# BIDIRECTIONAL SIGNALLING AND PHOSPHORYLATION OF CD11/CD18-INTEGRINS IN T CELLS

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## ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals I-IV:

I. Valmu, L.*, Fagerholm, S.*, Suila H. and Gahmberg, C. G. (1999) The cytoskeletal association of CD11/CD18 leukocyte integrins in phorbol ester activated T cells correlates with CD18 phosphorylation. *Eur. J. Immunol.* 29: 2107-2118.

II. Fagerholm, S., Prescott, A., Cohen, P. and Gahmberg, C. G. (2001) An essential role for calmodulin in regulating human T cell aggregation. *FEBS Lett.* 491: 131-136.

III. Fagerholm, S., Morrice, N., Gahmberg, C. G. and Cohen, P. (2002) Phosphorylation of the cytoplasmic domain of the integrin CD18 chain by protein kinase C isoforms in leukocytes. *J. Biol. Chem.* 277: 1728-1738.

IV. Fagerholm S.*, Hilden, T.* and Gahmberg, C. G. (2002) Lck tyrosine kinase is important for activation of the CD11/CD18-integrins in human T lymphocytes. *Eur J. Immunol. In press.*

* These authors have contributed equally to the work.

## ABBREVATIONS

| | |
|---|---|
| APC | antigen presenting cell |
| ARF | ADP-ribosylation factor |
| CaMKII | Ca-calmodulin-dependent kinase II |
| cAMP | cyclic AMP |
| DAG | diacyl glycerol |
| FAK | focal adhesion kinase |
| GAP | GTPase activating protein |
| GDI | guanine nucleotide dissociation inhibitor |
| GEF | guanine nucleotide exchange factor |
| GPI | glycosyl phosphatidylinositol |
| HEV | high endothelial venule |
| ICAM | intercellular adhesion molecule |
| IL | interleukin |
| JAB-1 | Jun-activation domain-binding protein-1 |
| JNK | c-Jun N-terminal kinases |
| LAD | leukocyte adhesion deficiency |
| LOK | lymphocyte-oriented kinase |
| LPS | lipopolysaccharide |
| MadCAM | mucosal addressin cell adhesion molecule |
| MAP kinase | mitogen-activated protein kinase |
| MARCKS | myristoylated alanine-rich C kinase substrate |
| MHC | major histocompatibility complex |
| MIDAS | metal ion dependent adhesion site |
| MLCK | myosin light chain kinase |
| MW | molecular weight |
| PAK | p21-activated kinase |
| PDBu | phorbol 12,13 dibutyrate |
| PH | pleckstrin homology |
| PI 3-kinase | phosphoinositide 3-OH kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| pLN | peripheral lymph node |
| PMA | phorbol 12-myristate 13-acetate |
| PMN | polymorphonuclear cell |
| Rack | receptor for activated protein kinase C |
| SDF-1 | stromal cell-derived factor-1 |
| SH2 | Src homology 2 |
| sICAM | soluble ICAM |
| TCR | T cell receptor |
| TNF | tumour necrosis factor |
| VCAM | vascular cell adhesion molecule |

**SUMMARY**

T cell adhesion is of critical importance for the function of the immune system. It is needed for the formation of the immunological synapse between the T cell and an antigen-presenting cell (APC) to initiate the immune response. It is also required for T cell cytotoxicity and T cell recirculation in the body, and during inflammation. One group of adhesion molecules involved in T cell adhesion is the leukocyte integrins, CD11/CD18 or $\beta_2$-integrins, that bind to the intercellular adhesion molecules (ICAMs). These interactions are highly regulated, and can be initiated for example by the ligation of the T cell receptor, by phorbol esters or by a chemokine signal. Intracellular signalling pathways are then activated, leading to increased avidity of integrins for their ligands. The integrins also activate intracellular signalling pathways through outside-in signalling. This process is important for example during T cell costimulation. The integrin CD18 cytoplasmic domain is important for integrin regulation and becomes phosphorylated on serine and functionally important threonine residues after cell activation, providing a possible mechanism to regulate integrin function.

In this study, signalling pathways regulating integrins in human T cells have been examined. Lck, a protein tyrosine kinase, and calmodulin, a calcium-binding protein, have been identified as novel components of the signalling pathways involved in regulating CD11/CD18-integrin-dependent adhesion and cell aggregation. Integrin-proximal events have been further characterised. We have identified the main kinase activity that phosphorylates CD18 on serine and threonine residues as protein kinase C, the main cellular target for phorbol esters. A novel phosphorylation site in CD18 has been found, and the phosphorylation sites in CD18 have been further characterised *in vitro* and *in vivo*. A putative mechanism for integrin avidity-regulation has been proposed, in which phosphorylated integrins bind to the actin cytoskeleton, possibly through the interaction with 14-3-3 proteins. This could provide a mechanism for integrin-mediated cytoskeletal changes of T cells and cell spreading, which leads to adhesion strengthening.

CD11/CD18-integrins are involved in regulated adhesive interactions of T cells and other leukocytes. The identification of components of the regulatory cascade and the investigation of the mechansim of integrin regulation is important, since deregulation of leukocyte integrin-mediated adhesion leads to fundamental defects of the immune system.

## INTRODUCTION

The human immune system helps to protect us from the hostile environment we live in. Although immune cells are generally thought of as nonadherent cells, dynamic cell adhesion actually regulates most aspects of the immune system. The T cell is an adhesion-dependent component of the adaptive immune system. To be effective, T cells need to circulate freely in the blood, but when activated, they need to quickly adhere to other cells via membrane receptors to mediate the immune response. For example, cytotoxic T cells need to bind to their target cells in order for killing to take place and helper T cells adhere to B cells in the initiation of the antibody production process. Cell adhesion is also necessary for lymphocyte recirculation through the body, and for recruitment of leukocytes to sites of inflammation. There are several families of adhesion molecules involved in cell binding, for example cadherin, selectin, immunoglobulin, proteoglycan and integrin superfamilies. For leukocyte adhesion processes, the selectins (L-, P- and E-selectin), the immunoglobulin superfamily (ICAMs, VCAM and MadCAM) and the integrins (CD11/CD18- and $\alpha_4$-integrins) are especially important.

CD11/CD18-integrins are only expressed on the surface of leukocytes and can thus be termed leukocyte integrins. These integrins are highly specialized in transient, dynamic interactions with their cellular ligands, the intercellular adhesion molecules (ICAMs). CD11/CD18-integrins are heterodimeric membrane proteins, consisting of an $\alpha$ (CD11a, b, c or d) and a $\beta$ (CD18) chain, with large extracellular domains and short intracellular domains devoid of enzymatic activity. The intracellular tails are nevertheless crucial for the regulation of integrin function. Integrin-mediated adhesion is regulated by intracellular signalling pathways initiated, for example, by T cell receptor engagement or by soluble chemokines. Several different signalling pathways have been implicated in integrin regulation.

The mechanism of integrin activation involves both changes in affinity of individual receptors and changes in lateral surface motility and clustering of integrins to achieve increased avidity for the ligands. Different modes of activation seem to use different mechanisms at the molecular level. Adhesion strengthening presumably occurs by cytoskeletal rearrangements and altered interactions with the actin cytoskeleton. Evidence is accumulating that integrins also function as signalling receptors, providing cells with costimulatory signals during immune responses. For example, CD11/CD18-integrins can function as coreceptors for the T cell receptor during T cell-APC interactions. The integrins are thus crucial for the function of the immune system, as shown by a rare, and severe, genetic disease called leukocyte adhesion deficiency, where the integrins are reduced in amount or missing. The signalling pathways and the proximal events mediating integrin activation and integrin outside-in signalling, and thus regulation of T cell function in the immune system, have remained largely undefined on the molecular level.

In this study, signalling pathways that regulate the integrin CD11a/CD18-integrins in human T cells have been studied. Additionally, integrin proximal events, i.e. integrin phosphorylation has been studied in detail. New signalling pathways involved in regulation of T cell adhesion through integrins are described, and the main kinase activity that phosphorylates the CD18-chain in leukocytes is identified. CD18 phosphorylation sites are studied in detail. Additionally, the role of phosphorylation in CD11/CD18-integrin regulation is discussed.

## REVIEW OF THE LITERATURE

### 1. T CELL ADHESION

Most leukocytes (white blood cells), in contrast to most other cell types, are normally nonadherent, and circulate freely in the bloodstream as passive, round, nonpolarized cells. However, they also need to be able to interact with other cells in diverse ways. Lymphocytes recirculate through the blood and peripheral lymphoid organs and tissue during immune surveillance, continuously looking for antigens (reviewed by Bradley and Watson, 1996). Naïve lymphocytes recirculate preferentially through peripheral lymph nodes (pLN), and this requires the interaction with the endothelium in high endothelial venules (HEV), which is specialized in recruiting large amounts of lymphocytes. In the pLN, the lymphocyte may encounter antigens, presented by an antigen presenting cell (APC), which induces lymphocyte proliferation and activation. The activated lymphocytes enter the bloodstream and may subsequently be recruited to inflammation sites to perform effector functions, again through interaction with endothelium.

The interaction of the leukocyte with blood vessel endothelium that initiates the recruitment of leukocytes into tissue is a multistep-process (Fig 1) involving several families of cell surface receptors (reviewed in Springer, 1994, Worthylake and Burridge, 2001). Leukocytes first attach loosely to endothelium by the interactions of selectins with carbohydrate ligands on the endothelium. In HEVs, L-selectin appears to play a crucial role in lymphocyte tethering and rolling (Warnock et al, 1998). In some instances, the $\alpha_4$-integrins can contribute to this process of tethering and rolling along the endothelium

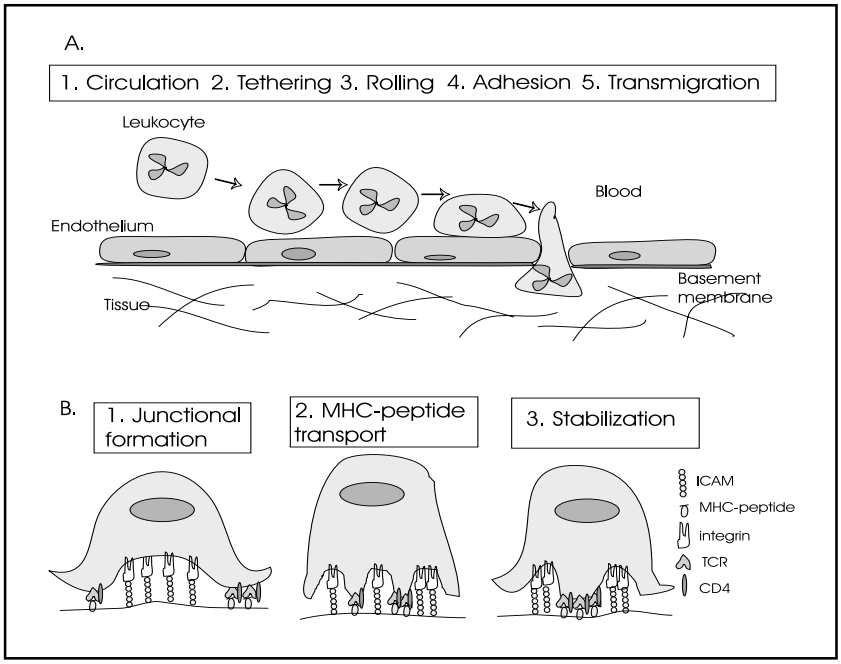


**Figure 1. Adhesive contacts of a T cell.** (A) The multistep model of leukocyte interactions with the endothelium. Adapted from Springer, 1994. See text for details. (B) Steps in the formation of the immunological synapse. Adapted from van der Merwe et al, 2000. Additionally, other molecules, like DC-SIGN (Bleijs et al, 2001) and chemokine-receptors may be involved, but how they are distributed in the synapse is so far unknown.

(Alon et al, 1995). When interacting with the endothelium, the leukocytes will become susceptible to signals that trigger tight, integrin-mediated cell adhesion. Chemokines are a group of extracellular signalling molecules that bind to chemokine receptors on leukocytes, and regulate leukocyte trafficking in the body and the adhesive state of integrins (Baggioni et al, 1998). Integrin activation by chemokines is crucial for leukocyte transmigration (Bargatze and Butcher, 1993, Warnock et al, 1998). Also the selectins or $\alpha_4\beta_1$-integrins may provide the activating signal (Worthylake and Burridge, 2001). The integrin-ligand pairs involved in firm adhesion are $\alpha_4\beta_1$-VCAM, $\alpha_4\beta_7$-VCAM/MadCAM and/or CD11a,b/CD18-ICAM 1-5 (Warnock et al, 1998, Berlin-Rufenach et al, 1999, Andrew et al, 1998). The adhesion process starts with polarization of the leukocyte; a leading edge with a membrane protrusion (lamellipodium) is formed, followed by the cell body containing the nucleus, and an elongated uropod, the rear end of the cell (Sanchez-Madrid and del Pozo, 1999). Intracellular signalling pathways and the actin cytoskeleton play a major part in the polarization.

The firm cell attachment is followed by migration along the endothelium and invasion between neighboring endothelial cells. Leukocyte migration is comparable to amoeboid movement of *Dictyostelium* (Friedl et al, 2001). The five-step process of migration includes pseudopod extension and attachment to the surface, cell polarization, contraction of the cell body, release of rear attachment sites, and uropod retraction. Actin polymerization leads to forward movement of membrane protrusions, which are stabilized by adhesive contacts at the leading edge of the cell (Worthylake and Burridge, 2001, Ridley, 2001). The process is regulated by integrins and multiple different intracellular signalling pathways. The mechanism of leukocyte transmigration through the endothelium is not as well known as the initial steps of rolling and adhesion. During the process, endothelial cell junctions may be partially dissassembled, and the leukocyte may interact with junctional proteins during the invasion process, which is probably also guided by chemokines (Bianchi et al, 1997, Johnson-Leger et al, 2000). The CD11a/CD18-integrin (van Epps et al, 1989, Kavanaugh et al, 1991), and the immunoglobulin superfamily members, PECAM-1 (CD31), and JAM-1, in addition to CD99 (Muller et al, 1993, Ostermann et al, 2002, Aurrand-Lions et al, 2002) are crucial for the transmigration step. It has been postulated that some of these proteins, which are positioned in the the cleft between endothelial cells, may interact directly with leukocytes to guide the cell into the junction, and to avoid endothelial layer disruption (Aurrand-Lions et al, 2002). After transmigration, the leukocyte can invade the tissue by a process that requires proteolytic activity (Bianchi et al, 1997).

Lymphocytes also interact with other cell types. T cells interact with APCs in the initiation of an immune response. The formation of the so called immunological synapse (Fig 1) is a multistep process (reviewed in Bromley et al, 2001, Dustin and Cooper, 2000, Dustin and Chan, 2000). It results in a stable supramolecular activation cluster (Monks et al, 1998), containing surface receptors, intracellular signalling proteins, and a cytoskeletal scaffold. The immunological synapse, in contrast to the interaction between lymphocyte and endothelium, can be stable for several hours. The process is again initiated by chemokines, or other signals that trigger the polarization of the T cell. This brings the plasma membranes of the two cells into close proximity, and initiates cell adhesion between the two cells, mediated by CD11a/CD18 and ICAM-1, and other adhesion receptors, such as DC-SIGN and other ICAMs (Bleijs et al, 2001). At this stage, the T cell receives a signal from the T cell receptor (TCR) to stop migration (Dustin et al,

1997). A contact region is formed at the interaction site between T cell and APC, with the integrin in the center and the TCR engaged to peptide-MHC complexes at the periphery. After this, the TCRs are transported to the center of the contact region, together with intracellular signalling proteins like Lck and PKCθ, and the integrins are excluded from this region (Monks et al, 1998). Myosin, PI 3-kinase and coreceptors, like integrins and CD28, are needed for this transport of cell surface receptors to the immunological synapse (Wulfing and Davis, 1998). The immunological synapse achieves the stable structure required for sustained signalling and, ultimately, T cell activation. Alternatively, sequential interaction with several APCs can lead to the same end-result (reviewed by Friedl and Gunzer, 2001).

## 2. CD11/CD18-INTEGRINS

### 2.1. Structure of the CD11/CD18-integrins

*Structure*

The CD11/CD18-integrins belong to the integrin superfamily of cell surface receptors. Integrins are heterodimeric, type 1 transmembrane glycoproteins present on the surface of most cells (reviewed in Hynes, 1992). They mediate adhesion to both the extracellular matrix and to other cells, via binding to diverse ligands. Integrins consist of two polypeptide chains, the α-chain and the β-chain, that are noncovalently linked to each other. 18 known α-subunits and 8 known β subunits associate to form at least 24 known heterodimers (Figure 2), with extremely diverse cell distribution and ligand binding capacities. The CD11/CD18-integrins share the same β-subunit (CD18) but have different α-subunits (CD11a, b, c, d) (reviewed in Gahmberg et al, 1997, Gahmberg, 1997, Harris et al, 2000), and since they are only expressed on leukocytes, they can be termed "leukocyte integrins". The integrin subunits were cloned in the late 1980's (Law et al, 1987, Corbi et al, 1987, Arnaout et al, 1988, Larson et al, 1989). The CD11a/CD18-integrin was first described using monoclonal antibodies that could inhibit cytotoxic T cell killing (Davignon et al, 1981, Sanchez-Madrid et al, 1982) and was found to be enriched on lymphocytes (Kurzinger et al, 1981). The CD11b/CD18-integrin was described to be a receptor for

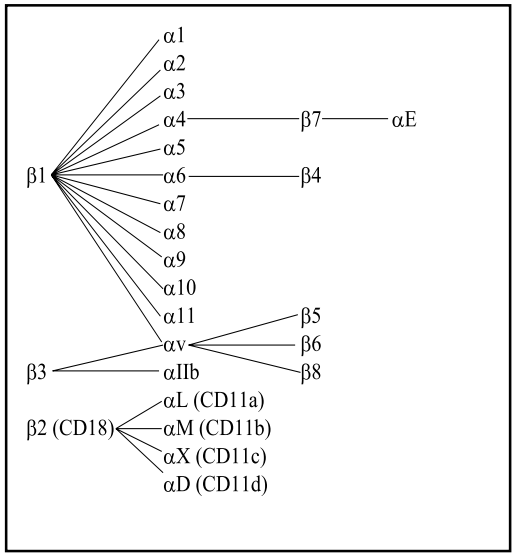


**Figure 2. The integrin superfamily.** Integrins consist of α and β chains in different combinations. At least 24 different heterodimers are known. The β2 or CD18-family is only found in leukocytes and can thus be termed leukocyte integrins.

complement (Beller et al, 1982) and is found mainly in macrophages and polymorphonuclear leukocytes (Springer et al, 1979), and the CD11c/CD18-integrin in monocytes and macrophages (Miller et al, 1986). The CD11d/CD18-integrin was found later, and seems to be expressed mainly on macrophages (Danilenko et al, 1994, van der Vieren et al, 1995), and eosinophils (Grayson et al, 1998).

