signalling. PLCγ is also activated by chemokine triggering. All these pathways have been implicated in LFA-1 regulation. TCR-ligation also leads to recruitment of adapter protein to the plasma membrane, such as SLAP-130/Fyb, that regulates LFA-1 clustering on the cell surface. Vav is GEF for Rho GTPases that transduces TCR-signals to LFA-1. PI3K is activated after chemokine triggering and TCR-ligation and it probably contribute to LFA-1 activation through cytohesin. Rap1 is activated in T cells by multiple pathways and it is important for LFA-1 regulation.

Phosphoinositide 3-kinases (PI3Ks) are a family of intracellular signal transducing enzymes that share the capacity to phosphorylate phosphatidylinositol lipids (Kapeller and Cantley, 1994). PI3K has been demonstrated to play a role in TCR-induced adhesion and chemokine-induced clustering of LFA-1 (Nagel et al, 1998, Katagiri et al, 2000, Constantin et al, 2000). In contrast, a catalytically inactive PI3K does not affect TCR-induced adhesion of mouse T cells to the LFA-1 ligand ICAM-1 (Okkenhaug et al, 2002). One proposed mechanism for PI3K signalling is through cytohesin-1, an integrin-binding ARF-GEF (Nagel et al, 1998, Geiger et al, 2000).

Members of the Ras/Rho superfamily of small GTPases regulate the actin cytoskeleton in many cell types, including leukocytes (reviewed in Vicente-Manzanares and Sancher-Madrid, 2004). Members of the Ras/ Rho family have been reported to have both a positive and a negative role in integrin-mediated adhesion. For example, RhoA has been reported to control the induction of LFA-1 high-affinity state and rapid lateral mobility induced by chemokines (Giagulli et al, 2004), whereas RhoH has been demonstrated to maintain LFA-1 in a nonadhesive state (Cherry et al, 2004). Interestingly, Rap1, a small GTPase, has emerged as an important regulator of integrin adhesiveness (Kinashi and Katagiri, 2005). Rap1 is activated by a number of extracellular stimuli, including TCR-ligation as well as chemokine and phorbol ester triggering (Katagiri et al, 2000, Katagiri et al, 2002, Shimonaki et al, 2003). Expression of a dominant-active form of Rap1 (Rap1V12) enhances LFA-clustering and adhesion by increasing both affinity and avidity of LFA-1 (Katakiri et al, 2000, Sebzda et al, 2002). Rap1 is believed to act at least in part, through RAPL (regulator of cell polarization and

adhesion enriched in lymphoid tissues), Rap1-interacting protein (Katagiri et al, 2003). Another protein clearly implicated in Rap1-induced integrin-mediated cell adhesion and spreading is Riam, an interactor of Rap-GTP. Overexpression of Riam promotes the active conformation of β1 and β2 integrins and integrin-mediated adhesion, and knock-down of Riam abolishes Rap-induced adhesion (Lafuente et al, 2004). Moreover, several other proteins have been identified that interact with the active form of Rap1, including Arap3 and Afadin (Bos, 2005). Interestingly, Rap1 has recently been shown to associate with β1 integrins through protein kinase D1 after phorbol ester or TCR-stimulation. This association was shown to be critical for activation-dependent β1 integrin clustering and adhesion in T cells (Medeiros et al, 2005).

In addition, guanine nucleotide exchange factors (GEF) for GTPases play a role in integrin regulation. Vav1 is GEF for Rho GTPases that has recently been shown to transduce TCR-signals required for LFA-1 function (Ardouin et al, 2003). Vav1 has been shown to require multiple LFA-1-indused functions, including adhesion and spreading, but not agonist-induced inside-out activation (Gakidis et al, 2004).

2. INTEGRIN STRUCTURE AND AFFINITY CHANGES

2.1. Structure of the integrin ectodomain

The overall shape of the integrin extracellular domain has been known from electron microscopy studies (Weisel et al, 1992, Du et al, 1993): a globular amino-terminal "headpiece" binds ligand and two long carboxy-terminal rod-like "legs" connect the head to the transmembrane and cytoplasmic domains (Figure 4A). In 2001 the first X-ray crystal structure of the extracellular domain of an integrin was published at 3.1 Å resolution (Xiong et al, 2001). The structure was from the I-domain lacking integrin αVβ3, a receptor that participates in cardiovascular and bone functions. The big surprise was that the structure assumed a bent conformation, in which the ligand-binding headpiece was folded back onto the legs of the molecule. A second integrin crystal structure followed closely after the first, in which a cyclic Arg-Gly-Asp (RGD) peptide was soaked in the presence of manganese into pre-existing integrin crystals (Xiong, 2002). The location of the ligand binding pocket was found to be at the junction of the β-propeller and the β I-like domain in the integrin head. Although αVβ3 is a member of the subclass of integrins that lacks an I-domain, its structure also predicted the I-domain insertion site to be within a loop on the surface of the β-propeller that faces the β subunit. A complementary NMR structure of β2 integrin fragment reveals the structure of the some missing domains and defines the disposition of residues important in integrin activation (Beglova et al, 2002).

The α-subunit

The N-terminal region of all integrin α subunits contains seven segments of about 60 amino acids with weak homology to one another. The seven repeats have been predicted to fold into a seven-bladed β-propeller domain (Springer, 1997). Half of the 18 integrin α subunits, including αL, contain a 200-residue module

homologous to the cation-binding A-domain of von Willebrand factor (called the “inserted” I-domain). In I domain-containing integrins the I domain is the major ligand binding domain and recognizes ligand directly (Michishita et al, 1993, Diamond et al, 1993). The I-domain is predicted to be inserted between the second and the third β-strand of the β-propeller (Springer, 1997). Crystal and NMR structures have been determined for I-domains from the integrin αM (Lee et al, 1995, Baldwin et al, 1998), αL (Qu and Leahy, 1995, 1996, Legge et al, 2000), α2 (Emsley et al, 1997) and α1 subunits (Nolte et al, 1999). These showed that the I domain has a dinucleotide-binding or Rossmann fold, with a central hydrophobic β-sheet, containing five parallel and one anti-parallel β-strand, surrounded by amphipathic α-helices (Figure 4B). There are six major α-helices and several short α-helices that differ between I domains. The top face of the I-domain contains a unique divalent cation (Mg^{2+}) coordination site designated the metal ion-dependent adhesion site (MIDAS). The metal ion is required for ligand binding by integrins, and is coordinated by five amino acids in the integrin, while the sixth coordinating residue has been proposed to come from the ligand (Casasnovas et al, 1997, Xiong et al, 2001).

The region C-terminal to the β-propeller domain comprises a large portion of the α subunit extracellular domain of about 500 amino acids, so-called stalk region. These regions provide the crucial link between the transmembrane and cytoplasmic domains and the conformational changes that occur in the ligand-binding head region. The stalk region of the α subunit is composed of three β-sandwich domains: an Ig-like “thigh” domain and two very similar domains that form the “calf” module (Figure 4A) (Xiong, 2001). Between the thigh domain and the first calf domain is a highly flexible “knee” (genu), which adopts a striking about 135° bend in the crystal structure.

The β-subunit

The β subunit ectodomain consists of eight domains; the N-terminal PSI domain (for Plexins, Semaphorins, and Integrins), an Ig-like “hybrid” domain (with the I-like domain emerging from the loop connecting its two β-sheets), four epidermal growth factor (EGF)-like repeats and a novel cystatin-like fold (Figure 4A) (reviewed in Hynes, 2002). The PSI domain is a cysteine-rich region of residues 1-50 which shares sequence homology with membrane proteins including plexins and semaphorins (Bork et al, 1999). The integrin PSI domain forms a two-stranded anti-parallel β-sheet, with two flanking short helices, connected by disulfides to the central sheet, and an N-terminal segment that may also be helical (Xiong et al, 2004). The structure is stabilized by four disulfide bridges. Although the PSI domain is at the N-terminus of the integrin primary sequence, it is not at the distal end of the molecule. Instead, the β subunit portion of the integrin “head” is composed of an Ig-like hybrid domain and the I-like domain inserted into the loop of the hybrid domain (Xiong, 2001). This I-like domain contains a putative metal-binding DXSXS sequence motif similar to that of the MIDAS in the I domain, a similar secondary structure (Lee et al, 1995), but weak sequence homology to the I domain (Ponting et al, 2000); therefore, it has been termed the I-like domain. The I-like domain contains two long loops, including one that is important for determining ligand specificity, and referred to as the specificity-determining loop (Takagi et al, 1997). The I-like domain has an extra cation-binding site

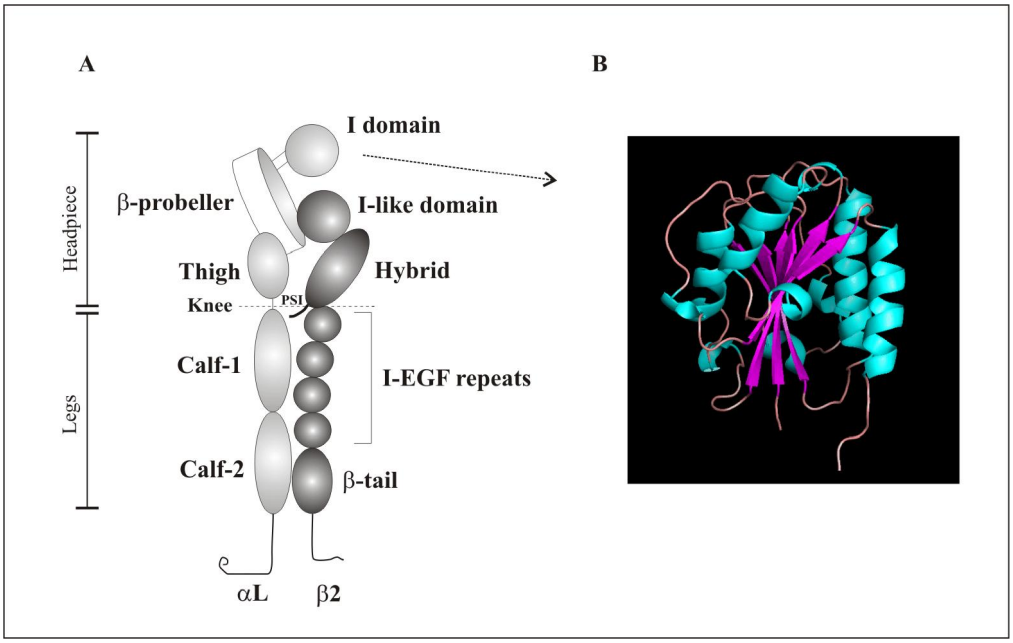

Figure 4. The overall structure of LFA-1. A. Structural scheme of LFA-1. The knee differentiates the headpiece from the legs. B. Ribbon diagram of the αL I domain (PDB code 1LFA). The β-strands (violet) and α-helices (light cyan) are labeled. The Mg^{2+} -binding site is at the top.

adjacent to the MIDAS of the I-like domain (known as ADMIDAS) (Xiong et al, 2001) and this site is likely to be the inhibitory Ca^{2+}- binding site (Mould et al, 2003). The I-like domain together with β-propeller directly binds ligand in integrins that lack I domains in the α subunit, and indirectly regulate ligand binding in integrins that contain I domains (Kamata et al, 2002, reviewed in Shimaoka et al, 2002).

Recently the crystal structure of the PSI domain / hybrid domain / I-EGF1 segment from the β2 subunit was solved (Shi et al, 2005). The structure reveals an elongated molecule with a rigid architecture stabilized by nine disulfide bridges. The PSI domain is located centrally in the 3D structure, below the hybrid "head" domain, and participates in the formation of extended interfaces with both the hybrid domain and I-EGF1 domains. The β subunit "leg" is made up of four tandem cysteine-rich repeats. The first and second are poorly resolved in the crystals, but the third and fourth are clearly folded into EGF-like folds (Xiong, 2001), termed integrin-EGF (I-EGF) domains (Takagi et al, 2001a). The NMR structure of the second and third cysteine-rich repeats of β2 reveals similar EGF-like shapes (Beglova et al, 2002). The fourth I-EGF repeats are followed by a C-terminal disulfide bonded β-sheet domain termed the transmembrane-proximal β-tail domain (βTD) (Xiong, 2001). Interestingly, many activating antibodies, or antibodies that bind only when integrins are activated, bind to the stalk region or "leg" of the β subunit, indicating the importance of this region in regulating ligand binding in the headpiece (reviewed by Humphries, 2004).

