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b2 INTEGRIN SIGNALING IN LEUKOCYTES

Karim Dib and Tommy Andersson

Division of Experimental Pathology, Department of Laboratory Medicine, Lund University, Malmö University Hospital, Entrance 78, SE–20 502 Malmö, Sweden

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1. ABSTRACT

Members of the β2 integrin family are the dominating integrins expressed on leukocytes, and they play a major role in leukocyte cell-cell and cell-matrix adhesions during inflammation and other immune responses. β2 integrins are signaling receptors, but they are also targets of and are functionally affected by intracellular signals. Accordingly, researchers usually discuss two types of signaling by β2 integrins (and integrins in general): transmission of signals into the cell following binding of ligands or counter-receptors to the integrins (outside-in signaling), and regulation of the avidity and conformation of integrins by signals generated by other receptors within the cell (inside-out signaling). In this review, our aim is to summarize what is known about the capacity of β2 integrins to generate outside-in signaling in leukocytes, in particular polymorphonuclear neutrophils. Results in the literature clearly demonstrate that one of the earliest events in β2 integrin signaling is activation of non-receptor tyrosine kinases, which in turn triggers downstream activation of various signaling pathways that affect different functional responses of the cell. We also discuss molecules of potential importance in β2 integrin signaling.

2. INTRODUCTION

Integrins belong to a widely expressed family of cell-surface glycoproteins. These adhesion receptors bind specific ligands that are extracellular matrix proteins, soluble ligands, or counter-receptors on other cells. Integrins are heterodimeric transmembrane receptors that consist of an α subunit non-covalently associated with a β subunit. Each subunit has a large N-terminal extracellular domain, a hydrophobic transmembrane region, and a short C-terminal cytoplasmic domain. In vertebrates, at least seventeen different α and eight β chains have been cloned and shown to form at least twenty-four αβ dimers (1-3). Integrins are divided into eight subfamilies, and the β2 integrins constitute one such group. The most striking feature of the β2 integrins is that they are expressed exclusively on leukocytes, which is why they are also

referred to as "leukointegrins" (4, 5). The β2 integrins are designated CD11/CD18, because they are composed of a common β chain, CD18 (6), and one of four unique α chains, CD11a (α_L subunit), CD11b (α_M subunit), CD11c (α_x subunit), or CD11d (α_d subunit). The four αβ pairs are denominated LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), gp150/95 (CD11c/CD18) (4, 5), and $\alpha_d\beta 2$ (CD11d/CD18) (7, 8). Note that in this review, we will use the CD11/CD18 nomenclature. The CD11a chain is expressed on virtually all leukocytes and many leukocyte-derived cells, such as macrophages. T and B lymphocytes normally express only CD11a/CD18 (4). Polymorphonuclear neutrophils (PMNs) express three of the leukocyte integrins: CD11b/CD18 at a significantly higher level than CD11a/CD18 and CD11c/CD18 (5, 9). The most prominent CD11 chains on activated granulocytes and tissue macrophages are CD11b and CD11c, respectively (4). CD11d/CD18 is abundant on the $CD8^+$ lymphocyte subpopulation in peripheral blood and on macrophages present in specialized tissue compartments (7, 8). Additional details regarding the distribution of β2 integrins on leukocytes and leukocyte-derived cells have been published by Arnaout (4) and Gahmberg and colleagues (5).

The CD11b/CD18 integrins of circulating leukocytes are found mainly in specific granules and secretory vesicles and to a limited extent on the cell surface. In human PMNs activated with cytokines (e.g., TNFα), chemoattractants (fMLP, leukotriene B4), or phorbol 12-myristate 13-acetate (PMA), CD11b/CD18 integrins are translocated from secretory vesicles to the surface of the plasma membrane (10). It is assumed that this causes the integrins on the surface to adopt an active conformation. The way in which agonists induce a change in the avidity/conformation of integrins is referred to as "inside-out" signaling. Such signaling properties of β2 integrins have been thoroughly reviewed by other authors (2, 11-15) and will therefore not be given further consideration here.

Integrins themselves can also generate intracellular signals after interacting with ligands or counter-receptors on other cells ("outside-in" signaling). The objective of this review is to describe what is known about the nature of the intracellular signals that β2 integrins initiate in leukocytes. It is now evident that β2 integrins activate non-receptor tyrosine kinases, including the src family tyrosine kinases, which seem to play a key role in regulation of leukocyte functions. Activation of these kinases and their downstream effectors is essential for the regulating effects of integrins on cell shape and migration.

3. DISCUSSION

3.1. b2 integrin engagement induces activation of tyrosine kinases

Numerous investigators have suggested that one of the initial events in β2 integrin-mediated signaling in leukocytes is activation of various non-receptor tyrosine kinases. These include three members of the src family, $p58^{c\text{-}fgr}$ (Fgr), $p59/61^{hck}$ (Hck), and $p53/56^{Lyn}$ (Lyn) (16-20), and the non-src $p72^{syk}$ (Syk) tyrosine kinase (21-22). In addition, members of the focal adhesion kinase (FAK) family, $p125^{FAK}$ (23-25), as well as the focal adhesion kinase-related protein B (fakB) (26, 27) and PYk2 (28, 29) have been implicated. However, the precise mechanisms by which β2 integrins initiate activation of these tyrosine kinases are unknown.