The integrins consist of a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain (Figure 3). Both subunits contain many extracellular disulphide bonds, and are heavily N-glycosylated, mainly containing high mannose and high molecular weight complex type sugars (Asada et al, 1991). A major advance in the understanding of integrin structure and function was recently made, when the crystal structure of the extracellular region of the $\alpha_V\beta_3$-integrin was determined (Xiong et al, 2001). The "head" of the integrin is made up of a seven-bladed β-propeller domain (homologous to the domain found for example in heterotrimeric G-proteins), interacting with the A (I, inserted)-domain from the β subunit (Figure 3). The ligand binding is proposed to occur at the top of the integrin head. In the CD11-chains, and certain other integrin α-chains, there is an additional I-domain of approximately 200 amino acids (Corbi et al, 1987, Arnaout et al, 1988, Larson et al, 1989). This domain is important for ligand binding, and has been proposed to lie on top of the β-propeller, leaving it accessible for ligand binding (Springer, 1997). The structures of the I-domain of CD11a/CD18 and CD11b/CD18 have been solved (Qu and Leahy, 1995, Lee et al, 1995), and found to be very similar. They have a classical Rossmann fold, with six β-strands surrounded by seven α-helices and containing a MIDAS (metal ion dependent adhesion site) site that can bind both $Mg^{2+}$ and $Mn^{2+}$. The metal ion is necessary for adhesion, and is coordinated by five amino acids in the integrin, while the sixth coordinating residue has been proposed to come from the ligand. The β-chain I-like domain has been shown to adopt a very similar structure to the α-chain I-domain (Xiong et al, 2001).

*Ligand binding sites*

The ligand binding sites in leukocyte integrins has been studied by mutational experiments and by monoclonal antibody-mapping studies. The main ligand-binding sites in the leukocyte integrin extracellular domains are the I domain of the α-chain, the I-like domain in the β-chains and the EF-hand-like motifs in the α-chain (reviewed in Binnerts and van Kooyk, 1999). CD11a/CD18, for example, can bind multiple intercellular adhesion molecules (see below), and the binding sites in the integrin are partially overlapping, but nevertheless structurally distinct (van Kooyk et al, 1996). The I-domain in the α-chain is essential for ligand binding of CD11/CD18-integrins. There is an ICAM-1 and an ICAM-3 binding site in the CD11a/CD18-integrin I-domain (Randi and Hogg, 1994, van Kooyk et al, 1996). Binding of CD11b/CD18 to iC3b, fibrinogen and ICAM-1 occurs via the I-domain, via overlapping but not identical sites (Diamond et al, 1993). However, also the β-propeller domain has been implicated in binding. Deletion of the I-domain abolishes CD11a/CD18-integrin binding to ICAM-1 and 3 ( Leitinger and Hogg, 2000, Yamanchili et al, 2000), and CD11b/CD18-integrin binding to some, but not all, soluble ligands (Yalamanchili et al, 2000). The I-domain is thus absolutely essential for CD11a/CD18-integrin binding to its ligands (while other sites may modulate the interaction), while the more promiscuous CD11b/CD18-integrin also uses other sites for ligand binding.

Also the I-like domain with its MIDAS-motif in the β-subunit is important for ligand binding (Bajt

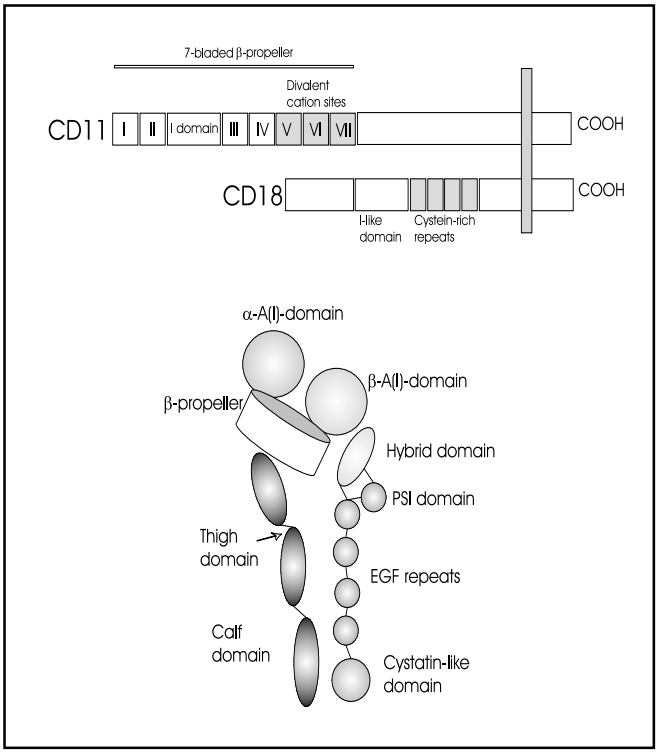


**Figure 3. CD11/CD18-integrin structure.** (A) Schematic structure of the integrin primary structure. (B) Tertiary structure of an I-domain containing integrin, adapted from Humphries and Mould, 2001.

et al, 1995). The coordination of the metal-ion is not identical to the αI-domain, and the βI domain has been found to be in an "open" conformation (Xiong et al, 2001), as opposed to the I-domain in the α-chain, which can adopt both an "open" and a "closed" conformation, with different affinities for ligand (reviewed in Hogg and Leitinger, 2001). Additionally, the βI-domain contains an extra metal-ion binding site (ADMIDAS), that can bind $Ca^{2+}$, conferring additional complexity to ligand binding.

The EF-hand-like domains in the α-subunit have also been implicated in ligand binding (Stanley et al, 1994), but since they are located at the bottom of the β-propeller domain, the interaction with ligand may not be direct (Xiong et al, 2001).

### 2.2. CD11/CD18-integrin ligands and ligand binding

All CD18-integrins bind intercellular adhesion molecules (ICAMs), belonging to the immunoglobulin superfamily (reviewed in Gahmberg et al, 1997, Gahmberg, 1997). At the moment, five different ICAMs are known (Fig 4).

ICAM-1 (reviewed in van de Stolpe and van der Saag, 1996) was the first of the intercellular adhesion molecules to be identified as a ligand for leukocyte integrins (Rothlein et al, 1986, Marlin and Springer, 1987, Patarroyo et al, 1987, Simmons et al, 1988), and revealed, for the first time, an interaction between immunoglobulin and integrin families of adhesion receptors (Staunton et al, 1988). ICAM-1 is a type I transmembrane glycoprotein (see Fig 4). The crystal structure of the first two immunoglobulin (Ig)-domains has been determined (Casasnovas et al, 1998). Only ICAM-1 has been shown to form dimers at

the cell surface (Miller et al, 1995, Reilly et al, 1995, Casasnovas et al, 1998), which may explain the higher binding capacity of ICAM-1 than ICAM-2 and ICAM-3 to CD11a/CD18-integrins (Binnerts et al, 1994). The CD11a/CD18-integrin binding site in ICAM-1 is in the first domain, on a relatively flat binding face, while CD11b/CD18 binds to the third Ig domain of ICAM-1 (Diamond et al, 1991). Also CD11c/CD18 has been reported to bind to ICAM-1 (Blackford et al, 1996). The cytoplasmic domain of ICAM-1 is thought to initiate intracellular signalling (reviewed in Hubbard and Rothlein, 2000) and both ICAM-1 and ICAM-2 bind to the cytoskeletal proteins α-actinin and ezrin (Carpen et al, 1992, Heiska et al, 1996, Heiska et al, 1998, Helander et al, 1996). ICAM-1 is widely expressed and can be transcriptionally upregulated by inflammatory mediators. It is thought to recruit leukocytes into inflamed tissue and plays a crucial role in T cell-APC interactions. (Hubbard and Rothlein, 2000).

Antibodies against ICAM-1 cannot inhibit all leukocyte integrin dependent cell-cell interactions. This led to the speculation that also other ICAMs exist. ICAM-2 was subsequently found (Staunton et al, 1989). It is expressed at high levels on unstimulated endothelium, and is thus implicated in lymphocyte recirculation through lymphatic tissues (Nortamo et al, 1991a, b). ICAM-2 is also expressed at low levels in most leukocytes. It binds to CD11a/CD18 and CD11b/CD18. The crystal structure of the immunoglobulin domains of ICAM-2 has been determined (Casasnovas et al, 1997). The integrin binding site is in the first domain of ICAM-2 and is similar to the binding site in ICAM-1, and has been speculated to dock into a groove in the CD11a/CD18 I-domain (Casasnovas et al, 1999). Very recently, ICAM-2 was found to be a PKB-activating anti-apoptotic factor (Perez et al, 2002).

ICAM-3 is highly expressed on resting leukocytes (de Fougerolles and Springer, 1992), and may thus be involved in initiation of the immune response. Of all ICAMs, it is most closely related to ICAM-1 (Fawcett et al, 1992, de Fougerolles et al, 1993). It binds CD11a/CD18 (de Fougerolles and Springer, 1992) and CD11d/CD18 (van der Vieren et al, 1995). The binding sites for CD11a/CD18 are located on both faces of the first Ig-domain, and thus differ from the corresponding sites in ICAM-1 and ICAM-2, even if some residues implicated in binding are conserved between the ICAMs (Holness et al, 1995, Bell et al, 1998). ICAM-3 interacts with the cytoskeletal protein moesin (Serrador et al, 1997). It has also recently been shown to be crucially involved in the initial, pre-antigen interaction between T cell and APC by binding to CD11a/CD18 (Montoya et al, 2002), and can act as a costimulatory molecule for the initiation of T cell signalling (Montoya et al, 2002, Berney et al, 1999, Hernandez-Caselles et al, 1993, Juan et al, 1994, Bleijs et al, 1999). It has low affinity for CD11a/CD18 as compared to ICAM-1 and ICAM-2, and could instead act as a signalling molecule to strengthen CD11a/CD18-ICAM-1 mediated adhesion (Bleijs et al, 2000).

ICAM-4 was originally named the LW blood group antigen, but was subsequently found to be homologous to the ICAM-family of proteins (Bailly et al, 1994). It is expressed on red blood cells and binds to leukocyte integrins CD11a/CD18 and CD11b/CD18 (Bailly et al, 1995, Hermand et al, 2000). It binds to CD11a/CD18 via its first Ig domain (Hermand et al, 2000). CD11b/CD18, however, binds to both the first and the second domain of ICAM-4 (Hermand et al, 2000). Binding between CD11a/CD18 and ICAM-4 seems to be different than the binding between CD11a/CD18 and ICAM1-3 (Hermand et al, 2000). The physiological functions of ICAM-4 remain to be determined, however, it has been speculated to be involved in red cell turnover (Gahmberg et al, 1997).

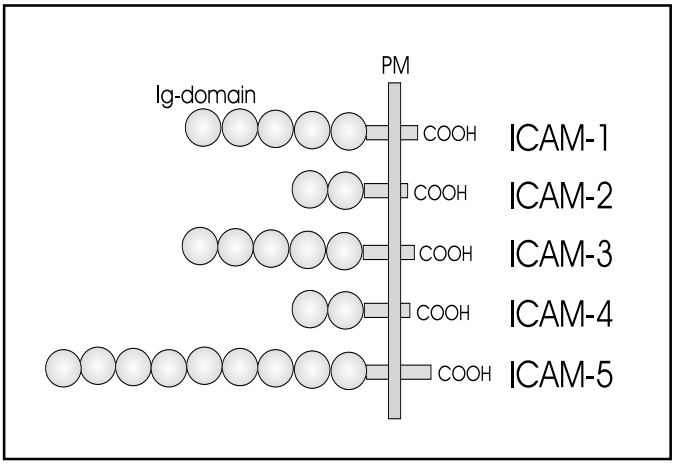


**Figure 4. Schematic structures of the ICAMs.** ICAM-1 (CD54) and ICAM-3 (CD50) have five Ig-domains, while ICAM-2 (CD102) and ICAM-4 (LW) have only two. ICAM-5 (telencephalin) is the largest ICAM, with nine Ig-domains, and is expressed in the brain.

ICAM-5 or telencephalin is the only ICAM expressed in the brain, where it is restricted to the telencephalon (Yoshihara et al, 1994). It binds to CD11a/CD18 (Mizuno et al, 1997, Tian et al, 1997). The main binding site for CD11a/CD18 is in the first domain (Tian et al, 2000a). ICAM-5 plays a role in neurite outgrowth by homophilic interactions (Tian et al, 2000b), and may switch binding partner after oligomerization during development to mediate binding to leukocytes. Interestingly, ICAM-5 has recently been found to interact with presenilins, proteins that have been implicated in Alzheimer's disease (Annaert et al, 2001).

CD11/CD18-integrins have also been found to interact with other proteins. CD11a/CD18 may bind E-selectin (Kotovuori et al, 1993) and it binds type I collagen (Garnotel et al, 1995) and JAM-1, a new ligand for the integrin implicated in transendothelial migration (Ostermann et al, 2002). CD11b/CD18 is the most promiscuous of the leukocyte integrins and binds diverse ligands in addition to the ICAMs. These include iC3b (inactivated complement factor) (Beller et al, 1982, Wright et al, 1983), fibrinogen (Altieri et al, 1988), CD23 (Lecoanet-Henchoz et al, 1995) and factor X (Altieri and Edgington, 1988). CD11c/CD18 binds to iC3b (Bilsland et al, 1994), fibrinogen (Loike et al, 1991), LPS (Ingalls and Golenbock, 1995), CD23 (Lecoanet-Henchoz et al, 1995) and type I collagen, and CD11d/CD18 to VCAM (Grayson et al, 1998).

### 2.3. CD11/CD18-integrin function – leukocyte adhesion deficiency and transgenic models

The role of CD11a/CD18-integrins in T cell cytotoxicity, antigen-stimulated proliferation and T helper cell functions was implied already early on, by the use of integrin-blocking antibodies (Davignon et al, 1981, Sanchez-Madrid et al, 1982, Krensky et al, 1984). The CD11b/CD18- and CD11c/CD18-integrins, on the other hand, were initially described as complement receptors (Beller et al, 1982, Bilsland et al, 1994).

A rare human inherited disease, leukocyte adhesion deficiency-I (LAD-I), emphasizes the importance of leukocyte integrins (reviewed in Arnaout, 1990, Hogg and Bates, 2000). It is characterised by decreased

surface expression levels of CD11/CD18 integrins, and the severity of the disease is determined by the levels of expression or function of the leukocyte integrins. The patients suffer from recurrent bacterial infections, massive leukocytosis, impaired wound healing and severe gingivitis. The main cell type affected is the neutrophils, because they lack significant levels of other integrins to compensate for CD18-integrin loss.

Also targeted gene deletions (so called "knock-outs") of integrin genes in mice have been reported, and these models reveal much about the function of individual integrins *in vivo* (reviewed in Bouvard et al, 2001). CD18 null mice exhibit a phenotype closely resembling human LAD-I (Sharffetter-Kochanek et al, 1998, Mizgerd et al, 1997). The degree of neutrophil extravasation into different tissue depends on the model of inflammation, however, and indicates that other integrins can substitute for CD18-integrins in some circumstances. Inactivation of leukocyte integrin $\alpha$-chains have revealed individual functions for CD11a/CD18 and CD11b/CD18, respectively. Neutrophil adhesion to endothelium occurs mainly via CD11a/CD18, and less via CD11b/CD18 (Ding et al, 1999). Leukocytes lacking CD11a/CD18 can still fight viral infection, but T cell proliferation and cytotoxicity in response to alloantigen are severely impaired, implicating additional roles for CD11a/CD18-integrins (Schmits et al, 1996, Shier et al, 1996). CD11a/CD18-deficiency in mice leads to unability to reject tumors (Schmits et al, 1996, Shier et al, 1996, Shier et al, 1999) and CD11a/CD18-integrins are also important for T cell recirculation (Andrew et al, 1998, Berlin-Rufenach et al, 1999). CD11b/CD18-deficient neutrophil adhesion to endothelium, phagocytosis and degranulation is impaired (Coxon et al, 1996, Lu et al, 1997), and in addition, mast cell number in certain locations is reduced and their function is impaired (Rosenkranz et al, 1998). Interestingly, CD11b/CD18 knockout mice also revealed an unexpected phenotype; the mice are obese (Dong et al, 1997). The same phenotype was seen for the ICAM-1 knockout, indicating that this receptor-ligand pair somehow regulates adipose tissue metabolism (Bouvard et al, 2001).

CD11c/CD18 and CD11d/CD18-integrin knockouts have not been reported. CD11c/CD18 is thought to be involved in adhesion of monocytes and neutrophils to endothelium, other cells and substrates, similarly to CD11b/CD18-integrins, and has also been reported to mediate T cell homotypic aggregation (Blackford et al, 1996). The CD11d/CD18 integrin is still being characterized.

### 2.4. Regulation of CD11/CD18-integrins

*Integrin avidity regulation*

The mechanism of CD11/CD18-integrin activation has been most extensively studied for the CD11a/CD18-integrin (reviewed in Stewart and Hogg, 1996). Even if the exact mechanism remains to be determined, there are emerging paradigms for the regulation of adhesion through this integrin. CD11a/CD18 is not active on resting cells. Activation of T cells through the TCR or with phorbol ester, cell permeable analogs of diacyl glycerol, leads to increased adhesion to ICAM-1 coated on plastic (Dustin and Springer, 1989) or on microspheres (Welder et al, 1993, Pyszniak et al, 1994), and to increased integrin-ICAM-mediated cell-cell-adhesion (Patarroyo et al, 1985, Rothlein and Springer, 1986). TCR-induced integrin activation is transient and dependent on temperature and intracellular signalling (see below). It is regulated on the level of CD11a/CD18-integrin (rather than ICAM-1) avidity for its ligand.

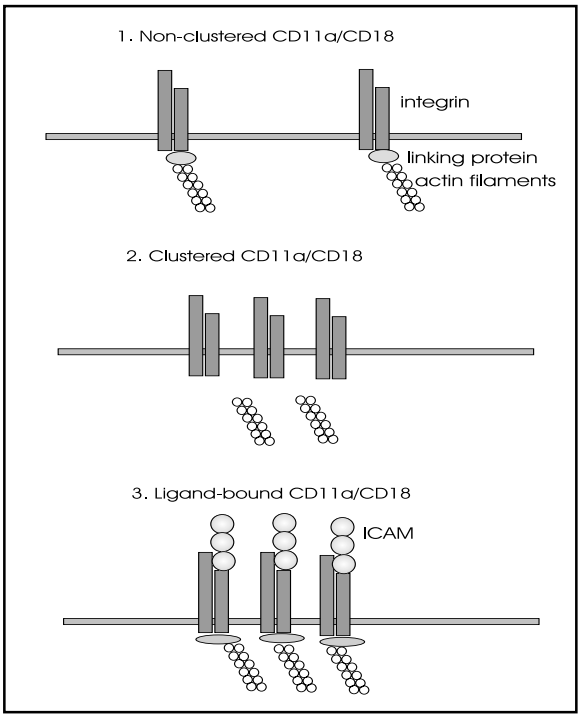


**Figure 5. Schematic model of integrin avidity regulation**, adapted from van Kooyk and Figdor, 2000. In resting cells (1), integrins are locked in a non-active conformation by interactions with the actin cytoskeleton through linking proteins. When cells are activated (2), the cytoskeletal restraint is released, and the integrins can move freely on the surface and cluster. Subsequently (3), integrins are ligand bound and have reattached to the actin cytoskeleton, possibly through a different linker protein. Additionally, changes in affinity and conformation may occur both before and after ligand binding.