2.2. Structure of the cytoplasmic tails

Integrin cytoplasmic domains are normally <50 amino acid in length, with the β subunit sequences exhibiting greater homology to each other than the α subunits. Several NMR structures of integrin α and β subunit

cytoplasmic tails have been reported, mainly of the αIIbβ3 integrin (Li et al, 2001, Ulmer et al, 2001, Vinogradova et al, 2000, 2002, 2004, Weljie et al, 2002). Although some studies have failed to detect an α-β association (Li et al, 2001, Ulmer et al, 2001), the latest NMR analyses confirm that the subunits interact (Vinogradova et al, 2002, 2004, Weljie et al, 2002). The first ten residues of the α subunits cytoplasmic domain after the transmembrane segment appear to form an α-helix that is terminated by a proline residue (Figure 5B). Also, the first 20 amino acids of the β subunit cytoplasmic domains are α-helical, and thus, two membrane-proximal helices mediate a weak link between the subunits via a series of hydrophobic and electrostatic contacts. The α subunit helix is followed by a turn, allowing the acidic C-terminal loop to fold back and interact with the positively charged N-terminal region, and the final 25 residues following the β subunit membrane-proximal helix are disordered in an aqueous environment, except one or two NPxY-like motif (Figure 5A), the first of which has propensity to form a β turn (Ulmer et al, 2001). Such β turn-forming sequences frequently serve to bind to phosphotyrosine-binding (PTB) domains (Van der Geer et al, 1995). Interestingly, β3 tail has been shown to have additional membrane binding site at the NPxY motif (Vinogradova et al, 2004).

The head domain of the large cytoskeleton protein talin has been demonstrated to be a major player in activation of different integrins, including LFA-1 (Kim et al, 2003, Tadokoro et al, 2003). It binds directly to the β chains of integrins cytoplasmic domains, and the crystal structure of the integrin-binding portion of talin (F3-domain) linked to a 12-residue fragment of the β cytoplasmic domain has been determined (Garcia-Alvarez et al, 2003). The structure shows that residue 740-742 of β3 (DTA before the first NPxY-motif) form a β strand that is incorporated into the β5-β7 sheet of talin F3. NPxY-motif form a reverse turn, with Tyr747 pointing into an acidic pocket of talin F3. An unusual aspect of the β3-talin F3 complex from the typical PTB-domain interactions is a pocket occupied by the side chain of a conserved β-tail tryptophan (W739 in β3). This structure is believed to represent a prototype for integrin associations with other signalling molecules (Calderwood et al, 2003).

The interaction between the α and β subunit membrane-proximal cytoplasmic regions is unclasped by activating mutations (Hughes et al, 1996, Lu and Springer, 1997) and by the talin head domain (Calderwood et al, 1999), suggesting that cytoplasmic interactions between the α and β subunits regulate the integrin affinity (Figure 5C). A model for integrin activation was proposed in which the spatial separation of the cytoplasmic and/or transmembrane domains was a general mechanism for initiating integrin inside-out activation (Takagi et al, 2001b, Vinogradova et al, 2002, 2004). This movement would enable structural changes to be transmitted to the extracellular domain. The membrane anchoring of the NPxY region has an important functional role during activation since the membrane anchoring may restrict the movement of the β tail during tails separation. Importantly, fluorescence resonance energy transfer (FRET) studies or replacement of the αL and β2 cytoplasmic domains with acidic and/or basic peptides support this model also for LFA-1 (Kim et al, 2003, Lu et al, 2001a).

Furthermore, glycosylation mapping studies have suggested that the membrane-proximal domains of the α and β subunits can reside within the membrane bilayer and that certain activating mutations in this region can displace them from the membrane, thereby shortening the transmembrane domains (Armulik et al, 1999, Stefansson et al, 2004). An upward movement of membrane-proximal helices has also been suggested during talin binding (Vinogradova et al, 2004), indicating that changes in the membrane insertion of membrane-proximal domains of the α and β subunits can be a one mechanism to regulate integrin activation.

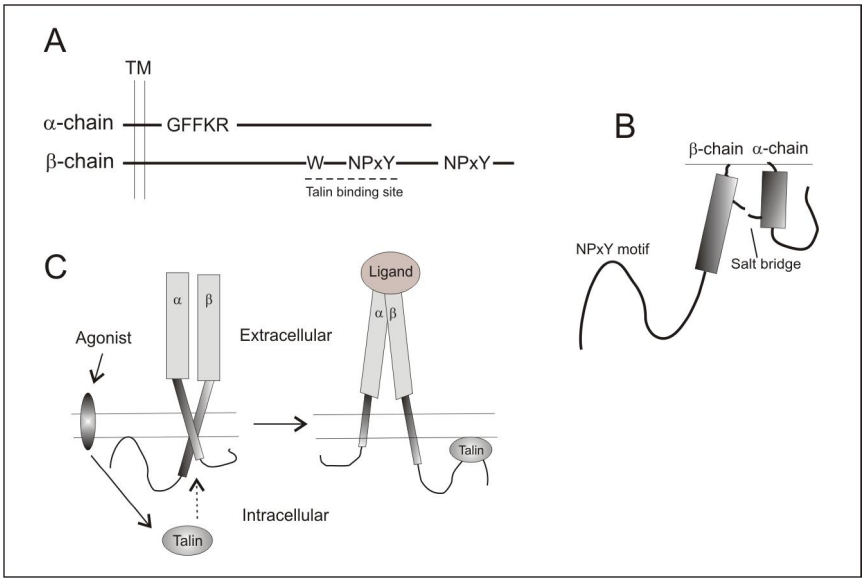

Figure 5. Structure of the integrin cytoplasmic tails. A. Schematic figure of the integrin cytoplasmic domains. (TM = transmembrane) B. Schematic representation of the cytoplasmic domain of the integrin. α-helices are shown as the tetragons. The membrane-proximal regions are shown at the top. C. Model for talin-induced integrin activation. See text for details.

2.3. Models for LFA-1 ligand binding and activation

Structural basis of LFA-1 ligand-binding

LFA-1 binds ICAMs, members of the IgSF of which ICAM-1 is the most widely studied. Although the extracellular domain of LFA-1 is large and structurally complex, the ligand-binding site is contained solely within the I domain of αL (reviewed in Springer and Wang, 2004, Lu et al, 2001b). Crystal structures for two different I-domain conformations, termed open and closed, have been obtained for the αM and α2 I-domains, and it has been speculated that these represent the "high affinity" and "low affinity" conformations, respectively (Lee et al, 1995a, 1995b, Emsley et al, 2000). The two conformations differ in the side chains that coordinate the Mg^{2+} ion in the MIDAS to make the Mg^{2+} ion more electrophilic for an acidic residue from the ligand. The metal ion is central in the binding site and directly coordinates a Glu residue in the ICAM-1 (Lee et al, 1995b). Also, compared to the closed conformation, the open conformation exhibits a large 10 Å movement of the C-terminal α-helix (α7) down the side of the I domain, and a rearrangement of the loop connecting the α7-helix to the preceding β-strand. The structure of the αL I-domain has been

determined only in the closed conformation (Qu and Leahy, 1995, 1996, Legge et al, 1999); however, direct evidence of conformational changes has been obtained by NMR spectroscopy (Huth et al, 2000).

The structural basis of a LFA-1 – ligand complex has been studied using an I-domain which is locked in the open or closed conformation by disulfide bridges (Figure 6A) (Lu et al, 2001b, 2001c, Shimaoka et al, 2001). The open conformation of the αL I-domain was modeled by using the open αM I-domain as a template. Locking the I-domain in the open conformation resulted in a 9000-fold increase in affinity for ICAM-1 (Shimaoka et al, 2001). By contrast, the closed conformation has similar affinity to ICAM-1 as the wild type, and it prevents activation of cell adhesion by Mn^{2+} or an activating antibody (Lu et al, 2001b), supporting the idea that conformation regulates affinity. The crystal structure of the αL I-domain and its complex with ICAM-1 (IgSF domains 1-3) was determined for intermediate-affinity and high-affinity I-domain mutants (Shimaoka et al, 2003). The I-domain binds only to the side of domain 1 of ICAM-1, making no contacts with the flexible loops at the N-terminal end of domain 1 (Casasnovas et al, 1998) or with domain 2. The key integrin-binding residue Glu-34 at the end of the β strand C of ICAM-1 directly coordinates to the Mg^{2+} ion on the MIDAS site at the center of the binding groove. The bond between the Mg^{2+} and Glu-34 is surrounded by a ring of hydrophobic contacts, and around this area, there are polar interactions involving hydrogen bonds and salt bridges that appear to orient optimal contact of ICAM-1 with the I-domain. Also, the crystal structure of ICAM-3 (IgSF domain 1) in complex with the αL I-domain has been determined (Song et al, 2005). The structure suggests a common docking mode for all ICAMs that bind to LFA-1, and the markedly different affinity among ICAMs are regulated by the hydrophobic interactions surrounding the metal coordination bond.

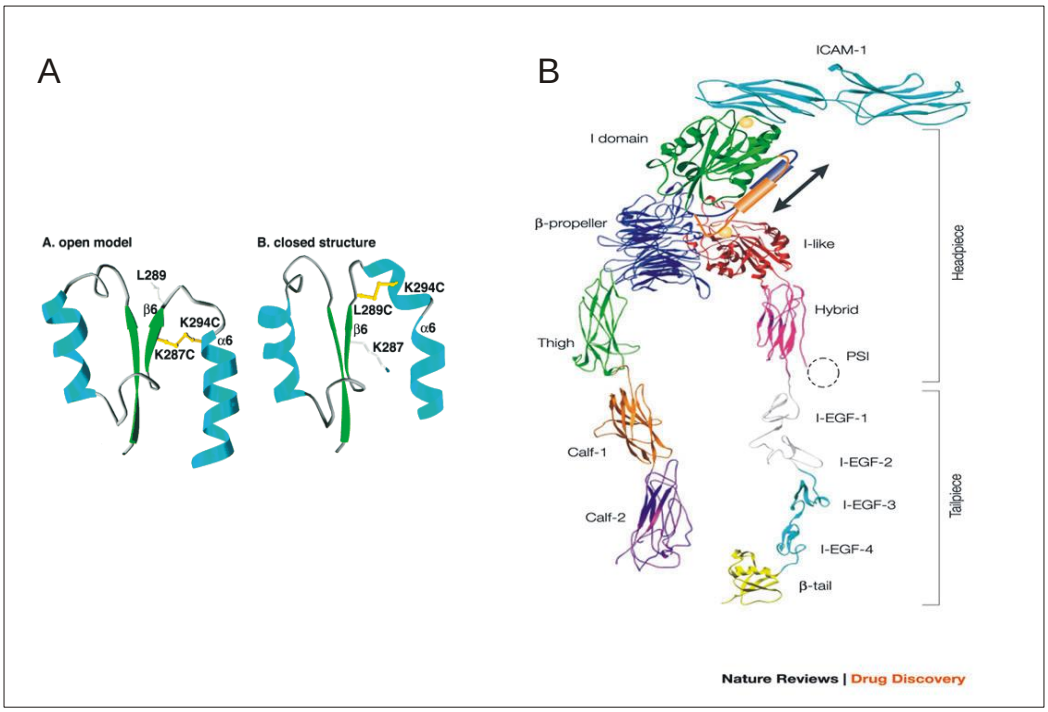

Figure 6. The structural basis of a LFA-1–ligand complex. A. Open and closed structure of the αL I-domain. Adapted from Lu et al, 2001a. B. Model of LFA-1 bound to ICAM-1. The I-domain α7-helix is represented by a cylinder in its low-affinity (blue) and high affinity (orange) conformation. Mg^{2+} ions at the MIDAS and ADMIDAS are gold spheres. Adapted from Shimaoka and Springer, 2003.

