

The Role of Protein Tyrosine Phosphorylation in Integrin-mediated Gene Induction in Monocytes

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Abstract. Integrin-mediated cell adhesion, or cross-linking of integrins using antibodies, often results in the enhanced tyrosine phosphorylation of certain intracellular proteins, suggesting that integrins may play a role in signal transduction processes. In fibroblasts, platelets, and carcinoma cells, a novel tyrosine kinase termed pp125^{FAK} has been implicated in integrin-mediated tyrosine phosphorylation. In some cell types, integrin ligation or cell adhesion has also been shown to result in the increased expression of certain genes. Although it seems reasonable to hypothesize that integrin-mediated tyrosine phosphorylation and integrin-mediated gene induction are related, until now, there has been no direct evidence supporting this hypothesis. In the current report, we explore the relationship between integrin-mediated tyrosine phosphorylation and gene induction in human monocytes. We demonstrate that monocyte adherence to tissue culture dishes or to extracellular matrix proteins is followed by a rapid and profound increase in tyrosine phosphorylation, with the predominant phosphorylated component being a protein of 76 kD (pp76). Tyrosine

phosphorylation of pp76 and other monocyte proteins can also be triggered by incubation of monocytes with antibodies to the integrin $\beta 1$ subunit, or by F(ab)₂ fragments of such antibodies, but not by F(ab) fragments. The ligation of $\beta 1$ integrins with antibodies or F(ab)₂ fragments also induces the expression of immediate-early (IE) genes such as IL-1 β . When adhering monocytes are treated with the tyrosine kinase inhibitors genistein or herbimycin, both phosphorylation of pp76 and induction of IL-1 β message are blocked in a dose-dependent fashion. Similarly, treatment with genistein or herbimycin can block tyrosine phosphorylation of pp76 and IL-1 β message induction mediated by ligation of $\beta 1$ integrin with antibodies. These observations suggest that protein tyrosine phosphorylation is an important aspect of integrin-mediated IE gene induction in monocytes. The cytoplasmic tyrosine kinase pp125^{FAK}, although important in integrin signaling in other cell types, seems not to play a role in monocytes because this protein could not be detected in these cells.

SPECIFIC cell-cell and cell-extracellular matrix (ECM)¹ adhesion events are fundamental to many different biological processes. In inflammatory reactions, monocytes are directed to sites of immunologic challenge by chemotactic factors. However, the migration of monocytes into tissues also involves adhesive interactions with the vascular endothelium and subsequently with ECM components and connective tissue cells (Zimmerman et al., 1992; Butcher, 1991; Osborn, 1990). The proteins that mediate many of the adhesive reactions of monocytes belong to the integrin family of cell surface receptors. Integrins are non-

covalently linked glycoprotein α/β heterodimers; each subunit has a large extracellular domain, a single α -helical transmembrane region, and usually a short cytoplasmic domain (Hemler, 1990; Hynes, 1992; Ruoslahti, 1991). There are at least 9 β subunits and 15 α subunits that can associate in various combinations (Arnout, 1993). The specificity of ligand binding by integrins is dependent on α/β heterodimer association, although there is considerable overlap of ligand recognition capabilities (Hemler, 1990; Hynes, 1992).

Recent studies have clearly shown that integrins not only establish a physical link between cells and the ECM, but can also transduce signals into the cells. Binding of adhesive ligands to integrins can induce protein tyrosine phosphorylation in fibroblasts, carcinoma cells, T lymphocytes, B cells, platelets, and neutrophils (Golden et al., 1990; Kornberg et al., 1991; Guan et al., 1991; Nojima et al., 1992; Kanner et al., 1993; Kapron-Bras et al., 1993; Freedman et al.,

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; FAK, focal adhesion kinase; IE, immediate-early.

1993). These changes in tyrosine phosphorylation are likely caused by the activation of integrin-stimulated tyrosine kinases. A protein of 125 kD that is tyrosine phosphorylated in response to integrin ligation has been cloned and identified as a novel protein tyrosine kinase; based on its focal contact localization, this protein has been termed p125^{FAK} (focal adhesion kinase) (Schaller et al., 1992; Hanks et al., 1992; Hildebrand et al., 1993). In fibroblasts, platelets, and carcinoma cells, FAK becomes activated and tyrosine phosphorylated when cells adhere to fibronectin or are ligated with anti-integrin antibodies (Kornberg et al., 1992; Lipfert et al., 1992; Burridge et al., 1992); alternatively, FAK can be activated by certain mitogens (Rankin and Rozengurt, 1994) or by transformation with src (Guan and Shalloway, 1992). Tyrosine phosphorylation caused by FAK or by other as yet unidentified integrin-responsive tyrosine kinases may represent the proximal portions of integrin-mediated signal transduction pathways. In addition to changes in tyrosine phosphorylation, a number of other integrin-mediated signaling events have been described. For example, ligation of integrins can activate protein kinase C (Vuori and Ruoslahti, 1993), activate the Ca²⁺-dependent protease calpain (Fox et al., 1993), affect the Na⁺/H⁺ antiporter (Schwartz et al., 1992), influence the subcellular distribution of phosphoinositide-3-kinase (Zhang et al., 1992), and modulate the level of Ca²⁺ and cAMP (Ng-Sikorski et al., 1991; Hendey et al., 1992; Nathan and Sanchez, 1990). The relation between these events and integrin-mediated tyrosine phosphorylation is unclear at present.