The cell surface expression of the integrin or the ligand was not changed during the functional upregulation (Dustin and Springer, 1989). Different monoclonal antibodies that recognize so called "activation epitopes" have been used extensively to detect activated integrins on the cell surface, and they have been thought to detect high-affinity integrins. However, Bazzoni and Hemler (1998) have emphazised that these epitopes do not always correlate with high affinity binding, and thus soluble ICAM-1 (sICAM-1) binding is a more correct way of measuring integrin affinity. Using this method, it has been established that neither TCR engagement nor phorbol ester or calcium ionophores induce affinity changes in the CD11a/CD18-integrin (Stewart et al, 1996, Stewart et al, 1998, Kotovuori et al, 1999).

The mechanism of CD11a/CD18-integrin activation after TCR-engagement seems to involve the actin cytoskeleton. CD3- or phorbol ester-induced T cell adhesion to coated ICAM-1 is abolished by treatment of cells with high concentrations of cytochalasin-D, which disrupts actin filaments, and both CD3 and phorbol ester activation induce cell spreading (Stewart et al, 1996). On the other hand low concentrations of cytochalasin-D actually activate cell adhesion, and have been shown to increase motility of the CD11a/CD18-integrin on the cell surface (Kucik et al, 1996). The lateral mobility of integrins on the cell surface is 10-fold increased after cell activation with phorbol ester. In addition, phorbol ester, calcium ionophore and CD3-antibodies all induce clustering of CD11a/CD18-integrins (Stewart et al, 1998). Based on these results, a model has been suggested (Lub et al, 1995, Stewart et al, 1998, van Kooyk et al, 1999, reviewed in van Kooyk and Figdor, 2000), in which integrins are maintained in a low-avidity state on the resting cell surface by tethering of the integrin to the actin cytoskeleton (Figure 5). Disruption of this linkage is required for the integrin to move freely on the cell surface, to cluster and

increase probability of ligand encounter. The actin cytoskeleton appears to play a dual role in integrin regulation, as a new actin-integrin linkage probably needs to be formed subsequent to ligand binding, to allow cells to spread, and to maintain cell adhesion. This would explain the effect of different concentrations of cytochalasin D on cell adhesion. Integrins have been shown to associate with the cytoskeleton after cell activation (Pardi et al, 1992), and cell spreading has been implicated in adhesion to ICAM-1 in some, but not all cases (Peter and O'Toole, 1995, Stewart et al, 1996, van Kooyk et al, 1999). Cell spreading could influence adhesion in multiple ways, by moulding the two surfaces to fit, for example, or by reducing shear applied to the cells in the non-spread state. The use of cell spreading to promote adhesion possibly depends on whether coated ligand or ligand microspheres are used as the adhesive substrate.

Also the dynamic microtubule cytoskeleton has recently been implicated in integrin activation and cell spreading (Zhou et al, 2001). Another possible mechanism of regulating integrin distribution on the cell surface and ligand binding is by clustering of so called membrane rafts, specialized lipid domains in the plasma membrane that are enriched in cholesterol and certain membrane proteins and could serve as signalling platforms in T cell activation. Indeed, integrins are excluded from lipid rafts in resting cells, but clustering of membrane rafts leads to activation of CD11a/CD18-integrin binding to ICAM-1, and activated integrins seem to reside in rafts (Krauss and Altevogt, 1999, Leitinger and Hogg, 2002). The actin cytoskeleton plays an active role in this process.

*Integrin affinity regulation*

Chemokines induce very rapid and transient activation of integrins, triggering the firm adhesion of leukocytes interacting with blood vessel endothelium under flow (Campbell et al, 1998). This form of adhesion has different features as compared to the slower and more sustained integrin-mediated adhesion that occurs, for example, in T cell-APC contact formation. The chemokines SLC, ELC and SDF-1$\alpha$ have been shown to induce arrest of lymphocytes on vascular endothelium, and this process is dependent on CD11a/CD18-integrins (Constantin et al, 2000). Interestingly, these chemokines also trigger binding of sICAM-1 to the integrin, indicating that an affinity-increase takes place (Constantin et al, 2000). In addition, the chemokines trigger redistribution of CD11a/CD18-integrins on the cell surface to clusters similar to those seen in TCR-induced activation of integrins, and also this may contribute to the increased adhesion. However, it appears only to do so at low, but not high, ligand density, and to contribute to adhesion rather than to play the major role in it (Constantin et al, 2000). It seems plausible that the different modes of integrin activation predominate in different biological contexts. For certain other integrins, affinity regulation seems to be the main mechanism of activation (reviewed in Hughes and Pfaff, 1998).

In addition to chemokine-induced affinity-increases, it is believed that conformational changes occur in the integrin after ligand binding (Cabanas and Hogg, 1993, Kotovuori et al, 1999). This may stabilize the integrin-ligand interaction. Indeed, very high amounts of sICAM-1 has been shown to block binding of of an integrin-binding antibody to phorbol ester-activated T cells, indicating an affinity-increase in the integrin (Lollo et al, 1995). Since phorbol esters do not induce affinity-changes in CD11a/CD18 (Stewart et al, 1996), the effect may be due to high concentrations of sICAM-1 inducing a conformational change

in the CD11a/CD18-integrin leading to integrin activation. Additionally, it has been shown that a peptide derived from ICAM-2 can bind to both CD11a/CD18- and CD11b/CD18-integrins and induce binding of sICAM-1 to the integrin (Li et al, 1993a, b, Li et al, 1995, Kotovuori et al, 1999). Alternatively, ligand binding may induce intracellular signalling that leads to activation of other CD11/CD18-integrins on the same cell, inducing binding to the same or different ICAMs (reviewed in Bazzoni and Hemler, 1998, Soede et al, 1998, Soede et al, 1999, Kotovuori et al, 1999, Bleijs et al, 2000).

Certain monoclonal antibodies to integrin extracellular domains seem to stabilize a high-affinity form of the integrin and thus lead to integrin-ligand binding through an extracellular effect (Keizer et al, 1988, van Kooyk et al, 1991, Andrew et al, 1993, Landis et al, 1993, Petruzzeli et al, 1995). Also other changes that contribute to cell-cell adhesion take place either after, or independently of, ligand binding. It should be emphasized that ligand binding on its own is not enough to cause cell-cell adhesion, which is a more complex, multistep process.

Extracellular $Mg^{2+}$ ions can induce T cell adhesion to coated ICAM-1 without intracellular signalling. This activation-protocol has been shown to induce a high-affinity form of CD11a/CD18, which can bind to sICAM-1 (Cabanas and Hogg, 1993, Stewart et al, 1996). Divalent cations also have other functions in integrin regulation (reviewed in Leitinger et al, 2000). $Ca^{2+}$ seems to have a dual role. It can act both as an activator of adhesion, which occurs via receptor clustering (van Kooyk et al, 1994) and as an inhibitor of adhesion (Dransfield et al, 1992). This may be explained by the presence of multiple $Ca^{2+}$-binding sites in the integrin extracellular domain (Gahmberg et al, 1988), as revealed by the crystal structure (Xiong et al, 2001). However, it is likely that divalent cations play a structural, rather than a regulatory role in adhesion.

Table 1. summarises the different modes of CD11a/CD18-integrin activation and their characteristics.

| **Activating agent** | **sICAM-1 binding** | **integrin clustering** | **intracellular signalling** | **cytoskeleton, cell spreading-dependency** |
|---|---|---|---|---|
| TCR crosslinking | no | yes | yes | yes |
| phorbol ester | no | yes | yes | yes |
| calcium ionophore | no | yes | yes | yes |
| chemokines | yes | yes | yes | calpeptin inhibits |
| $Mg^{2+}$ | yes | no | no | no |
| ligand | yes | N. D. | no | yes |

**Table 1. Characteristics fo CD11/CD18-integrin activation by different activating agents.**

## 3. BIDIRECTIONAL SIGNALLING THROUGH CD11/CD18-INTEGRINS

### 3.1. Cytoplasmic domains in the regulation of CD11/CD18 integrin function

*Integrin cytoplasmic domains*

The cytoplasmic domains of integrins are short and devoid of any known enzymatic activity, but nevertheless play important roles in the regulation of integrin function (Hibbs et al, 1991, Ylänne et al,

1995, Bodeau et al, 2001, reviewed in Ylänne, 1998). The integrin β-chains show a high degree of sequence homology, while the α-chains are highly conserved between species, but not homologous to each other, except for the membrane proximal KXGFFKR-sequence. The integrin β-chains have both unique and conserved functions, while α-chains may play specific roles, unique for each integrin.

A structural model has been developed for the $\alpha_{IIb}\beta_3$-integrin cytoplasmic domain (Haas and Plow, 1997). The β-chain structure was predicted to have a β-turn at its NPXY-motif (β3 744-747), and to be α-helical in the membrane proximal region. The two cytoplasmic tails of the integrin could interact via a number of possible docking sites, and the juxta-membrane region of $\beta_3$ was found to be conformationally labile, adopting a number of different structures. Additionally, because of the β-turn in the β-cytoplasmic domain, the C-terminus of the integrins resides in close proximity to this labile region. The GFFKR-region of the α-chain may also be interacting with this region. Stabilizing any of these structures by interaction with different proteins may be a way of regulating integrins. A salt bridge has also been proposed to exist between the α and the β subunit membrane proximal domains, locking the integrin in an inactive state (Hughes et al, 1996).

*The CD18 cytoplasmic domain*

The integrin CD18 cytoplasmic domain has been studied by mutational analysis (Figure 6). It has been shown to be essential for activation of the integrins (Hibbs et al, 1991). Truncation of the cytoplasmic domain eliminates binding to ICAM-1, while complete deletion results in spontaneous clustering and activation of CD11a/CD18. More specifically, a phenylalanine at position 754 (Fabbri et al, 1999) and 766 (Hibbs et al, 1991) and a threonine triplet (758-760) (Hibbs et al, 1991) are important for binding to ICAM-1. The mutation of the TTT-domain decreases the default adhesion to ICAM-1 but adhesion can still be stimulated by phorbol ester, except if all of the threonines are mutated (Hibbs et al, 1991). A TTT to AAA mutation additionally inhibits reorganisation of the actin cytoskeleton and cell spreading, so called integrin postreceptor events (Peter and O'Toole, 1995), but does not influence integrin affinity (Peter and O'Toole, 1995) or integrin-mediated transendothelial migration (Weber et al, 1997).

Expressing integrins in different cells may confer different phenotypes to the integrins. For example, CD11a/CD18-integrins expressed in the erythroleukemic cell line K562 cells cannot be activated by phorbol ester, while exchanging the CD18 cytoplasmic domain to the $\beta_1$ cytoplasmic domain restores the PMA-responsiveness of the integrin (Lub et al, 1997, Bleijs et al, 2001). This implies that different integrin β-chains have different functions, and that lymphocyte-specific regulatory elements may exist. Interestingly, a single amino-acid exchange of $\beta_1$-$\beta_2$ (L732R) in the cytoplasmic domain of CD18, conferred calpain-dependent PMA-responsiveness to the integrin in K562-cells (Bleijs et al, 2001). However, this was also dependent on other residues in the integrin cytoplasmic tail, i.e. Thr758.

Arg733-Lys742 (the so called cyto1-sequence in $\beta_1$) is important for cytoskeletal association, ER retention of uncomplexed integrins, assembly of heterodimers and cell surface expression of integrins (Pardi et al, 1995). More specifically, Tyr735 in the cyto1-region has been shown to be important for recycling of receptors to the plasma membrane (Fabbri et al, 1999), while Phe754 in the cyto2-region is important for cell migration (Fabbri et al, 1999).

Interestingly, it has recently been shown that the C-terminal NPXF-motif, and especially Phe766, is

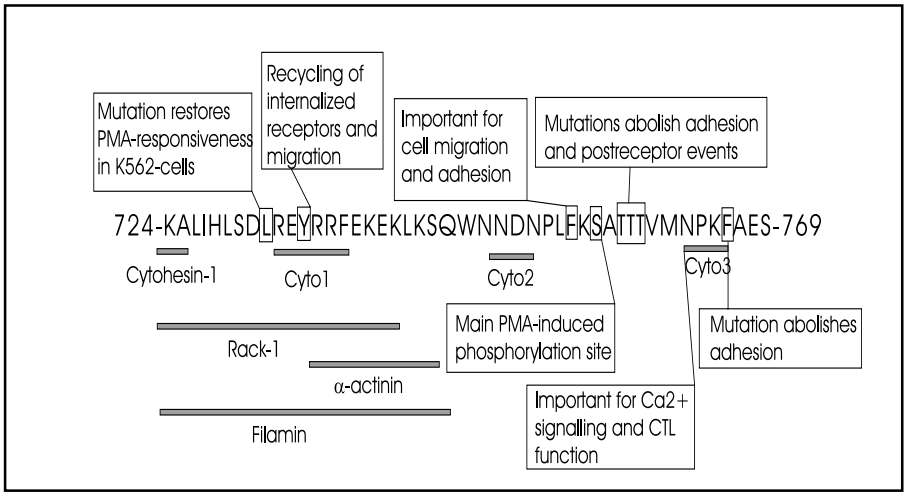


**Figure 6. Schematic figure of the CD18 cytoplasmic domain.** The areas in the CD18 sequence implicated in integrin functions and binding to cytoplasmic proteins are indicated. Cyto1, 2, 3, areas implicated in integrin function in other integrins (reviewed in Ylänne et al, 1998).

important for calcium-signalling and cytotoxic killing of target cells in response to clustering of integrin cytoplasmic domains (Sirim et al, 2001). This is the first report describing areas in the CD18 cytoplasmic domain involved in outside-in signalling functions of the integrin.

*The CD11 cytoplasmic domains*

The CD11/CD18-integrin α-chains have been less extensively studied. Interestingly, CD11a and CD11b cytoplasmic domains have been shown to have unique functions in chemokine-induced activation of the CD11/CD18-integrins (Weber et al, 1999). The conserved GFFKR-motif in the membrane-proximal region of the α-chain is believed to be involved in maintaining the integrin in a low-affinity form by interacting with a corresponding region in the integrin β-chain, since distruption of this region leads to constitutive activation of the integrin and a high affinity/avidity form (Peter and O'Toole, 1995, Lu and Springer, 1997, van Kooyk et al, 1999). However, truncations after this motif resulted in reduced binding to ICAM-1 after PMA-stimulation (Lu and Springer, 1997). Additionally, the GFFKR-motif is important for stability of the integrin heterodimer, maybe by direct interaction with the β-chain cytoplasmic domain (Pardi et al, 1995). Interestingly, neither constitutively active (lacking the GFFKR-sequence) or inactive (truncated after the GFFKR-sequence) integrins could support transendothelial migration (Weber et al, 1997), indicating that dynamic avidity modulation is important for cell migration. The α-chain cytoplasmic domain is not needed for cytoskeletal attachment of the integrins (Pardi et al, 1995).

### 3.2. Binding of intracellular proteins to CD11/CD18-integrin cytoplasmic domains

The regulatory role of integrin cytoplasmic domains is presumably mediated by their binding to other proteins. A lot of research has focused on identifying factors interacting specifically with the integrin cytoplasmic tails, and especially factors that are cell type specific, since regulation of integrin-mediated adhesion is a cell type-specific events (Lub et al, 1997). Integrins interact with a variety of intracellular proteins (see table 1) (reviewed in Liu et al, 2000). The CD11/CD18-integrins have been described to

interact with several different proteins, which presumably mediate different functions of the integrins. Interestingly, no proteins binding specifically to either the C-terminal region of the integrin containing the important threonine-triplet and the NPXF-motif involved in calcium-signalling, or the α-tails of the CD11/CD18-integrins have yet been described, although calreticulin has been proposed to bind to the conserved GFFKR-motif in integrin α-subunits.

*Cytoskeletal proteins*

Activation of integrins could potentially be influenced by the cell cytoskeleton in multiple ways (Calderwood et al, 2000). It could increase the local concentration or activity of certain factors in the proximity of integrins, regulate the integrin activation state directly or regulate integrin avidity by clustering integrin or aligning membranes of the opposing cells. The cytoskeletal protein α-actinin interacts directly with the CD18 cytoplasmic domain both *in vitro* and *in vivo* in neutrophils activated with the chemotactic peptide fMLP-, but not by the phorbol ester PMA (Pavalko and LaRoche, 1993). The α-actinin-binding site in CD18 has been mapped to residues 736-746 (Sampath et al, 1998). Also filamin interacts directly with CD18, binding to residues 724-247, but the binding sites for α-actinin and filamin are not identical (Sharma et al, 1995).

Talin has also been described to interact with the CD18 cytoplasmic tail, but the interaction site in CD18 has not been mapped (Sampath et al, 1998). In a model of the regulation of CD11a/CD18-integrin mediated PMN adhesion in response to fMLP, talin interacts with the CD18 tail in resting cells. After cell activation, talin dissociates from the CD18-tail and CD18 reassociates with α-actinin (Sampath et al, 1998). This could explain the dual role of the cytoskeleton in regulation of adhesion: inhibition of adhesion in unstimulated cells, and stimulation of adhesion in activated cells.