The optimal interaction between the I-domain and ICAMs requires the critical rearrangements in the I-domain by pulling down the C-terminal α7 helix, and interestingly, ICAM-1 itself induces this movement by binding to the I domain (Figure 6B) (Shimaoka et al, 2003). Moreover, the I-like domain undergoes similar conformational changes during activation that allosterically regulate binding through the αL I-domain (Lu et al, 2001c, Kamata et al, 2002, Yang et al, 2004a, 2004b).

Global conformational changes in the integrin ectodomain

The crystal structure of the extracellular domains of αVβ3 revealed an unexpected bent conformation of the integrin (Xiong et al, 2001); the integrin head domain was folded down onto the legs. Based on evidence from the NMR structure of the β2 subunit I-EGF domains (Beglova et al, 2002), together with mapping of activation–dependent or activation-inducing antibodies (Lu et al, 2001d, Zang and Springer, 2001), the bent conformation was proposed to represent a low-affinity state of the integrin, and a switchblade-like (or pocket-knife model) opening was proposed to occur upon activation (Figure 7) (Takagi et al, 2002, Liddington, 2002). In the bent conformation only the closed conformation of the headpiece (I- or I-like domain) is present (Xiong et al, 2001) and the extension re-orients the ligand binding face to the open conformation and at the same time exposes activation epitopes in the tailpiece (Takagi et al, 2002, 2003, Lu et al, 2001d, Xie et al, 2004). An extended form with a closed headpiece conformation, known as an intermediate affinity state, has also been demonstrated (Takagi et al, 2002, 2003). FRET measurements between an α4β1-bound peptide and the plasma membrane have been used to reveal that such movements actually take place in living cells (Chigaev et al, 2003). Transition to the extended state from a bent state involves separation of the α and β subunits at their cytoplasmic, transmembrane and leg domains (Takagi et al, 2002, Vinogradova et al, 2002, Kim et al, 2003, Luo et al, 2004). A recent atomic structure of αIIbβ3 integrin head fragment in complex with a ligand mimetic suggested the outward swing of the hybrid domain. The hybrid and PSI domains act as a rigid lever that transmits and amplifies this motion, resulting in separation of the α and β legs at their knees to favor leg extension (Xiao et al, 2004). This is agreement with studies that showed the exposure of activation-dependent antibody epitopes in the hybrid and PSI domains upon activation (Tng et al, 2004, Mould et al, 2005, Tang et al, 2005).

In solution, and apparently on the cell surface as well, integrins are not fixed in a particular conformation, but equilibrate between them (Takagi et al, 2002). Whether or not the equilibrium favors the bent, an intermediate or the extended conformation is affected by the presence of activating intracellular factors and the concentration of extracellular ligands. For example, on the surface of the rolling leukocytes, LFA-1 has been shown to be in an extended conformation and the αL I-domain is not in a high-affinity state, whereas the conformation of LFA-1 that mediates firm adhesion appears to most closely correspond to an extended conformation with a high-affinity I-domain (Salas et al, 2004). Similarly, endothelium-presented chemokines triggered a fast extension of bent LFA-1, which mediated lymphocyte rolling on high endothelial venules (Shamri et al, 2005). To support firm adhesion, this extended LFA-1 conformation required immediate activation by its ligand, ICAM-1.

However, some studies have suggested that a switchblade movement is not required to render the integrin competent to bind physiological ligands (Calzada et al, 2002, Butta et al, 2003), and an alternative "deadbolt" model has been proposed (Xiong et al, 2003). This model proposes that the βTD and the β I-like domains interact and the interaction holds the integrin in a non-ligand-binding form. Inside-out activation is then accomplished by releasing this deadbolt allosterically, ~40 Å from the membrane, via cytoplasmic / transmembrane movements. This model assumes that the bent integrin can stably bind ligand. This matches the crystal structure of the αVβ3 in complex with an RGD peptide (Xiong et al, 2002). Additionally, recent reports showed that the bent αVβ3 stably binds a physiological ligand in solution in a Mn^{2+} -dependent manner (Adair et al, 2005), and that the conformational change in the I domain of αL can occur independently of the conversion from a bent to an extended form (Larson et al, 2005), suggesting more diverseness in integrin activation.

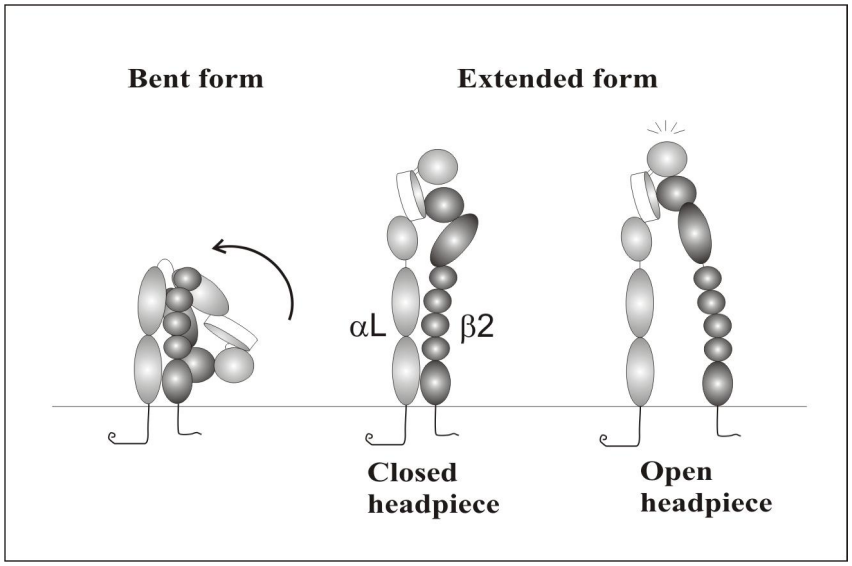

Figure 7. Global conformational changes of the extracellular portion of integrins. The bent conformation represents a low-affinity state of the integrins, the extended form with a closed headpiece represents the intermediate affinity state and the extended form with an open headpiece the high affinity state.

3. CYTOPLASMIC DOMAINS AND REGULATION OF AVIDITY

3.1. Important motifs in the integrin cytoplasmic tails

Although it has undoubtedly been established that integrins can undergo conformational changes during activation, which increase the affinity of individual integrins for their ligands (reviewed by Shimaoka et al, 2002, Springer and Wang, 2004, Humphries et al, 2003), additional affinity-independent mechanisms have also been proposed to contribute to the control of integrin-mediated adhesion (reviewed by van Kooyk and Figdor, 2000, Hogg at al, 2002, Calderwood, 2004). Affinity-independent mechanisms involve integrin clustering, resulting in increased avidity and providing stronger adhesion at sites of cell-cell contact, but also, interactions with and reorganization of the cytoskeleton. Although the integrin α and β cytoplasmic domain are relative

short and do not contain any intrinsic enzymatic activity, they are important for affinity and avidity regulation. The β cytoplasmic tails are closely related to each other, whereas the α cytoplasmic tails have more diversity in structure (reviewed in Williams et al, 1994, Ylänne, 1998). However, important motifs are found in both tails (Figure 8).

The α subunit cytoplasmic domains are well conserved among different species but share little homology with each other (Hynes, 1992). Thus, α subunit cytoplasmic tails may mediate the direct specific responses after cell stimulation. For example, the α4 cytoplasmic domain promotes β1 integrin-mediated cell migration, whereas the α2 and α5 cytoplasmic domains facilitate collagen gel contraction and spreading (Chan et al, 1992, Kassner et al, 1995, Na et al, 2003). Likewise, αL and αM have been shown to have unique functions in chemokine-induced activation of the β2 integrins (Weber et al, 1999). The membrane-proximal GFF(K/R)R motif in the cytoplasmic tail of the α subunit is strictly conserved among integrin-family members, except α8, α10 and α11. Truncation of the α cytoplasmic domain before, but not after, the conserved GFF(K/R)R motif, or deletion of this sequence rendered, the integrin constitutively active with a high affinity conformation (Hibbs et al, 1991a, O'Toole et al, 1994, Peter and O'Toole, 1995, Lu and Springer, 1997, van Kooyk et al, 1999). The arginine residue in GFFKR and an aspartic –acid residue at the corresponding position in the β chain form a salt bridge, which holds the integrin in a low affinity form (Hughes et al, 1996), and breaking this interaction activates the integrin (Lu and Springer, 1997, Takagi et al, 2001b). Additionally, the GFFKR motif stabilizes the integrin heterodimer, probably because of its direct interaction with the β chain (Pardi et al, 1995).

The β subunit cytoplasmic domains are generally considered important in the regulation of integrin functions (Williams et al, 1994). Complete deletion of the β2, β3 or β7 cytoplasmic domains results in constitutively active integrins (Lub et al, 1997, Hughes et al, 1995, Crowe et al, 1994), probably because the link between the α and β subunit is disrupted. In contrast, truncation of the β2 domain after 6 amino acids from the transmembrane domain eliminated LFA-1 binding to ICAM-1 and sensitivity to phorbol ester (Hibbs et al, 1991a), indicating that LFA-1 adhesiveness is controlled by the cytoplasmic domain of the β2 subunit. Mutation studies have revealed a number of different functionally important areas within the β2 cytoplasmic domain.

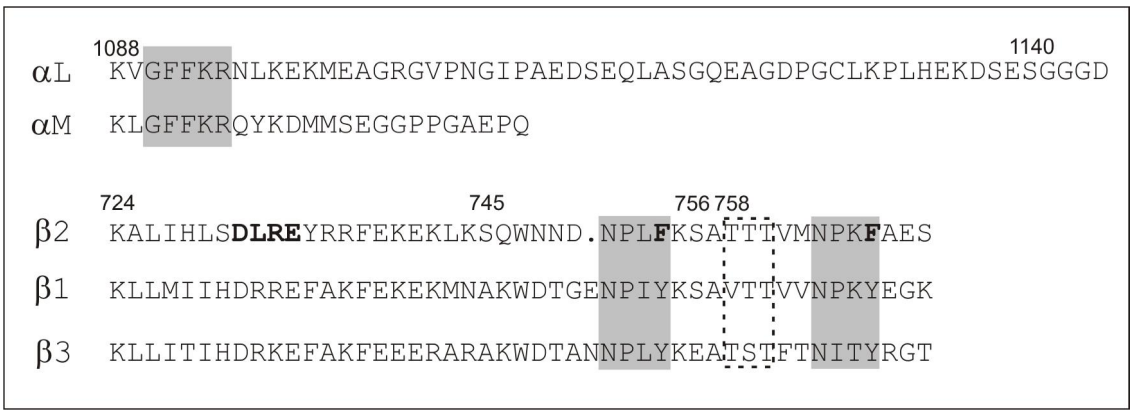

Figure 8. Amino acid sequences of integrin α and β subunits. Conserved motifs that are important for integrin functions are highlighted.

A cluster of three threonine residues has been shown to be necessary for ligand binding (Hibbs et al, 1991b). This threonine triplet is also required for actin cytoskeleton reorganization and cell spreading, so called postreceptor events (Peter and O'Toole, 1995), but has no effect on LFA-1 mediated transendothelial migration induced by chemokines (Weber et al, 1997). Corresponding threonine residues in β1 and β3 cytoplasmic domains have also shown to be of critical importance for integrin function (Wennerberg et al, 1998, Stroeken et al, 2000, Bordeau et al, 2001).

Despite the similarity of β1 and β2 cytoplasmic tails, integrins are differently regulated by inside-out signals. Wild type LFA-1, when expressed in the erythroleukemic cell line K562 (Andersson et al, 1979), is not clustered and is defective for phorbol ester activation. Replacement of the β2 cytoplasmic domain with the β1, but not the β7 cytoplasmic tail, restored the clustered cell surface distribution and sensitivity to phorbol ester (Lub et al, 1997). This indicated that β2 and β7 integrins use different lymphocyte-specific signal elements to activate integrins. Interestingly, substitution of a single leucine residue to arginine in the β2 DLRE motif together with an intact TTT sequence is sufficient to completely restore phorbol ester responsiveness of LFA-1 expressed in K562 cells (Bleijs et al, 2001).