In human monocytes, cell adherence to plastic tissue culture dishes results in a rapid induction of multiple inflammatory mediator genes, while adherence to dishes coated with ECM components such as fibronectin, laminin, or collagen results in a relatively selective pattern of gene induction (Haskill et al., 1988; Eierman et al., 1989; Sporn et al., 1990; Haskill et al., 1991). Although multiple cell surface components may be involved in adherence to tissue culture plastic, it is the $\beta 1$ subfamily of integrins that is likely to be primarily responsible for the adherence of cells to the ECM components. To investigate whether $\beta 1$ integrin-mediated signaling would cause induction of inflammatory mediator genes, nonadherent monocytes were treated with anti- $\beta 1$ antibodies. Engagement of $\beta 1$ integrins induced the expression of multiple inflammatory mediator genes, whereas engagement of $\beta 2$ integrins produced no response (Yurochko et al., 1992). Among the immediate-early (IE) genes induced by adherence or by antibody ligation of $\beta 1$ integrins are cytokines including IL-1 β , tumor necrosis factor- α , and IL-8, as well as transcription-associated factors including I κ B, A20 (MAD-6), *c-fos*, and *c-jun* (Juliano and Haskill, 1993). These results suggest that $\beta 1$ integrin-dependent ligand binding and adherence may provide an important signaling pathway for initiation of the inflammatory response in monocytes.

Integrin-mediated signaling in monocytes results in the increased expression of a number of IE genes. As discussed above, in other cell types, integrin signaling has been shown to lead to changes in patterns of tyrosine phosphorylation. In the present study, we have investigated integrin-stimulated tyrosine phosphorylation in monocytes and its possible relationship to integrin-mediated IE gene induction. We demonstrate that either cell adhesion or $\beta 1$ integrin ligation by antibodies triggers a marked increase in tyrosine phosphory-

lation of several proteins, especially a component migrating at 76 kD. We also examine the effects of integrin ligation or cell adhesion on the expression of the IE gene IL-1 β . The increases in both tyrosine phosphorylation and IL-1 β message expression induced either by cell adhesion or by $\beta 1$ integrin ligation could be inhibited by the tyrosine kinase inhibitors herbimycin or genistein. These results suggest that integrin-mediated tyrosine phosphorylation plays an important role in the induction of IE genes in monocytes, a vital part of the inflammatory response.

Materials and Methods

Materials

Mouse monoclonal anti-phosphotyrosine antibody (PT-66), and human collagen type I and type IV were purchased from Sigma Immunochemicals (St. Louis, MO). Mouse anti-FAK ascites (2A7) for immunoprecipitation was kindly given by Dr. J. T. Parsons (University of Virginia, Charlottesville, VA), and mouse anti-FAK for immunoblotting was purchased from Transduction Laboratories (Lexington, KY). The mouse anti- $\beta 1$ antibody TS2/16 as ascites fluid was a generous gift of Dr. F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). TS2/16 F(ab')₂ and F(ab) fragments were prepared by proteolytic digestion using kits from Pierce Chemical Co. (Rockford, IL), following the manufacturer's directions. Removal of intact antibody or Fc fragments was accomplished using a protein G affinity column; the purity of the F(ab')₂ and F(ab) fragments were evaluated by SDS-PAGE before their use in experiments. The rat anti-integrin $\beta 1$ subunit antibody DH12 and the mouse anti- $\beta 2$ antibody 60.3 were kindly provided by Dr. J. J. Cassiman (University of Leuven, Leuven, Belgium) and Dr. J. M. Harlan (University of Washington, Seattle, WA), respectively. Rabbit anti-mouse IgG and goat (F(ab')₂ fragment anti-mouse IgG were obtained from Cappel Laboratories (Durham, NC). [³²P]dCTP (6,000 Ci/mmol) and [³²P]dCTP (6,000 Ci/mmol) were provided by Du Pont NEN (Boston, MA) and Amersham Corp. (Arlington Heights, IL), respectively. Herbimycin and genistein were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Protein G-Sepharose was from Pharmacia LKB (Piscataway, NJ). Human fibronectin and laminin were obtained from Collaborative Biomedical (Bedford, MA). The tissue culture reagents were from Gibco Laboratories (Grand Island, NY).

Isolation of Monocytes

Human monocytes were isolated from normal donors as previously described (Haskill et al., 1988). The samples contained >85% monocytes, of which >95% were viable, as determined by myeloperoxidase and trypan blue staining.

Cell Culture

Monocytes were cultured in RPMI 1640 medium at 37°C and 5% CO₂ either adherent to polystyrene tissue culture dishes (Corning Inc., Corning, NY) or in suspension in polypropylene tubes (Costar Corp., Cambridge, MA), with constant rocking. Human carcinoma cells (line KB) were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 50 μ g/ml of streptomycin, and 50 U/ml of penicillin. NIH 3T3 cells were maintained in DME containing 10% fetal calf serum, 50 μ g/ml of streptomycin, and 50 U/ml of penicillin. Substratum-coated dishes were prepared by incubating 50 μ g/ml of fibronectin, collagen, or laminin in tissue culture dishes at 4°C overnight. The dishes were blocked with 0.1% BSA and washed with PBS before use.

Integrin Clustering

Monocytes were incubated for 45 min on ice in RPMI 1640 medium or medium containing intact anti- $\beta 1$ IgG, F(ab')₂ or F(ab) fragments, washed twice with cold medium, and then incubated at 37°C in RPMI 1640 medium or medium containing a secondary antibody. In some cases, monocytes were treated with first or a second antibody alone. For SDS-PAGE, cells were directly lysed in sample buffer. For immunoprecipitation and kinase assay, cells were lysed in a buffer containing 20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 μ g/ml aprotinin, 0.3 M sucrose,

0.1% Triton X-100, and 0.1% sodium deoxycholate, and the lysates were cleared by centrifugation at 30,000 *g* for 30 min at 4°C. Protein concentration in the lysates was determined using the bicinchoninic acid assay (Pierce Chemical Co.).

Immunoblotting

Total cell lysates from equivalent cell numbers, or immunoprecipitated proteins were separated by SDS-PAGE (8%) under reducing conditions. The proteins were transferred electrophoretically onto polyvinylidene fluoride membranes (Immobilon P; Millipore Corp., Bedford, MA). The membranes were blocked with 3% BSA in PBS. The membranes were subsequently probed with anti-phosphotyrosine antibody (1:2,500 dilution) in PBS containing 3% BSA. The antibody-antigen complexes were detected by using rabbit anti-mouse IgG (1 µg/ml) followed by ¹²⁵I-protein A (0.2 µCi/ml). The blots were visualized by exposing the washed and dried membranes to films (X-Omat AR; Eastman Kodak Co., Rochester, NY) at -80°C.

