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Adhesion to fibronectin promotes the activation of the p125^{FAK}/Zap-70 complex in human T cells

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SUMMARY

The β1 integrins are a family of heterodimeric adhesion receptors involved in cell-to-cell contacts and cell-to-extracellular matrix interactions. Through their adhesive role, integrins participate in transduction of outside/inside signals and contribute to trigger a multitude of cellular events such as differentiation, cell activation, and motility. The fibronectin integrin receptors, $\alpha_4\beta_1$ and $\alpha_5\beta_1$, can function as costimulatory molecules in T-cell receptor (TCR)-dependent T-cell activation. In the current study the Jurkat T-cell line was used as a model system to investigate the TCR-independent role of cell adhesion to fibronectin in the activation of Zap-70, a central molecule in the signalling events in T cells. Upon adhesion to plastic immobilized fibronectin but not to bovine serum albumin (BSA) the phosphorylation of p125^{FAK}, a protein kinase that localizes to focal adhesion sites, was induced. Moreover, clustering of fibronectin receptors led to the detection of a p125^{FAK}/Zap-70 complex. Finally, while the complex between fak-B, another protein kinase localized to focal adhesion sites, and Zap-70 was detected in cells plated either on BSA or on fibronectin, the formation of the p125^{FAK}/Zap-70 complex appeared specifically induced following fibronectin-mediated integrin clustering. These data suggest the existence of a high degree of specificity when the members of the β1 integrin family mediate signalling pathways in T cells.

INTRODUCTION

The integrin family of cell surface receptors has long been known to play an essential role in the physical aspects of cell adhesion: these molecules represent the principal receptors for extracellular matrix (ECM) proteins but also serve as transmembrane bridges between the ECM and the actin containing filaments of the cytoskeleton. Co-ordinate regulation of integrin-mediated functions is of fundamental importance not only to cell adhesion and migration, but also to the overall cellular architecture, survival and function.[1] Engagement of integrins by their extracellular ligands transduce signals mediating cytoplasmic alkalinization, increase in intracellular Ca^{2+}, and tyrosine phosphorylation.[2–4] In many cell types there is a prominent tyrosine phosphorylation of proteins of 105–130 000 MW following integrin cross-linking.[5,6] A large body of experimental data support the concept that the autophosphorylation of a protein kinase, p125^{FAK}, that localizes to the focal adhesion contacts, is involved in the reorganization of the cytoskeleton and in the regulation of cell shape, and this enzymatic step is considered to be one of the key regulatory points in signal transduction following cell adhesion.[7–9] In fact, association of the cytoplasmic components tensin and paxillin to the focal adhesion depends upon the autophosphorylation of p125^{FAK}. On the other hand, after cell activation p125^{FAK} is bound by src family kinase pp60^{src}, pp59^{fyn} and pp51^{csk} through SH2 domains and these domains also link p125^{FAK} to the Grb2 adaptor protein and then to the Ras pathway[10] and thus integrins' ligation transduce external stimuli from the ECM to the nucleus.

Several members of the integrin family that regulate cell adhesion to ECM are also expressed on the T-cell surface and, apart from a role in cell adhesion, recent observations have shown that they participate in transduction of outside/inside signals and trigger a multitude of cellular events such as activation, differentiation and functional responses[11] of T cells. While it is known that binding of T cells to fibronectin (FN) via β_1 integrins leads to the generation of costimulatory signals and that T-cell adhesion to various ECM proteins can potentiate the response to substimulatory amounts of anti-CD3 or

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Abbreviations: ECM, extracellular matrix; FN, fibronectin.