3.1.1. Activation of src family tyrosine kinases

The primary structure of src tyrosine kinases is organized into the following series of domains: an N-terminal domain which is lipid-modified; the src homology (SH)3 and SH2 domains; a catalytic domain that is bilobal and a C-terminal regulatory end that contains a regulatory tyrosine residue (Tyr^{527} in src) which is conserved in all members of the src family. Tyrosine kinase activity is decreased when Tyr^{527} is phosphorylated and bound to the SH2 domain whereas phosphorylation of Tyr^{416} in the catalytic domain increases tyrosine kinase activity (30, 31). Williams and colleagues (31) recently reported that the contact between the SH3 domain of the molecule and the catalytic domain also participates in regulation of the kinase activity. Moreover, Cooper and Howell (30) have stated: "Oligomerization of the receptor-src kinase complex may stabilize the activated conformation of the src kinase and/or promote autophosphorylation in its kinase domain. This activation process could be further amplified by recruiting other cytoplasmic tyrosine kinases to the complex." It has been shown that clustering of β2 integrins on human PMNs causes these integrins to associate with Hck (32) and Syk (21). It is possible that other src tyrosine kinases can also become directly or indirectly associated with β2 integrins, because Yan and coworkers (21) have found that PMNs spreading on fibrinogen in response to TNF-α gave rise to a protein complex containing Fgr/Lyn and Syk. Thus it seems that β2 integrins have the potential to interact with several different tyrosine kinases, possibly resulting in a multi-protein-signaling complex. Thus, the src kinases may very well be directly activated by their recruitment to β2 integrins, as discussed below.

3.1.2. Activation of Syk

Syk is a 72 kDa protein tyrosine kinase involved in the transduction of signals initiated by many receptors in hemopoietic cells. Syk has been reported to be tyrosine phosphorylated on six tyrosine residues following B cell activation : one located between the tandem SH2 domains (Tyr^{130}), three in the linker region (Tyr^{317}, Tyr^{342}, Tyr^{346}), and two in the catalytic domain (Tyr^{519}, Tyr^{520}) (33). At least, two models could account for the activation of Syk. In B lymphocytes, aggregation of surface immunoglobulins induces the phosphorylation of the antigen receptor immunoreceptor tyrosine-based activation motifs (ITAMs), and this reaction is catalyzed by src family tyrosine kinases. Syk binds then to the phospho-ITAMs via its SH2 domains. The recruitment of Syk to the ITAMs could then stimulate Syk-catalyzed autophosphorylation of Tyr^{130}, $Tyr^{519}$and Tyr^{520} (33). Moreover, tyrosine residues in the linker region (Tyr^{317}, Tyr^{342}, Tyr^{346}) are the principal sites on Syk phosphorylated by Lyn but not by Syk. The phospho-tyrosine residues in the linker region are docking sites for the binding of Syk-interacting proteins such as Cbl, Vav

and PLC-γ. Interestingly, it has been shown in PMNs that both the α and β chains of β2 integrins are tyrosine phosphorylated upon engagement (see section 3.2) but it has not been proven whether these tyrosine phosphorylated sites are involved in the recruitement of Syk to the β2 integrins. Another model for Syk activation proposed an initial Lyn-catalyzed phosphorylation of $Tyr^{519}$and Tyr^{520} (catalytic domain) (34) which would then initiate a cascade of Syk-catalyzed intermolecular phosphorylation of tyrosine residues in the catalytic domain ($Tyr^{519}$and Tyr^{520}). Formation of a multi-protein complex containing both src tyrosine kinases and Syk has been observed after clustering of β2 integrins (21), which is compatible with the mechanism of src-dependent activation of Syk.

Consequently, it is difficult to distinguish the enzymes (Src kinases and/or Syk) that are involved in the tyrosine phosphorylation of proteins that occurs upon engagement of β2 integrins. Studies addressing that question have often used inhibitors of src family tyrosine kinases, such as (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo(3,4-d(pyrimidine) (PP1) (35), and agents that block Syk, for example (3,4,3',5'-tetrahydroxy-trans-stilbene) (piceatannol) (36). PP1 is a highly potent and selective inhibitor of src family tyrosine kinases (in *in vitro* kinase assays: IC_{50}= 5nM for Lck; 6nM for Lyn, 5nM for Hck, and 170 nM for src) whereas this component is essentially inactive for inhibition of ZAP-70 (35), a tyrosine kinase closely related to Syk. The potency of PP1 is decreased *in vivo*: PP1 blocks by 50% the anti-CD3-induced tyrosine phosphorylation of proteins at 0.5 μM (35). Piceatannol is less specific, although it does preferentially inhibit the activity of Syk as compared to Lyn (in *in vitro* kinase assays: IC_{50}= 10 μM for Syk and 100 μM for Lyn) (37).

Associations of tyrosine kinases with β2 integrins have been detected by Western blot analysis of tyrosine kinases in anti-CD18 immunoprecipitates, which indicates it is possible that such interaction may not be direct but instead involves "docking" proteins. Indeed, integrins are linked to the actin-based cytoskeleton via several actin-binding proteins, including α-actinin, talin, and filamin. β2 integrins are constitutively bound to filamin (38) and talin (39), whereas they must be clustered in order to bind to α-actinin (38). Both β2 integrin-dependent adhesion of human PMNs to fibrinogen and antibody-induced clustering of β2 integrins have been found to induce redistribution of Fgr, Lyn, and Syk to a Triton X-100-insoluble fraction containing a majority of the cytoskeletal proteins as well as caveolae, which are subcellular structures that are enriched in glycosylphosphatidylinositol-linked proteins and cytoplasmic signaling proteins (17, 18, 20, 21). The cited authors observed that this redistribution was associated with augmented tyrosine kinase activity and increased tyrosine phosphorylation of the tyrosine kinases. Thus, it is obvious that association of β2 integrins with the cytoskeleton is intimately related to activation of src tyrosine kinases and Syk. Similar activation of src tyrosine kinases has been induced in human PMNs by treatment with the protein kinase C (PKC) activator PMA (20) or fMLP (which exerts its effects by activating a G-protein-coupled receptor) (40). Taken together, these results support the notion that interaction of β2 integrins with the cytoskeleton is necessary for activation of the tyrosine kinases. However, such interplay *per se* is not enough to trigger signaling by these integrins, because, even in resting PMNs, there is substantial association of CD11b/CD18 with the actin cytoskeleton (38, 39, 41). As mentioned above, it is possible that a precise hierarchy of events controls the recruitment of cytoskeletal proteins and tyrosine kinases to a multi-protein signaling complex that is generated upon clustering of β2 integrins (18).