Protein Tyrosine Kinase Assay

Cell lysates (0.35 µg) were incubated with 10 µg of random copolymer of glutamate and tyrosine (molar ratio, 4:1; Sigma Immunochemicals) for 10 min at 30°C in a final volume of 30 µl kinase assay buffer. The kinase assay buffer contained 25 mM Hepes, pH 7.4, 10 mM MgCl₂, 10 µM ATP and 2 µCi[γ-³²P]ATP. 25-µl aliquots of reaction mixtures were spotted on 3-MM cellulose disc filter papers (Whatman Inc., Clifton, NJ). The filters were washed four times with 10% trichloroacetic acid, and the radioactivity was measured. Each assay was performed in duplicate.

Immunoprecipitation of FAK

The lysates were precleared by incubation with protein G-Sepharose. The cleared lysates were first incubated with anti-FAK antibody for 3 h at 4°C, followed by the addition of protein G-Sepharose and incubation for an additional 3 h at 4°C. The precipitates were washed extensively with lysis buffer, and were subsequently boiled with sample treatment buffer to dissociate the proteins.

Flow Cytometry

Cells (5 × 10⁵) were pretreated with normal goat IgG (2 mg/ml) for 15 min on ice. Cells were then incubated with primary antibodies (2 µg/ml), washed, and treated with goat F(ab)₂ fragment anti-mouse IgG coupled to fluorescein (1:20 dilution; Cappel Laboratories) for 45 min on ice. After three washes, cells were resuspended in PBS and analyzed for fluorescence using a flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Background staining was assessed by omitting the primary antibody.

RNA Isolation and Northern Analysis

Total cellular RNA was isolated by the guanidium isothiocyanate-cesium chloride method (Chirgwin et al., 1979). Northern analysis was performed according to a published procedure (Thomas, 1980), with modification. Briefly, total RNA was denatured and electrophoresed on a 1.2% agarose-formaldehyde gel, transferred onto polyvinylidene fluoride membrane (Immobilon N; Millipore Corp.), and fixed to the membrane by UV irradiation. Human cDNA probes for IL-1β and β-actin (Yurochko et al., 1992) were labeled using a random primer kit (U.S. Biochemical Corp., Cleveland, OH) with [α-³²P]dCTP to specific activities of 3–5 × 10⁸ dpm/µg. After prehybridization for 2–4 h, the membrane was hybridized with the denatured probe at 60°C overnight in a solution containing 0.5 M sodium phosphate, pH 7.0, 1% BSA, 1 mM EDTA, and 7% SDS. The membrane was then washed with 40 mM sodium phosphate, pH 6.8, 1 mM EDTA, 5% SDS, and 1% BSA once, and 40 mM sodium phosphate, pH 6.8, 1 mM EDTA, and 1% SDS twice at 60°C. The blot was visualized by exposing the membranes to Kodak X-Omat AR film at -80°C.

Results

Cell Adhesion Induces the Tyrosine Phosphorylation of a 76-kD Protein in Monocytes

Previous reports have demonstrated that cell adhesion to

plastic tissue culture dishes induces inflammatory mediator genes in monocytes (Haskill et al., 1988, 1991). Therefore, we investigated the possibility that cell adhesion could also stimulate tyrosine phosphorylation. Monocytes were incubated in suspension or plated on plastic tissue culture dishes for different periods of time (4 min to 3 h) at 37°C. Lysates from adherent cells were examined for proteins phosphorylated on tyrosine residues by using anti-phosphotyrosine immunoblotting. As shown in Fig. 1 A, cell adhesion induced a significant increase in the tyrosine phosphorylation of a protein with an apparent molecular mass of 76 kD (pp76). When the autoradiograms were exposed for longer periods, other proteins were also found to be tyrosine phosphorylated; their apparent molecular masses were 119, 99, 66, 61, 52.5, 51.5, 43, 39, and 36 kD (Fig. 1 B). The increase in tyrosine phosphorylation of pp76 was detected 4 min after cells were plated on to the dishes, and reached a maximal level after 15 min, the level of phosphorylation declined slightly after 1 h, but was still significantly elevated for ≥3 h. The kinetics of pp76 tyrosine phosphorylation probably reflects the rate of cell attachment to the dishes because immunofluorescence microscopy of adhering cells using anti-phosphotyrosyl antibodies indicated that tyrosine phosphorylation appeared immediately as the cells attached (data not shown). To further confirm the specificity of the anti-phosphotyrosine Western blotting, excess of phenyl phosphate (20 mM) was added to the anti-phosphotyrosine antibody-containing solution during the incubation. No signal was detected in the blot after coincubation of anti-phosphotyrosine antibody with phenyl phosphate (data not shown), indicating that the proteins detected contain the phosphotyrosine residue. Pretreatment of cells with cycloheximide (20 µM, 2 h) had no effect on the induction of tyrosine phosphorylation on pp76 (data not shown), indicating that the stimulation of pp76 tyrosine phosphorylation did not require an increase in protein synthesis. Initial cell fractionation experiments have suggested that pp76 is primarily located in the cytosol (data not shown). Lysates from adherent monocytes displayed a marked increase in tyrosine kinase activity, as compared to lysates from nonadherent cells, using the poly-amino acid poly(Glu,Tyr) as a specific substrate (Table I). This suggests that the observed increase in tyrosine phosphorylation was caused, at least in part, by activation of a tyrosine kinase(s). Thus, monocytes undergo an increase in protein tyrosine phosphorylation under the same conditions (Haskill et al., 1988) that produce IE gene induction.