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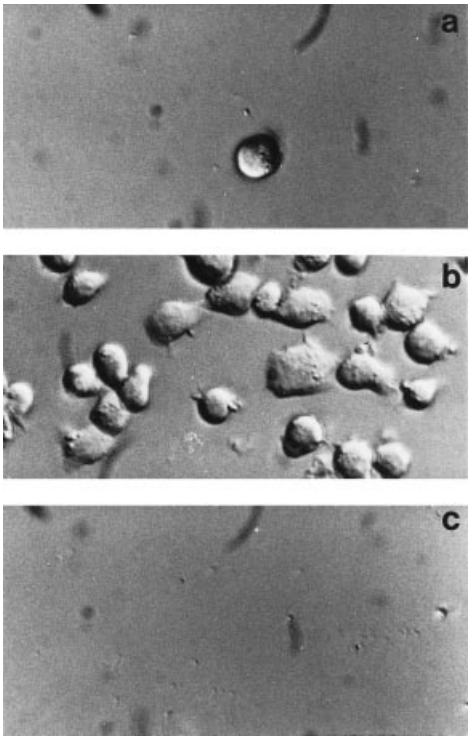

Figure 1. Cell adhesion to fibronectin. Jurkat cells were added to plastic-immobilized BSA (10 μg/ml) (a) or FN (10 μg/ml) in the absence (b) or in the presence (c) of the anti β1 functional mAb 4B4. Adherent cells were analysed with an inverted microscope and micrographs were made using Nomarski optics.

anti-T-cell receptor (TCR),[5,12] the signals that are required to induce cytoskeletal changes needed for increased adhesion remain largely unknown. Studies with human CD4$^+$ peripheral T cells suggest that α4β1 and α5β1 account for all of the adhesion to FN.[5,12,13] Engagement of TCR by antigen activates several protein kinases of which Zap-70 is one of the most relevant.[14–19] We have recently demonstrated that strong adhesion of T cells to FN up-regulated the levels of nuclear factor-κB (NF-κB) and that the increased NF-κB binding activity was inhibited by calphostin C, an inhibitor of protein kinase C (PKC).[20] Using FN and the Jurkat T-cell line as a prototype model, in the current study we demonstrate that FN stimulates tyrosine phosphorylation of p125^{FAK} and that cross-linking of β1 integrins by insoluble FN results in a p125^{FAK}/Zap-70 complex and in the induction of tyrosine phosphorylation.

MATERIALS AND METHODS

Materials

FN was purified on gelatin–Sepharose as described.[21] Phorbol myristate acetate (PMA) and sodium orthovanadate were purchased from Sigma (Sigma Chemical Co., St Louis, MO). Leupeptin and aprotinin were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech Inc. (Uppsala, Sweden).

Antibodies

Rabbit polyclonal antibodies against peptides at the C-terminus[22] of murine p125^{FAK} were generously provided by Dr G. Tarone, University of Torino. One of these antibodies is specific for p125^{FAK} and does not recognize other kinase-related members; the second polyclonal antibody, raised against a closely localized peptide at the C-terminus,[22] could not discriminate between p125^{FAK} and the closely related fak-B member of the FAK family (Dr G. Tarone, personal communication). Anti-phosphotyrosine mouse monoclonal antibody (mAb) PY20 and the anti Zap-70 mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mAb (4B4) against the β1 integrin subunit was purchased from Ortho (Ortho Diagnostic Systems, Raritan, NJ). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G (IgG) antibodies were purchased from Sigma.

Cell line

Jurkat T cells were originally obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI-1640 (BioWhittaker Inc., Walkersville, MD) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD), 100 U/ml penicillin/streptomycin and 2 mM L-glutamine and grown to a density of $3–5 \times 10^5$/ml. Prior to the use in our assays, a clone of Jurkat cells was selected through several rounds of cell adhesion onto FN.

Adhesion assay

Twenty-four-well tissue-culture plates were coated overnight at 4° with 0·3 ml of carbonate buffer, pH 9·4, containing 10 μg/ml FN. The wells were blocked with 0·5% bovine serum albumin and washed with phosphate-buffered saline (PBS) prior to use. Jurkat cells were washed, resuspended in serum free RPMI-1640 and added to FN-coated wells (7×10^5 cells/well), centrifuged at 150 ***g*** and incubated at 37° for different times, time zero correspondng to the end of the centrifugation. Cells were collected and washed with cold PBS containing 1 mM sodium orthovanadate. For inhibition experiments, cells were plated onto FN as above, in the presence of function-blocking or control antibodies. Adherent cells were analysed with an inverted microscope (DM IRB, Leica, Densheim, Germany) equipped with a differential interference contrast objective.