Studies of PMNs have demonstrated that the src tyrosine kinases Fgr and Hck play important roles in the β2 integrin signaling system. For example, PMNs from mice in which the genes for Hck and Fgr had been deleted were unable to spread and exhibited defective activation of specific adhesion-dependent functions (19, 42), which suggests that activation of tyrosine kinases occurs upstream of cytoskeletal rearrangements. In addition, it has been reported that the Syk inhibitor piceatannol, which also inhibits src tyrosine kinases to a certain degree, blocked the spreading of human PMNs on a fibrinogen-coated surface and production of H_2O_2 (22). Furthermore, genistein has been found to impede β2 integrin-induced actin polymerization in PMNs (43). Those findings indicate that activation of tyrosine kinases is necessary for β2 integrin-dependent modulation of the actin cytoskeleton

3.1.3. Activation of tyrosine kinases of the FAK family

FAK is a non-receptor tyrosine kinase that associates with integrins at focal contacts (44). Using an in vitro assay, it was observed (45) that FAK binds to β1, β2 and β3 integrins, and that such interaction involves an aspartic acid and a glutamic acid residue within the membrane proximal region of the β2 cytoplasmic domain. In addition, Rodriguez-Fernandez and colleagues (25) recently found that interaction of the β2 integrin CD11a/CD18 with its ligand, intercellular adhesion molecule 1 (ICAM-1), induced remodeling of T lymphocyte morphology and activation and redistribution of PYk2 and FAK from the cytoplasm to a microtubule-organizing center. Interestingly, increased tyrosine phosphorylation of FAK has been detected in human PMNs that were plated on a fibrinogen-coated surface and exposed to the chemoattractant fMLP, but this phosphorylation was not accompanied by increased tyrosine kinase activity of FAK (22). This suggests that the primary role of FAK in PMNs could be to serve as a scaffold protein for the recruitement of src tyrosine kinases as discussed previously (46). Moreover, piceatannol has been reported to block β2 integrin-induced tyrosine phosphorylation of FAK (22), which implies that Syk and/or src tyrosine kinases (see above) are directly or indirectly involved in such phosphorylation of FAK. At present, there is no real evidence that FAK plays a role in activation of specific adhesion-dependent functions in PMNs (47), but this issue has as yet not been fully explored.

Less is known about how fakB and PYk2 are activated, although Yan and Novak (28) observed that Lyn

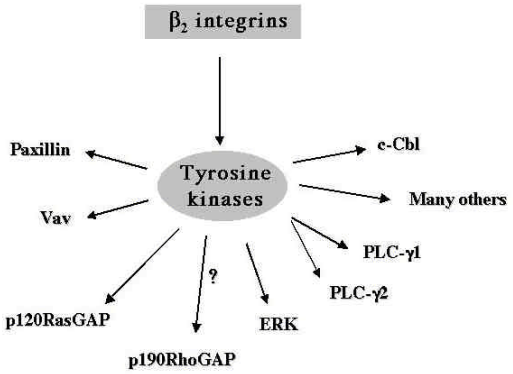

Figure 1. The different proteins that are tyrosine phosphorylated upon engagement of β2 integrins in leukocytes (For further details, please see the discussion).

and Syk may be required for β2 integrin-induced tyrosine phosphorylation of PYk2 in human PMNs. Furthermore, those investigators found that the activation of PYk2 occurred downstream of cytoskeletal rearrangement.

3.2. Proteins that are tyrosine phosphorylated upon b2 integrin engagement in leukocytes

As shown in Figure 1, a number of proteins are tyrosine phosphorylated in leukocytes upon engagement of the β2 integrins. These include, among others, the guanine nucleotide exchange factor Vav (48), the docking protein Cbl (Melander et al, unpublished data), the cytoskeletal protein paxillin (47), the extracellular regulated kinase ERK (49), the GTPase activating protein p120RasGAP (Dib et al, unpublished data) and possibly also the GTPase activating protein p190RhoGAP (50), and both CD11a and CD18 cytoplasmic chains (51). The contributions of some of these proteins to the β2 integrin signaling pathway are discussed in the following subsections.

3.2.1. The docking protein Cbl

Cbl is a multi-domain protein originally identified as the cellular homologue of the Cas NS-1 murine leukemia retroviral oncogene v-cbl (52). Cbl is expressed in hematopoietic cells, and it possesses proline-rich motifs and multiple potential tyrosine phosphorylation sites, which could mediate association with proteins that contain SH3 or SH2 domains respectively. Tyrosine phosphorylation of c-Cbl has been found to occur in response to clustering of different types of integrins in various kinds of cells: β1 integrins in macrophages, leukocytes (53-55) and the promegakaryocytic cell line MO7e (56); β2 integrins in eosinophils (57), the M07e cell line (56), and human PMNs (Melander et al, unpublished data). Several tyrosine kinases have been implicated in the integrin-induced phosphorylation of Cbl. More specifically, Fyn, Yes, and Syk have been shown to be the major kinases involved in such phosphorylation in T cells (58), whereas Fgr and Lyn were found associated with phosphorylated Cbl following β1 integrin engagement in macrophages (53). Interestingly, there is definite evidence that Cbl is the docking protein responsible for recruiting PI 3-kinase in the β1 integrin signaling system (see section 3.3.2 below; and refs 54, 55). In addition, recent reports support the view that Cbl acts as a negative regulator of syk (59) and Fyn (60). The Cbl-dependent negative regulation of Fyn in Jurkat T-lymphocyte was correlated with enhanced protein turnover (60). This is consistent with the finding that cell lines derived from Cbl (-/-) mice had elevated levels of Fyn as compared with wild type controls (60). In T lymphocytes, Cbl-b is a negative regulator of cell activation and autoimmunity (61, 62). Clearly, more work is needed to evaluate the role that phosphorylated Cbl plays in the β2 integrin signaling pathway in leukocytes.