Several β subunits have two NPxY motifs within their cytoplasmic domain, but in β2 both of these tyrosines are replaced by phenylalanines. Importantly, both phenylalanines have shown to be essential for β2 integrin activity. Mutations of the phenylalanines to non-conservative alanines abrogated ligand-dependent adhesion, but the conservative Phe to Tyr substitution had no effect (Hibbs et al, 1991b, Fabbri et al, 1999), suggesting conservation of the general structure of the NPxY motif but loss of its possible phosphorylation state. The NPXF motif in β2 cytoplasmic domain has also been shown to trigger intracellular Ca^{2+} mobilization and therefore contribute to T cell signalling (Sirim et al, 2001).

3.2. LFA-1 interacts with a variety of intracellular proteins

The critical role of the cytoplasmic domains in integrin function implicates the importance of cytoplasmic proteins that modify or bind to integrin cytoplasmic tails. LFA-1 has been described to interact with several proteins, including cytoskeletal proteins such as talin, α-actinin and filamin, signalling proteins such as cytohesin, and adaptor proteins such as Rack-1 (Table 1) (reviewed in Liu et al, 2000). β cytoplasmic tails play crucial roles in cytoskeletal interactions, whereas in most cases the α cytoplasmic domains play regulatory roles (Calderwood et al, 2004).

Actin-binding proteins

The connection of integrin receptors to the actin cytoskeleton regulates many functions of integrins, in particular cell adhesion and migration (reviewed in Wiesner et al, 2005). However, actin filaments cannot bind directly to integrins. Instead, integrins are linked indirectly to actin filaments via several actin-binding proteins, including talin, the first cytoplasmic protein shown to bind to integrins (Horwitc et al, 1986). Talin is an abundant cytoskeletal protein that plays a crucial role in integrin activation (Tadokoro et al, 2003). It binds

strongly to β1A, β1D, β2 and β3 integrin tails and weakly to the β7 tails (Horwitch et al, 1986, Knezevic et al, 1996, Sampath et al, 1998). The major integrin-binding site lies within the talin globular head domain (Calderwood et al, 1999, Patil et al, 1999), although the C-terminal rod domain also contains a lower-affinity integrin-binding site (Calderwood et al, 1999, Yan et al, 2001). The talin head domain containing a FERM domain (Band four-point-one, ezrin, radixin, moesin) activates β1, β2 and β3 integrins by binding to their NPxY motif in the β cytoplasmic tails (Calverwood et al, 1999, Calderwood et al, 2002, Kim et al, 2003). The binding causes a spatial separation of the αL and β2 cytoplasmic domains and therefore integrin activation (Kim et al, 2003). This is consistent with NMR studies on dissociation of the αIIbβ3 cytoplasmic complex by the talin head domain (Vinogradova et al, 2002). Thus, talin binding to integrin is proposed to be a final step in integrin activation. In addition to this activation function, talin expression and localization are critical for localization of the active LFA-1, ICAM-binding stability and T cell migration (Smith et al, 2005).

Additionally, at least two other cytoskeletal proteins, α-actinin and filamin, have been shown to directly interact with the β2 cytoplasmic domain (Pavalko and LaRoche, 1993, Sharma et al, 1995). The α-actinin-binding site is in the membrane-proximal half of the β2 integrin tail, while the C-terminal portion of the tail inhibits this interaction (Sampath et al, 1998). Thus, the association of α-actinin with β2 integrins is probably regulated by conformational changes in the cytoplasmic domain that unmask the α-actinin binding site. Filamin has also been shown to interact with the β cytoplasmic domain of several integrins, including β2 (Sharma et al, 1995). Filamin crosslinks actin, forming either loose microfilaments networks or tight actin bundles, depending on the actin:filamin ratio, but filamin also acts as an adaptor protein for a number of signalling proteins that can regulate cytoskeletal dynamics (Stossel et al, 2001). Binding of filamin to β integrin tails has been shown to be important in the regulation of the cell migration, as the β7 integrin tail, which binds strongly to filamin, inhibited cell migration, whereas β1 tails that bind weakly to filamin supported cell migration (Calderwood et al, 2001). Interestingly, filamin binds to the important TTT-region of the β cytoplasmic tail (Calderwood et al, 2001), and it is possible that this interaction is regulated activation-dependent manner, for example by phosphorylation.

14-3-3 adaptor proteins, a highly conserved family of phospho-serine and –threonine binding proteins, have been shown to interact with a synthetic β2-integrin peptide phosphorylated on the threonine residue (Thr758) of the TTT-region (Fagerholm et al, 2002). Additionally, β1 and β4 has been shown to interact with 14-3-3, but the interaction between β1 and 14-3-3 occurs outside of the amphipathic groove which is involved in 14-3-3 interactions with other ligands, and the association was not thought to be phosphorylation-dependent (Han et al, 2001, Santoro et al, 2003). However, the functional amphipathic groove of 14-3-3, rather than its interaction with β1 integrin, is required for 14-3-3 regulation of cell spreading and migration (Rodriguez and Guan, 2005).

Several other cytoskeletal proteins have been identified as integrin-binding proteins; however, interaction with β2 has not been reported. Platelet myosin has phosphorylation-dependent associations with β3 tails (Jenkins et al, 1998), skelemin binds to β1 and β3 tails in non-muscle cells (Reddy et al, 1998), and tensin has shown to bind to β3, β5, β7 and more weakly to β1 cytoplasmic tails (Calderwood et al, 2003).

Cell signalling proteins

In addition to providing a link to the actin cytoskeleton, integrin β tail-binding proteins also regulate signalling. Because the integrin intracellular domains have no catalytic function, the interaction with other signal transducing molecules is crucial for integrin-mediated signalling. One of these signalling molecules is cytohesin-1 (Kolanus et al, 1996), which has a GEF activity for the ARF family of small GTPases (Meacci et al, 1997). Cytohesin-1 specifically binds selectively to the membrane-proximal residues in β2 cytoplasmic tails, and mutation of these residues inhibits LFA-1 mediated adhesion (Geiger et al, 2000). Overexpression of cytohesin-1 increased unstimulated LFA-1 binding to ICAM-1, whereas its pleckstrin homology (PH)-domain or antisense cytohesin-1 oligonucleotides reduced β2-mediated cell adhesion (Kolanus et al, 1996, Korthäuer et al, 2000, Hmama et al, 1999). Cytohesin-1 is believed to have two independent roles in LFA-1 regulation; direct modulation of LFA-1 avidity by molecular interactions, and by its GTPase activity, which is more important for transmigration (Greiger et al, 2000, Weber et al, 2001).

The Trp-Asp (WD) repeat protein Rack1 (receptor for activated protein kinase C) has also been identified as a β2 tail-binding protein (Liliental and Chang, 1998). Rack1 is composed of seven WD repeats, and it was originally identified based on its ability to bind to the activated form of PKC (Ron et al, 1994). The interaction of Rack1 with integrins requires stimulation with phorbol ester, which indicates that the Rack1 may link PKC directly to integrins and mediate integrin bidirectional signalling (Liliental and Chang, 1998, Buensuceso et al, 2001). Moreover, Rack1 has been shown to regulate integrin-mediated adhesion, protrusion, and chemotactic migration through its interaction with Src family kinases (Cox et al, 2003). The other integrin-binding WD repeat protein, WAIT-1 (WD protein associated with integrin tails), binds to both α and β tails, specifically β7, α4 and αE, but not β2 or αL (Rietzler et al, 1998). Both Rack1 and WAIT-1 interact with a membrane-proximal region of the β subunit. However, no functional data suggesting a role for WAIT-1 interaction with β tail has been provided.

JAB-1 is a transcriptional coactivator for the Jun family of activator protein-1 (AP-1) transcription factors, which has been shown to constitutively interact with the cytoplasmic domain of the β2 integrin (Bianchi et al, 2000). JAB-1 is found in the nucleus and in the cytoplasm, and the β2-interaction is believed to keep JAB-1 in cytoplasm. Interestingly, Ser745 phosphorylation of β2 leads to disengagement of JAB-1 from LFA-1 and induces Jun activation, which indicates that signalling through the LFA-1 might also regulate the gene expression (Perez et al, 2003).

In contrast to the β subunits, there are only a few descriptions of interactions between cytoplasmic proteins and integrin α subunits. Calreticulin, a luminal endoplasmic reticulum calcium-binding protein, directly interacts with the KxGFF(K/R)R motif of several α cytoplasmic domains and has important roles in several cell functions including integrin signalling (reviewed in Coppolino and Dedhar, 1998). However, interaction with the αL-tail has not been described. Very recently the αL tail has been shown to interact with the transmembrane receptor-like protein tyrosine phosphatase CD45 (Geng et al, 2005), but the physiological significance of this interaction is yet unclear. Thus, the most interesting αL-binding cytoplasmic protein is RAPL, a Rap1-binding molecule that may mediate Rap1-induced cell adhesion. RAPL overexpression increases the

adhesiveness of LFA-1 through both affinity and avidity regulation (Katagiri et al, 2003). In addition, T and B cells isolated from RAPL knock-down mice are much less adherent to ICAM-1 and do not show the characteristic cell polarizarization and patchy appearance of integrins after chemokine stimulation (Katagiri et al, 2004). RAPL, which is highly expressed in lymphocytes, binds to active Rap1-GTP upon stimulation with TCR or SDF-1. Activated RAPL then forms a complex with the αL subunit and moves to the leading edge, forming large patch-like clusters (Katagiri et al, 2003). RAPL binds αL close to the membrane-proximal GFFKR motif, which is known to stabilize α and β subunit heterodimer formation, and disruption of this link between the subunits could be a mechanism how RAPL affects LFA-1 mediated cell adhesion (reviewed in Kinashi and Katagiri, 2004).

Binding protein	**Binding site**	**Effect on cells**	**Reference**
Talin	β2, membrane distal, NPxY-motif	integrin activation, cytoskeletal interaction	Horwitz et al, 1986
α-actinin	β2, membrane proximal (736-746)	?	Pavalko and LaRoche, 1993
Filamin	β2, membrane proximal (724-747)	Migration regulation?	Sharma et al, 1995
14-3-3	β2, TTT-motif	cytoskeletal interaction?	Fagerholm et al, 2002
Cytohesin 1 and 3	β2, membrane proximal (724-725)	Adhesion and migration regulation?	Kolanus et al, 1996
Rack1	β2, membrane proximal	Link PKC to integrin ?	Liliental and Ghang, 1998
JAB-1	β2, Ser745	Signalling	Bianchi et al, 2000
Calreticulin	α-tail, KxGFFKR-motif	Signalling	Coppolino et al, 1997
CD45	αL, membrane distal	?	Geng et al, 2005
RAPL	αL, membrane proximal	Integrin activation	Katagiri et al, 2003

Table 1. Proteins interacting with LFA-1 cytoplasmic tails

3.3. Regulation of avidity

It is becoming evident that the cytoskeleton is involved in the dynamic regulation of the adhesive state of LFA-1 (reviewed in Carman and Springer, 2003, van Kooyk and Figdor, 2000). The actin cytoskeleton is not only important for driving membrane remodeling (which can either increase or decrease LFA-1 avidity) but also acts as a platform to bring together surface receptors and recruit required signalling molecules. The importance of the cytoskeleton as a dynamic regulator of leukocyte adhesion came to light using agents, which inhibit actin disassembly, such as cytochalasin D. TCR-ligation or phorbol ester-induced T cell adhesion is abolished by treatment of cells with high concentrations of cytochalasin D (Stewart et al, 1996). On the other hand, low concentration of cytochalasin D has the capacity to promote integrin-mediated adhesion by allowing lateral movement of the LFA-1 on the cell surface (Kucik et al, 1996). In additional, several stimuli that increase cell adhesion, such as phorbol ester or TCR-ligation, increase diffusion of LFA-1 in the cell membrane without a detectable change in the affinity (Kucik et al, 1996, Stewart et al, 1996, van Kooyk and Figdor, 2000).