*Signalling proteins*

Several signalling proteins have been identified that interact with integrin cytoplasmic domains. Cytohesin-1 was initially identified in a two-hybrid screen of the CD18 cytoplasmic domain (Kolanus et al, 1996), and was subsequently identified as a member of the ARF-GEF-family (guanine nucleotide exchange proteins for ADP-ribosylation factor) (Meacci et al, 1997). The ARFs are small GTPases that mediate vesicle trafficking and possibly actin remodelling in cells. The binding site for cytohesin-1 in the CD18-cytoplasmic domain was mapped to the membrane proximal part of the integrin (723-731), and a triple mutation (WKA723-725TRG) failed to interact with cytohesin-1 (Geiger et al, 2000). The same mutation inhibited cell adhesion to ICAM-1, suggesting that the direct binding of cytohesin-1 to integrins may regulate adhesion. Interestingly, overexpression of cytohesin-1 or its Sec7-domain leads to an increase in CD11a/CD18-integrin mediated binding to ICAM-1, while its pleckstrin homology (PH)-domain inhibited adhesion (Kolanus et al, 1996). Cytohesin-1 and its PH-domain also plays a role in leukocyte arrest on endothelium (Weber et al, 2001), while transmigration also requires the GEF-activity (Weber et al, 2001). Additionally, the ARF-GEF activity was shown to be necessary for integrin mediated adhesion and cell spreading (Geiger et al, 2000). Active phosphoinositide 3-OH (PI 3)-kinase enhances membrane association of cytohesin-1 via its PH-domain (Nagel et al, 1998), indicating that this lipid kinase acts upstream of cytohesin-1 in inducing adhesion. Interestingly, phorbol ester has been shown to induce

| Protein | Integrin chain | Binding site | Reference |
|---|---|---|---|
| calreticulin | α-tails | KXGFFKR | Coppolino et al, 1997 |
| filamin | β1, β2, β7 | 724-747 | Sharma et al, 1995, Pavalko et al, 1998 |
| α-actinin | β2, β1 | 736-746 (β2)<br>762-774 (β1) | Pavalko and LaRoche, 199, Sampath et al, 1998 |
| Rack-1 | β1, β2, β3 | 724-743 (β2) | Lilienthal and Chang, 1998 |
| 14-3-3 | β1 | 776-790 | Han et al, 2001 |
| talin | β1A, β1D, β2, β3, β7 | membrane distal, several areas | Knezevic et al, 1996 Sampath et al, 1998, Pfaff et al, 1998, Calderwood et al, 2001 |
| ILK | β1, β2, β3 | N. D. | Hannigan et al, 1996 |
| JAB-1 | β2 | N. D. | Bianchi et al, 2000 |
| FAK | β1, β2, β3 | membrane proximal | Schaller et al, 1995 |
| paxillin | β1, β3, α4, α9 | β subunit membrane proximal, α subunit 983-991 | Schaller et al, 1995, Tanaka et al, 1996 Liu et al, 1999, Young et al, 2001 |
| Phospholipase Cγ | β1 | conserved membrane proximal region, α-chain GFFKR | Vossmeyer et al, 2002 |
| myosin | β3 | TyrP β3 | Jenkins et al, 1998 |
| Shc | β3 | TyrP β3 | Cowan et al, 2000 |
| DRAL/FHL2 | α3A, α3B, α7A, β | β: C-terminal NXXY<br>α: 12 aa next to membrane proximal | Wixler et al, 2000 |
| cytohesin 1 and 3 | β2 | 723-725 | Kolanus et al, 1996, Geiger et al, 2000 |
| β3 endonexin | β3 | membrane distal | Shattil et al, 1995 |
| ICAP-1 | β1 | 785-799 | Chang et al, 1997 Zhang and Hemler, 1999, Degani et al, 2002 |
| PI 3-kinase | β1 | pTyr-peptide | Johansson et al, 1994 |
| CIB | αIIb | N. D. | Naik et al, 1997, Tsuboi, 2002 |
| IRS-1 | αVβ3 | N. D: | Vuori and Ruoslahti, 1994 |
| melusin | β1A, β1D | 26 aa membrane proximal | Brancaccio et al, 1999 |
| MIBP | β1A, β1B, β1D | membrane proximal | Li et al, 1999 |
| Mss4 | α2, α3A, α5, α6A | KXGFFKR | Wixler et al, 1999 |
| BP180 | β4 | several areas | Schaapveld et al, 1998 |
| plectin | β4 | multiple regions | Rezniczek et al, 1998 |
| skelemin | β1, β3 | membrane proximal | Reddy et al, 1998, Reddy et al, 2001 |
| WAIT-1 | β7, α4, αE | 729-737 in β7 | Rietzler et al, 1998 |
| TAP-20 | β5 | N. D. | Tang et al, 1999 |
| nischarin | α5 | N. D. | Alahari et al, 2000 |

**Table 2. Proteins interacting with integrin cytoplasmic tails**.

phosphorylation of cytohesin-1, and the phosphorylation may mediate the interaction of cytohesin-1 with the actin cytoskeleton (Dierks et al, 2001), adding another level of possible regulation of integrins by cytohesin. The downstream element of cytohesin-1 ARF-GEF-mediated functions remains to be identified.

Rack-1, the receptor for activated protein kinase C, is an adaptor protein for PKCβ, which is involved in shuttling the activated enzyme in cells (Ron et al, 1999). It has been reported to bind directly to integrin β cytoplasmic tails ($\beta_1$, $\beta_2$ and $\beta_5$) in the membrane-proximal region, and to associate with CD18-integrins in PMA-stimulated leukocytes (Lilienthal and Chang, 1998). This could potentially position the activated kinase in the direct proximity of the integrin and thus regulate both inside-out and outside in signalling. However, other reports have questioned the specificity of the Rack-1-integrin β-chain interaction, since Rack-1 was also found to interact with $\alpha_V$ and $\alpha_4$ cytoplasmic domains (Zhang and Hemler, 1999). An alternative explanation is that both subunits of integrins interact with the cytoplasmic protein, and differentially regulate the binding, as has been reported for PLCγ binding to $\alpha_1\beta_1$ and $\alpha_5\beta_1$-integrin (Vossmeyer et al, 2002).

JAB-1 (Jun activation domain-binding protein-1) is a transcriptional coactivator which interacts directly with the CD18 cytoplasmic tail (Bianchi et al, 2000). This potentially couples the integrin-ICAM-1 interaction directly to the regulation of gene transcription.

*Lateral associations of integrins*

Integrins also interact laterally with other membrane proteins (reviewed in Porter and Hogg, 1998, Woods and Couchman, 2000), like tetraspanins, syndecans and growth factor receptors, and these interactions may modulate integrin-mediated cellular functions. Growth factor receptors have not so far been shown to interact with the leukocyte integrins, but the CD11b/CD18-integrin associates *in cis* with several other membrane proteins (Porter and Hogg, 1998). These include the tetraspanin protein CD63 (Skubitz et al, 1996), FcγRIIIB (which is involved in phagocytosis), CD14 (a GPI-linked protein which binds bacterial lipopolysaccharide) and uPAR (which is also a GPI-linked protein and regulates pericellular proteolysis and migration, Xue et al, 1994, Bohuslav et al, 1995). Additionally, CD11a/CD18 has been implicated in an interaction with the leukocyte adhesion molecule DNAX accessory molecule-1 (DNAM-1), which is necessary for DNAM-1 function, but whether this link is direct or indirect is not known (Shibuya et al, 1999). These different membrane proteins may regulate/collaborate with CD11/CD18-integrin in different ways, by forming functional units with the integrin and associated intracellular/extracellular proteins, or by directing the integrins to specialized membrane domains.

### 3.3. Inside-out signalling through CD11/CD18-integrins

3.3.1. Introduction

Integrin inside-out signalling is the process where ligation of different cell surface receptors initiates intracellular signalling events that ultimately result in increased integrin binding to ligands. Multiple signalling pathways are involved in inside-out signalling through CD11/CD18-integrins. They differ for different integrin family members, cell types and activating stimuli. It can also be assumed that affinity-

and avidity regulation of integrins involve different mechanisms and different signalling pathways. In addition to signalling that activates the binding of integrin to ICAM-1, additional signalling pathways are involved in adhesion-strengthening events (Petruzzelli et al, 1998). The importance of signalling in the regulation of adhesion has been emphasized by the discovery of an alternative form of LAD, in which the integrins are normally expressed on the cell surface, but cannot become activated by inside-out signalling. This is presumably due to a signalling defect, since integrin activation can still be achieved by divalent cations, but the defective pathway is so far unknown (Hogg et al, 2001). For now, the main ways of studying signalling pathways involved in integrin signalling rely on the use of cell-permeable inhibitors and activators of different signalling pathways, transfection experiments with constitutively active or inactive signalling components, and targeted gene deletions of signalling components. A combination of these approaches has been used to identify different pathways involved in regulating CD11/CD18-integrin mediated adhesion. The main emphasis will be put on describing inside-out signalling initiated by the TCR.

### 3.3.2. Activation of leukocyte integrins by cell surface receptors

Adhesion of leukocytes is triggered by a number of different surface receptors. Several of these stimulating receptors can be assumed to be involved in regulating T cell-APC contact. The initial finding was that triggering of the TCR induces CD11a/CD18-integrin binding to ICAM-1 (Dustin and Springer, 1989, van Kooyk et al, 1989). Since then, ligation of numerous other leukocyte surface proteins has been shown to be involved in regulating adhesion, however, for many of them, the signalling events induced and the functional importance is not known. Among others, ligation of CD2 (van Kooyk et al, 1989), CD44 (Koopman et al, 1990, Vermot-Desroches et al, 1995), CD45 (Spertini et al, 1994, Lorenz et al, 1994), CD98 (Suga et al, 2001), CD73 (Airas et al, 2000) and the interleukin-2 (IL-2)-receptor (Nielsen et al, 1996) have been shown to induce CD11/CD18-integrin-mediated adhesion.

In addition to these signals, multiple inflammatory mediators have been shown to induce adhesion, which is presumably relevant in the recruitment of leukocytes to the site of tissue inflammation. Several different chemokines, including SDF-1, fMLP and MIP-3β have been shown to initiate rapid, CD11a/CD18-integrin mediated T cell adhesion to endothelium (Campbell et al, 1998, Constantin et al, 2000). This process occurs through the activation of pertussis-sensitive heterotrimeric G-proteins. IL-8 triggers firm adhesion of monocytes to endothelium (Gerstzen et al, 1999). Also direct activation of heterotrimeric G-proteins by $AIF_4$- trigger activation CD11a/CD18-integrin-mediated adhesion to ICAM-1 (Driessens et al, 1997). L-selectin, P-selectin and $\alpha_4\beta_1$-integrins can activate CD11a/CD18-integrins, which may bypass or contribute to chemokine-induced arrest on endothelium (Worthylake and Burridge, 2001).

CD14 is the receptor for bacterial lipopolysaccharide, and CD14-engagement has been shown to induce both CD11a/CD18- and CD11b/CD18-dependent adhesion in monocytes and polymorphonuclear leukocytes (Lauener et al, 1990, Hmama et al, 1998, Detmers et al, 1998). It is worthwhile noting that both CD14 and CD73 are GPI-linked receptors, and clustering them with antibodies may be involved in regulating lipid rafts on the plasma membrane. However, the role of rafts in the regulation of adhesion by these receptors has so far not been investigated.

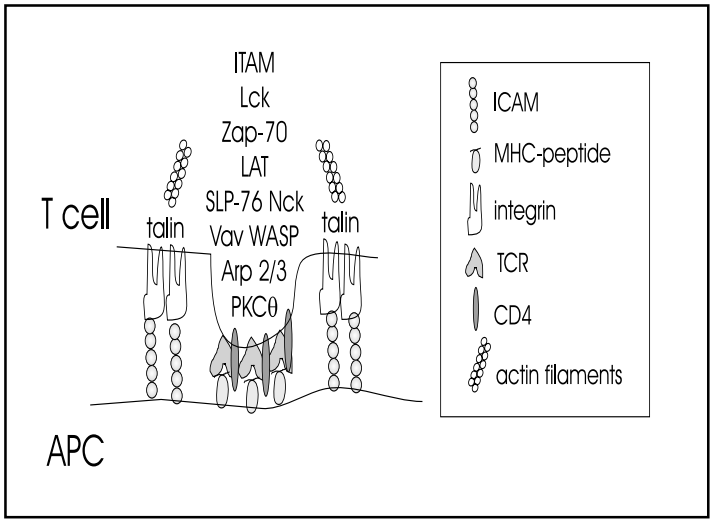


**Figure 7. The mature immunological synapse.** The engaged TCR-molecules are positioned in the center of the synapse. Lck phosphorylation of ITAMs leads to the recruitment of ZAP-70 to the synapse. Initial tyrosine phosphorylation events then lead to the recruitment of adapter proteins, like LAT and SLP-76. SLP-76 recruits Vav (a GEF for Rac and Cdc2) and Nck, and subsequently WASP is recruited. These proteins are involved in the recruitment and activation of the crucial Arp2/3 complex, which then initiates actin polymerization. Myosin (not shown) is thought to be involved in the contraction of the actin network to form the central cluster of engaged TCRs. Then, other signalling proteins, like PKCθ, are selectively recruited to the actin scaffold. The CD11a/CD18-integrin is positioned in the periphery of the synapse, linking to the actin cytoskeleton via talin, a cytoskeleton-membrane linking protein (adapted from Bromley et al, 2001, Dustin and Cooper, 2000).

### 3.3.3 Summary of TCR signalling

TCR engagement by peptide-MHC and subsequent signalling is associated with the formation of an immunological synapse, a stable organisation of membrane receptors, cytoskeletal components and intracellular signalling proteins (Fig 7). This is the result of cytoskeletal reorganisation, raft clustering and recruitment of other signalling components to these structures (Dustin and Cooper, 2000, Bromley et al, 2001). Crosslinking of the TCR initially activates tyrosine kinases, and tyrosine phosphorylation events leads to recruitment of different adapter proteins (proteins devoid of intrinsic enzymatic activity that instead mediate protein-protein interactions) to the vicinity of the TCR. This is followed by the activation of phospholipase C, that initiates $Ca^{2+}$ signalling and protein kinase C activation, and PI 3-kinase, that leads to recruitment of PH-domain containing proteins to the plasma membrane. Small GTP-binding proteins, like Ras and proteins of the Rho-family, are also activated, and can initiate MAP kinase pathways and rearrangement of the actin cytoskeleton. Several signalling pathways, the main ones being PI 3-kinase, PKC and Rap-1, have been implicated in leukocyte integrin activation (Fig 8). The pathways may converge in the vicinity of the integrin to regulate common downstream elements.

### 3.3.4. Signalling pathways involved in CD11/CD18-integrin activation

*Lck and other tyrosine kinases*

The triggering of the TCR initially leads to the activation of protein tyrosine kinases like Lck. Lck plays a fundamental role in T cell activation (Straus and Weiss, 1992), and initiates very early signalling

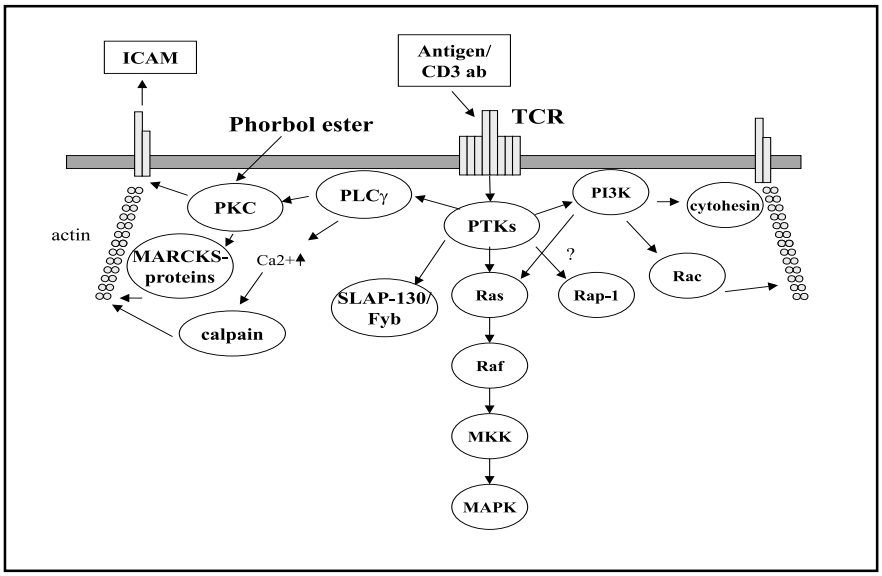


**Figure 8. Schematic figure of signalling pathways implicated in CD11/CD18-integrin activation in T cells.** T cells are activated through the TCR by binding to peptide-MHC or with TCR-ligating antibodies. This leads to protein tyrosine kinase (PTK) activity and recruitment of adapter proteins to the plasma membrane, resulting in the activation of multiple downstream pathways. SLAP-130/Fyb is an adapter protein that regulates integrin clustering on the cell surface. PLC generates DAG and $IP_3$, activating PKC and $Ca^{2+}$-signalling, respectively. Both have been implicated in integrin regulation, possibly through regulation of MARCKS and calpain. The Ras-MAPK pathway is activated downstream of PTK-signalling and may also play a role in integrin activation. PTK activity also results in PI 3-kinase activation, and recruitment of PH-domain containing proteins to the plasma membrane. Cytohesin and Rac are downstream mediators of PI 3-kinase implicated in integrin activation. Multiple signalling pathways have been implicated in regulation of the actin cytoskeleton, that plays a crucial role in integrin regulation. Rap-1 is activated in T cells after MHC-peptide stimulation and is important for integrin regulation.

events in T cells, like $Ca^{2+}$ signals and tyrosine phosphorylation (reviewed in Dustin and Chan, 2000). Lck is dynamically regulated by positive and negative tyrosine phosphorylation events (Hermiston et al, 2002). After activation, Lck phosphorylates so called ITAM-motifs in the TCR chains. This leads to the recruitment and activation of ZAP-70, another protein tyrosine kinase, which phosphorylates further downstream substrates. The role of TCR proximal components in regulation of cell adhesion has not been extensively studied. Lck is involved in superantigen-induced T cell-B cell conjugation, a process which is known to be CD11a/CD18-integrin-dependent, and Lck-deficiency led to impaired integrin-clustering on the cell surface (Morgan et al, 2001). Lck also regulates the affinity of the $\alpha_4\beta_1$-integrin in T cells (Feigelson et al, 2001). Tyrosine kinases are required for the initiation of signalling leading to increased adhesion, for example by raft clustering (Krauss and Altevogt, 1999).

Early tyrosine phosphorylation events lead to the recruitment of adaptor proteins to the vicinity of the TCR (reviewed in Peterson et al, 1998). One of the proteins that is recruited to the TCR is SLAP-130/ Fyb, which has been implicated in T cell actin reorganisation. A knock-out of SLAP-130/Fyb recently revealed that this adapter protein is involved in coupling the TCR to integrin-mediated adhesion to ICAM-1 (Peterson et al, 2001). This was shown to be due to defective clustering of CD11a/CD18-integrins on the cell surface, even if actin polymerization was normal (Peterson et al, 2001).

*PKC*

Tyrosine phosphorylation events also leads to the activation of, among other enzymes, phospholipase C. This enzyme generates the crucial second messengers diacylglycerol (DAG) and inositol triphosphate ($IP_3$) from phosphatidylinositol 4,5 bisphosphate. DAG, in turn, activates the ubiquitous kinase, protein kinase C (PKC). At least 12 different isozymes of PKC have been described (Dempsey et al, 2000). They differ in structure, cofactor-dependence, expression and cellular localization. The cPKCs (conventional PKCs) (PKCα, βI, βII and γ) are activated by DAG and calcium, while the nPKCs (novel PKCs) (δ, ε, η and θ) are calcium-independent, and the aPKCs (atypical PKCs) (ξ, ι, λ) do not require DAG for activity. The PKCs are additionally regulated by phosphorylation and by subcellular location mediated by PKC binding proteins. Interestingly, one of the isoforms, PKCθ, is selectively expressed in T cells and localizes to the immunological synapse, and plays a fundamental role in T cell activation (Altman et al, 2000).

Phorbol esters, cell permeable analogs of DAG, had already earlier been reported to stimulate cell-cell adhesion mediated by CD11a/CD18-ICAM-1 interaction (Patarroyo et al, 1985, Rothlein and Springer, 1986). Activation of integrins via phorbol ester bypasses the requirement for tyrosine phosphorylation (Petruzzelli et al, 1998). A recent report has shown that transfection of consitutively active PKC isoforms α, β, γ and δ, but not PCKξ can induce CD11a/CD18-integrin-mediated binding to coated ICAM-1 in a model cell system (Katagiri et al, 2000), providing further evidence that PKC is involved in the regulation of adhesion. One putative downstream effector of PKC in macrophages is the protein MacMARCKS (macrophage-enriched myristoylated alanine-rich C kinase substrate), which has been shown to be essential for the increased surface motility of CD18-integrins that initiates adhesion after phorbol ester-stimulation (Zhou and Li, 2000). The MARCKS family of proteins regulates actin-membrane interactions (Aderem et al, 1992), but the role of other MARCKS-family members in regulation of adhesion in other cell types than macrophages has so far not been investigated.