3.2.2. The guanine nucleotide exchange factor Vav

Small GTP-binding proteins cycle between an inactive GDP-bound and an active GTP-bound form (63). In the latter conformation, these GTPases interact with specific effectors to initiate downstream signals that regulate various cellular functions. Guanine nucleotide exchange factors (GEFs), activated by extracellular stimuli, are responsible for the GDP-GTP exchange and thereby for activation of the GTPases. The two GEF proteins Vav and son of sevenless (Sos) have been found in leukocytes. Vav and Sos belong to the subset of GEFs that contain both a Dbl and a pleckstrin homology (PH) domain. In addition, Vav but not Sos contains two SH3 and one SH2 domain. Vav (which exists in two forms, designated Vav1 and Vav2) acts as a GEF for the small GTPases Rac1, RhoA, and RhoG (64-66). Two Sos proteins designated Sos1 and Sos2 have been found (67, 68). Sos proteins catalyse the GDP-GTP exchange for the small GTPase $p21^{Ras}$ (Ras) (68). The activities of Vav and Sos are regulated by a tyrosine phosphorylation event: Vav is activated by direct tyrosine phosphorylation (64), whereas Sos forms a complex with Grb2, a docking protein that binds to the tyrosine-phosphorylated form of Shc (69). The subsequent hydrolysis of bound GTP to GDP turns off the activity of the small GTPase, and that reaction is catalyzed by GTPase-activating proteins (GAPs); the GAPs are required, because the small GTPases have low intrinsic GTPase activity (63). Antibody engagement of β2 integrins on PMNs has been found to induce tyrosine phosphorylation of Vav (48), and, after such a change, Vav would be able to control the activity of the GTPase RhoA (see section 3.5). Several different tyrosine kinases have been shown to phosphorylate Vav in leukocytes, for example, Lyn (70), the syk-related tyrosine kinase Zap 70 in T lymphocytes (71), and Syk in B lymphocytes (71). Thus, Syk and src tyrosine kinases are again implicated in downstream signaling of β2 integrins in leukocytes, in this case regarding regulation of Vav.

3.2.3. p190RhoGAP and p120RasGAP

The best-characterized Rho-specific GAP is p190RhoGAP. It was originally found to co-precipitate with p120RasGAP and was assumed to be a tyrosyl-phosphorylated protein (72). In our experiments on human PMNs (50), we found that β2 integrin engagement increased the GAP activity of p190RhoGAP on RhoA, an effect that was totally blocked by a pretreatment with PP1. This finding is compatible with the idea that tyrosine phosphorylation of p190RhoGAP is necessary for β2 integrin-induced deactivation of RhoA in PMNs. Alternatively, the change in p190RhoGAP activity could be due to β2 integrin-induced tyrosine phosphorylation of p190RhoGAP-interacting proteins, such as p120RasGAP. This issue is presently tested in our laboratory.

3.3. Activation of phosphoinositide 3-kinase by β2 integrins

Phosphoinositide (PI) 3-kinase is a lipid kinase that phosphorylates phosphoinositides at the 3' position of the inositol ring. It is presumed that one of the primary events catalyzed by PI 3-kinase in intact cells is phosphorylation of phosphatidylinositol-(4,5)-bisphosphate (PIP_2), resulting in the formation of phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3). This enzyme consists of a regulatory (p85) and a catalytic (p110) subunit (73, 74). Two isoforms of p85 (p85α and p85β) and three splice variants of the p85α gene have been identified, and the p85 subunit contains one SH3 and two SH2 domains. Four isoforms of p110 have been cloned and are designated α, β, γ, and δ. The α, β, and δ forms of p110 (constituting class IA) occur as heterodimers with p85 subunits, and, notably, p110δ is expressed predominantly in leukocytes (75). In contrast, p110γ (class IB) associates not with p85, but instead with another regulatory subunit, p101, and the resulting heterodimer can be directly activated by βγ subunits of heterotrimeric GTP-binding proteins, which is not the case for the class IA PI 3-kinases (76). Two highly similar Gβγ-activated PI 3-kinases have been purified from pig PMN cytosol, and both of these are made up of a catalytic subunit (120 or 117 kDa) and a 101-kDa regulatory subunit. The p101 subunit has been found to potentiate the effect of βγ on PI 3-kinase activity (76).

PI 3-kinase appears to be involved in numerous aspects of leukocyte activity (77-79), including β2 integrin signaling in PMNs (80). Löfgren and coworkers (80) found that engagement of β2 integrins increased the activity of PI 3-kinase, as revealed by formation of PIP_3. Furthermore, Axelsson and coworkers (32) recently noted that clustering of β2 integrins induced phosphorylation and activation of protein kinase B/Akt, a downstream target of PI 3-kinase. Several different mechanisms have been proposed to regulate the activity of PI 3-kinase that occurs in response to extracellular stimuli (73, 74), but very little is known about how this kinase is activated in the β2 integrin signaling pathway of leukocytes, which is discussed in the following sections.

3.3.1. Association of PI 3-kinase with β2 integrins

It is well known that tyrosine-phosphorylated receptors, such as the platelet-derived growth factor (PDGF) receptor (81) and the T cell receptor (CD28) (78), can bind to the SH2 domains of p85 and thereby increase the catalytic activity of PI 3-kinase (73, 74). It has been shown in PMNs that both the α and β chains of β2 integrins are tyrosine phosphorylated upon engagement (51), but there is no evidence of a direct interaction between PI 3-kinase and the integrins. However, such a direct or indirect association has been observed in osteoclasts in which PI 3-kinase was co-immunoprecipitated with αvβ3 integrins (82).

3.3.2. Association of PI 3-kinase with docking proteins

As mentioned above, β2 integrins have the potential to use the docking proteins Cbl and/or FAK to generate downstream signals, and, as discussed below (section 3.4), they may also use insulin receptor substrate (IRS) proteins for that purpose. Once phosphorylated on tyrosine, the indicated docking proteins bind to and activate various signaling proteins that contain SH2 domains. Cbl (55), IRS (83, 84), and FAK (85) have all been shown (in various cell models) to bind and activate PI 3-kinase in response to cell adhesion or integrin engagement.