However, the mechanism of clustering and its role in adhesion remain unclear. One possibility is that lymphocyte activation involves release of receptors from the cytoskeleton that simply facilitates ligand-dependent accumulation of integrins into the site of contact (Kucik et al, 1996). The expression level of calpain, a specific Ca^{2+}-dependent protease, is increased after T cell activation, and it may be responsible for releasing LFA-1 from the cytoskeleton (Stewart et al, 1998). The potential target of calpain involving in LFA-1 regulating is talin, since calpain-mediated proteolysis of talin has been identified as a mechanism by which adhesion dynamics are regulated (Franco et al, 2004). An alternative hypothesis is that the dynamic recruitment of leukocyte integrins into lipid rafts regulates LFA-1 avidity; however, the reports are still controversial. LFA-1 on mouse thymocytes was shown to associate with lipid rafts (Krauss and Altevogt, 1999) whereas LFA-1 transfected into a human T cell line showed that LFA-1 does not associate with rafts unless it is activated by Mn^{2+} (Leitinger and Hogg, 2002). Moreover, raft disruption by cholesterol depletion did not inhibit LFA-1 mediated adhesion of human peripheral blood T cells (Shamri et al, 2002) but adhesion of murine T cells was strongly decreased (Marwali et al, 2003). The actin cytoskeleton plays an active role also in this process. Another suggested mechanism for active integrin clustering includes formation of homotypic associations between transmembrane domains of adjacent integrins upon transition to the extended conformation (Li et al, 2001, 2003).

Interestingly, a recent report presented the novel finding that clustering of LFA-1 follows and does not precede ligand binding (Kim et al, 2005). Therefore, it was speculated that the conformational change of LFA-1 plays a critical and limiting role in the formation of integrin-ligand bonds that initiate firm adhesion, after which LFA-1 accumulates at the substrate contact interface, resulting in adhesion strengthening.

4. PHOSPHORYLATION OF THE INTEGRIN CYTOPLASMIC TAILS

4.1. Integrin β-chain phosphorylation

Serine/threonine phosphorylation

Reversible phosphorylation is a key mechanism regulating many cellular activities. Protein phosphorylation can rapidly *switch* the activity of proteins from one state to another. It may modify protein activity directly by changing the conformation but it can also modulate the structure of protein motif to favor protein-protein interaction. Phosphorylation of integrin cytoplasmic domains could therefore be a mechanism for regulating integrin activity.

Integrin phosphorylation has been studied for several years. Early studies of β2 integrins showed that serine phosphorylation occurs only after cell stimulation by phorbol ester (Figure 9) (Hara and Fu, 1986, Chatila and Geha, 1988, Chatila et al, 1989, Buyon et al, 1990) or by TCR-ligation (Pardi et al, 1992, Valmu and Gahmberg, 1995), and the main phorbol-ester induced phosphorylation site was found to be Ser756 (Hibbs et al, 1991b). The function of this Ser756 phosphorylation in the regulation of β2 is still unknown; however, it

does not affect cell adhesion to ICAM-1 (Hibbs et al, 1991b). The corresponding serine is not found in the β3 polypeptide, but β1 has been reported to be phosphorylated on Ser785, and dephosphorylation coincides with the activation of the integrin during differentiation (Dahl and Grabel, 1989). β1 serine phosphorylation has been suggested to regulate integrin localization because the serine/threonine phosphatase inhibitor, okadaic acid, has been shown to increase the level of β1 phosphorylation and cause the selective removal of β1 from focal adhesion sites (Mulrooney et al, 2000). Additionally, the Ser to Asp mutation, which mimics a constitutively phosphorylated state, leads to reduced β1 localization to focal contacts (Reszka et al, 1992, Barreuther and Grabel, 1996). Moreover, serine phosphorylation of β1 is reported to regulate cell spreading and migration (Mulrooney et al, 2001).

β2 integrin has a unique, non-conserved serine residue in the cytoplasmic tail (Ser745), which has been shown to become phosphorylated in T cells, in response to phorbol ester stimulation (Fagerholm et al, 2002) or by stimulation of the cells by adding ICAM-2 (Perez et al, 2003). Ser745 phosphorylation releases JAB-1 from LFA-1 and induces the activation AP-1-driven transcription (Perez et al, 2003).

The threonine triplet (Thr758-760) in the β2 cytoplasmic domain, and its the corresponding motifs in β1 and β3, are necessary for integrin functions (Hibbs et al, 1991b, Valmu et al, 1991, Peter and O'Toole, 1995, Wennerberg et al, 1998, Mastrangelo et al, 1999, Bodeau et al, 2001). Phosphorylation of the threonine triplet in the β2 cytoplasmic tail is a very dynamic process; it can be revealed only when the serine/threonine phosphatases in the activated T cells are inhibited by okadaic acid (Valmu and Gahmberg, 1995). The kinases responsible for phosphorylation of the β2 threonine residues, as well as Ser745, were identified as PKC isoforms (Fagerholm et al, 2002). Mutation studies have showed that the β2 threonine triplet is vital for β2-mediated cell adhesion (Hibbs et al, 1991b), and it is involved in so called postreceptor events, such as spreading and the cytoskeletal association (Peter and O´Toole, 1995). Threonine phosphorylated β2 integrins distribute preferentially to the actin cytoskeleton (Valmu et al, 1999) and the linkage between integrin and actin-binding proteins might be mediated by 14-3-3 proteins (Fagerholm et al, 2002).

Recently, the β1 integrin has been showed to be phosphorylated at two threonine residues during muscle differentiation (Kim et al, 2004). β3 becomes phosphorylated on threonine residues after platelet activation with thrombin, phorbol myristate, calculin A or platelet-activation factor (Parise et al, 1990, Lerea et al, 1999, van Willigen et al, 1996). The phospholipid-dependent kinases protein kinase B and phospholipid-dependent kinase 1 have been shown to phosphorylate β3 threonine residues in vitro (Kirk et al, 2000). The mutation studies have indicated that the threonine residues in β1 and β3 integrins are of critical importance in integrin extracellular domain conformational changes, cell adhesion and spreading, and integrin signalling (Wennerberg et al, 1998, Mastrangelo et al, 1999, Lerea et al, 1999, Bodeau et al, 2001). In addition, β1 threonine phosphorylation has been speculated to regulate integrin association with the actin cytoskeleton during mitosis and muscle differentiation (Suzuki and Takahashi, 2003, Kim et al, 2004). Interestingly, β3 threonine phosphorylation has been reported to inhibit outside-in signalling events by blocking recruitment of the tyrosine kinase Shc, suggesting that the threonine phosphorylation of β3 may be an important negative regulator of integrin (Kirk et al, 2000).

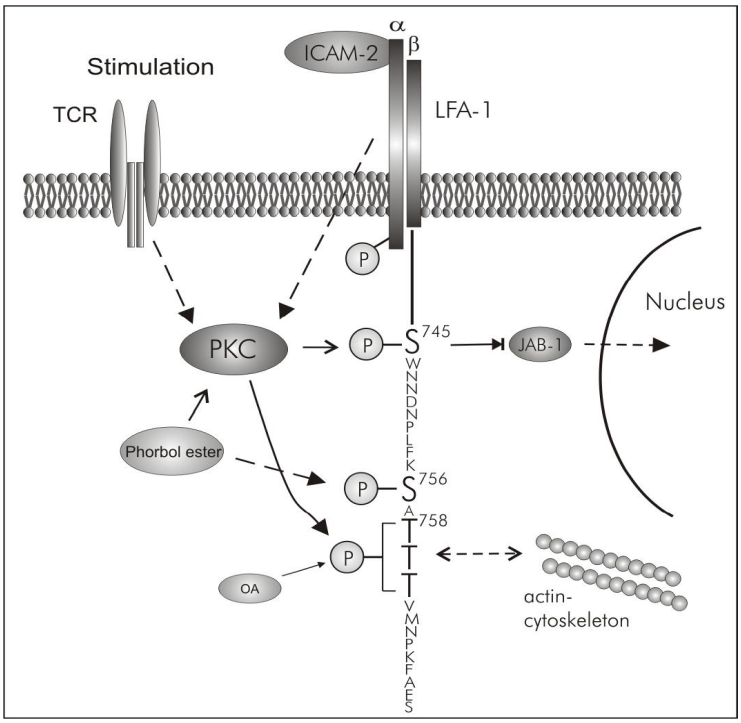

Figure 9. Serine/threonine phosphorylation of LFA-1. TCR-ligation and phorbol ester-stimulation of cells induce serine and threonine phosphorylation of β2. Ser756 is the main phosphorylation site after phorbol ester activation but the function of Ser756, and kinase that phosphorylates Ser756-phosphorylation are unknown Ser745 becomes phosphorylated in response to phorbol ester and it is a direct target of PKC. Ser745 is also phosphorylated after ICAM-2 binding to LFA-1, and integrin phosphorylation at this site induces the release of the transcriptional coactivator JAB-1 from the integrin, allowing it to participate in downstream signaling. Thr758-Thr760 are important for integrin-mediated adhesion and cytoskeletal reorganization, and these residues are phosphorylated by PKC-isoforms after phorbol ester stimulation or TCR-ligation. The Thr-phosphorylation is labile, and can only be detected in the presence of phosphatase inhibitors.

Tyrosine phosphorylation

Like β2, on resting cells β3 integrins are inactive but when cells are exposed to agonists they undergo changes to the extended, active conformation. Outside-in signalling mediated by the β3 integrin is critical to platelet function and has been shown to involve the phosphorylation of tyrosine residues on the cytoplasmic tails of β3 (Law et al, 1996). The β3 cytoplasmic tail contains two tyrosine residues, each in a NPxY motif, a well known recognition sequence for proteins containing PTB domains (Van der Geer et al, 1995). The tyrosine phosphorylation of β3 in platelets is a dynamic process which is initiated upon platelet aggregation (Law et al, 1996), or by adhesion of platelets to β3 specific ligand (Blystone et al 1996, Prasad et al, 2003). β3 tyrosine phosphorylation has been demonstrated to be induced by Src-family kinases (Datta et al, 2002). A mouse model that expresses a β3, in which the tyrosine residues in the integrin cytoplasmic domain have been substituted with phenylalanines, displays a phenotype of selectively abrogated outside-in signalling, defective aggregation of platelets clot-retraction response *in vitro*, and a bleeding defect *in vivo* (Law et al, 1999). Additionally, mutation studies have shown that the tyrosine residues are essential for cell spreading and recruitment of integrins to focal adhesions (Ylänne et al, 1995). Phosphorylation is dependent on the ligand, and β3-mediated adhesion to vitronectin (Blystone et al, 1997), but interestingly, not to fibronectin

(Datta et al, 2002, Butler et al, 2003), requires tyrosine phosphorylation. Phosphorylation is believed to exert its effects through phosphorylation-dependent protein-protein interactions because several signalling and cytoskeletal proteins have been shown to selectively interact with either tyrosine-phosphorylated or non-phosphorylated β3 cytoplasmic tails. The signalling proteins Grb2 and Shc bind to tyrosine phosphorylated β3 (Law et al, 1996, Blystone et al, 1996, Cowan et al, 2000), and Pyk2 has recently been identified as a phosphorylated β3 binding partner (Butler and Scott, 2005). In addition, Vav1, a GEF for Rac and Rho, has been shown to have phosphorylation-dependent association with β3 (Gao et al, 2005). Furthermore, the cytoskeletal protein myosin associates with a double-tyrosine-phosphorylated β3 peptide (Jenkins et al, 1998), and this linkage might mediate transmission of force to the fibrin clot during the process of clot retraction. β3 tyrosine phosphorylation has also shown to be necessary for the Arp3-organization into adhesion contacts (Chandhoke et al, 2003).