Preparation, of cell lysates, immunoprecipitation and immunoblotting

After stimulation, whole cell lysates from 1×10^7 cells were prepared for immunoprecipitation. Cells were washed once with cold PBS plus 1 mM sodium orthovanadate, resuspended in 300 ml of lysis buffer containing 50 mM Tris–HCl, pH 7·5, 50 mM NaCl, 1% Triton-X-100, 2 mM ethylenediamine tetra-acetic acid (EDTA), 50 mM NaF, 1 mM phenylmethylsulphonyl fluoride (PMSF) 10 mg/ml aprotinin, 10 mg/ml leupeptin, and incubated on ice for 20 min. After lysis the samples were centrifuged at 12 000 **g** for 20 min and the supernatants were used for immunoprecipitation. Immune complexes were precipitated with protein A Sepharose beads and fractionated on 10% polyacrylamide gels under reducing conditions. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, non-specific sites were blocked with

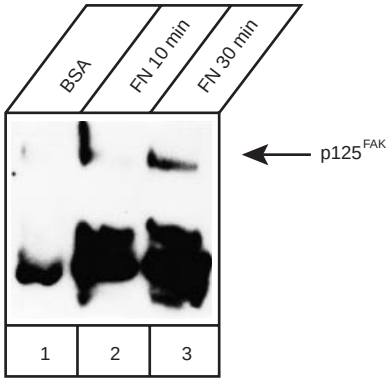

Figure 2. Induction of $p125^{FAK}$ tyrosine phosphorylation following adhesion to fibronectin Phosphorylation of $p125^{FAK}$ was examined in Jurkat cells adherent to plastic-immobilized fibronectin (10 μg/ml) for 10 or 30 min or incubated onto BSA-treated dishes for 30 min. Aliquots of cell lysate containing equivalent amounts of proteins as determined by the Lowry assay were used for immunoprecipitation. $p125^{FAK}$ was incubated with specific antibodies and immunocomplexes were attached to protein A; then, immunoprecipitated complexes were fractionated on a 10% polyacrylamide gel under reducing conditions and immunoblotted with an anti-phosphotyrosine mAb.

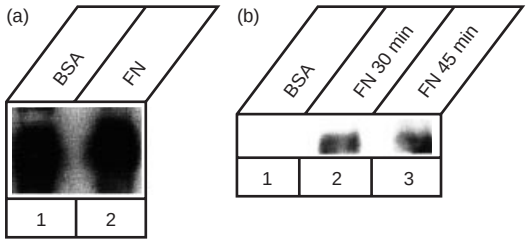

Figure 3. Coprecipitation of the $p125^{FAK}$/Zap-70 complex following adhesion to FN. (a) Association of FAK-related members with Zap-70 was examined in Jurkat cells plated for 30 min onto BSA- or FN-coated (10 μg/ml) dishes. Cell lysates were incubated with the anti-$p125^{FAK}$ antibodies and immunocomplexes were attached to protein A–Sepharose, fractionated on a 10% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with an anti Zap-70 antibody. (b) Association of $p125^{FAK}$ with Zap-70 was examined in Jurkat cells plated for 30 min onto BSA- (10 μg/ml) and for 30 or 45 min onto FN-coated (10 μg/ml) dishes. Cell lysates were incubated with anti-$p125^{FAK}$ specific antibodies and immunocomplexes were processed as in (a). The samples contained equal protein amounts by protein assay and equal quantities of $p125^{FAK}$ by Western blotting.

blocking buffer (1% low-fat dry milk in PBS). The blots were then stained with anti-phosphotyrosine or anti Zap-70 antibodies, washed extensively, stained for 1 hr with secondary antibody and visualized by the enhanced chemiluminescence (ECL) system (Amersham).