3.3.3. Association of PI 3-kinase with src tyrosine kinases

Liu and colleagues (86) have previously proposed that activation of PI 3-kinase entails direct interaction of the proline-rich domain of p85 with the SH3 domain of src family tyrosine kinases. Furthermore, we have recently found that antibody-induced clustering of β2 integrins on PMNs triggered an association of Fgr and Hck with the p85 subunit of PI 3-kinase (32). Thus, it seems that β2 integrin-induced activation of PI 3-kinase in PMNs to some extent involves interaction between the p85 subunit and src family tyrosine kinases.

3.3.4. Association of PI 3-kinase with small GTP-binding proteins

It is well established that the small GTP-binding protein Ras can bind the catalytic p110 subunits of PI 3-kinase in a GTP-dependent manner (87, 88), and that event induces the activity of the p110 subunit. Furthermore, it has been demonstrated that antibody-induced β2 integrin engagement in PMNs results in activation of Ras (48), and we have observed association of PI 3-kinase with the GTP-bound form of Ras following clustering of β2 integrins (32). Together, these results support the idea that β2 integrins can activate PI 3-kinase through an interaction between active Ras and p110 subunits.

It has been shown that binding of the small GTP-binding proteins Rac and Cdc42 to the bcr domain of the p85 subunit leads to activation of PI 3-kinase (89). Although it is known that both Rac and Cdc42 are present and activable in PMNs (90), nothing is known about the potential roles of these GTPases as mediators of integrin-induced activation of PI 3-kinase in leukocytes.

Obviously, leukocytes do seem to exhibit various mechanisms of β2 integrin-induced activation of PI 3-kinase, and that conclusion is compatible with the model in which optimal activation of PI 3-kinase requires input from both p85 and p110 subunits (88).

3.4. Possible cross-talk between the β2 integrins and the insulin/IGF-I signaling pathway

There is growing evidence that insulin receptor (IR) signaling and integrin signaling can interact and converge at the level of the insulin receptor substrate (IRS). The IRS molecules (IRS-1 and 2) (MW 170–190 kDa) are docking proteins that are known to be tyrosine phosphorylated on multiple residues upon stimulation of cells with insulin or IGF-I (91). These phosphorylated tyrosine residues create binding sites for proteins that contain SH2 domains, such as the p85 regulatory subunit of PI 3-kinase, the adaptor protein Grb2, and the tyrosine phosphatase Syp (91). The first study indicating an interaction between integrins and the IR was conducted by

Vuori and Ruoslahti (92), who found that insulin stimulation led to the association of IRS-1 with αvβ3 integrins. Subsequent research has revealed that αvβ3 integrins can associate with activated IRs (93), and also that integrin engagement induces tyrosine phosphorylation of IRS-1 (83, 84). Furthermore, insulin potently activates α5β1 integrin-mediated cell adhesion, and, in turn, IR kinase activity is enhanced by α5β1 integrins (83). Leukocytes express the components of the insulin/IGF-I signaling system, as evidenced by the findings that insulin and cytokines induce tyrosine phosphorylation of IRS-2 (IRS-1 is not expressed) in primary B and T cells, macrophages, and the murine myeloid cell line FD-5 (94). The phosphorylated IRS-2 can then recruit and activate signaling proteins such as PI 3-kinase. Reports also indicate that circulating human PMNs express IRs on their surface (95), and that insulin stimulates chemokinesis (96) and chemotaxis in these cells (97). Thus, it is tempting to suggest that, like β1/β3 integrins, the β2 integrins use the IR signaling system in leukocytes. However, to substantiate that possibility, it must first be determined whether cross-talk actually occurs between integrin and IR signaling pathways in leukocytes.

3.5. β2 integrins regulate the small GTP-binding proteins Ras and RhoA

The profound alteration in morphology that occurs in connection with the spreading and locomotion of PMNs is associated with β2 integrin-induced intracellular signals that, among other things, cause rearrangement of the actin cytoskeleton (80). Little is known about how β2 integrins regulate such rearrangement. However, in other cell types, small GTP-binding proteins belonging to the Rho subfamily (Rho A, B, and C; Rac 1 and 2; Cdc42 or G25K; RhoE, G, and D; TC10) and members of the Ras superfamily of small GTPases have been strongly implicated in cytoskeletal modulations generated by growth factors and integrins (98, 99).

Ras was originally identified as a molecular switch that relays proliferative signals from cell surface receptors to the nucleus. It is now known that Ras also regulates focal adhesion and stress fiber turnover and mediates a signal that is essential for cell movement during embryogenesis (100). Presently available data indicate that remodeling of the actin cytoskeleton in the motile but non-mitotic PMNs could be controlled by Ras in cooperation with the GTPases of the Rho subfamily.

3.5.1. β2 integrins regulate Ras in PMNs

In one of our studies (48), Ras was markedly activated in human PMNs plated on a surface coated with anti-CD18 antibodies. Moreover, genistein (a non-specific tyrosine kinase inhibitor), but not U73122 (a PLC inhibitor), blocked the β2 integrin-induced activation of Ras, indicating that a tyrosine kinase(s), but not a Ca^{2+} signal, is involved in this activation process. In addition, such activation was not significantly affected by the PI 3-kinase inhibitor wortmannin. In the cited investigation, we initially proposed that activation of Ras could be at least partly due to the activation of Vav, because we found that β2 integrin engagement in PMNs induced tyrosine phosphorylation of Vav and an association between Vav and Ras. That finding was in good agreement with results reported in the literature at the time, which seemed to imply that Vav is a guanine nucleotide exchange factor for Ras (101). However, more recent investigations have convincingly shown that Vav is a phosphorylation-regulated GEF for both Rac-1 (64, 65) and the Rho subfamily (66), but not for Ras. Thus, what we originally assumed to be an association of Ras with Vav following β2 integrin engagement in PMN, may instead represent an interaction between the Ras and the Rho signaling pathways. Obviously, the mechanisms by which β2 integrins activate Ras have not been completely elucidated, therefore it would be very interesting to ascertain whether or not such activation is due to tyrosine phosphorylation of Shc (another docking protein) and the formation of a Shc-Grb2-Sos complex. Indeed, tyrosine phosphorylation of Shc occurs after integrin clustering of α1β1, α5β1, αvβ3 (102), and α6β4 (103), suggesting a role for the Shc-Grb2-Sos complex in Ras activation elicited by β1 and β3 integrins. Furthermore, it has been suggested that Shc phosphorylation makes at least a partial contribution to α6β4-mediated activation of MAP Kinase (103).