Also β1 integrin tyrosine phosphorylation has been reported but only in fibroblasts that are transformed by v-src (Hirst et al, 1986, Tapley et al, 1989, Johansson et al, 1994). The tyrosine phosphorylation leads to integrin displacement from focal contacts (Johansson et al, 1994), and it has major effects on the organization of focal adhesions and the cytoskeleton, and on directional cell motility (Sakai et al, 1998), and cell transformation (Sakai et al, 2001, Datta et al 2001). The importance of the NPxY motifs of the β1 cytoplasmic tail has also been shown in integrin-mediated adhesion in T lymphocytes but the implication of phosphorylation in this process has not been studied (Romzek et al, 1998). In addition, the tyrosine residues are involved in FAK-activation and cell spreading (Wennerberg et al, 2000). Although tyrosine phosphorylation normally increases the binding to PTB domains, the phosphorylation of the NPxY motif in β1 inhibits talin binding (Tapley et al, 1989). The interactions between the integrin membrane proximal NPxY motif and talin are largely hydrophobic (Garcia-Alvarez et al, 2003), and the lack of positively charged residues in talin is consistent with recognition of non-phosphorylated tyrosine and disruption of integrin binding by phosphorylation. Integrin tyrosine phosphorylation may therefore be an important negative regulation of β1 integrin activation.

In β2 integrins, including LFA-1, the tyrosine residues in the NPxY motif are replaced by phenylalanines. There is, however, an additional tyrosine in the membrane-proximal tyrosine-based endocytosis motif (YXXØ, where X is any amino acid and Ø is an amino acid with bulky hydrophobic residue). Tyrosine phosphorylation of β2 has been reported in IL-2 stimulated NK cells (Umehara, et al, 1993) and in polymorphonuclear cell after stimulation by type I collagen (Garnotel et al, 1995). The tyrosine residue in the β2 cytoplasmic tail has been shown to play a critical role in the internalization process and LFA-1 recycling to the ruffling membrane (Fabbri et al, 1999, Tohyama et al, 2003) but the role of phosphorylation of these processes has not been reported.

4.2. Integrin α-chain phosphorylation

Integrin α chains, including αL, αM, αX, α3A, α4 and α6A, also become phosphorylated, mostly on serine. αL, αM and α4 are constitutively phosphorylated but α3A or α6A integrin phosphorylation is increased upon cell treatment with phorbol ester (Hara and Fu, 1986, Chatila et al, 1989, Buyon et al, 1990, Shaw et al,

1990, Valmu et al, 1991, Hogervorst et al, 1993a, Han et al, 2001). The phosphorylated serine of α3A and α6A occur within a QPSXXE motif highly conserved among the α3A, α6A and α7A tails in all animal species examined. Upon mutation of a critical serine in the QPSXXE motif in the α6A tail, there was no loss of cell adhesion, in contrast, transfectants witch express the serine to alanin mutation adhere to laminin more extensively than the wild-type transfectants (Hogervorst et al, 1993b). Likewise, neither constitutive nor phorbol ester stimulated cell adhesion was altered by the serine mutation in the α3A tail (Zhang et al, 2001). Thus, phosphorylation of the α3A and α6A tails has no obvious effect on inside-out integrin signalling. However, α3A phosphorylation is functionally relevant because phosphorylation has been shown to strongly influence cell signalling, morhphology, and motility, most likely by affecting integrin-dependent cytoskeletal organization (Zhang et al, 2001).

α4 integrins mediate increased cell migration and decreased cell spreading because the α4 cytoplasmic domain binds tightly to paxillin, a signal adapter protein (Liu et al, 1999). Phosphorylation of the α4 cytoplasmic tail by protein kinase A at a serine residue within the paxillin binding site regulates paxillin binding (Liu and Ginsberg, 2000, Han et al, 2001). Both phosphorylation and dephosphorylation of α4 is important for optimal cell migration (Han et al, 2003), probably because of spatio-temporal regulation of paxillin binding to the α4 tail. In migrating cells the phosphorylated α4 accumulated along the leading edge, whereas unphosphorylated α4 and paxillin colocalized along the lateral edges of those cells (Goldfinger et al, 2003). The paxillin-α4 interaction mediates the recruitment of an ADP-ribosylation factor GTPase-activating protein (Arf-GAP) that decreases Arf activity, thereby inhibiting small GTPase Rac, which mediates formation of the leading lamellipodium (Nishiya et al, 2005). The localized formation of the α4-paxillin Arf-GAP complex mediates the polarization of Rac activity to the leading edge and promotes directional cell migration (Nishiya et al, 2005). Interestingly, like α4, the α9 cytoplasmic domain enhanced cell migration and inhibited cell spreading (Young et al, 2001). Paxillin also specifically binds to the α9 tails, but in contrast to α4, the interaction has no effect on cell migration. The possible role of phosphorylation in paxillin-α9 interaction has not been studied. αL, αM and αX integrins can be phosphorylated on serine residues, but the phosphorylated sites and possible functions have remained unknown.

SUMMARY OF THE STUDY

5. AIMS OF THE STUDY

Phosphorylation is a common mechanism for the regulation of integrin functions, but its role in LFA-1 activation has remained incompletely understood. The aims of the present study were:

1. To examine the role of the tyrosine kinase Lck in the LFA-1 functions.
2. To characterize the induced β2 threonine phosphorylation *in vivo*.
3. To map the phosphorylation site(s) of αL subunit.
4. To determine the role of integrin phosphorylation in the regulation of LFA-1 activation.

6. EXPERIMENTAL PROCEDURES

Detailed description of the materials and methods are found in the original publications.

Materials and methods	**Original publications**			
Antibodies	I	II	III	IV
Peptide synthesis		II		IV
cDNA constructs and mutagenesis			III	IV
Transfection			III	IV
T cell isolation and cell lines	I	II	III	IV
Radioactive cell labelling and cell activation	I	II	III	IV
Immunoprecipitation	I	II	III	IV
SDS-PAGE and immunoblotting	I	II	III	IV
Phosphopeptide mapping		II	III	
Determination of the stoichiometry of phosphorylation		II		IV
Cell adhesion and aggregation assays	I		III	IV
Flow cytometry	I			IV
Endoglycosidase H treatment	I			
Phosphoamino acid analysis			III	
Manual radiosequencing of phosphopeptides			III	IV
Peptide affinity chromatography				IV
14-3-3 affinity chromatography				IV
Co-immunoprecipitation				IV
Immunofluorescence staining				IV
Soluble ligand-binding assay				IV

7. RESULTS

7.1. The role of the tyrosine kinase Lck in the regulation of LFA-1 activation in human T lymphocytes (I)

Despite extensive studies the signalling pathways and molecular mechanisms involved in integrin regulation, the role of TCR proximal signalling events is poorly understood. Lck has been reported to be involved in early T cell activation events via ITAM-phosphorylation and ZAP-70-activation, thereby inducing tyrosine phosphorylation and activating different signalling pathways (Straus and Weiss, 1992). Lck is dynamically regulated by positive and negative tyrosine phosphorylation events, and the CD45 tyrosine phosphatase has been shown to regulate Lck activity (reviewed in Mustelin and Tasken, 2003). To examine whether Lck regulates LFA-1 activation, we used the Src kinase inhibitor PP2, which inhibits Lck kinase activity. PP2 inhibited adhesion induced by TCR-ligation, but not phorbol ester induced adhesion to coated ICAM-1, at the same concentration (10μM) that completely inhibited phosphorylation of mitogen-activated protein (MAP)-kinase on the pThr-pTyr motif that is important for catalytic activity (I, Fig1). MAP kinase has also been shown to be involved in LFA-1 inside-out signalling initiated by TCR (O'Rourke et al, 1998). Since PP2 not only inhibits Lck, but also other Src-family kinases such as Fyn, we used the Jurkat cell line deficient in Lck (JCaM1.6) to verify the results. JCaM1.6 cells did not adhere to ICAM-1 nor aggregate when activated either through TCR or, surprisingly, with phorbol ester (I, Fig2). Retransfection of JCaM1.6 cells with human Lck restored both TCR- and phorbol ester –induced adhesion to wild-type levels.

Next we examined whether reduced adhesion was due to altered expression levels of LFA-1. Indeed, JCaM1.6 cells were found to express significantly lower levels of functional heterodimers on the cell surface (I, Fig3). Further investigations showed that the protein expression of both subunits was normal, but the β2 polypeptide was immaturely glycosylated and uncomplexed with αL and therefore inefficiently transported to the cell surface (I, Fig4). Retransfection of Lck did not lead to increased LFA-1 on the cell surface.

In addition, we examined the role of CD45 in LFA-1 mediated adhesion using CD45-deficient Jurkat T cells, J45.01. J45.01 cells were able to bind ICAM-1 and aggregate when stimulated with phorbol ester but not when stimulated through TCR, as expected because of the crucial role of CD45 in TCR signalling. We also examined whether Lck and CD45 influenced Ser756 phosphorylation of β2 cytoplasmic domain, but the β2 subunit became normally phosphorylated on Ser756 in both JCaM1.6 and J45.01 cells (I, Fig5). PP2 had no effect on Ser756 phosphorylation in cells treated with PP2 before phorbol ester activation. These results indicate that Ser756 phosphorylation of β2 happens independently of Lck in Jurkat cells.

7.2. Characteristics of LFA-1 β chain phosphorylation (II, III)

Since protein phosphorylation is known to often regulate protein activity, we wanted to examine the role of LFA-1 phosphorylation in integrin regulation. The β2 subunit of LFA-1 has previously been shown to become phosphorylated after activation both on serine residues (Hibbs et al, 1991, Fagerholm et al, 2002) and very

dynamically on threonine residues (Valmu et al, 1995) but the biological role of phosphorylation was not clear. To identify the specific amino acids phosphorylated in phorbol ester activated T cells, we first performed tryptic phosphopeptide mapping. The phosphorylated β2 tryptic peptides were run on cellulose plates with synthetic marker peptides. Ser756 was shown to be a major phosphorylated site after phorbol ester stimulation, as observed earlier (Figure 9) (Hibbs et al, 1991, Fagerholm et al, 2002). After activation of T cells by phorbol ester together with the serine/threonine phosphatase inhibitor okadaic acid (OA), Ser756 and two of the threonine residues in the β2 threonine triplet become phosphorylated (II, Fig1). The phosphorylated peptide was isolated from the phosphopeptide map and subjected to Edman degradation. The result showed that all three threonine residues (758-760) become phosphorylated but only two at a time (III, Fig1). TCR-ligation also leads to a strong threonine phosphorylation, but interestingly, differed from phorbol ester activation in that phosphorylation occurred only on the first threonine (Thr758) of the threonine triplet (III, Fig2).
The other leukocyte-specific integrin, β7, has a serine residue in the position corresponding to Ser756 in β2 and a similar threonine triplet to β2. Interestingly, also β7 became phosphorylated on the first threonine after phorbol ester activation (III, Fig6). However, mutation of this threonine had no effect on β7-mediated adhesion to vascular cell adhesion molecule-1 (VCAM-1) (III, Fig7).
For determination of the stoichiometry of β2 phosphorylation, the relative amount of radioactive phosphate incorporated into the γ-position of ATP first had to be quantified. The radioactivity of ATP from ^{32}P-labelled T cells was present only in the β and γ-phosphoryl groups, and in 1:1 proportion (II, Fig3 and 4). The stoichiometry of β2 phosphorylation was then calculated by comparing the incorporation of ^{32}P-label from the γ-phosphoryl group of [^{32}P]ATP into the β2 molecule with the absolute amount of β2 polypeptide. The stoichiometry of phosphorylation of β2 in phorbol ester stimulated T cells was 0.12 mol of phosphate / mol of β2 subunit and the phorbol ester stimulated T cells in the presence of OA was 0.92 mol of phosphate / mol of β2 subunit (II, Table 1).

7.3. Identification of the phosphorylation site and stoichiometry in the αL chain (IV)

αL has been shown to be constitutively phosphorylated in T cells, but the phosphorylation site and possible functions has not been reported. To examine the role of LFA-1 α subunit phosphorylation, we first mapped the phosphorylation site(s). Tryptic phosphopeptide maps from resting (IV, Fig1) and from phorbol ester activated cells (data not shown) were performed. The maps were identical, thus, no additional sites become phosphorylated after cell activation. Identification of the serine phosphorylation site was made by Edman degradation, and this showed that Ser1140 is the major αL phosphorylation site (Figure 8) (IV, Fig1).
To assess the stoichiometry of αL phosphorylation in T cells, we first generated a phospho-specific antibody against the phosphorylated form of αL. The antibody was shown to be sequence and phospho-specific (IV, Fig2). This phospho-specific αL antibody immunoprecipitated about 40 % of the total heterodimeric αL from resting T cells (IV, Fig2), indicating that approximately 40 % of surface αL was phosphorylated.