Adherence to fibronectin and collagen have also been reported to result in the induction of inflammatory mediator gene expression, although the pattern of expression is selective (Sporn et al., 1990). Thus we examined whether tyrosine phosphorylation was induced by cell adhesion to dishes coated with different ECM components. As seen in Fig. 2, cell adhesion to tissue culture dishes, as well as to dishes coated with fibronectin, laminin, collagen type I, or collagen type IV, all stimulated tyrosine phosphorylation of pp76. The intensity of the pp76 bands were measured by a densitometer and compared. Cell adhesion to bare culture dishes resulted in the highest level (taken as 100%) of tyrosine phosphorylation on pp76, while cell adhesion to fibronectin, laminin, collagen type I, or collagen type IV-induced levels of 75, 70, 30, and 60%, respectively. Some differences were noted in

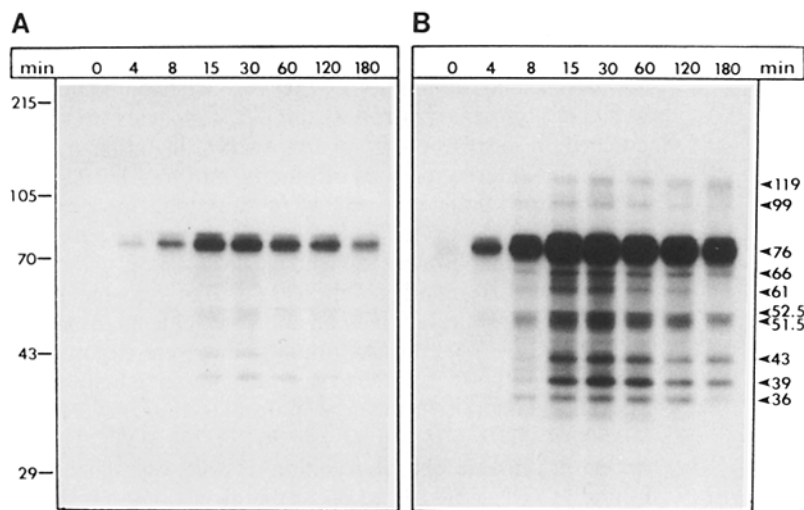


Figure 1. Induction of tyrosine phosphorylation by cell adhesion in human monocytes. Human monocytes in RPMI 1640 medium were plated on plastic tissue culture dishes for the times indicated at 37°C. After the incubations, cell lysates from 2×10^5 cells were analyzed for phosphotyrosyl-containing proteins by Western blot as described in Materials and Methods. The film was exposed to the blot for 8 h (A), or for 40 h (B). Molecular mass references are indicated.

the tyrosine phosphorylation patterns of the minor bands when the cells were adherent to various ECM proteins; these differences could conceivably relate to the different patterns of monocyte gene expression induced by individual ECM proteins, however, this relationship has not been definitively established.

Ligation of $\beta 1$ Integrins with Antibody Induces Tyrosine Phosphorylation of pp76 and IL-1 β Expression

Since the $\beta 1$ subfamily of integrins is considered to be primarily responsible for the adherence of cells to ECM components such as fibronectin, laminin and collagen, we examined whether ligation of $\beta 1$ integrins on monocytes could induce the same pattern of tyrosine phosphorylation as that caused by cell adhesion. Monocytes were incubated with anti-integrin $\beta 1$ antibody (TS2/16) or its purified F(ab) $_2$ or F(ab) fragments, and then, in some cases, further incubated with the F(ab) $_2$ fragment of goat anti-mouse IgG. Phosphotyrosine-containing proteins in cell lysates were then analyzed by immunoblotting. As shown in Fig. 3 (*top panel*), ligation of $\beta 1$ integrins with whole antibody TS2/16 (lane 2) or its divalent F(ab) $_2$ fragment (lane 3) resulted in increased tyrosine phosphorylation of pp76, while the monovalent TS2/16 F(ab) (lane 4) had no effect. Treatment with secondary antibody did not enhance the increase in tyrosine

phosphorylation of pp76 induced by TS2/16 (lane 6) or TS2/16 F(ab) $_2$ (lane 7); however, a significant induction of tyrosine phosphorylation was detected in the TS2/16 F(ab) samples that were further incubated with a secondary antibody (lane 8). Fig. 3 (*middle panel*) shows mRNA levels for IL-1 β , a prototype of the inflammatory mediator genes expressed after monocyte stimulation. As with the tyrosine phosphorylation response, ligation of $\beta 1$ integrins with TS2/16 or TS2/16 F(ab) $_2$, but not with TS2/16 F(ab), induced IL-1 β message expression. Additional incubation with secondary antibody slightly increased the expression of IL-1 β in TS2/16 and TS2/16 F(ab) $_2$ -treated samples, but signifi-

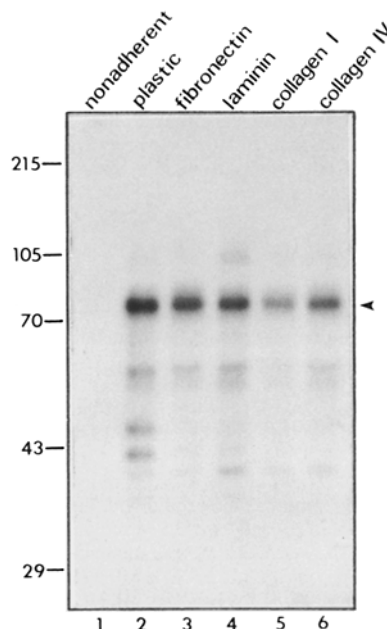


Figure 2. Induction of tyrosine phosphorylation by cell adhesion to various ECM components. Human monocytes in RPMI 1640 were incubated nonadherently or plated for 15 min at 37°C on culture dishes, or culture dishes treated with fibronectin, laminin, collagen type I, or collagen type IV. Cell lysates from 3×10^5 cells were analyzed for phosphotyrosyl-containing proteins by Western blot. Molecular mass references are indicated at the left.