3.5.2. β2 integrins regulate RhoA in PMNs

We recently observed that PMNs plated on a surface coated with anti-β2 integrin antibodies exhibited a transient increase in [^{32}P-GTP] bound to RhoA (50). A possible explanation for such an increase is that clustering of β2 integrins induces tyrosine phosphorylation/activation of Vav (48), which is a GEF for the Rho family of small GTPases (66). Alternatively, Vav may be activated by binding of D-3 phosphoinositides to its PH domain (104). The fact that PI 3-kinase is activated upon clustering of β2 integrins (see part 3.3) suggests that lipid products generated by PI 3-kinase could mediate the β2 integrin-stimulated activation of RhoA in PMNs.

In addition to the rise in [^{32}P-GTP] associated with RhoA, we found that β2 integrins induced an increase in [^{32}P-GDP] bound to RhoA (50). This latter increase is probably explained by an inducible intrinsic GTPase activity in PMNs whereby the exchange of GDP for GTP on RhoA is immediately counteracted by conversion of bound GTP to GDP, as has been suggested by Laudanna and coworkers (105). Our data also suggest that the β2 integrin-induced increase of RhoGAP activity is due to augmented tyrosine phosphorylation of p190RhoGAP or one of its associated protein (p120RasGAP) (50). Based on these findings, we concluded that engagement of β2 integrins in PMNs induces a rapid cycling of RhoA between its inactive and active states (Figure 2), a feature that might explain the key role this protein plays in regulating the dynamic alterations of the actin cytoskeleton that occur during cell locomotion.

Thus far, only Ras and RhoA are known to be activated by engagement of β2 integrins, although it is likely that other small leukocyte GTP-binding proteins, such as Rac1, Rac2, and Cdc42, are activated in the same

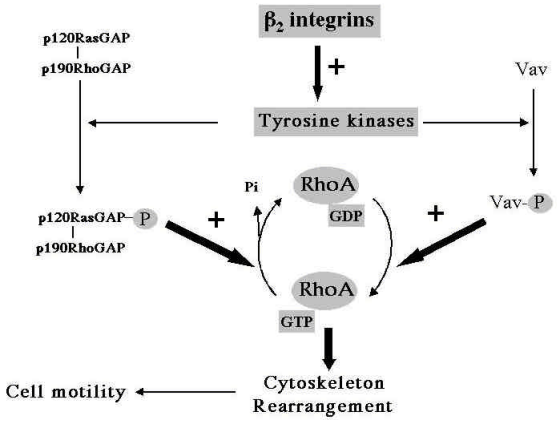

Figure 2. β2 integrin engagement in PMNs induces rapid cycling of RhoA between its inactive GDP-bound form to its active GTP-bound form. β2 integrin engagement in PMNs leads to tyrosine phosphorylation and activation of Vav (a GEF for RhoA). Tyrosine phosphorylated Vav catalyzes the GDP-GTP exchange on RhoA. β2 integrin engagement in PMNs also increases the phosphorylation content of p120RasGAP and perhaps to a limited extent of its associated partner p190RhoGAP. This would increase the GAP activity of p190RhoGAP on RhoA. Thus, we propose that it is not active GTP-bound RhoA per se that generates the dynamic changes in the actin cytoskeleton, but rather the rate of turnover between GDP-bound and GTP-bound RhoA that gives rise to PMN motility.

way. This issue is presently being investigated in our laboratory.

3.6. Calcium signaling

Early experiments revealed that spreading of human PMNs is accompanied by a large rise in cytoplasmic free Ca^{2+} (106). Furthermore, Jaconi and colleagues (107) detected spontaneous elevations in the level of cytosolic free Ca^{2+} in PMNs adhering to albumin- or fibrinogen-coated surfaces but not in suspended PMNs. In subsequent studies, two different approaches were used to demonstrate the participation of β2 integrins in the adhesion-induced Ca^{2+} signaling. First, pre-incubation with antibodies directed against CD11b and/or CD18 (108, 109) was found to block the adhesion-induced elevations in cytosolic free Ca^{2+} in PMNs. Secondly, antibody-induced engagement of CD11b or CD18 in suspended PMNs (a situation mimicking adhesion-induced clustering of integrins) (110, 111) and stimulation of PMNs with type I collagen (51) were observed to induce a transient increase in the cytosolic free Ca^{2+} concentration. Such transient augmentation of the Ca^{2+} signal in these cells is due to both a release of Ca^{2+} from intracellular stores and an influx of Ca^{2+} across the plasma membrane (110, 111). Similar Ca^{2+} responses have been found to occur upon antibody engagement of β2 integrins in cells of the monocytic line THP-1 (which express large amounts of CD11b/CD18) (112) and HL60 cells differentiated into neutrophil-like granulocytes (113, 114).