*PI 3-kinase*

TCR-engagement and chemokines activate different PI 3-kinase isoforms in T cells (Wymann et al, 2000). PI 3-kinase is a lipid kinase that phosphorylates inositol-lipids in the 3-OH position, thus creating phosphatidylinositol triphosphate ($PIP_3$) a crucial signal intermediate, which is involved in recruiting proteins with PH-domains to the plasma membrane (Shimizu and Hunt, 1996, Klarlund et al, 1997). PI 3-kinase also plays a role in TCR-induced adhesion (Nagel et al, 1998, Katagiri et al, 2001). Interestingly, the PI 3-kinase γ knockout mouse has revealed a LAD like phenotype, showing that PI 3-kinase γ plays a crucial role in recruitment of cells in inflammation (Sasaki et al, 2000, Hirsch et al, 2000, reviewed in Wymann et al, 2000). Indeed, PI 3-kinase regulates at least some CD11/CD18 adhesion-pathways regulated by proinflammatory mediators, mainly through regulating the actin cytoskeleton and integrin avidity (Nielsen et al, 1996, Capodici et al, 1998, Hmama et al, 1998, Jones et al, 1998). Recently, chemokines have been shown to induce clustering of the integrin CD11a/CD18 on lymphocytes, an event that is dependent on PI 3 kinase (Constantin et al, 2000). The PI 3-kinase-dependent receptor clustering appears to be important at low ligand densities, and may play a role for example in the recruitment of lymphocytes to inflamed endothelium where ICAM-1 has not yet been upregulated (Constantin et al, 2000). PI 3-

kinase activity is also necessary for integrin-mediated adhesion initiated by raft-clustering (Krauss and Altevogt, 1999). Cytohesin-1, the ARF-GEF described earlier, is one of the mediators of integrin-mediated adhesion which lies downstream of PI 3-kinase (Kolanus et al, 1996, Nagel et al, 1998, Geiger et al, 2000, Hmama et al, 1998).

*MAP kinase*

Early tyrosine phosphorylation events lead to the activation of the classical Ras-MAP(mitogen-activated protein)-kinase pathway in T cells after T cell receptor crosslinking. The general view of MAP-kinase signalling is that it is mainly involved in changes in gene transcription, rather than in cytoplasmic events. However, two reports has implicated the role of the classical MAP kinase (Erk) pathway in the regulation of CD11a/CD18-integrins in T cells and CD11b/CD18 in neutrophils (O'Rourke et al, 1998, Pillinger et al, 1998). Interestingly, salicylates (the active component in aspirin) inhibited both MAP kinase activity and cell adhesion and transmigration, indicating that salicylates may, in part, excert their anti-inflammatory effects by inhibiting integrins (Pillinger et al, 1998, Gerli et al, 1998). However, inhibition of both MEK (MAPK/extracellular signal-regulated kinase, the upstream activator of MAP kinase) and PI 3-kinase by chemical inhibitors were required for the complete inhibition of T cell adhesion to ICAM-1. This result indicates that these two pathways converge proximal to the integrin (O'Rourke et al, 1998). Interestingly, a mutant T cell line that was reported not to mediate integrin-mediated adhesion to ICAM-1, was found to have an unusual form of MAP kinase (Mobley et al, 1996).

The MAP kinase family has also been implicated in adhesion induced by proinflammatory mediators. p38 MAP kinase (stress-activated, mitogen-activated protein kinase) has been implicated in LPS- and TNF-induced CD11b/CD18-integrin mediated neturophil adhesion and the neutrophil oxidative burst (Detmers et al, 1998). L-selectin-induced neutrophil adhesion also seems to be dependent on p38 MAP kinase (Simon et al, 1999, Simon et al, 2000, Smolen et al, 2000).

In addition to these protein kinase pathways, also protein phosphatases have been implicated in the regulation of inside-out signalling (Hedman and Lundgren, Valmu and Gahmberg, 1995, Petruzzelli et al, 1998).

*$Ca^{2+}$-signalling*

$IP_3$, produced by phospholipase C, triggers the release of calcium from intracellular calcium-stores, and $Ca^{2+}$ has been shown to be crucial for the induction of CD11a/CD18-integrin mediated adhesion (Rothlein and Springer, 1986, Stewart et al, 1996, Stewart et al, 1998). $Ca^{2+}$ is involved in regulating many cellular events, like stabilization of the contact between T cell and APC through changes in the cytoskeleton (Lewis, 2001). The effects of $Ca^{2+}$ are mediated through calcium-binding proteins, like calmodulin and calpain (Pettit and Fay, 1998). Calmodulin, in its calcium-bound form, binds to downstream effectors and modulates their function, and has multiple cellular effects. Its involvement in CD11/CD18-integrin-mediated adhesion has not been investigated, but it should be noted that one of the downstream effectors of calmodulin is the integrin-modulating factor, MARCKS (Aderem et al, 1992).

Calpain, a calcium-dependent protease, is thought to be involved in the regulation of CD11a/CD18 clustering on the plasma membrane after cell activation. It was shown that calcium-mobilizers, as well as

other cellular stimuli, induced clustering of CD11a/CD18 on the cell membrane, and adhesion to ICAM-1, possibly by releasing the integrin from cytoskeletal restraint. This could be inhibited by the use of calpeptin, an inhibitor of calpain (Stewart et al, 1998, Constantin et al, 2000, Airas et al, 2000). However, one of the two calpain isoforms, μ-calpain has been shown not to be involved in regulating adhesion through $\alpha_{IIb}\beta_3$-integrins, since a μ-calpain-knockout has defects in late stages, but not early stages of platelet aggregation (Azam et al, 2001). The other calpain-enzyme, m-calpain is a potential activator of adhesion, but the knockout has so far not been reported. Also, the potential target of calpain has not been determined, even if numerous cytoskeletal proteins are substrates for calpain. A potential cytoskeletal protein being cleaved by this protease is talin, the head-domain of which has been shown to increase adhesion via $\alpha_{IIb}\beta_3$ integrins (Calderwood et al, 1999, Yan et al, 2001).

*Small GTP-binding proteins*

Small GTPases of the Rho family have been extensively studied as regulators of the actin cytoskeleton, integrins and cell adhesion and migration (reviewed in Ridley, 2001). The Rho family of proteins are inactive in the GDP-bound form and become activating when binding GTP, after which they interact with and activate diverse cellular target proteins. They are regulated by proteins (guanine-nucleotide exchange factors, or GEFs, GTPase-activating proteins, or GAPs, and guanine-nucleotide-dissociation inhibitors, or GDIs) that modulate the guanine nucleotide binding or hydrolysis. These factors are in turn regulated by, for example, second messengers or tyrosine phosphorylation. The active forms of Ras and Rac, two small GTP-binding proteins involved in MAP kinase activation and actin cytoskeleton reorganisation, respectively, were found to induce CD11a/CD18-induced adhesion to ICAM-1 downstream of PI 3-kinase (Katagiri et al, 2000). A knockout of Rac-2, a Rac isoform only expressed in hematopoietic cells, gives a LAD-like phenotype, where neutrophil recruitment to the site of inflammation is impaired, and their function is disturbed (Roberts et al, 1999). Rac probably functions downstream of PI 3-kinase also in this system, since PI 3-kinase lipid products regulate Rac activation at the leading edge of a migrating cell (Ridley, 2001).

In T cells, Rho seems to be involved mainly in postreceptor events during cell-cell adhesion, rather than in direct CD11/CD18-integrin activation (Tominaga et al, 1993, Katagiri et al, 2000, Petruzzelli et al, 1998). On the other hand, blocking Rho or its downstream effector Rock in T cells actually leads to CD11a/CD18-integrin activation by integrin clustering at the plasma membrane (Rodriguez-Fernandez et al, 2001), implicating that Rho has many different roles. Indeed, Rho has been implicated in both CD11a/CD18- and CD11b/CD18-integrin-mediated adhesion in response to proinflammatory mediators, possibly acting upstream of PI 3-kinase (Hmama et al, 1998, Laudanna et al, 1996, Soede et al, 2001). H-Ras, in turn seems to play a complicated role in integrin regulation by chemokines, by activating both a positive and a negative pathway of integrin-regulation, and has been speculated to be involved in integrin regulation during migration (Weber et al, 2001).

Very recently, Rap1 (reviewed in Bos et al, 2001), a small GTPase which is the closest relative to Ras, was found to be a crucial factor in the regulation of CD11a/CD18-integrin-mediated adhesion to ICAM-1, independent of PKC and PI 3-kinase (Katagiri et al, 2000). Expression of a constitutively active form of Rap1 activated adhesion, and, interestingly, led to binding of sICAM-1 to CD11a/CD18,

indicating that an affinity-change took place. Dominant negative Rap1, and a Rap1 specific GTPase activating protein, inhibited TCR-induced adhesion, both when induced by receptor crosslinking, or in a more physiological setting, during antigen presentation (Katagiri et al, 2000, Katagiri et al, 2002). Also CD31-induced CD11a/CD18-integrin adhesion to ICAM-1 and LPS-induced integrin-dependent macrophage spreading is mediated by Rap-1 (Reedquist et al, 2000, Schmidt et al, 2001). Rap-1 thus seems to be a crucial component regulating integrins, and it would be of substantial interest to find the downstream effectors of Rap1, but they are so far unknown.

*Negative regulation of adhesion*

TCR-induced CD11a/CD18-integrin activation is transient. It needs to be downregulated when T cells detach from APCs. The downregulation of adhesion has not been extensively studied, however, there are some implications of the signalling pathways involved. CyclicAMP (cAMP) was found early on to inhibit T cell adhesion to ICAM-1, and it was hypothesized that early TCR-signalling events would lead to adhesion, while later signalling would lead to deadhesion (Dustin and Springer, 1989). Subsequently, it was revealed that TCR and CD11a/CD18-integrin coligation leads to increase in cAMP levels in cells, leading to PKA (protein kinase A) activation and deadhesion of integrin from its ligand via actin disassembly (Rovere et al, 1996). This is a feedback loop regulating the transient adhesion of T cells. Also other pathways have recently been implicated in the negative regulation of CD11a/CD18-integrin adhesion to ICAM-1 in response to CD4-ligation (Mazerolles et al, 1994, Mazerolles et al, 1996). This has been suggested to be important to avoid unfruitful interactions between T cell and APC. CD4 ligation was shown to induce dissociation of cytohesin from CD18-integrins, this being a potential mechanism for downregulation of adhesion (Mezzarolles et al, 2002). Another potential negative regulator of CD11a/CD18-integrins is the PAK-family member LOK (lymphocyte oriented kinase), in the absence of which lymphocytes aggregate extensively and integrins cluster at the cell surface (Endo et al, 2000).

Also CD28-ligation has been implicated as a negative regulator of CD11a/CD18-integrin mediated adhesion. Coligation of the TCR and CD28 led to a decrease in Rap1 activity and also a decrease in CD11a/CD18-integrin adhesion to ICAM-1 (Katagiri et al, 2002). Since CD28 is a potent costimulatory molecule in T cells, the modulation of adhesion via CD28-ligation seems reasonable, to avoid too stringent contact of T cell-APC.

### 3.4. Outside-in signalling by CD11a/CD18-integrins

3.4.1. Introduction

Integrin outside-in signalling has been most extensively studied in nonlymphoid cells (reviewed in Giancotti and Ruoslahti, 1999). Integrin ligation leads to the formation of focal adhesions. Focal adhesions are stable complexes formed at the plasma membrane consisting of integrins, cytoskeletal proteins and signalling proteins, linking the inside of the cell to the extracellular matrix by strong adhesive contacts and conferring anchorage dependence to cells. Integrin ligation in this context signals survival. In migrating cells, smaller and more transient complexes are formed, so called focal complexes. These need to be rapidly assembled and disassembled to allow short-term contact between the cell and the substratum, assembling at the leading edge of the migrating cell and disassembling at the uropod.

Integrin ligation activates several signalling pathways in cells that alter gene expression, influencing cell survival, differentiation, migration, morphology, etc (Giancotti and Ruoslahti, 1999). Signalling pathways activated by $\beta_1$- and $\beta_3$-integrins include activation of focal adhesion kinase (FAK), a tyrosine kinase that localises to focal adhesions, is regulated by integrins and mediates cell spreading and morphological changes. FAK contains many binding sites for SH2 and SH3-domain containing proteins, and may function as an adapter molecule to build up signalling complexes. Integrins also activate MAP kinase pathways through the recruitment of the Grb/Sos-complex, which activates Ras, and they have been implicated both upstream and downstream of small GTP-binding proteins like Rho, Rac and Cdc42 (Ridley, 2001). Integrins also work in concert with other cell surface receptors, like growth factor receptors, to influence the behaviour of the cell (Giancotti and Ruoslahti, 1999, Eliceiri, 2001). The proximal events involved in regulating initiation of cell signalling are still largely unknown. The initiating event could be the release of α-β integrin chain association after integrin ligand binding to allow the β chain cytoplasmic domain to initiate signalling. Tyrosine phosphorylation of the β-chain and clustering of integrin tails have also been proposed as ways of initiating signalling complexes. Alternatively, integrins may interact *in cis* with other membrane receptors to initiate signalling.

### 3.4.2. CD11/CD18-integrins and costimulation

The main area of research on CD11/CD18-integrin outside-in signalling has been focusing on the role of CD11a/CD18 in costimulation of T lymphocytes. T cells are activated through the TCR upon engagement of a MHC-peptide complex on the antigen presenting cell. This initiates intracellular signalling pathways, leading ultimately to T cell activation, proliferation and clonal expansion. However, ligation of the TCR alone (signal 1) cannot sufficiently activate the T cell. Instead, it needs a signal 2, the costimulatory signal, to avoid anergy (nonresponsiveness). The signal 2 is provided by a number of different cell surface receptors, of which CD28 is considered to be the most important (Dustin and Shaw, 1999). However, lack of CD28 does not lead to T cell anergy in all cases. It has become increasingly apparent that also other cell surface receptors can act as costimulatory molecules for T cells, and that T cells encounter different molecules on different APCs at different developmental and differentiation stages. The CD11a/CD18-integrin has been proposed to act as one of these molecules, as coligation of the integrin (by crosslinking antibody or ligand) and TCR induces T cell proliferation and IL-2 production (van Noesel et al, 1988, van Seventer et al, 1990). It has also been shown in several reports that the lack of CD11a/CD18-integrins leads to profound defects in T cell signalling and proliferation (van Noesel et al, 1988, Voss et al, 1991, Scharffetter-Kochanek et al, 1998, Shier et al, 1999). However, it has been problematic to distinguish the role of the integrin as an adhesion receptor from its role as a costimulatory molecule, and there is still some controversy about the subject. One model proposes that CD11a/CD18 only functions to increase adhesion between the T cell and the APC, thus promoting cell-cell contact and antigen-MHC-TCR interaction, and lowering the amount of antigen needed for T cell activation (Bachmann et al, 1997). However, integrins can provide a costimulatory signal also in the absence of cell-cell contact (van Seventer et al, 1990). Also, in some instances (CD8+ T cells stimulated by alloantigen), integrins are crucial for IL-2 production, proliferation and cytolytic function of the cells, and increased TCR signalling cannot compensate for the absence of the integrin (Shier et al, 1999). However, ligation of

integrins, even if it does induce the production of IL-2, ultimately leads to cell death at least in naïve CD4+ T cells (Zuckerman et al, 1998), so it is still unclear if also additional receptors are required for costimulation by CD11a/CD18-integrins. It is clear that CD28 and CD11a/CD18-molecules do not provide the same signal for T cells, but instead have unique functions in T cell costimulation (Bachmann et al 1997, Zuckerman et al, 1998, Sedwick et al, 1999). Additionally, ligation of CD11a/CD18-integrins by different ICAMs lead to different cell responses and a different cytokine repertoire, creating additional diversity for the integrin as a costimulatory molecule (Bleijs et al, 1999).

Interestingly, it has been suggested that CD11/CD18-integrins also play a costimulatory role in other immune cells (reviewed by Lowell and Berton, 1999). In neutrophils, the system is activated by pro-inflammatory agonists, which upregulate integrin-mediated adhesion and cell spreading. The adhesion and spreading of PMN give a second signal to the cell, which leads to the activation of cell signalling pathways. This system is potentially easier to study than T cell costimulation, since at least some of the pathways activated by integrins are not activated by the initial stimuli alone. Integrin signalling leads to actin cytoskeleton rearrangements, cell migration, degranulation and respiratory burst in myeloid leukocytes (Lowell and Berton, 1999). Cells derived from LAD-patients have been very useful in the study of integrin outside-in signal transduction in human neutrophils, where the CD11/CD18-integrins are the predominant integrins. Indeed, it has been shown that these cells cannot adhere, rearrange their cytoskeleton, migrate out of the vasculature or become fully activated, hallmarks of integrin-dependent costimulation (Lowell and Berton, 1999). CD11/CD18-integrins can also act as costimulatory signals for LPS/taxol-signalling pathways. LPS has long been known to bind to CD11/CD18-integrins (Wright and Jong, 1986), but its functional significance has remained unclear. However, it has recently been shown that expression of three surface receptors together, namely CD14, Toll-like receptor 4 and CD11b/CD18 is required for full LPS and taxol-inducible gene expression of a variety of LPS-sensitive genes (Perera et al, 2001). The function of CD11/CD18-integrins as receptors for LPS complicates the issue of LPS-induced regulation of adhesion. CD11/CD18-integrins also collaborate with an Fc-receptor to produce LTB4 in neutrophils after immune complex activation (Graham et al, 1993), showing that these integrins can collaborate with numerous cell surface receptors to induce intracellular signalling leading to diverse cellular outcomes.

Interestingly, costimulation both of neutrophils (Coxon et al, 1996, Avdi et al, 2001) and T cells (Zuckerman et al, 1998) by CD11/CD18-integrins has been linked to apoptosis. This would provide the integrins with a fundamentally different role than that of $\beta_1$- and $\beta_3$-integrins, which signal survival.