Table I. Increase of Protein Tyrosine Kinase Activity by Cell Adhesion in Human Monocytes

	Poly(Glu, Tyr)	Counts per minute
Nonadherent	—	3175 \pm 196
	+	23,512 \pm 2,317*
Adherent	—	3,632 \pm 250
	+	30,262 \pm 2,917*

Cell lysates from nonadherent or adherent monocytes were incubated in the presence (+) or absence (—) of a random copolymer of glutamate and tyrosine (poly(Glu,Tyr)) for 10 min at 30°C in kinase assay buffer. The kinase activity was determined by measuring the incorporation of 32 P from [γ - 32 P]ATP into poly(Glu,Tyr) as described in Materials and Methods.

The values represent the mean \pm SD of three experiments. * $P < 0.01$ (Student's t test).

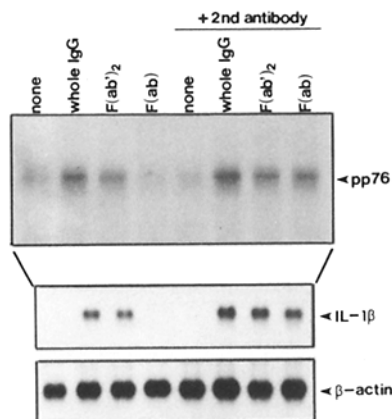


Figure 3. Induction of tyrosine phosphorylation and IL-1 β mRNA expression by integrin clustering in human monocytes. Human monocytes were incubated in RPMI 1640 medium or medium containing anti- β 1 antibody TS2/16, or its F(ab)₂ or F(ab) fragment (2 μ g/ml) for 45 min on ice. The cells were washed and incubated with RPMI 1640 medium alone or medium containing goat F(ab)₂ fragment anti-mouse IgG (1 μ g/ml) at 37°C. For Western blotting, cell lysates harvested after 15 min incubation were probed with anti-phosphotyrosine antibody (2×10^5 cells/lane). For Northern blotting, total cellular RNA isolated after 1 h incubation was probed with IL-1 β or β -actin probes (4 μ g total RNA/lane).

cantly induced IL-1 β expression in the TS2/16 F(ab)-treated samples. These observations suggest that cross-linking or clustering of β 1 integrins can produce major increases in IL-1 β mRNA and in tyrosine phosphorylation of pp76 because binding of monovalent F(ab) fragments did not stimulate a response, but treatment of F(ab) plus a secondary antibody resulted in a significant response. The observed antibody effects on tyrosine phosphorylation and gene expression are unlikely to result from engagement of monocyte Fc receptors because TS2/16 F(ab)₂ was as effective as intact antibody.

We previously have demonstrated that ligation of integrins with three different anti- β 2 antibodies did not induce IL-1 β

gene expression (Yurochko et al., 1992). Here we have examined the effect of antibody 60.3, an anti- β 2 reagent, on tyrosine phosphorylation. As seen in Fig. 4 A, binding of TS2/16 produced a substantial increase in tyrosine phosphorylation of pp76, while treatment with similar amounts of 60.3 produced no response. To exclude the possibility that the lack of effect of 60.3 was caused by poor binding of the antibody, a flow cytometry experiment was carried out. As shown in Fig. 4 B, both antibodies TS2/16 and 60.3 bound substantially to monocytes, although TS2/16 displayed somewhat higher binding than 60.3. We have observed that other anti- β 1 antibodies, such as DH12, also trigger tyrosine phosphorylation even when bound to monocytes at levels lower than that observed for the anti- β 2 antibody 60.3 (data not shown). These results suggest that the induction of tyrosine phosphorylation is mediated by engagement of β 1 integrins, but not β 2 integrins, as is also true of IE gene induction (Yurochko et al., 1992).

Herbimycin and Genistein Inhibit Tyrosine Phosphorylation of pp76 and IL-1 β Message Expression Induced by Cell Adhesion or by Ligation of β 1 Integrins

The tyrosine phosphorylation of pp76 caused by cell adherence suggests that there may be a critical role for tyrosine kinases in the signaling pathway leading to gene induction in monocytes. This possibility was tested by the use of tyrosine kinase inhibitors. Herbimycin and genistein have been used to inhibit the activity of protein tyrosine kinases, and to study the role of tyrosine phosphorylation during transmembrane signaling. These two agents have distinct mechanisms of action; genistein is a competitive inhibitor with respect to ATP, while herbimycin attacks critical sulfhydryl groups in tyrosine kinases (Akiyama and Ogawara, 1991; Uehara and Fukazawa, 1991). We examined the effects of herbimycin and genistein on integrin-mediated tyrosine phosphorylation and IE gene induction in monocytes. Fig. 5 A demonstrates that pretreatment of monocytes with her-