RESULTS AND DISCUSSION

To study the signal events generated following triggering of FN integrin receptors an *in vitro* system using a clone of Jurkat T cells selected for the ability to strongly adhere onto FN was employed. FN-coated wells readily promoted adhesion of Jurkat cells with a rapid time-course and at 20 min 90% of the cells projected small processes, appeared flattened and spread out and lost homotypic aggregation (Fig. 1b). Cells plated onto BSA did not show any attachment to the substrate (Fig. 1a). $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are the major β1 integrins expressed on Jurkat cells (23 and our unpublished observations). Although expression of this type of receptors is not always a predictor of integrin usage, in this case the addition of the β_1 specific 4B4 mAb (Fig. 1c) or of both anti α_4 and α_5 mAbs (data not shown) could completely abrogate cell adhesion. These constitutively expressed integrins co-operated in promoting adhesion of Jurkat cells, the α4β1 integrin displaying a higher efficiency when used on the intact FN ligand molecule, while the α5β1 integrin was more efficient when only the FN central cell-binding domain was used as a substrate (unpublished data). Thus, binding of our subline of Jurkat cells to FN can be accounted for completely by the β1 integrins α4β1 and α5β1.

Several lines of evidence indicate that integrin ligation induces tyrosine phosphorylation of numerous intracellular substrates including $p125^{FAK}$: this molecule is then involved in signal transduction to the cytoskeleton and to the nucleus.[3] A rapid and sustained synergistic increase in tyrosine phosphorylation of $p125^{FAK}$ had been demonstrated in Jurkat cells after the simultaneous triggering of the TCR–CD3 complex and of the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin-mediated binding to immobilized FN.[24] Initially and to confirm that adhesion to FN was able to induce activation of $p125^{FAK}$ also in the present Jurkat subline, cells incubated on plastic surfaces coated with BSA or FN were solubilized and analysed for tyrosine phosphorylation of $p125^{FAK}$. Cell lysates were immunoprecipitated with anti-$p125^{FAK}$ antibodies and then analysed by Western blotting with the anti-phosphotyrosine mAb PY20 (Fig. 2): already at 10 min of adhesion (lane 2) an increased phosphorylation, compared to BSA-stimulated cells (lane 1), was detected that further increased at 30 min (lane 3). In the same blot other co-immunoprecipitated bands of higher mobility were stained by mAb PY20. One phosphoprotein, absent from the BSA-stimulated sample and migrating with an approximate molecular mass of about 70 000 MW, led us to hypothesize that it could represent Zap-70. The strong band migrating around 50 000 MW very likely represented the heavy chain of the primary antibody. Solid phase cross-linking of $\alpha_4\beta_1$ using specific antibodies or the CS-1 region of FN stimulated tyrosine phosphorylation of several proteins in Jurkat cells including PLCγ, $p125^{FAK}$, paxillin, $p59^{fyn}$/$p56^{lck}$ and MAP kinase.[24,25] However, neither protein associations nor the involvement of Zap-70 were investigated in that study and therefore it was not determined whether triggering via the $\alpha_4\beta_1$ receptor was sufficient for the formation of a complex between $p125^{FAK}$ and Zap-70. In a different study Rabinowich and colleagues were able to demonstrate that cross-linking of either $\alpha_4\beta_1$ or $\alpha_5\beta_1$ in NK cells was sufficient to induce phosphorylation and physical association of $p59^{fyn}$ and Zap-70 with $p125^{FAK}$.[26] The signal transduction pathways initiated by integrin ligation involve cytoskeletal-dependent activation of tyrosine kinases and phosphorylation of a number of substrates.[5,6] Tyrosine phosphorylation of $p125^{FAK}$ has been observed in a variety of cell types, suggesting that this kinase is part of a common pathway for integrin signalling. Moreover, related focal adhesion tyrosine kinases are phosphorylated when B or T cells are activated: Pyk-2 or RAFTK, is tyrosine phosphorylated after β1 integrin stimulation in B cells,[27] or after β3