Integrins also have stimulatory properties independent of costimulation. This occurs at least during so called integrin crosstalk, whereby integrins regulate the function of other integrins. CD11a/CD18-integrin outside-in signalling, initiated by binding of ICAM-1 or by deleting the integrin I-domain, includes modulation of the activity of $\beta_1$-integrins on T cells (Porter and Hogg, 1997, Leitinger and Hogg, 2000). The molecular mechanism for this integrin crosstalk is unknown, but it includes the cytoskeleton, and clustering of the $\beta_1$-integrins (Leitinger and Hogg, 2000). This form of integrin crosstalk may be involved in regulating increased transendothelial migration after CD11a/CD18-integrin ligation (Porter and Hogg, 1997). Conversely, $\alpha_4\beta_1$-integrin engagement has also been shown to activate CD11/CD18-integrins (Chan et al, 2000, May et al, 2000, Hyduk and Cybulsky, 2002). Activation of CD11a/

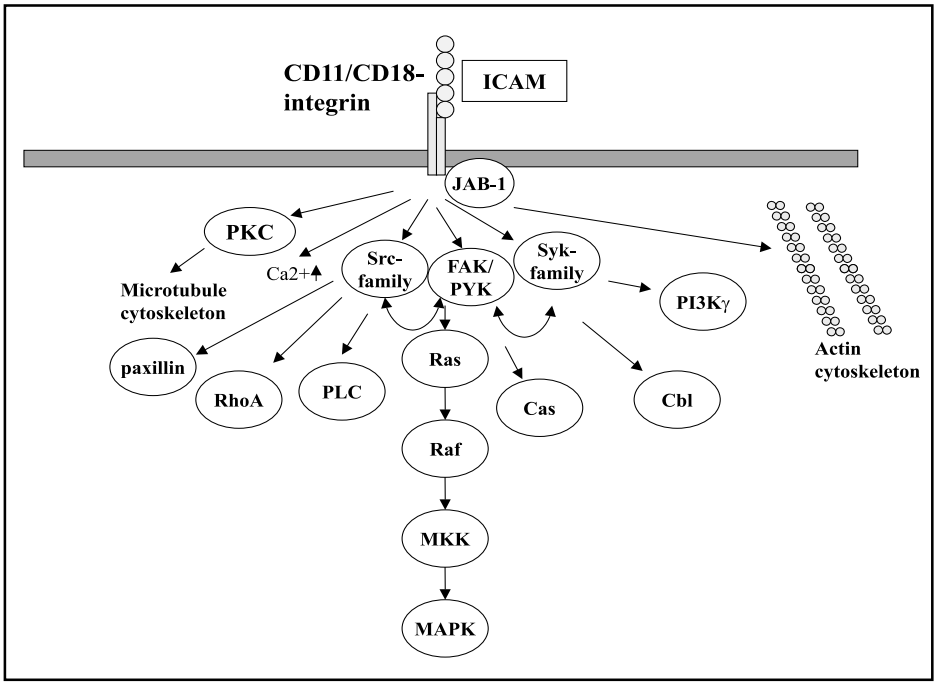


**Figure 9. Schematic figure of CD11/CD18-integrin outside-in signalling.** Integrin ligation results in the activation of multiple downstream effectors. Early on, $Ca^{2+}$ transients and PTK-activity (Src-family, FAK/PYK and Syk-family kinases) is induced. Crosstalk between these tyrosine kinases is common. The PTKs are involved in regulating the adapter proteins paxillin, Cbl and Cas. Also PLC, PI 3-kinase, RhoA, the Ras-MAPK pathway and other MAP kinases lie downstream of PTK-activation. PKC-activation after integrin-ligation is involved in cell migration, possibly by influencing the microtubule cytoskeleton. Integrins also reorganize the cortical actin cytoskeleton after ligation. JAB-1 is a transcription factor that has been shown to directly associate with the CD18 cytoplasmic tail.

CD18-integrins by $\alpha_4$-integrins may be involved in regulating firm adhesion after initial tethering and rolling of leukocytes by $\alpha_4\beta_1$-integrin (Chan et al, 2000), and thus, integrin crosstalk modulates the multistep cascade involved in regulating leukocyte recruitment to inflamed tissue.

### 3.4.3. Intracellular signalling pathways activated by CD11/CD18-integrins

*Early signalling events*

The CD11/CD18-integrins could act as costimulatory molecules in T cells (and other leukocytes) in different ways, either through directly acting as signalling molecules, or by influencing actin reorganisation events and the organisation of the immunological synapse, thus indirectly affecting TCR signalling. The first evidence that CD11/CD18-integrins could act as signalling molecules as well as adhesion molecules came from studies demonstrating a role for these receptors in phagocytosis (Lowell and Berton, 1999). Subsequently, it was found that integrin ligation with antibodies or ligand could activate second messenger pathways like $Ca^{2+}$ transients and phosphatidylinositol turnover (Pardi et al, 1989, Altieri et al, 1992), and could inititate tyrosine phosphorylation in T cells, PMN and B cells (Kanner et al, 1993, Berton et al, 1994, Wong et al, 1995). Many of these events could be further increased in T cells by the ligation of the TCR (see Figure 9 for intracellular signalling pathways activated by CD11/CD18-integrins). Ligation/

activation of CD11/CD18-integrins also induces phospholipase Cγ activation (Kanner et al, 1993) and tyrosine phosphorylation of the adapter proteins p130Cas and c-Cbl (Petruzzeli et al, 1996, Willeke et al, 2000). The activation of tyrosine kinases is indeed the first event that occurs after integrin ligation.

*Tyrosine kinases*

The three main families of tyrosine kinases associated with integrin outside-in signalling are the Src-family kinases, the FAK/PYK-2 kinases and kinases of the Syk family (Lowell and Berton, 1999). These families are intimately linked together: In T cells, the Src-family kinase Lck is crucial for the activation of the downstream effector Zap-70 of the Syk-family, and the initiation of T cell activation downstream of the TCR. In fibroblasts, FAK and the Src-kinases form complexes and together initiate downstream signalling events. Integrin ligation has been shown to lead to activation of the Src-family kinase Fgr, Lyn and Hck (Berton et al, 1994, Yan et al, 1997, Piccardoni et al, 2001). Knockouts of the myeloid Src-family kinases Hck/Fgr/Lyn have revealed that these kinases are intimately linked to integrin-dependent cell spreading, migration and superoxide production in neutrophils and macrophages (reviewed in Lowell and Berton, 1999). This is possibly through their role in activating D3 phosphoinositide-production and thus activation of the small GTP-binding proteins Rho, Rac and Cdc42, which have crucial roles in the regulation of the actin cytoskeleton. Indeed, P-selectin has been proposed to activate CD11b/CD18-integrin mediated adhesion, which in turn would activate Hck/Lyn and lead to a rearrangement of the integrin-cytoskeleton linkage to strengthen adhesion in a feedback loop (Piccardoni et al, 2001). Additionally, engagement of CD11b/CD18 in neutrophils leads to activation both of RhoA and p190RhoGAP, at least partially through the Src-family kinases (Dib et al, 2001).

Integrin ligation also lead to activation of the tyrosine kinases FAK, fakB and Pyk-2 (Rodriguez-Fernandez et al, 1999, Kanner, 1996). In addition, Syk is activated and forms a complex with CD18-integrins and Src in PMN spreading on fibrinogen (Yan et al, 1997). Zap-70 has been implicated downstream of CD11a/CD18-integrin ligation in T cells (Soede et al, 1999). Interestingly, FAK has been shown to interact with peptides from the cytoplasmic domain of $\beta_1$, $\beta_2$ and $\beta_3$-integrins (Schaller et al, 1995), which may play a role in the activation of this kinase in response to integrin engagement. One possible downstream effector of these tyrosine kinases is the adapter/cytoskeletal protein paxillin. CD11/CD18-integrins have been shown to be crucial for the tyrosine phosphorylation of paxillin in PMN in the response to numerous stimuli, as shown by the absence of paxillin tyrosine phosphorylation in LAD cells (Graham et al, 1994, Fuortes et al, 1994). However, integrin-independent paxillin-phosphorylation pathways also exist (Fernandez et al, 1997).

*Other downstream signalling events*

Crosslinking of CD11/CD18 integrins on PMN can lead to tyrosine-kinase dependent activation of the small GTP binding protein Ras (Zheng et al, 1996), which links to numerous crucial downstream effectors, like PI 3-kinase and the classical MAP kinase pathway. The CD11a/CD18-integrin also works in concert with T cell receptor engagement to activate the classical MAP-kinase/Erk pathway and JNK (c-Jun-N-terminal kinase), another MAP kinase family member (Geginat et al, 1999, Ni et al, 1999). JNK can also be activated in adherent neutrophils stimulated by TNF (tumour necrosis factor), a process which is also

dependent on integrin costimulation, and involves the tyrosine kinases Syk and PYK-2 (Avdi et al, 2001). Additionally, CD11b/CD18-integrins are crucial for the activation of MAP kinase family members in response to taxol (Perera et al, 2001), and both CD11b/CD18-integrin and CD11c/CD18-integrin engagement induces MAP kinase pathway activation (Rezzonico et al, 2000) and subsequent production of inflammatory cytokines (Lecoanet-Henchoz et al, 1995, Rezzonico et al, 2000).

Costimulation through integrin and TCR also activates PI 3-kinase (Ni et al, 1999). PI 3-kinase activity is required for integrin-mediated costimulation of CD8+ T cell proliferation, by regulating integrin-induced IL-2 receptor α-subunit expression (Ni et al, 1999, 2001).

An interesting new mode of integrins to regulate signalling pathways is demonstated by the direct interaction of CD11a/CD18 with the transcriptional coactivator JAB-1 (Bianchi et al, 2000). The ligation of the integrin was shown to increase the nuclear localisation of JAB1 and activation of c-Jun dependent transcription.

3.4.4. The role of integrin-cytoskeleton interactions

Integrins link to the actin cytoskeleton and have been shown to fundamentally influence actin cytoskeleton reorganisation and the organisation of the immunological synapse. Ligation of CD11a/CD18-integrins with crosslinking antibodies on activated T cells leads to a dendritic phenotype, and crosslinking of CD18-integrins in PMN leads to actin polymerization (Kelleher et al, 1995, Lofgren et al, 1993). Additionally, coating activated T cells on ICAM-1 leads to cell polarization (Rodriguez-Fernandez et al, 1999). APCs expressing ICAM-1 binds integrins on T cells and the engagement leads to redistribution of talin to the T cell-APC contact zone independent of antigen (Sedwick et al, 1999), indicating that integrin-ligation leads to a redistribution of the cortical cytoskeleton. Importantly, the CD11a/CD18-ICAM-1 (or CD28-B7) interaction have been shown to induce a movement of cell surface receptors to the interface of the T cell-APC contact, a movement which is dependent on myosin motor proteins (Wulfing and Davis, 1998). Apparently, the cargo for this membrane transport is lipid rafts (Viola et al, 1999), leading to association of rafts and associated signalling molecules with the TCR (Dustin and Shaw, 1999).This shows that costimulatory molecules contribute to the formation of the immunological synapse and thus fundamentally affectTCR signalling (see also Fig 7).

Except linking to the actin cytoskeleton, integrins may also be involved in regulating the microtubule system at the rear end of the cell during cell migration. PKCs have been shown to important for the locomotory phenotype of T cells (Kelleher et al, 1995, Volkov et al, 2001). Induction of a locomotory phenotype of preactivated T cells by integrin ligation concomitantly leads to redistribution of PKCδ and PKCβI to the microtubule cytoskeleton (Volkov et al, 1998). Interstingly, PKCβI negative T cells cannot migrate in response to integrin-triggering, and their locomotory phenotype can be restored by transfection of these cells with PKCβI (Volkov et al, 2001). The system during in vivo migration of cells probably involves chemokines as the initial integrin-activating trigger (von Andrian, 2001).

Several of the signalling pathways activated by CD11/CD18-integrins require an intact cytoskeleton (Geginat et al, 1999, Rodriguez-Fernandez et al, 1999). Indeed, integrin-mediated costimulation of cell proliferation has been proposed to be due to integrin-dependent organization of the actin cytoskeleton and cell spreading in response to ICAM-1, which influences IL-2 production and subsequent S phase

entry (Geginat et al, 1999). It appears likely that activation of at least some of the signalling pathways initiated by integrin ligation are actually due to a fundamental reorganisation of the cell morphology and subcellular molecular composition that are initiated by integrin ligation. However, evidence suggests that both actin reorganisation and signalling events activated directly by CD11a/CD18-integrins play a role in T cell costimulation.

## 4. CD11/CD18-INTEGRIN PHOSPHORYLATION

### 4.1. Phosphorylation of CD11-chains

Reversible protein phosphorylation on serine, threonine or tyrosine residues is a common mechanism to regulate protein function. Phosphorylation may modulate protein function by directly regulating the conformation or activity of a protein. It may also create docking motifs for adapter proteins containing domains that bind to phosphorylated amino acid sequences, or otherwise regulate protein-protein interactions. Phosphorylation of the integrin cytosplasmic domains is a putative integrin regulatory mechanism.

The phosphorylation of CD11/CD18-integrins has been extensively studied. The integrin α-chains (CD11a, b and c) are constitutively phosphorylated on serine residues in monocytes and peripheral blood mononuclear cells (Hara and Fu, 1986, Chatila et al, 1989). Additionally, CD11c displayed some threonine phosphorylation (Chatila et al, 1989). The phosphorylation status of CD11d has not been investigated so far. The phosphorylation was not significantly increased upon cell activation with various stimuli, except in a single report, where hyperphosphorylation of CD11a in response to anti-CD3 treatment was shown (Pardi et al, 1992). The intracellular tails of the CD11-chains do not show extensive sequence homology, so it is quite interesting to note that they are all constitutively phosphorylated, indicating a function for CD11-phosphorylation in the regulation of integrins. Indeed, phosphorylation of other integrin α-chains has been shown to influence integrin function. Phosphorylation of the integrin $\alpha_3$-chain has been shown to influence integrin-mediated signalling, migration and cytoskeletal rearrangements (Zhang et al, 2001), while phosphorylation of the $\alpha_4$-integrin subunit regulates the binding of paxillin to this integrin subunit (Han et al, 2001). Paxillin binding to the $\alpha_4$-integrin has been implicated in the unusual characteristics of this integrin, which promotes migration and decreases cell spreading in contrast to many other integrin subunits (Chan et al, 1992, Liu et al, 1999, Liu and Ginsberg, 2000, Han et al, 2001). Recently, also $\alpha_9$, which is closely related to $\alpha_4$ and regulates cell behaviour in a similar way, has been shown to bind paxillin, but it is not yet known if phosphorylation also regulates this interaction (Young et al, 2001). Regarding the function of the phosphorylation of the CD11-chains, Buyon et al (1997) proposed that CD11-phosphorylation may be involved in regulating adhesion-competence at the cell membrane, since CD11b is not phosphorylated in granules in neutrophils. Upon translocation to the plasma membrane, the CD11b molecules become phosphorylated, which may act as a tag to identify adhesion-competent integrins.

### 4.2. Phosphorylation of the CD18-chain

*Serine phosphorylation*

The cytoplasmic domain of the integrin CD18-chain has been shown to be intimately linked to the

regulation of adhesion. Interestingly, it also becomes strongly phosphorylated on serine residues after activation of different cell types with phorbol ester (Hara and Fu, 1985, Chatila and Geha, 1988, Chatila et al, 1989, Buyon et al, 1990, Valmu et al, 1991). The chemotactic peptide fMLP as well as RANTES, has been reported to induce weak phosphorylation of the CD18-chain, but much more transiently than phorbol ester (Chatila et al, 1989, Merrill et al, 1990, Szabo et al, 1997). This raised the possibility that serine phosphorylation may be important in the regulation of adhesion. However, when the main phorbol ester induced serine phosphorylation site (Ser756) was mapped by mutational studies (Hibbs et al, 1991), it was shown that the mutation of this serine to alanine did not affect phorbol ester induced adhesion to coated ICAM-1. Phorbol ester-induced serine phosphorylation of the CD18-chain has been proposed to have an alternative role; to modulate the outside-in signalling by CD11/CD18-integrins (Hellberg et al, 1995). The corresponding serine is not found in $\beta_3$, but the $\beta_1$-integrin has been reported to be phosphorylated on Ser785 (corresponding to Ser756 in CD18) in undifferentiated F9 teratocarcinoma cells, and it becomes dephosphorylated upon differentiation (Dahl and Grabel, 1989). An attempt to mimic the phosphorylated integrin by a Ser-Asp mutation showed reduced localisation at focal contacts (Barreuther and Grabel, 1996). This mutation has also been shown to promote cell attachment to laminin, but to reduce cell migration and spreading (Mulrooney et al, 2001).

*Threonine phosphorylation*

In the mutational study of the putative phosphorylation sites of CD18 (Hibbs et al, 1991) it was noted that the threonine triplet, Thr758-760 in the integrin CD18 cytoplasmic domain, was intimately linked to adhesion. Mutation of these threonines either alone or in combination reduced phorbol ester-induced binding of cells to coated ICAM-1. However, the integrin still retained the ability to be phorbol ester stimulated, unless all three threonines were mutated. Interestingly, the threonine triplet has since been shown to be transiently phosphorylated when T cells are stimulated with either phorbol ester or an antibody against the TCR-CD3-complex in the presence of okadaic acid, a phosphatase inhibitor (Valmu and Gahmberg, 1995). In another report, okadaic acid by itself was reported to induce phosphorylation of CD18, this time in neutrophils (Merrill et al, 1994). The effect was shown to be additive in the presence of phorbol ester. Okadaic acid, on the other hand, inhibits T cell aggregation, indicating that dynamic phosphorylation may be important for the regulation of adhesion (Valmu and Gahmberg, 1995). The threonine triplet has been shown to be involved in regulating postreceptor events through the CD11/CD18-integrins, like cell spreading (Peter and O'Toole, 1995). Additionally, in response to thrombin, PMA or calyculin A (a phosphatase inhibitor) the $\beta_3$-integrin has been found to be phosphorylated mainly on threonine, with trace amount of serine-phosphorylation (Parise et al, 1990, Lerea et al, 1999). The calyculin A-induced phosphorylation sites were mapped and determined to be Thr751 and Thr753 (corresponding to the first and the last threonine, Thr758 and Thr760, of the threonine triplet in CD18). Threonine phosphorylation was speculated to have a negative role on $\beta_3$-integrin inside-out and outside-in signalling (Lerea et al, 1999), possibly by inhibiting the binding of tyrosine-phosphorylated integrins to Shc, a signalling protein (Kirk et al, 2000). However, the role of phosphorylation in the regulation of $\beta_3$-integrins remains inconclusive, since phosphorylation of $\beta_3$ has also been reported to expose ligand-binding sites on $\alpha_{IIb}\beta_3$-integrin in the response to platelet-activating factor (van Willigen et al, 1996).

Mutation of residues 751-753 in the integrin $\beta_3$ cytoplasmic domain has been reported to inhibit cell attachment, cell spreading and extracellular domain conformation changes (Mastrangelo et al, 1999, Bodeau et al, 2001), but to not have a completely detrimental effect on signalling induced by $\beta_3$-integrins (Bodeau et al, 2001). Threonine phosphorylation of $\beta_1$-integrins has not been reported so far, but the VTT-sequence in the $\beta_1$-integrin cytoplasmic domain has been shown to be important for a number of integrin-dependent functions, including signalling, conformational changes in the extracellular domain as detected by an activation epitope, cell attachment and cell spreading (Wennerberg et al, 1998, Bodeau et al, 2001), and is thus very important for proper integrin function.

*Tyrosine phosphorylation*

$\beta_1$- (Hirst et al, 1986, Johansson et al, 1994, Takahashi, 2001) and $\beta_3$-integrins (reviewed in Phillips et al, 2001) have been shown to become tyrosine phosphorylated, which may influence their interactions with signalling and cytoskeletal proteins, or regulate integrin activity (Jenkins et al, 1998, Law et al, 1999, Cowan et al, 2000, Phillips et al, 2001, Datta et al, 2001). The CD18-chain do not contain the conserved tyrosines present in $\beta_1$ and $\beta_3$ integrins. Instead, they have been replaced by phenylalanines. There is one tyrosine in the CD18 cytoplasmic domain, and tyrosine phosphorylation of CD18 has been shown to occur when natural killer-cells are cultured with IL-2 to gain lymphokine-activated killer-activity (Umehara et al, 1992). Additionally, collagen binding to CD11a/CD18-integrins has been shown to induce tyrosine phosphorylation both of the CD11a and the CD18-chains (Garnotel et al, 1995).

Phosphorylation of the leukocyte integrins has been studied widely, but individual phosphorylation sites have been only poorly documented. Additionally, it is entirely unknown which signalling cascades regulate phosphorylation, and which kinases phosphorylate these residues in vivo, or the exact function of the phosphorylation sites in the regulation of integrins.