3.6.1. Phospholipase C-g is involved in the b2 integrin-induced Ca^{2+} signal

In general, all isoforms of PLC can catalyze the hydrolysis of phosphatidylinositol (4, 5) biphosphate into two second messenger molecules: diacylglycerol (DAG) and inositol trisphosphate [Ins(1,4,5)P_3]. The water-soluble [Ins(1,4,5)P_3] molecule stimulates the release of Ca^{2+} from internal stores (115), whereas the DAG molecule remains in the membrane where it serves as a natural activator of PKC, which is a Ca^{2+}- and phosphatidylserine-dependent Ser/Thr kinase (116). Several lines of evidence support the concept that activation of phospholipase C-γ (PLC-γ) is responsible for the β2 integrin-induced Ca^{2+} signal. Reports indicate that engagement of β2 integrins in T cells (117) and human PMNs (114) is directly linked to tyrosine phosphorylation of PLC-γ1 and PLC-γ2, respectively. Moreover, it is well known that such a covalent modification of PLC-γ is associated with activation of this isoform and thereby also with increased production of [Ins(1,4,5)P_3] (118, 119). Furthermore, it has been shown that β2 integrin-induced release of intracellular Ca^{2+} originates from thapsigargin-sensitive stores (120), and that U73122, a membrane-permeable aminosteroid that inhibits PLC signaling, dose-dependently inhibits β2 integrin-induced release of Ca^{2+} from intracellular stores (114). Garnotel and coworkers (51) have also found that stimulation of PMNs with type I collagen, which is presumed to activate the β2 integrin CD11a/CD18, induces a concomitant increase in both [Ins(1,4,5)P_3] and intracellular Ca^{2+}. Despite these findings, several investigators have detected only modest formation of [Ins(1,4,5)P_3] in TNF-stimulated adherent PMNs (121) and in PMNs with β2 integrins engaged in different ways (114, 122). However, Petersen and coworkers (111) observed that, in contrast to stimulation with chemotactic factors, clustering of PMN β2 integrins resulted in only a localized cellular increase in cytosolic free Ca^{2+}, which suggests that the local concentration of [Ins(1,4,5)P_3] might very well be sufficient to induce such a Ca^{2+} signal. Consequently, taken together, the cited data indicate that engagement of β2 integrins generates a PLC-γ- and [Ins(1,4,5)P_3]-mediated Ca^{2+} signal in leukocytes (**figure 3**).

3.6.2 . Tyrosine kinases are required for the b2 integrin-induced Ca^{2+} signal

The two tyrosine kinase inhibitors herbimycin A (117) and methyl-2,5-dihydroxycinnamate (113, 114) have both been found to block β2 integrin-induced Ca^{2+} signaling, which supports the notion that one or several tyrosine kinases are involved in generation of the Ca^{2+} signal, presumably via phosphorylation and activation of PLC-γ (figure 3). In other cell types and for different receptors, it has been shown that PLC-γ is a substrate for $pp60^{src}$ (119) as well as Syk (123). Moreover, engagement of β1 integrins on platelets appears to induce phosphorylation of Syk and a concomitant activation of PLC-γ2 (124). However, although engagement of β2 integrins on PMNs causes activation of several members of the src tyrosine family (Fgr, Hck, and Lyn) and Syk (see section 3.1), it cannot be ruled out that some other enzymes are responsible for the β2 integrin-induced activation of

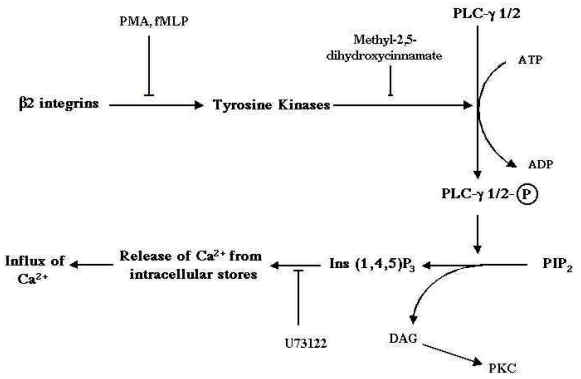

Figure 3. The mechanisms by which β2 integrins generate a Ca^{2+} signal in leukocytes. β2 integrin engagement induces activation of tyrosine kinases which in turn tyrosine phosphorylate and thereby increase the catalytic activity of PLC-γ. PLC-γ catalyzes the hydrolysis of phosphatidylinositol (4,5) biphosphate (PIP_2) into two second messenger molecules: diacylglycerol (DAG) and inositol trisphosphate [Ins(1,4,5)P_3]. [Ins(1,4,5)P_3] stimulates the release of Ca^{2+} from internal stores, which is followed by an influx of Ca^{2+}. In human PMNs, treatment with PMA, fMLP, or tyrosine kinase inhibitors (Metyl-2,5-dihydroxycinnamate, herbimycin A) blocks the β2 integrin-induced Ca^{2+}signal.

PLC-γ in these cells. One such alternative is indicated by the results of experiments on T-lymphocytes (26), showing that the tyrosine kinase fakB is activated after engagement of the CD11a/CD18 β2 integrin and subsequently interacts with the SH3 domain of PLC-γ1 as well as the phosphoprotein pp35-36 to form a multi-protein complex. In addition, it was recently shown that tyrosine-phosphorylated FAK associates with PLC-γ in human hepatic cells (125). Consequently, since β2 integrin engagement leads to tyrosine phosphorylation of FAK in human PMNs (23, 25), formation of a FAK-PLC-γ signaling complex may contribute to the β2 integrin-induced Ca^{2+} signal in leukocytes. However, research is needed to determine whether signaling by such a complex actually occurs, and, if so, whether it is responsible for the β2 integrin-induced Ca^{2+} signal.

3.6.3 . Involvement of PKC in b2 integrin signaling

Clustering of β2 integrins on PMNs achieved by use of anti-CD18 antibodies mounted on particles has been shown to activate first phospholipase D and thereafter PKC (126), and that signaling event was found to play an important role in regulating phagocytosis of complement-opsonized particles (122). PKC may be able to participate in that process, because it can induce phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS), an effect that renders MARCKS unable to mediate a connection between the cytoskeleton and the plasma membrane.

Notwithstanding, it is also clear that the PKC signal that is caused by β2 integrins is part of the mechanism that terminates the β2 integrin-induced Ca^{2+} signal. In support of that, treatment of granulocytic cells with PMA (113) or human PMNs with the chemotactic substance fMLP (120) prior to β2 integrin engagement has been observed to abrogate the Ca^{2+} signaling capacity of β2 integrins. The ability of PMA to inhibit the β2 integrin-induced Ca^{2+} signal was correlated with PMA-induced phosphorylation of the CD18 chain (113), which agrees well with the results of other studies in which treating leukocytes with fMLP (127) or PMA (128, 129) caused Ser/Thr phosphorylation of CD18. In the investigation by Hellberg and colleagues (113), PMA-induced Ser/Thr phosphorylation of CD18 was accompanied by impairment of β2 integrin-induced tyrosine phosphorylation of proteins, which implies that the indicated phosphorylation weakens a very early step in the β2 integrin signal pathway (figure 3). However it is difficult to reconcile the indicated finding of Hellberg and coworkers with the fact that treatment of PMNs with PMA causes src tyrosine kinases to redistribute to the cytoskeleton (see section 3.1.1), a phenomena that occurs in parallel with an increased tyrosine kinase activity. Additional studies are needed to clarify these somewhat contradictory findings.