7.4. LFA-1 phosphorylation in the regulation of integrin activation (IV)

We then went on to study the LFA-1 phosphorylation sites in cells. Thr758, the first threonine in the TTT-motif, was determined as a major phosphorylation site of β2 after TCR-ligation. Interestingly, 14-3-3 adapter proteins from leukocyte lysates have previously been shown to interact with a synthetic β2 peptide phosphorylated on Thr758 (Fagerholm et al, 2002). To investigate the association in more detail, we used recombinant 14-3-3 proteins and examined the binding of these proteins to the phosphorylated β2 cytoplasmic peptides. We showed that the β2 peptide binds to purified 14-3-3-proteins directly and in a phosphorylation-dependent manner and the binding occurs through the canonical 14-3-3-phosphopeptide binding motif (IV, Fig7). Furthermore, endogenous 14-3-3-proteins and β2 integrins could be coprecipitated from TCR-stimulated, but importantly, not unstimulated human T cells (IV, Fig7), indicating that phosphorylation-dependent interaction occurs also *in vivo*.

Mutation studies were then used to investigate the role of the 14-3-3-association with the β2 integrin. The Thr758Ala mutation, which reduces the association with 14-3-3 (IV, Fig7), significantly reduced the constitutive integrin-mediated adhesion of transfected COS1-cells to ICAM-1 (IV, Fig8). MEM-83, an activating αL antibody, could still activate the Thr-mutated integrin, showing that activating conformational changes could still occur also for the mutated integrin. To examine whether the effects of the Thr758 mutation seen were due to the blocking of binding of 14-3-3 proteins to the integrin, we cotransfected cells with wild-type LFA-1 and an R18-construct, which blocks 14-3-3 interactions with its cellular ligands by binding to the phosphopeptide-binding groove in 14-3-3 (Jin et al, 2004). The R18 peptide clearly reduced cell adhesion to ICAM-1 (IV, Fig8). The TTT-motif of the integrin has been closely associated with actin reorganization events, but not affinity-changes in integrins (Peter and O'Toole, 1995). Thus, we wanted to examine whether 14-3-3 association with β2 was involved in cell spreading. Indeed, the Thr758 mutation was shown to significantly reduce cell spreading on ICAM-1 as examined by FITC-phalloidin staining of polymerized actin (IV, Fig9). In addition, R18-transfected cell spreading on ICAM-1 was almost completely abolished.

The role of αL phosphorylation in the regulation of LFA-1 activation was also investigated. We generated stable Jurkat Jβ2.7 cell transfectants expressing wild-type and Ser1140Ala αL. Mutation did not affect the heterodimerization or cell surface expression of LFA-1 (IV, Fig3). The binding of both wild-type and Ser1140Ala αL transfectants to immobilized ICAM-1 was increased by TCR-ligation and by phorbol ester treatment as compared to resting cells (IV, Fig.4). In contrast, mutant cells bound less efficiently to ICAM-1 when activated by MEM-83 or Mg/EGTA. These activators probably activate LFA-1 binding to ICAM-1 by inducing conformational changes in the extracellular head-domain of LFA-1 (Lu et al., 2004, Shimaoka et al, 2002). We also studied whether a negative charge at position 1140 (Ser to Asp-mutation) would be enough to affect adhesion, but the Ser-Asp mutation did not result in a different adhesion phenotype compared to the Ser-Ala mutation (IV, Fig4). To further study the mechanisms of the different modes of adhesion, a soluble ICAM-1Fc binding assay was used to measure the ligand-binding activity. MEM-83 greatly increased the binding of soluble ICAM-1 to wild-type αL transfectants, while only minimal binding was observed for

Ser1140Ala αL cells (IV, Fig4). Similar results were seen when affinity modulation of LFA-1 was detected by using the monoclonal antibody 24 (mAb24) (Dransfield and Hogg, 1989). These results suggest that αL phosphorylation could be involved in LFA-1 conformational changes needed for integrin binding to ligand.

Some physiological activators of LFA-1, including chemokines and ligands, have been shown to lead to extracellular conformation changes of LFA-1 (Constantin et al, 2000, Cabanas and Hogg, 1993, Li et al, 1993, Kotovuori et al, 1999). To determine whether Ser1140-mutation affected mAb24 expression, we stimulated cells with the chemokine SDF-1 or ICAM-2. Clear staining was detected on the wild-type cells but not on the Ser1140-mutant cells (IV, Fig5).

Furthermore, the small GTPase Rap1 is a potent activator of LFA-1 which has been shown to increase LFA-1 affinity (Katagiri et al, 2003). Importantly, Rap1 acts through the α chain cytoplasmic domain (Tohyama et al, 2003), and thus we wanted to examine the effect of active Rap1 (Rap1V12) on the wild-type and Ser1140-mutated cells. Rap1V12 was able to induce binding of wild-type cells but not Ser1140-mutant cells to coated ICAM-1.

The talin head-domain directly binds to the β2 cytoplasmic domain and presumably induces a separation of the cytoplasmic tails of the α and β chains, which leads to a conformational change in the extracellular domain (Tadokoro et al, 2003, Kim et al, 2003). Thus, we examined whether a talin head-domain can activate the Thr758Ala mutated β2 or Ser1140Ala mutated αL integrin. Talin head-domain was able to induced binding of wild-type and both mutant COS1-cells to ICAM-1 (IV, Fig10), indicating that the mechanism by phosphorylation regulated LFA-1 is not mediated by talin.

8. DISCUSSION

8.1. Lck is important for activation of LFA-1

Since the LFA-1 interaction with its ligands must be tightly regulated, several different signalling pathways are expected to participate in LFA-1 activation. Early tyrosine phosphorylation events after TCR-ligation are known to be crucial for T cell signalling, but the involvement of early signalling molecules in integrin activation still remains poorly understood. We have investigated the role of Lck in regulating LFA-1-mediated functions.

Lck is a lymphoid-specific cytosolic PTK, which plays a key role in TCR-linked signalling (Weiss and Littman, 1994). The activity of Lck is regulated by protein kinases and phosphatases, including CD45 (Mustelin and Tasken, 2003). Some Lck-dependent functions seem to be independent of Lck kinase activity, and presumably involve protein-protein interactions through the Src-homology 2- (SH2-), SH3- or unique domain of Lck (Xu and Littman, 1993, Park et al, 1995, Lee-Fruman et al, 1996). We have now shown that Lck is also involved in regulating LFA-1 functions. Firstly, PP2, a specific inhibitor of the Src-family kinases, inhibits TCR-induced LFA-1 adhesion to coated ICAM-1. However, PP2 did not inhibit phorbol ester-induced adhesion. Results indicate that Lck kinase activity is required for inside-out signalling induced by TCR-ligation but not by phorbol ester, which bypass proximal TCR signalling events. Secondly, the Lck-deficient cell line, JCaM1.6, did not adhere to coated ICAM-1 when stimulated either through the TCR or, interestingly, with phorbol ester. Also T cell aggregation was deficient in these cells. Importantly, Mg/EGTA-treatment could activate Lck-deficient cells (data not shown), indicating a defect in inside-out signalling. Since the LFA-1 was non-functional also after phorbol ester stimulation, it indicates that kinase-independent activity of Lck is needed for phorbol ester activation. Transfection of these cells with human Lck restored both TCR- and phorbol ester-induced adhesion to wild-type levels. And thirdly, JCaM1.6 was found to express significantly lower amounts of LFA-1 on the cell surface than wild type Jurkat cells. The β2 polypeptide was normally expressed but it was immaturely glycosylated and uncomplexed with the αL chain. This indicates that Lck is involved in regulating integrin cell heterodimer formation and surface expression. The CD45 null cell line, J-AS, has also significantly lover LFA-1 expression than in the parental Jurkat cells (manuscript by Turunen PM, Nurmi, SM, Autero, M, Gahmberg, CG, and Fagerholm, SF.), indicating the same signalling pathways as Lck in regulation LFA-1 surface expression. The small amount of CD45 present in the J45.01 cells is enough to restore surface expression almost to normal levels (I, Fig3). However, retransfection of JCaM1.6 with human Lck did not restore LFA-1 surface expression, even if it restored stimulus-induced cell adhesion. This might indicate that the localization of transfected Lck is not correct or the amount is in-sufficient for increased β2 surface expression.

The mechanism of integrin-regulation by Lck remains to be determined. Probably both kinase activity and an adapter function of Lck are needed for T cell adhesion. Interestingly, superantigen-induced LFA-1-meditated conjugation of T cell to APC has also been shown to require both Lck kinase activity and a

functional SH3 domain (Morgan et al, 2001). Activation of ZAP-70, the protein normally thought to lie directly downstream of Lck in the TCR signaling pathway, is dispensable for conjugation, but some other molecules that interact with the SH3 domain of Lck are probably needed for the inside-out signalling from TCR to LFA-1. One interesting possibility is that the Lck SH3 domain may affect adhesion via its interaction with proline-rich sequences in CD28 (Holdorf et al, 1999). CD28 is costimulatory molecule, which is engaged by APC during antigen presentation. CD28-Lck-interaction has been shown to regulate Rap1 activation (Carey et al, 2000) and Rap1, in turn, has been directly implicated in regulating LFA-1 dependent adhesion (Katagiri et al, 2000). Moreover, Lck recruitment to the IS depends on the CD28 proline-rich binding motif for Lck (Tavano et al, 2004). The SH3 domain of Lck has also been shown to be required for costimulation-dependent Lck reorganization of lipid rafts and TCR signal stabilization (Patel et al, 2001). LFA-1 is probably also recruited to lipid rafts upon T cell stimulation (Leitinger and Hogg, 2002), and thus, lipid rafts would provide a platform for functional interactions needed for LFA-1 activation. However, the role of lipid raft in LFA-1 regulation is still controversial.

Furthermore, one possible downstream signal molecule of Lck is Vav1, a GEF for Rho GTPases. Lck has been shown to regulate Vav1 activity (Han et al, 1997). Vav also has a role in lipid raft clustering (Villalba et al, 2001) and Lck recruitment into lipid rafts and accumulation at IS (Tavano et al, 2004), indicating that Vav1 may also be involved in Lck-signalling. In addition, Vav1 has been shown to be required for TCR induced LFA-1 activation (Ardouin et al, 2003), and it also regulates TCR-induced integrin clustering and cell adhesion (Krawczyk et al, 2002). Thus, Vav1 is a potential signalling molecule involved in Lck-signalling from the TCR to LFA-1.

8.2. Threonine phosphorylation of the β2 chain regulates LFA-1 activation through affinity-independent mechanisms

Phosphorylation of the cytoplasmic domains of LFA-1 was initially reported more than a decade ago (reviewed in Fagerholm et al., 2004), but the significance of these phosphorylation events in integrin regulation has remained unclear. Threonine phosphorylation has been reported to occur after stimulation of cells by TCR-ligation or phorbol ester (Valmu and Gahmberg, 1995), and it has also been shown that the threonine triplet in β2 (Thr758-760) is important both for adhesion and cytoskeletal reorganization mediated by LFA-1 (Hibbs et al., 1991, Peter and O'Toole, 1995). PKC has been shown to be the main β2 kinase in leukocytes, and many PKC isoforms are capable to phosphorylate the β2 subunit in vitro (Fagerholm et al, 2002). We have now mapped the threonine phosphorylation sites in the β2-cytoplasmic domain. After phorbol ester activation all three threonine residues act as substrates but importantly, only two at a time. In contrast, if T cells were stimulated through TCR, only the first threonine residue (Thr758) of the triplet becomes phosphorylated. These results indicate that different signalling events take place and different PKC isoforms are activated under different activation conditions. Since TCR-ligation induces phosphorylation of Thr758, PKCβ and PKCδ are probably involved, whereas phorbol ester also activates other PKC isoforms.