## SUMMARY OF THE STUDY

5. AIMS OF THE STUDY

The role of different signalling pathways and phosphorylation events in the regulation of CD11/CD18-integrins in T cells has remained unclear. The purpose of the present study was:

1. To examine different signalling pathways involved in regulating T cell adhesion through CD11/CD18-integrins.

2. To identify the kinase(s) responsible for CD18-phosphorylation.

3. To map integrin phosphorylation sites in vitro and in vivo in response to different stimuli.

4. To examine the role of integrin phosphorylation in the regulation of integrin-mediated cellular functions.

## 6. MATERIALS AND METHODS

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

| Materials and methods | Original publication |
|---|---|
| 1. Antibodies | I-IV |
| 2. Synthetic peptides | I, III |
| 3. T cell isolation and cell culture | I-IV |
| 4. Integrin purification | III |
| 5. Flow cytometry | IV |
| 6. Cell adhesion and aggregation assays | I, II, IV |
| 7. Radioactive cell labellings | I, IV |
| 8. Subcellular fractionation | I, II, III |
| 9. Immunoprecipitation | I, III, IV |
| 10. SDS-PAGE and immunoblotting | I-IV |
| 11. Phospho amino acid analysis | I, III |
| 12. Mass spectrometry | III |
| 13. Identification of phosphorylation sites in phosphorylated peptides | III |
| 14. Purification of integrin kinase | III |
| 15. Confocal microscopy | II |
| 16. Peptide affinity chromatography | I, III |
| 17. Phosphorylation of lipopeptide in vivo | I |
| 18. Protein band shift assays | II |
| 19. Protein kinase assay | III |
| 20. Endoglycosidase H treatment | IV |

7. RESULTS

### 7.1. Examination of signalling pathways involved in regulating CD11/CD18-integrins in T cells (II, IV)

Signalling pathways involved in regulating CD11/CD18-integrins in human T cells were examined using cell permeable inhibitors to known signalling pathways and mutant cell lines lacking specific signalling molecules (Figure 10 shows the signalling pathways investigated in this study).

The role of TCR proximal elements in the regulation of CD11/CD18-integrins is poorly understood. Lck is involved in early T cell activation by initiating tyrosine phosphorylation events and thereby inducing downstream signalling (Straus and Weiss, 1992). To examine the putative role of Lck in the regulation of CD11/CD18-integrins in T cells we used the specific Src kinase inhibitor PP2. PP2 inhibited CD3-induced, but not phorbol ester induced T cell adhesion to coated ICAM-1, at the same concentration (10 μM) that completely inhibited phosphorylation of MAP-kinase on the pThr-pTyr motif that is important for catalytic activity (IV, Fig 1). MAP kinase has previously been shown to be involved in regulation of integrin-mediated adhesion in T cells (O'Rourke et al, 1998), and is thus a putative downstream mediator of Lck in this system. By using cells deficient in Lck (JCaM1.6 cells), we wanted to verify the result of the kinase inhibitor, which also inhibits other Src kinase family members than Lck. JCaM1.6 cells, however, do not adhere to ICAM-1 or aggregate when activated either through the TCR or with phorbol ester, even if phorbol esters are generally thought to bypass early TCR signalling (IV, Fig 2). We therefore decided to examine the level of CD11/CD18-integrins expressed in these cells. Indeed, JCaM1.6 cells were found to express significantly reduced levels of functional integrin heterodimers on the cell surface, as examined by flow cytometry and cell surface labelling experiments (IV, Fig 3). As a control, the expression of CD3 was examined, and was found to be similar in all cell lines used (IV, Fig 3). To determine whether protein expression levels in the cells were normal, CD11/CD18-integrin protein expression in the cells was further studied. The JCaM1.6 cells were found to express high levels of a precursor form of CD18, that was immaturely glycosylated and not associated with CD11a, but instead existed as free, uncomplexed chains (IV; Fig 4). Transfection of JCaM1.6 cells with human, but not mouse (unpublished data), Lck, restored cell adhesion in response to CD3-triggering and phorbol ester, without affecting the low surface expression of integrins in these cells (IV, Fig 2, 3).

Also the role of the protein phosphatase CD45 in the regulation of integrin-mediated adhesion was studied (IV, Fig 2). CD45 is involved in the activation of Lck in T cells. CD45-deficient Jurkat T cells were able to bind to coated ICAM-1 and aggregate, in response to phorbol ester, but not in response to the the CD3-antibody.

Also other processes than integrin activation are involved in regulating cell adhesion. These are poorly understood, but presumably involve additional signalling pathways and cytoskeletal reorganisation. Therefore, we wanted to investigate signalling pathways involved in regulating cell-cell (rather than cell-coated ligand) adhesion. Chemical inhibitors of different signalling elements were examined for their effect on CD11/CD18-integrin-dependent T cell aggregation induced by phorbol ester and CD3-ligation. PI 3-kinase, which has previously been shown to be involved in the regulation of integrin-mediated cell adhesion, was confirmed to be involved in homotypic T cell aggregation induced by CD3-ligation by the use of two chemical inhibitors of PI 3-kinase, LY294002 and wortmannin (II, not shown).

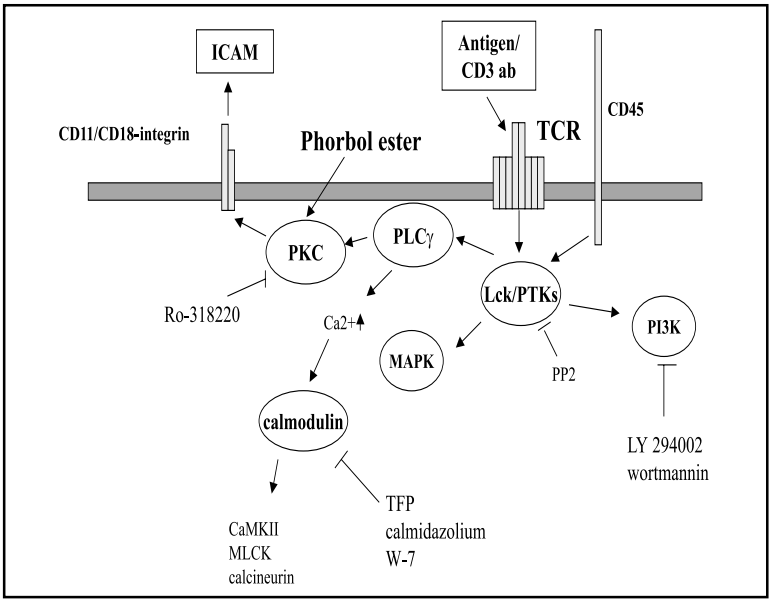


**Figure 10. Schematic figure of signalling pathways investigated in this study.** Anti-CD3 antibody or phorbol esters have been used to activate T cells. TCR-engagement leads to the activation of Lck and other Src-family kinases, and subsequently PLC, the MAPK pathways and PI 3-kinase. CD45, the transmembrane phosphatase, is involved in Lck activation. Phorbol esters directly activate PKC, while the $Ca^{2+}$-calmodulin-complex binds to and activates numerous downstream effectors, among them CaMKII, MLCK and calcineurin. The inhibitors to different signalling pathways used in this study are indicated. Trifluoperazine (TFP), calmidazolium and W-7 are calmodulin antagonists, while PP2 specifically inhibits the Src-family kinases and LY 294002 and wortmannin inhibit PI3 kinase. Ro318220 potently inhibits PKC.

The mammalian target of rapamycin (mTOR), a downstream effector of PI 3-kinase, was shown not to be involved in regulating aggregation (II, not shown).

Intracellular calcium has previously been shown to be important in the regulation of cell adhesion (Rothlein and Springer, 1986). Therefore, we wanted to examine whether the calcium-binding protein calmodulin, that regulates numerous calcium-dependent functions in cells, was involved in regulating cell adhesion. Indeed, W-7, trifluoperazine and calmidazolium, three structurally distinct calmodulin antagonists were shown to inhibit both CD3-induced and phorbol ester-induced CD11/CD18-dependent T cell aggregation (II; Fig 1). Downstream elements of calmodulin in T cells include myosin light chain kinase (MLCK), calcineurin and Ca-calmodulin kinase II (CaMKII). However, W-7, the calmodulin antagonist, at the concentration used to inhibit cell aggregation, does not influence dephosphorylation of the transcription factor NFAT (nuclear factor for activated T cells), which is induced by calcineurin, or phosphorylation of myosin light chains, induced by MLCK (II, Fig 2). Additionally, an inhibitor of CaMKII (KN-62) does not influence aggregation. Thus, these calmodulin-dependent enzymes were shown not to be involved in the calmodulin signalling pathway. However, both the calmodulin-antagonists and the PI3-kinase inhibitor affected integrin-dependent cell spreading and actin reorganisation in response to the CD3-antibody (II; Fig 4). Also the phorbol ester-induced cell spreading was inhibited by the calmodulin antagonists, but not by PI 3-kinase inhbitor (II, Fig 4). However, total integrin binding to the actin cytoskeleton was not affected by these inhibitors (II, Fig 3).

### 7.2. Identification of the kinase activity in leukocyte lysates that phosphorylates the CD18-integrin cytoplasmic tail (III)

Signalling pathways in T cells are involved in regulating integrin function through inside-out signalling and postereceptor events, regulating both integrin activity and adhesion strengthening. Integrin phosphorylation has been shown to occur in response to CD3-ligation and phorbol ester-stimulation (Hara and Fu, 1985, Valmu and Gahmberg, 1995), and may be a way to regulate integrin activation or other integrin-dependent events downstream of TCR initiated signalling pathways. To be able to examine the role of phosphorylation events in the regulation of integrins, we decided to purify the integrin kinase activity that could phosphorylate the integrin CD18-chain on serine and threonine residues. We therefore developed a kinase assay that could detect a kinase activity against a CD18 cytoplasmic peptide in leukocyte lysates. A kinase activity that phosphorylated the CD18 cytoplasmic domain on serine and threonine residues (not shown) was found in human leukocyte lysates (III, Fig 1). A purification protocol was developed to purify the kinase activity to near homogeneity using several chromatographic steps (III, Fig 2, 3). The proteins from the peak fractions of the final purification steps were subjected to tryptic mass fingerprinting and were identified as the catalytic domains of PKCδ and βI/II (III, Tables I and II). Catalytic domains of PKC are known to be active without the presence of lipid cofactors, explaining why they could be detected in the kinase assay with the CD18 cytoplasmic peptide. To confirm that PKC was the main kinase in leukocyte lysates that could phosphorylate CD18 on serine and threonine residues, the kinase activity in the column extracts was examined and found to be inhibited by PKC inhibitors (III, Fig 1).

### 7.3. Examination of integrin phosphorylation *in vitro* and *in vivo* (III, IV)

To examine the phosphorylation of the integrin CD18 cytoplasmic domain *in vitro*, seven different PKC isoenzymes were used to phosphorylate a peptide corresponding to the CD18 integrin cytoplasmic domain (III, Fig 4-6). The isoenzymes PKCα, a conventional PKC isoform and PKCη, a novel PKC isoform, appeared to be more active against the peptide than the other PKC isoforms βI, βII, δ, γ and ε (III, Fig 5A). Additionally, the PKC isoforms showed slightly different substrate specificity, inasmuch as PKCα, βII, δ and ε could phosphorylate the peptide both on serine and threonine, while PKCγ and βI phosphorylated the peptide almost exclusively on serine (III; Fig 5B). To examine the phosphorylation in more detail, the residues in the CD18 cytoplasmic peptide phosphorylated by PKC isoforms were determined by a combination of Edman sequencing, solid phase sequencing and mass spectrometry. PKCδ was shown to phosphorylate residues Ser745 and Thr758 in the integrin cytoplasmic domain, Ser745 being the major site (III, Fig 4). Interestingly, when PKCα was used to phosphorylate the peptide *in vitro*, it was shown to additionally phosphorylate residue Thr760, and possibly Thr759 (III, Fig 6).

To further investigate the relevance of the identified phosphorylation sites *in vivo*, phosphospecific antibodies were made against Ser745 and Ser756, a previously identified phosphorylation site in the integrin cytoplasmic domain. Ser756 could not be phosphorylated by PKC *in vitro* (III, Fig 4-6). We were not able to produce phosphospecific antibodies against Thr758 or Thr760 sufficiently sensitive to detect the phosphorylated integrins in cells. Ser756 and Ser745 phosphospecific antibodies were used to examine the *in vivo* phosphorylation of the integrin CD18-chain. We could confirm the result from previous mutational experiments (Hibbs et al, 1991) showing that Ser756 was an *in vivo* phosphorylation

site in the integrin (III, Fig 8). Ser756 phosphorylation was shown to be induced by both high amounts (200 nM) of phorbol ester alone, or low amounts (10nM) phorbol ester in the conjunction with the $Ca^{2+}$ ionophore A23178. The CD3-antibody OKT3 did not induce Ser756 phosphorylation either with or without pretreatment of cells with okadaic acid (III, Fig 8). Phosphorylation of Ser756 induced by both high amounts of phorbol ester alone, and by low amounts in conjunction with A23178, was inhibited by the PKC inhibitor, Ro318220. Additionally, the phosphorylation was significantly reduced by pretreatment of cells with a calmodulin antagonist, W-7 (III; Fig 8), that also inhibited cell spreading (II, Fig 4). Ser756 was found only to be phosphorylated in the intact integrin present on the surface of T cells, but not on the intracellular precursor form (IV, Fig 5), indicating that it can be used to distinguish mature and immature integrins.

Also Ser745 was found to be an *in vivo* phosphorylation site in the integrin CD18 cytoplasmic tail (III, Fig 7), induced by phorbol ester and strenghtened by the use of okadaic acid and the CD3-antibody together with phorbol ester. The phosphorylation of Ser745 was inhibited by the PKC inhibitor, Ro-318220, indicating that this phosphorylation event is mediated by PKC *in vivo*.

Also total integrin phosphorylation, induced by okadaic acid and phorbol ester in combination, which has been shown to induce both serine and Thr758-760 phosphorylation (Valmu and Gahmberg, 1995, Valmu et al, 1999), was inhibited by Ro318220. These results suggest that also the threonine phosphorylation of CD18 is mediated by PKC *in vivo* (III, not shown).

### 7.4. CD18-phosphorylation in the regulation of integrin binding to cytoplasmic proteins and cellular functions (I, II, III).

A putative way of regulating integrin function is by the binding of integrin cytoplasmic tails to different cytosolic proteins. Phosphorylation of integrin cytoplasmic domains has previously been shown to regulate the binding of the phosphorylated integrin to cytoplasmic molecules (Han et al, 2001, Jenkins et al, 1998, Cowan et al, 2000, Phillips et al, 2001, Kirk et al, 2000). Thus, we examined the role of integrin phosphorylation in the regulation of protein-protein interactions. A fractionation protocol of cells into soluble, nuclear and cytoskeletal (Triton X-100 insoluble) fractions was adapted (I; Fig 1). The cell surface CD11/CD18-integrin was found to exist mainly in the soluble fraction, while a small part of the integrins was found in the cytoskeletal fraction (I, Fig 2). Activation of the cells with phorbol ester in conjunction with okadaic acid did not significantly change the attachment of the integrin to the cytoskeleton (I, Fig 2), and the same was true for cells activated with the CD3 antibody, OKT3 (II, Fig 3). To examine the distribution of the phosphorylated integrin CD18-chain, $^{32}P$ labelled T-cells were fractionated into nuclear, soluble and cytoskeletal fractions, and the CD11/CD18-integrins were immunoprecipitated from the different fractions. Interestingly, serine- and threonine-phosphorylated integrins were shown to partitionate preferentially with the actin cytoskeleton (I, Fig 3), and threonine-phosphorylated integrins were shown to be enriched in the cytoskeletal fraction (I, Fig 4). We therefore wanted to investigate which cytoskeletal proteins could interact with the CD18 cytoplasmic domain. Peptide affinity chromatography experiments with a peptide corresponding to residues 746-769 of the cytoplasmic part of the integrin (containing Ser756 and Thr758-760) were performed. The cytoskeletal proteins talin and filamin, but not α-actinin or vinculin, were found to interact with this peptide (I, Fig 5). To confirm these

interactions *in vivo*, coprecipitation experiments were performed, and indeed, talin and filamin also coprecipitated with integrins in T cells (I, Fig 6). Talin and filamin are thus putative candidates for the regulated cytoskeletal interaction of the phosphorylated CD18-chain.

The role of threonine-phosphorylation in the regulation of protein-protein interactions of the integrin CD18-chain was further investigated using a Thr758 phosphorylated CD18-peptide. Two major protein bands from leukocyte lysates were observed to bind to a Thr758-phosphorylated peptide, but not to a non-phosphorylated peptide or a peptide phosphorylated on Ser756 (III, Fig 9). The interacting proteins were identified by tryptic mass fingerprinting as 14-3-3 isoforms (III, table III), proteins that interact with certain Ser- or Thr-phosphorylated sequences in proteins. This result was confirmed by immunoblotting with a 14-3-3 antibody (III; Fig 9). Additionally, 14-3-3 proteins from T cell cytoskeletal fractions, where the Thr-phosphorylated CD18-integrins preferentially reside (I, Fig 3, 4) were found to interact with the phosphorylated integrin peptide (III; Fig 9). Thus, 14-3-3 proteins are candidates for the regulated interaction between the phosphorylated integrin and the cytoskeleton.

We wanted to further study the role of CD18-phosphorylation in regulation of integrin-mediated adhesion. Therefore, a lipophilic peptide corresponding to the cytoplasmic part of the integrin CD18-chain was studied for its effect on CD11/CD18-integrin mediated adhesion to coated ICAM-1 and cell aggregation. Interestingly, the peptide was found to inhibit both T cell adhesion to coated ICAM-1 and cell aggregation in response to phorbol ester (I, Fig 8). Importantly, it also became phosphorylated inside cells (I, Fig 7).

8. DISCUSSION.

**8.1. Signalling pathways involved in regulating CD11/CD18-integrins.**

Lymphocyte adhesion is regulated at several levels. One of the levels is the regulation of integrin activation, the mechanism of which has remained poorly understood. Engagement of the TCR induces T cell adhesion to ICAM-1, which is important for T cell-APC contact and T cell killing events. Phorbol ester also triggers adhesion of CD11a/CD18-integrins to ICAMs. We have investigated the role of different signalling pathways in regulating T cell adhesion (Figure 10).

Lck is a tyrosine kinase that functions downstream of TCR triggering, and is crucial for the initial stages of T cell receptor signalling, like tyrosine phosphorylation and elevation of intracellular $Ca^{2+}$-levels (Straus and Weiss, 1992). However, the role of Lck in CD11/CD18-integrin activation has not been investigated. We have now found that PP2, a specific inhibitor of the Src-family kinases, inhibits CD3-induced CD11a/CD18-integrin-mediated adhesion of Jurkat T cells to coated ICAM-1. The inhibition of adhesion occurs with the same dose-dependency as MAP kinase inhibition, indicating that MAP kinase could be a downstream effector of Lck in regulating adhesion. MAP kinase has previously been shown to be involved in CD3-induced adhesion to ICAM-1 (O'Rourke et al, 1998). PP2 did not inhibit phorbol ester induced adhesion to ICAM-1. We further investigated the role of Lck in regulating integrins by using the JCaM1.6 cell line that is deficient in Lck. Intriguingly, we found that both phorbol ester-induced adhesion and CD3-induced adhesion is abolished in Lck-deficient Jurkat cells, even if phorbol esters are normally thought to bypass proximal TCR signalling events. Also T cell aggregation was deficient in these cells. Retransfection of JCaM1.6 cells with human, but not mouse, Lck, restored cell adhesion initiated both via phorbol ester- and CD3-stimulation. JCaM1.6 cells were therefore investigated for the surface expression of the CD11a/CD18-integrins and they were found to express significantly lower amounts of the integrins than wild type Jurkat cells. However, retransfection of Lck in JCaM1.6 cells did not restore CD18-expression to wild type levels. Lck overexpression in a cytotoxic T cell clone has previously been shown to increase the amount of CD11a/CD18-integrins on the cell surface (Torigoe et al, 1994), indicating that Lck is indeed involved in regulating integrin cell surface expression. The differences in these results may be due to differences in the amount of kinase expressed, or to differences in the cell lines used.