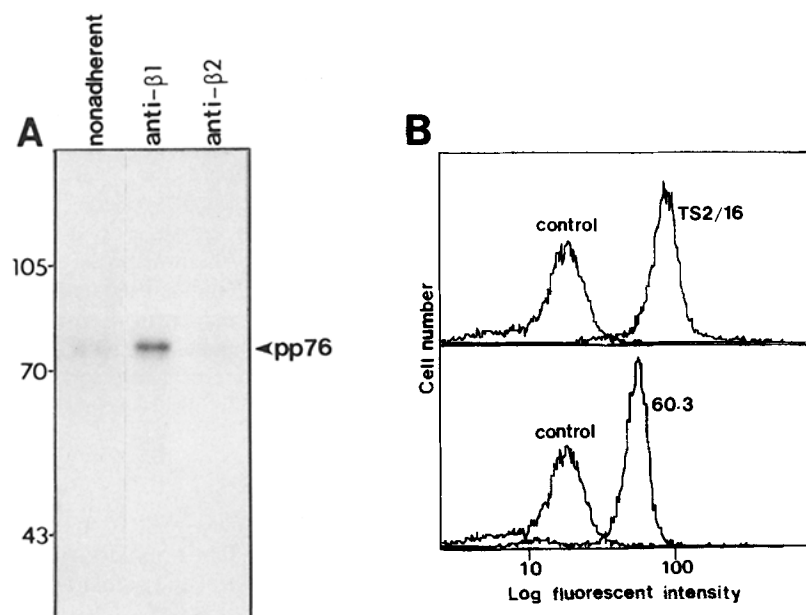


Figure 4. Ligation of integrins with anti- β 1 and anti- β 2 antibodies; effects on tyrosine phosphorylation, and quantitation by flow cytometric analysis. (A) Human monocytes were incubated nonadherently at 37°C, or incubated with anti- β 1 (TS2/16), or anti- β 2 (60.3) antibody (2 μ g/ml) for 45 min on ice, washed, and incubated with RPMI 1640 medium for 15 min at 37°C. After the treatments, cell lysates from 2×10^5 cells were analyzed for phosphotyrosyl-containing proteins by Western blot. Molecular mass references are indicated at the left. (B) Monocytes were pretreated with normal goat IgG. The cells were incubated with TS2/16 or 60.3 (2 μ g/ml), washed, and treated with goat F(ab)₂ fragment anti-mouse IgG coupled to fluorescein. After washing, cells were resuspended and analyzed using a flow cytometer. The control cells were stained with the secondary antibody alone.

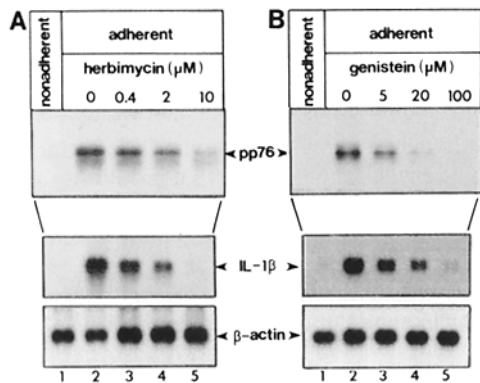


Figure 5. Inhibition of adhesion-induced tyrosine phosphorylation and IL-1 β mRNA expression by herbimycin or genistein. Human monocytes were pretreated with various concentrations of herbimycin for 1 h at 37°C (A), or genistein for 15 min at 37°C (B). The cells were further incubated nonadherently or plated on tissue culture dishes in the presence of inhibitors at 37°C. For Western blotting, cell lysates harvested after 15 min incubation were probed with antiphosphotyrosine antibody (1×10^5 cells/lane). For Northern blotting, total cellular RNA isolated after 1 h incubation was probed with IL-1 β or β -actin probes (3 μ g total RNA/lane).

bimycin for 1 h resulted in a dose-dependent inhibition of tyrosine phosphorylation of pp76 induced by cell adhesion. At 10 μ M, herbimycin strongly suppressed the induction of pp76 tyrosine phosphorylation, while a lesser degree of inhibition was detected at 0.4 and 2 μ M. To explore the functional relationship between protein tyrosine phosphorylation and IE gene induction, we examined the effect of herbimycin on the induction of IL-1 β message using Northern blot analysis. Expression of IL-1 β message was inhibited by herbimycin in a manner similar to the effect on tyrosine phosphorylation of pp76 (Fig. 5 A). Another protein tyrosine kinase inhibitor genistein was also used, and it showed similar inhibitory effects on tyrosine phosphorylation of pp76 and on IL-1 β expression (Fig. 5 B); the inhibitory effects of genistein were noticeable at 5 μ M and virtually complete at 100 μ M. Herbimycin and genistein did not affect cell viability or cell adhesion and spreading during the experiments (data not shown). Additional experiments indicated that other monocyte IE genes could also be blocked under the same conditions as IL-1 β (data not shown). These results indicate that the increased tyrosine phosphorylation observed upon monocyte adherence is an important intermediate for the subsequent induction of IE genes.

As in the case of adhesion-induced events, the effects of anti- β 1 antibody could be progressively blocked with herbimycin or genistein. As shown in Fig. 6, TS2/16-induced tyrosine phosphorylation was inhibited by herbimycin or genistein in a dose-dependent manner. When cells were examined for induction of IL-1 β message, treatment with TS2/16 produced a substantial increase in IL-1 β message, which could be blocked by herbimycin or genistein. Thus, ligation of β 1 integrins with antibody is sufficient to induce tyrosine phosphorylation and IE gene expression; both processes can be blocked by tyrosine kinase inhibitors, suggesting that integrin-induced IE gene expression requires protein tyrosine kinase activity.

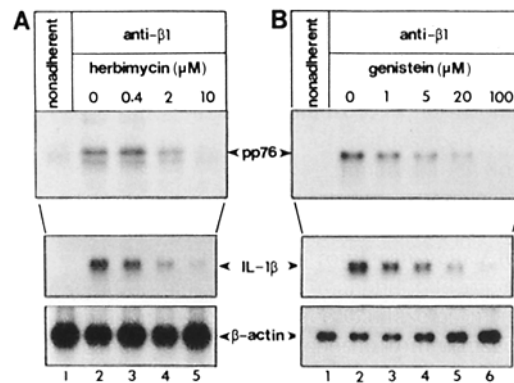


Figure 6. Inhibition of integrin ligation-induced tyrosine phosphorylation and IL-1 β mRNA expression by herbimycin and genistein. Human monocytes were pretreated with various concentrations of herbimycin for 1 h at 37°C (A) or genistein for 15 min at 37°C (B). The cells were incubated with RPMI 1640 medium alone or medium containing anti- β 1 antibody TS2/16 (2 μ g/ml) for 45 min on ice. The cells were washed and incubated with RPMI 1640 medium in the presence of inhibitors at 37°C. For Western blotting, cell lysates harvested after 15 min incubation were probed with antiphosphotyrosine antibody (2×10^5 cells/lane). For Northern blotting, total cellular RNA isolated after 1 h incubation was probed with IL-1 β or β -actin probes (3 μ g total RNA/lane).