integrin stumulation[28] in cytotoxic lymphocytes; another member of this family, fak-B,[22] can become phosphorylated in T-cell lines stimulated with interleukin-2 (IL-2) via a β2-integrin dependent signal pathway.[29,30] We assayed whether also in our T-cell system activation involved fak-B in a β1-integrin dependent fashion and whether this kinase would associate with Zap-70. Jurkat cells plated for 30 min onto BSA- or FN-coated dishes were lysed, and the cell lysates were incubated with polyvalent (i.e. able to recognize several members of the FAK family) or $p125^{FAK}$-specific antibodies. Blots of these precipitated complexes were immunodecorated with Zap-70 antibodies. Fak-B was constitutively associated with Zap-70 (Fig. 3a) and stimulation with FN did not augment significantly the level of this complex. On the other hand, the association of $p125^{FAK}$ with Zap-70 was specific for cells stimulated with FN for 30 min (Fig. 3b) and this association persisted almost unchanged also at 45 min

In our T-cell model $p125^{FAK}$ was tyrosine phosphorylated after a brief period of adhesion to FN and Zap-70 was co-immunoprecipitated with $p125^{FAK}$. It has also been reported that $p125^{FAK}$ is slightly phosphorylated in BSA-stimulated Jurkat and H9 cells but that its levels increase after adhesion to FN.[25] Furthermore, in a different experimental system, a $CD4^+$ clone stimulated with CD3 or the chemokine RANTES, $p125^{FAK}$ associated with two separate 70 000 MW proteins, paxillin and Zap-70.[31] In that case the trimolecular complex was preformed but the levels of tyrosine phosphorylation were markedly increased after stimulation with the chemokine. Although we could not detect Zap-70 in our BSA-stimulated cells, the phosphorylation and the association with $p125^{FAK}$ were readily detected after 10 min adhesion; these results are in agreement with the possibility that in the antigen-independent activation of T-cell adhesion to FN can lead to activation of Zap-70. $p125^{FAK}$ was found to associate with non-receptor kinases such as $p59^{fyn}$ and $p60^{src}$[32] via their SH2 binding domains. However, soluble fusion proteins containing the tandem SH2 domains of Zap-70 did not interact with any other phosphoprotein *in vitro* except TCRζ and CD3ε.[33,34] Therefore, a mechanism involving other elements such as paxillin will be required to explain the observed physical association between $p125^{FAK}$ and Zap-70 (32 and present report).

In this study we investigated the signal transduction mechanisms induced by integrin-mediated cell–ECM interactions in T lymphocytes. The overall results suggest that in antigen-independent conditions the cross-linking of both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins by immobilized FN induces a signalling cascade, not necessarily dependent upon both integrins, which involved the activation of a molecular complex between $p125^{FAK}$ and Zap-70. Recognition of fibronectin is rather more complex than previously supposed: multiple binding sites to which α4β1 binds, not only the classic IIICS peptide, but III-CS, RGD, and at least one synergistic site within the central cell binding domain have been described.[35–37] Thus, discriminating between downstream effects mediated by each individual integrin is not easily attained using fragments of fibronectin (i.e. the central cell-binding domain and the IIICS peptide to discriminate between binding to the α4β1 and α5β1 integrins), that have previously been considered 'specific' for either integrin. An alternative approach using solid phase cross-linking of individual integrins with integrin-specific mAbs has also been used to correlate activation of integrins with downstream events.[24,25] However, regardless of the 'artificial way' of integrin activation, it can not avoid cross-talks between the mAb-activated integrin and other integrins expressed by the cell under study.

In conclusion, while apparently more unique signalling pathways are activated by cross-linking $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins with FN in T cells such as the PKC-dependent modulation of the p50 and c-Rel components of NF-κB[20] and the down-regulation of GATA-3 (unpublished), other signalling proteins are shared with several receptor-mediated signalling pathways ($p125^{FAK}$ and Zap-70). Therefore, the generalized T-cell activation with enhanced cytokine gene expression and cytokine release along with induction of cell proliferation even in the absence of a specific antigen-mediated activation[38] detected during the course of inflammatory processes, could depend upon cross-talks between quite different but converging receptor-signalling pathways.

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