As suggested above, it is probable that PKC is the Ser/Thr kinase that is responsible for the phosphorylation of CD18. Two major observations support that idea: 1) PKC is a well known target of PMA (116), and 2) β2 integrin engagement has been found to activate PKC in PMNs via activation of PLD (126). Furthermore, Valmu and colleagues (130) have reported that β2 integrins can be phosphorylated *in vitro* by purified PKC. However, in addition to PKC, at least one other Ser/Thr kinase may be responsible for the phosphorylation of β2 integrins, namely the integrin-linked kinase (ILK). Experiments have revealed that ILK phosphorylates *in vitro* a peptide corresponding to the cytoplasmic part of the β1 integrin and co-immunoprecipitates with β1 integrin in lysate of cells (131).

3.6.4. Role of the cytoskeleton in b2 integrin signaling

Several kinds of experimental data indicate that the signaling capacity of β2 integrins is critically dependent on some sort of interaction between integrins and the cytoskeleton. Jaconi and colleagues (109) noted that the β2 integrin-induced Ca^{2+} response could be blunted in PMNs that were preincubated with a cytochalasin prior to engagement of the integrins. Cytochalasins bind to the barbed ends of actin filaments and thereby inhibit further growth/polymerization of those structures. However, it has been observed that β2 integrin-induced actin polymerization can be abolished without influencing the protein tyrosine phosphorylations or Ca^{2+} signaling caused by β2 integrins (132), which suggests that cyochalasins affect the signaling capacity of β2 integrins by a mechanism that is not directly related to inhibition of actin polymerization. This seems to be similar to association of chemotactic peptide receptors with the actin cytoskeleton, a process that does not require actin polymerization *per se*, but instead the presence of uncapped actin filaments (133). In support of such a relationship between β2 integrins and the cytoskeleton, we have found that disruption of the association between β2 integrins and the cortical

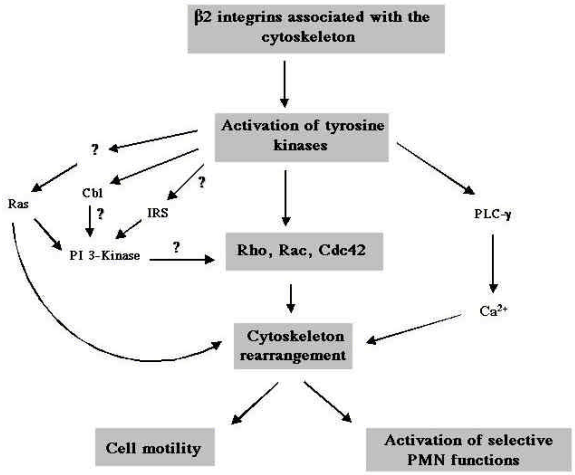

Figure 4. Overview of the cascade of events emanating from the β2 integrins. An interaction between β2 integrins and the cytoskeleton is required to elicit activation of tyrosine kinases. β2 integrin engagement induces redistribution of src family tyrosine kinases and Syk to the cytoskeleton and that process is associated with increased tyrosine kinase activity. The rapid cycling of the small GTPases RhoA and Rac is due to tyrosine phosphorylation of Vav and probably of p190RhoGAP or p190RhoGAP-associated proteins such as p120RasGAP. It is not known whether β2 integrin engagement induces activation of Rac and Cdc42 and, if so, what mechanisms are involved in that process. Likewise, it has not been shown how β2 integrins activate the small GTPase Ras, although that effect may involve the protein Shc (See section 3.5.1). It is possible that Ras plays a role in β2 integrin-induced activation of PI 3-kinase. β2 integrin-induced activation of Ras, RhoA, Rac and Cdc42 could regulate cytoskeletal rearrangement, an event that controls cell motility and activation of selective PMN functions. The two docking proteins Cbl and IRS may also be tyrosine phosphorylated in leukocytes following β2 integrin engagement. These proteins might recruit PI 3-kinase. The mechanisms by which β2 integrins generate a Ca^{2+} signal has been described in detail in Figure 3.

cytoskeleton abrogated the β2 integrin-induced Ca^{2+} signal and actin polymerization (41). Consequently, the observation that β2 integrins are associated with the cytoskeleton in freshly isolated and non-stimulated cells (38, 39, 41) implies that interaction of these integrins with the cytoskeleton is necessary for transmission of signals into the cell.

4. PERSPECTIVES

The conclusion from the studies reviewed above is that activation of src family tyrosine kinases and Syk is the initial signaling event triggered by β2 integrins on leukocytes. However, it is also evident that other non-src kinases, such as FAK, are activated. Although recent work implies FAK in the β2 integrin-signaling pathway, its precise role remains elusive. An important issue for the future is therefore to determine the contribution of FAK family tyrosine kinases in β2 integrin-dependent activation of selective leukocyte functions.

Engagement of β2 integrins on leukocytes is associated with a profound alteration of the cellular cytoskeleton, leading to cell spreading and locomotion. It is clear that activation of the small GTPases Ras and RhoA contributes to β2 integrin-induced regulation of the actin-based cytoskeleton. Whether other small GTPases such as Rac and Cdc42 are implicated in β2 integrin-induced modulation of the cytoskeleton is not know, nor are the downstream targets of these small GTPases identified. The future work needs to address these questions.

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Send correspondence to: Dr Karim Dib Division of Experimental Pathology, Department of Laboratory Medicine, Lund University, Malmö University Hospital, Entrance 78, SE–20 502 Malmö, Sweden, Tel: 46 40 332 593, Fax: 46 40 33 73 53, E-mail: Karim.Dib@exppat.mas.lu.se

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