The stoichiometry of phosphorylation of β2 integrin in phorbol ester stimulated T cells was determined to be 0.12 mol per mol of protein. This means that the phosphorylation occurred in approximate 12% of the integrins molecules if the phosphorylation took place only at a single serine residue. In the presence of serine/threonine phosphatase inhibitors, the stoichiometry of phosphorylation was 0.92 mol per mol of protein but since several sites become phosphorylated under these conditions each site is phosphorylated only at a low level. On the other hand, it is possible that the OA treatment is not highly efficient and in reality higher stoichiometry may occur. However, even a low stoichiometry of phosphorylation may play important roles by regulating protein-protein interactions.

Stimuli, which induce threonine phosphorylation of β2, have been shown to induce clustering of integrins on the cell surface and avidity rather that affinity (Stewart et al, 1996, 1998). In addition, the threonine phosphorylated LFA-1 has been shown to distribute preferentially to the actin cytoskeleton (Valmu et al, 1999), indicating cytoskeleton-dependent mechanisms of integrin activation. The linkage to the actin cytoskeleton could occur via actin-binding proteins that have been shown to associate with integrins or through specific adaptor proteins. 14-3-3 adaptor proteins bind to serine and threonine-phosphorylated proteins (Baldin et al, 2000) and mediate interactions with numerous intracellular proteins. 14-3-3 proteins from leukocyte lysates have been shown to interact with a synthetic β2 cytoplasmic peptide phosphorylated on Thr758 (Fagerholm et al, 2002), and we showed that the interaction occurs at the protein level, it is direct and phosphorylation-dependent. The binding occurs in T cells only after cell activation by stimuli that induced phosphorylation of Thr758, like phorbol ester and TCR-ligation. Mutation of Thr758 or blocking the 14-3-3-β2 interaction by R18-peptides inhibited LFA-1-mediated cell adhesion and spreading, indicating that the association is required for integrin functions.

14-3-3s are dimers, and both monomers can independently bind to phosphorylated targets either within the same protein or in different proteins (Tzivion and Avruch, 2002, MacKintosh, 2004). Thus, they would be ideal candidates to recruit other signaling proteins or cytoskeletal elements to the phosphorylated integrin to form larger complexes. LFA-1 has been reported to associate with several cytoskeletal proteins, including talin (Horwitz et al, 1986), α-actinin (Pavalko and Laroche, 1993) and filamin (Sharma et al, 1995). The head-domain of talin binds to the integrin β2 cytoplasmic domain and it has been shown to induce a separation of the LFA-1 α and β chains (Kim et al, 2003). However, the talin head-domain was capable to activate the β2 Thr758Ala mutant in cells, indicating that threonine phosphorylation is not needed for talin-induced activation. α-actinin has been shown to interact with the β2 cytoplasmic tail at a membrane-proximal site, and the C-terminal regulatory domain between residues 748-762, including the TTT-motif, inhibits the constitutive association of β2 with α-actinin (Sampath et al, 1998). However, point mutation Thr758Glu, which may mimic phosphorylation by introducing a negative charge, and Thr758Ala both enhanced α-actinin binding, suggesting that phosphorylation of Thr758 is not necessary for inducing binding of α-actinin. Interestingly, filamin has been shown to bind to the integrin in the region that contains the phosphorylatable threonines (Calderwood et al, 2001). Filamin is also a phosphoprotein and phosphorylation has been suggested to be one

possible mechanism that regulates its association with surface receptors, i.e. β2 integrins (Goldmann, 2001, Sharma and Goldmann, 2004). Filamin also binds to 14-3-3 proteins (Fagerholm S., personal communication), but whether β2, filamin and 14-3-3 are in the same complex, and whether phosphorylation regulates the interactions is not known.

Additionally, 14-3-3 proteins may promote cell adhesion and spreading by interacting with signalling molecules involved in LFA-1 regulation. Small GTPases of the Rho family have been shown to regulate the actin cytoskeleton, and cell adhesion and migration (reviewed in Ridley, 2001). 14-3-3ζ has been thought to provide a direct link between the cytoplasmic tails of integrin and signalling molecules involved in Rho GTPase activation, because 14-3-3ζ has been shown to mediate integrin-induced Cdc42 and Rac activation (Bialkowska et al, 2003). However, clear evidence of this interaction has not been reported. Another signalling molecule that has been implicated in integrin signalling and shown to interact with 14-3-3 proteins is $p130^{Cas}$ (Gracia-Guzman et al, 1999). 14-3-3 and $p130^{Cas}$ colocalized at membrane ruffles and lamellipodia during the initial integrin-mediated attachment of cells, supporting the proposal that the 14-3-3 proteins have a role in integrin signalling.

8.3. Serine phosphorylation of αL regulates LFA-1 activation through affinity-dependent mechanisms

We also established the role of LFA-1 α-chain phosphorylation in integrin regulation (Figure 10). We mapped the αL phosphorylation site to Ser1140 and showed that approximately 40 % of surface αL was phosphorylated in T cells. The α4 integrin has been shown to have a similar constitutive high stoichiometry of phosphorylation (Han et al, 2001), which plays profound roles in spatio-temporal regulation of integrin functions (Goldfinger et al, 2003). By mutation of the Ser1140 of αL to a non-phosphorylatable amino acid, we showed that phosphorylated Ser1140 is involved in conformational changes occurring in LFA-1 in response to several different affinity-increasing stimuli, such as an activating antibody, ligand or chemokines. Integrins have been shown to undergo a global conformational change from a bent to an extended form upon cell activation (Figure 7). In addition, the optimal interaction between LFA-1 and ligand requires critical rearrangements in the I-domain by pulling down the C-terminal α7 helix (Shimaoka et al, 2002). The Ser1140Ala mutant was able to undergo a conformational change from a bent form to an extended form similar to that of wild-type cells, as detected by the KIM127-antibody which detects the extended form of LFA-1 (unpublished data). This indicates that the αL phosphorylation is involved in I-domain rearrangements. How this integrin intracellular phosphorylation affects the conformation of extracellular I-domain remains to be established.
One possible mechanism is that the negative charge induced by phosphorylation facilitates the separation of the integrin cytoplasmic tails, leading to a conformational change in the extracellular domain (Kim et al, 2004, Adair et al, 2005). However, mutation of Ser1140 to a negatively charged Asp, did not lead to a different phenotype compared to the Ser1140 to Ala mutation, indicating that a negative charge alone is not enough the integrin activation. On the other hand, “locking” integrins in the phosphorylated state can have

negative effects on integrin activation since the dynamic nature of phosphorylation might be crucial for integrin function.

Alternatively, the αL phosphorylation site could act through cytoplasmic binding proteins that selectively bind to it or disconnect them from the phosphorylated form of the integrin. For example, α4 serine phosphorylation has been shown to inhibit the binding of paxillin, and therefore regulates cell migration (Han et al, 2001, 2003). Active Rap1 was unable to induce binding of Ser1140Ala-mutant cells to ICAM-1, suggesting that Rap1-induced activation requires αL-phosphorylation. Rap1 may possibly influence the binding of some cytoplasmic factors to the phosphorylated αL tail. One possible candidate is RAPL, a Rap1-binding molecule. RAPL has been claimed to have an important function in LFA-1 mediated cell adhesion, and importantly, it binds to the αL cytoplasmic tail (Katagiri et al, 2003). In the future, it would be interesting to study the effects of RAPL on S1140A-mutant cells. Other Rap1 effectors have also been implicated in Rap1-induced adhesion, for example Riam, but an interaction with LFA-1 has not reported (Bos, 2005). It is important to note that some leukocyte-specific restrictive factors that work through phosphorylated αL could be involved in LFA-1 regulation, because affinity-inducing stimuli of the integrin are functional also for the Ser1140Ala mutant in COS-1 cells.

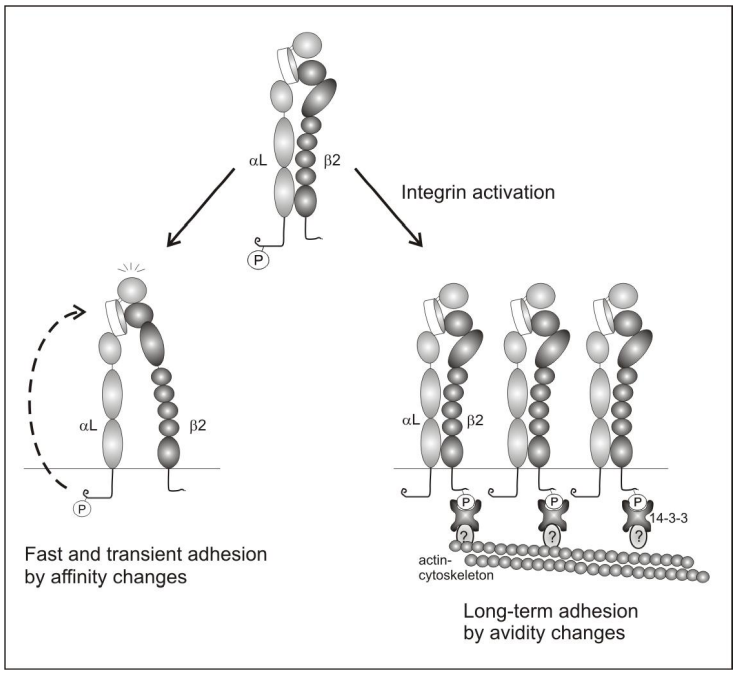

Figure 10. A model how α and β chain phosphorylation regulates LFA-1 activation. αL phosphorylation is required for adhesion events that involve rapid changes in the conformation and affinity of the LFA-1. β2 phosphorylation works through the interaction with 14-3-3-proteins and involves actin reorganization and integrin clustering.

9. CONCLUDING REMARKS

Dynamic adhesion is especially important in the immune system, where cells need to attach and detach continuously. The LFA-1 integrin is expressed exclusively in leukocytes and is of fundamental importance for the function of the immune system. LFA-1 mediates cell adhesion under various conditions, for example during immunological synapse formation between the T cell and the antigen presenting cell, and during leukocyte emigration from the bloodstream into tissues. Whereas the TCR-mediated adhesion is slow and sustained, chemokine-induced adhesion is fast and rapidly reversible. These different adhesive events may require different mechanisms of regulation. Both affinity-dependent and -independent mechanisms have been postulated to be important in the regulation of integrin activation. These mechanisms are not mutually exclusive, and different modes of integrin activation may involve different mechanisms alone or together. For example, TCR-induced activation of LFA-1 has not been shown to involve affinity regulation in the integrin, but instead has been closely correlated with a spreading phenotype of T cells and actin cytoskeleton rearrangements. In contrast, chemokines mediate rapid conformational changes in LFA-1 but also involves clustering of integrins.

The results of this thesis showed that the Lck tyrosine kinase is essential for proper integrin activation, and both Lck kinase activity and adapter functions are needed. Lck is involved both in the regulation of integrin cell surface expression and regulation of integrin inside-out activation. Thus, the Lck plays multiple roles in the regulation of T cell adhesion.

It was also shown that phosphorylation of both the α-chain and the β-chain of the integrin cytoplasmic domains plays a role in the molecular mechanisms involved in these different activation events (Figure 10). However, the αL and β2 polypeptides play distinctive roles in integrin activation. The integrin αL chain is constitutively phosphorylated and this phosphorylation site (Ser1140) is required for adhesion that involves rapid changes in the conformation and affinity of the integrin heterodimer. In contrast, the β2-Thr758 phosphorylation that is induced after physiological triggering of T cells through the TCR, and involves formation of the immunological synapse, works through the interaction with 14-3-3 proteins. This interaction is involved in integrin-mediated adhesion, actin reorganization and cell spreading.

Thus, different phosphorylation events participate in LFA-1 regulation. The different regulation mechanisms may depend on the surroundings and the context of adhesion, and thus enables regulation of both fast, transient adhesive events and long-term adhesion strengthening.

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