The results indicate that both Lck kinase activity and an adapter function of Lck are involved in regulating T cell adhesion. Lck may, for example, participate in the recruitment of PKC to membrane rafts, the signalling platforms in T cells where Lck resides normally, and where PKC isoforms (Parolini et al, 1999, Bi et al, 2001) and CD11a/CD18-integrins (Krauss and Altevogt, 1999, Leitinger and Hogg, 2002) are recruited upon T cell stimulation. Lck reorganisation of lipid rafts and T cell receptor signal stabilisation has been reported to be independent of the kinase activity, and instead require the Lck SH3-domain (Patel et al, 2001). The colocalisation of Lck, PKC and integrins in rafts would allow their functional interaction in the regulation of adhesion.

In addition to regulation of integrin activity, cell-cell adhesion is also regulated on other levels (Petruzzelli et al, 1998), like in adhesion strengthening during cell aggregation. Intracellullar calcium is known to be essential for adhesion (Rothlein and Springer, 1986). We found that calmodulin is a component

of the signalling pathways regulating integrin post-receptor events, since three independent calmodulin antagonists could efficiently abolish T cell aggregation both in response to CD3- and phorbol ester stimulation. Calmodulin-antagonists do not inhibit integrin-dependent adhesion to ICAM-1 induced by calcium-ionophores (Stewart et al, 1998), indicating that the inhibition occurs on the postreceptor level, rather than on the level of integrin activation. We have shown that the effect of the calmodulin antagonists on aggregation was due to diminished cell spreading, a process that is crucially dependent on integrins, as it does not occur in LAD-cells (Pardi et al, 1992). Also PI 3-kinase inhibitors were found to inhibit cell spreading in response to CD3- but not phorbol ester-induced stimulation. The downstream effector of calmodulin in this system was shown not to be MLCK, calcineurin or CaMKII, three common effectors of the $Ca^{2+}$-calmodulin pathway in cells. A potential downstream effector of calmodulin in the regulation of cell spreading and T cell aggregation is the actin-membrane linker protein, MARCKS. MARCKS is a protein that links the cortical actin cytoskeleton to the plasma membrane in resting cells, and crosslinks actin filaments (Hartwig et al, 1992). Upon cell stimulation, MARCKS is released from the plasma membrane to allow actin reorganisation and cell spreading to occur (Myat et al, 1997). This process is regulated by PKC phosphorylation of MARCKS and $Ca^{2+}$-calmodulin binding, which inhibits actin filament crosslinking (Hartwig et al, 1992). MARCKS dephosphorylation leads to subsequent membrane relocalisation. Very interestingly, MacMARCKS, a MARCKS relative in macrophages, has been shown to regulate integrin CD18 diffusion on the surface of cells (Zhou et al, 2000).

**8.2. Phosphorylation of the CD18 molecule**

The integrin CD18 cytoplasmic domain is important in regulating adhesion and also other functions of the leukocyte integrins, in analogy with the $\beta_1$- and $\beta_3$-integrin cytoplasmic domains (Bodeau et al, 2001). Phosphorylation of the integrin cytoplasmic domain after cell stimulation would be an ideal way of regulating integrin-mediated functions. The CD18-cytoplasmic domain has been found to be phosphorylated on serine (Hara and Fu, 1985, Valmu et al, 1991, Valmu and Gahmberg, 1995) and threonine (Valmu and Gahmberg, 1995) residues both after phorbol ester and CD3-ligation. Additionally, serine phosphorylation has been reported after fMLP and RANTES stimulation of cells (Chatila et al, 1989, Merrill et al, 1990, Szabo et al, 1997). The main phorbol ester-induced serine phosphorylation site was determined to be Ser756 in a mutational study (Hibbs et al, 1991), and the only threonines in the integrin cytoplasmic domain is the Thr758-Thr760-triplet.

We have now purified the main kinase activities in human leukocytes that can phosphorylate the integrin cytoplasmic domain on serine and threonine residues, and identified the kinases as different isoforms of PKC, a kinase that has long been known to be crucial for regulation of T cell adhesion. We have identified the phosphorylation sites in the integrin *in vitro*, and developed phosphospecific antibodies against these sites. The phosphorylation sites have then been investigated in detail *in vivo*.

We confirmed that Ser756 is a major phosphorylation site *in vivo* in T cells stimulated with phorbol ester. Ser756 is not phosphorylated by PKC, but instead by another, still unidentifed kinase. This kinase may be regulated by calmodulin, since calmodulin antagonists severely reduce Ser756 phosphorylation *in vivo*. The phosphorylation can be induced both by high amounts of phorbol ester alone, and by low amounts of phorbol ester in conjunction with calcium ionophore. This indicates that a calcium-dependent

PKC isoform may be involved in the signalling pathway upstream of the Ser756-kinase. However, no Ser756-phosphorylation can be seen after CD3-ligation either in the presence or absence of okadaic acid. Still, serine phosphorylation of CD18 occurs also after CD3-ligation (Valmu and Gahmberg, 1995), and thus, it must occur at a different site. Interestingly, we found that the main PKC phosphorylation site in the integrin cytoplasmic domain is Ser745, a previously unidentified site. This was found also to be phosphorylated *in vivo* in response to phorbol ester, and to be further enhanced after additional cell stimulation by CD3-ligation in the presence of okadaic acid. This indicates that this site is very transiently phosphorylated. Since the TCR-induced phosphorylation of CD18 is weak, and the antibody not very sensitive, we do not yet know if ligation of CD3 on its own causes Ser745-phosphorylation, but it does seem likely.

PKC isoforms, identified to be the major kinases in human leukocyte lysates that could phosphorylate the CD18-cytoplasmic domain, were also found to phosphorylate the threonine triplet that has been shown to be phosphorylated *in vivo* (Valmu and Gahmberg, 1995). Interestingly, different PKC isoforms were found to phosphorylate the TTT-triplet differently. PKCδ and β phosphorylated only Thr758, while PKCα could additionally phosphorylate Thr760 and possibly also Thr759. It has been shown that phorbol ester stimulation in conjunction with okadaic acid induces phosphorylation of two of the three threonines *in vivo* (Valmu et al, 1999), and that at least Thr758 and Thr760, and possibly Thr759 are phosphorylated under these circumstances (T. Hilden, personal communication). In contrast, CD3-stimulation of cells only leads to Thr758-phosphorylation (T. Hilden, personal communication). This indicates that different PKC isoforms can be activated by different cellular stimuli and phosphorylate different Thr-residues in the CD18-cytoplasmic tail. Since phorbol esters induce phosphorylation of the threonines in CD18, a cPKC or nPKC is probably involved. Interestingly, the nPKC, PKCθ is enriched in T cells and has been shown to localize to the central area of the immunological synapse (Altman et al, 2000), but there is so far no evidence for its colocalization with the CD11a/CD18-integrin. Another candidate PKC isoform is the cPKC, PKCβ. Rack1, an adaptor protein which binds to the active form of PKCβ and mediates its shuttling to specific locations in the cell (Ron et al, 1999), has been shown to bind to the membrane proximal part of the CD18-cytoplasmic tail in vivo after phorbol ester-stimulation of cells (Lilienthal and Chang, 1998). This would be an ideal way of regulating PKC positioning next to the phosphorylation sites in the integrin cytoplasmic domain. Additionally, Lck regulated PKC positioning in rafts, where integrins also reside, could be a way of regulating integrin phosphorylation. However, it is not yet known which PKC isoforms phosphorylate the CD18-chain on Ser745 and Thr758-760 *in vivo*.

### 8.3. The role of phosphorylation events in the regulation of CD11/CD18-integrins

The role of phosphorylation events in the regulation of integrins remains poorly understood. A mutational study revealed that mutation of individual phosphorylation sites in the CD18-cytoplasmic domain did not abolish binding of cells to coated ICAM-1 (Hibbs et al, 1991), even though mutation of the threonine triplet significantly reduced adhesion. Phosphorylation of the threonines occur after stimulation of cells by CD3-ligation or phorbol ester (Valmu and Gahmberg, 1995), stimuli which have been shown to induce clustering of integrins on the cell surface and avidity rather than affinity changes in the integrins

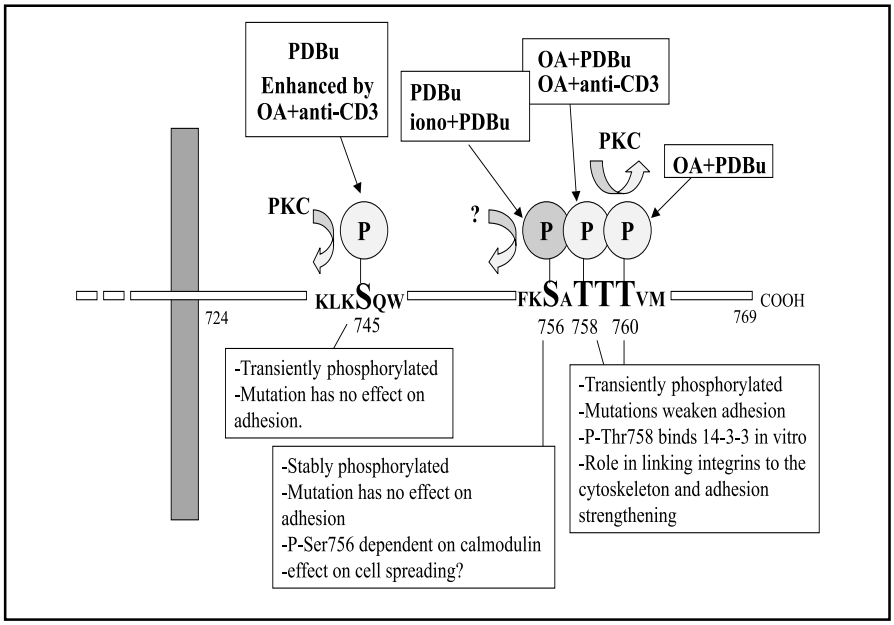


**Figure 11. Summary of the phosphorylation sites of CD18 investigated in this study.** The stimuli used for induction of phosphorylation of the different sites are indicated, as are the kinases involved. OA, okadaic acid, PDBu, a phorbol ester, iono, a calcium ionophore, anti-CD3, a CD3 antibody. The (putative) functions of the phosphorylation sites are in boxes.

(Stewart et al, 1998, Stewart et al, 1996, Kotovuori et al, 1999). Interestingly, mutation of the threonine triplet leads to reduced cytoskeletal association and actin reorganisation defects in cells (Peter and O'Toole, 1995).

We have shown that threonine-phosphorylated integrins distibute preferentially to the actin cytoskeleton *in vivo*. In light of the current model of integrin regulation, this process could be involved either in integrin activation, or in cytoskeleton-dependent adhesion strengthening that occurs after the initial release from the actin cytoskeleton to allow receptor clustering. Mutation of the threonines affect cell spreading and actin reorganisation, and adhesion strengthening (Hibbs et al, 1991, Peter and O'Toole, 1995). We have shown that PKC can phosphorylate the TTT-region in vitro, and PKC inhibitors inhibit CD18 threonine phosphorylation *in vivo*. Interestingly, overexpression of the PKC adaptor protein, Rack-1, that binds to CD18 and could localise the active kinase to the integrin (Lilienthal and Chang, 1998), leads to increased number of actin stress fibers and focal contacts in CHO-cells (Buensuceso et al, 2001), an effect that was dependent on PKC. This indeed would fit a model where threonine phosphorylation of the integrin CD18-chain by Rack-1-localised PKC, could be involved in adhesion-strengthening effects by inducing altered binding to the actin cytoskeleton and cytoskeletal reorganisation.

The linkage to the actin cytoskeleton could occur via talin or filamin, actin-binding proteins that have been implicated in the regulation of integrin-cytoskeleton interaction and integrin function (Calderwood et al, 1999, Sharma et al, 1995). Talin has also been speculated to be involved in keeping CD11a/CD18-integrins in the periphery of the immunological synapse by linking to the actin cytoskeleton

(Dustin and Cooper, 2000). We have shown that talin and filamin both bind to the integrin in the region that contains the phosphorylatable threonines. In coprecipitation studies, we have also found that both talin and filamin bind to the integrin *in vivo*. Whether phosphorylation regulates the interaction is not known. Filamin has been shown to interact with the corresponding region also in $\beta_1$ and $\beta_7$-integrins, and threonine-phosphorylation has been speculated to regulate the interaction, and, subsequently, cell migration (Calderwood et al, 2001).

It is possible that the detergent-insoluble fraction investigated in this study also contains lipid rafts, which have been shown to be Triton X-100-insoluble at +4°C, and thus possibly would cosediment with the isolated actin cytoskeletal fraction. Integrins have been shown to redistribute to lipid rafts after cell stimulation (Leitinger and Hogg, 2002), and the actin cytoskeleton plays a role in these events (Krauss and Altevogt, 1999). It may be that phosphorylation plays a role in integrin raft localisation, or in raft-cytoskeleton linkages.

Another possibility is that the cytoskeleton-integrin linkage is mediated by 14-3-3 proteins, which we found could interact specifically with Thr758-phosphorylated CD18-peptides.14-3-3s are proteins that bind to serine or threonine-phosphorylated proteins (Baldin et al, 2000), in analogy with SH2-domains that bind phosphotyrosine containing proteins. They can dimerize and thus function as adaptor proteins, and interact with numerous intracellular proteins. One of these proteins that binds 14-3-3s is Cbl, a ubiquitin ligase (reviewed in Liu and Gu, 2002). Cbl becomes serine-phosphorylated after T cell activation (Liu et al, 1997), and has been shown to be involved in $\beta_1$-integrin-mediated cell spreading in macrophages (Meng and Lowell, 1998). It could thus be a potential candidate for the linkage of threonine-phosphorylated CD18-molecules to the actin cytoskeleton via 14-3-3 proteins. We have indeed shown that a fraction of 14-3-3s in T cells was associated with the actin cytoskeleton, where the threonine-phosphorylated integrins also reside. Alternatively, 14-3-3s could be involved in building up signalling complexes at the site of the activated, phosphorylated integrin, or in actually clustering integrins by dimerizing with other integrin-bound 14-3-3 proteins.

The mutation of Ser756 in the integrin cytoplasmic domain does not reduce cell adhesion to ICAM-1 (Hibbs et al, 1991). Ser756 is also not phosphorylated in response to TCR-engagement. It is possible that this means that Ser756 phosphorylation is an artefact induced by phorbol ester stimulation of cells, and that Ser756-phosphorylation does not play a role in integrin regulation. However, there could be alternative explanations. The serine is quite well conserved in other β-integrin chains, implicating that it could have a function. Additionally, phorbol ester-stimulation does not necessarily mimic CD3-stimulation in the context of adhesion. Phorbol ester has actually been proposed to be an inducer of a migratory, rather than a stationary phenotype (Dustin et al, 1997, Volkov et al, 2001). The serine(s) phosphorylated after chemokine-activation of leukocytes has not been mapped, but could possibly be Ser756, and thus Ser756 could play a role in cell migration. Calmodulin-antagonists that reduce Ser756 phosphorylation have also been shown to reduce cell aggregation and spreading, that are regulated by phorbol ester. Additionally, Ser756 phosphorylation is induced by low amounts of phorbol ester in the presence of calcium ionophore, conditions that have previously been described to induce cell aggregation to the same degree as high amounts of phorbol ester alone (Valmu et al, 1991). These results implicate Ser756-phosphorylation in cell aggregation, i.e. postreceptor events that are regulated by different signalling

events than integrin activation (Petruzzelli et al, 1998). However, this has not yet been studied, and an alternative explanation is that calmodulin-induced rearrangements of the actin cytoskeleton may regulate the accessibility of the Ser756 kinase to the integrin cytoplasmic tail.

The role of Ser745-phosphorylation remains completely unknown. Ser745 is not conserved in other integrins and may thus be involved in CD11/CD18-integrin and leukocyte specific events. It is interesting to note that so many phosphorylation sites exist in the cytoplasmic part of the integrin CD18-chain (see Figure 11 for a summary). They may be phosphorylated in response to different cellular stimuli and have different functions, for example regulate binding to different cytoplasmic proteins. It is interesting that a lipophilic peptide, containing the whole integrin cytoplasmic domain, inhibits adhesion of T cells to coated ICAM-1, and also becomes phosphorylated within cells. It does imply that phosphorylation may play a role in regulating adhesion. However, integrins are also involved in several other functions in cells, like cell spreading, migration, and outside-in signalling, and phosphorylation may be involved in any of these events.

## CONCLUDING REMARKS

The immune system relies on dynamic adhesion events for function. The CD11/CD18-integrins are of pivotal importance for these processes. They are also essential for efficient costimulation of different immune cells. However, the regulation of integrin functions by signalling pathways, and integrin proximal events in the process leading to adhesion and signalling, have remained poorly understood.

Cell adhesion in the immune system is highly variable, and thus multiple mechanisms for regulation are likely to be employed. Cell adhesion events can be transient, for example when a T cell is migrating, or scanning the surface of an antigen presenting cell. Adhesion can also be very stable, like in the contact between T cell and APC. These different adhesive events may require different mechanisms of regulation. In stable adhesions, adhesion-strengthening via ligand-induced conformational changes and outside-in signalling to induce cellular changes is likely to be important. In the T cell-APC-interactions, it seems likely that a chemokine-signal first induces upregulation of integrin affinity, after which the TCR is engaged, leading to avidity increase via integrin clustering on the cell surface. We have shown that Lck is essential for proper integrin-activation, and both Lck kinase activity and adapter functions are needed. After integrin activation, adhesion is strengthened by cytoskeleton rearrangements and outside-in signalling of integrins, or secondary TCR signalling. We have shown that calmodulin is important for this process, by regulating cell spreading. These adhesion strengthening events may not be important during transient interactions, in contrast, very stable adhesion reduces the migration of cells.

Phosphorylation of the CD18-chain by PKC and other kinases on several sites that have been characterised in this study, is a potential integrin-proximal event that could regulate some of these diverse molecular processes involved in adhesion. Additionally, integrin phosphorylation may regulate the interaction of the integrin cytoplasmic tail with a number of cytoplasmic molecules, and could provide a platform for signalling complexes proximal to the activated integrin at the cell membrane. In the future, it would be important to mutate the identified individual phosphorylation sites to characterise their functions *in vivo*, in different integrin-mediated events.

Deregulation of CD11/CD18-integrin function has detrimental effects, leading to autoimmune disease or a nonfunctional immune system. Thus, understanding the regulation of integrin function is essential. The results presented here have revealed central regulatory mechnisms involved in integrin function, and provide a basis for further studies.

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