Monocyte Adhesion Does Not Induce Tyrosine Phosphorylation of pp125^{FAK}

In several cell types, there is a clear relationship between integrin ligation and the tyrosine phosphorylation of FAK (Kornberg et al., 1992; Burridge et al., 1992). We examined whether or not FAK was tyrosine-phosphorylated during cell adhesion in monocytes. Although the major phosphotyrosine-containing protein induced by adhesion of monocytes was pp76, longer exposures of the film revealed that a tyrosine-phosphorylated protein of \sim 119 kD was also present (Fig. 1 B). To determine whether the protein at 119 kD was FAK, total cell lysates from nonadherent or adherent monocytes were immunoprecipitated with a monoclonal anti-FAK antibody, and were analyzed by immunoblotting with anti-phosphotyrosine antibody. As indicated in Fig. 7 A, cell adhesion failed to induce tyrosine phosphorylation of FAK (lane 4). We used lysates from KB cells as a positive control; these showed stimulation of tyrosine phosphorylation of FAK caused by cross-linking with anti- β 1 antibody (lane 2), as previously described (Kornberg et al., 1992). To determine whether the lack of detection of tyrosine-phosphorylated FAK was simply caused by the absence of FAK in monocytes, we examined FAK protein expression in monocytes by Western blotting. Fig. 7 B indicates that monocytes expressed little, if any, FAK in comparison with the abundant levels of FAK in KB carcinoma cells and NIH 3T3 fibroblasts. Thus, we found no evidence of a role for FAK in the increased tyrosine phosphorylation observed in adhering monocytes.

Discussion

In this report, we have demonstrated that protein tyrosine phosphorylation is an important aspect of the signal trans-

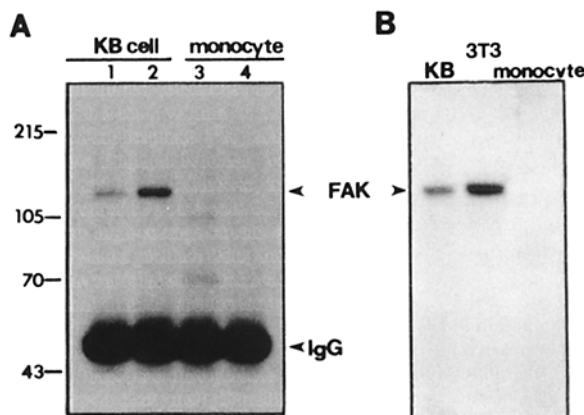


Figure 7. Analysis of FAK tyrosine phosphorylation and protein expression in human monocytes. (A) KB cells were untreated (lane 1), or treated essentially as previously described (Kornberg et al., 1991) with anti- $\beta 1$ antibody followed by secondary antibody (lane 2). Human monocytes were incubated nonadherently (lane 3) or plated on a tissue culture dish for 15 min at 37°C (lane 4). After the treatments, the cells were harvested and lysed. The cell lysates (150 μ g) were incubated with FAK antibody (2A7), followed by protein G-Sepharose. The precipitated immunocomplexes were analyzed for phosphotyrosyl-containing proteins by Western blot. The bands at ~ 55 kD are the heavy chain of mouse IgG recognized by 125 I-protein A. Molecular mass references are indicated at the left. (B) Total cell lysates from KB cells (50 μ g), NIH 3T3 cells (50 μ g), and monocytes (100 μ g) were subjected to electrophoresis and electrotransfer to polyvinylidene fluoride membrane, and the blots were probed with anti-FAK antibody, followed by 125 I-protein A.

duction cascade leading to integrin-mediated gene expression in monocytes. During inflammation, extravasating monocytes interact with vascular and connective tissue cells and with the ECM using integrins and other adhesion receptors (Butcher, 1991; Osborn, 1990). These adherence steps can result in the expression of key inflammatory mediator genes, indicating that cell adhesion is an important regulatory stimulus, and that adhesion triggers a signal transducing pathway (Yurochko et al., 1992; Sporn et al., 1990). Here we have provided evidence that the integrin signaling pathway in monocytes involves tyrosine phosphorylation. After monocyte adherence, several proteins, including a prominent component at 76 kD, are rapidly and intensely phosphorylated on tyrosine residues. This phenomenon also occurs when monocyte $\beta 1$ integrins are ligated by antibodies under nonadherent conditions. Because of the rapid tyrosine phosphorylation and the lack of effect of cycloheximide, it is apparent that this response does not require newly synthesized proteins and, thus, may be an early event in the gene induction pathway. There are no apparent major differences in the pattern of tyrosine phosphorylation caused by cell adhesion to plastic tissue culture dishes, adhesion to various ECM components, or antibody ligation of $\beta 1$ integrin, i.e., pp76 is the dominant tyrosine phosphorylated protein (although subtle differences in minor bands are observed). Quantitatively, however, the degree of pp76 tyrosine phosphorylation is dependent on the nature of the treatment; adherence to bare culture dishes always induces the highest level of pp76 tyrosine phosphorylation, but the reason for this is not clear at the present time. Since adherence to plas-

tic dishes presumably results in the nonspecific engagement of many different cell surface proteins, it may trigger several monocyte receptors known to cause changes in tyrosine phosphorylation, e.g., the receptors for lipopolysaccharide or for the IgG Fc domain (Scholl et al., 1992; Rankin et al., 1993; Weinstein et al., 1991), as well as integrins. By contrast, adherence to a specific ECM protein or treatment with an anti- $\beta 1$ integrin antibody should activate only integrin receptors, and thus produce a more modest tyrosine phosphorylation of pp76 and other monocyte proteins.

Protein tyrosine phosphorylation seems to be a common and important aspect of signaling pathways for many different receptors, including integrins (Argetsinger et al., 1993; Ullrich and Schlessinger, 1990; Juliano and Haskill, 1993). Clustering of integrin receptors by antibodies can stimulate protein tyrosine phosphorylation in several cell types (Kornberg et al., 1992; Kanner et al., 1993; Nojima et al., 1992; Freedman et al., 1993). To define the role of tyrosine phosphorylation in the induction of IE gene expression in monocytes, we inhibited adherence-induced and $\beta 1$ integrin ligation-induced pp76 tyrosine phosphorylation, and we examined the effect on IL-1 β mRNA expression. We found that treatment with two different types of tyrosine kinase inhibitors, herbimycin and genistein, suppressed both tyrosine phosphorylation and IL-1 β gene expression induced either by adherence or by $\beta 1$ integrin ligation. It seems unlikely that these two chemically distinct agents would cause similar "side effects" independent of their ability to inhibit tyrosine kinases. Thus, tyrosine phosphorylation seems to be an important aspect of the signaling pathway leading from integrins to IE gene expression in monocytes. Adherence or $\beta 1$ integrin engagement with antibodies both lead to induction of multiple IE genes, while integrin-mediated adherence to ECM proteins results in a more selective pattern of gene expression (Sporn et al., 1990; Eierman et al., 1989; Yurochko et al., 1992). However, each of these events resulted in the tyrosine phosphorylation of pp76. Thus, the major tyrosine phosphorylation response per se cannot account for matrix-specific patterns of gene induction, suggesting that other elements of the signaling cascade may be required for specificity. The subtle differences observed in the phosphorylation patterns of minor bands on different ECM substrata could contribute to specific responses, but this remains to be defined.

Signals transmitted through other surface receptors also activate tyrosine phosphorylation and subsequent downstream events in monocytic cells. For example, lipopolysaccharide induces tyrosine phosphorylation of a number of proteins in macrophages; this is followed by release of arachidonate metabolites and cytokines (Weinstein et al., 1991; Geng et al., 1993). Ligation of Fc receptors in monocytic cells also induces tyrosine phosphorylation followed by induction of messages for IE genes; both processes are blocked by tyrosine kinase inhibitors (Scholl et al., 1992; Weinstein et al., 1991). Thus, integrin-triggered signals, as well as signals triggered through other receptors, may converge on a transduction pathway that involves tyrosine phosphorylation in the regulation of subsequent downstream events.

Tyrosine phosphorylation of pp125^{FAK} has been observed subsequent to integrin clustering in several cell types, in-

cluding fibroblasts, platelets, and KB cells (Kornberg et al., 1992; Lipfert et al., 1992; Burridge et al., 1992). This might suggest that FAK functions as part of a general signaling pathway for integrin-mediated responses. However, this is apparently not the case in human monocytes. There was a minor adhesion-induced tyrosine phosphorylation of a protein at a molecular mass of 119 kD which was detectable after prolonged exposure of the immunoblot and which could possibly be FAK. However, immunoprecipitation with anti-FAK antibody followed by immunoblotting with anti-phosphotyrosine antibody failed to demonstrate that the tyrosine phosphorylated protein is FAK. Further investigation has found that monocytes express very little, if any FAK. This is consistent with recent evidence at the RNA level indicating that FAK is not expressed in monocytic cells (Choi et al., 1993). Therefore, protein tyrosine phosphorylation stimulated by cell adherence or by ligation of $\beta 1$ integrins in monocytes seems to result from the activation of specific protein tyrosine kinase(s) other than FAK. While it seems likely that FAK may play a central role in integrin signaling processes in fibroblasts, platelets, and carcinoma cells, this situation in hematopoietic cells may be more complex. In different T cell lines, antibody ligation of $\beta 1$ integrins has been reported to induce tyrosine phosphorylation of proteins of molecular mass 105 kD (Nojima et al., 1992) and 47–52 kD (Kapron-Bras et al., 1993), while ligation of $\beta 2$ integrins in T cells led to tyrosine phosphorylation of phospholipase C- $\gamma 1$ and an unknown 80-kD protein (Kanner et al., 1993). As shown here, in monocytes ligation of $\beta 1$ but not $\beta 2$ integrins results in tyrosine phosphorylation of a 76-kD substrate. In each of the cases mentioned above there is no evidence for the involvement of FAK, suggesting that integrins may couple with other tyrosine kinases in hematopoietic cells.

The enhanced protein tyrosine phosphorylation observed subsequent to monocyte adherence is apparently caused by the activation of one or more tyrosine kinases because increased kinase activity can be detected in lysates from adherent cells. At this point, the identity of the adhesion-activated kinase is unknown. Monocytes possess a large number of tyrosine kinases including cell surface receptor kinases such as c-fms (Sherr, 1991) and trk (Ehrhard et al., 1993), tyrosine kinases associated with cytokine and interferon signaling pathways (Igarashi et al., 1993; Stahl and Yancopoulos, 1993), and a number of nonreceptor tyrosine kinases, including members of the src family such as lyn, hck, and fgr (Stefanova et al., 1993), the syk kinase (Taniguchi et al., 1993), and the fes kinase (Greer et al., 1990). Since tyrosine kinase activation often results in prominent autophosphorylation, it seems possible that pp76 may itself be a tyrosine kinase; alternatively, it could be a particularly abundant or reactive substrate. Thus far, we have not been able to identify the kinase responsible for the increases in tyrosine phosphorylation subsequent to integrin ligation or cell adhesion in monocytes. However, the fact that increased tyrosine kinase activity persists in lysates from adherent monocytes suggests that we may be able to eventually purify and characterize this enzyme.

Integrin-mediated gene induction in monocytes seems to parallel and depend on changes in protein tyrosine phosphorylation. However, it is probably premature to rule out other contributions to the signaling cascade that results in IE gene expression. For example, monocyte adherence and

spreading has also been reported to lead to the activation of Ca^{2+} -independent phospholipases resulting in the release of arachidonate; the increase of arachidonate, in turn, causes an increase in intracytoplasmic Ca^{2+} and activation of Ca^{2+} -dependent phospholipases (Lefkowitz et al., 1992). As discussed above, a number of other integrin-triggered signals have been detected in other cell types. It will be important to learn what contributions these signals might make to the process of integrin-mediated gene induction in monocytes and other cells.

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