Activation of cytoplasmic tyrosine kinases is an important aspect of signal transduction mediated by integrins. In the human monocytic cell line THP-1, either integrin-dependent cell adhesion to fibronectin or ligation of β1 integrins with antibodies causes a rapid and intense tyrosine phosphorylation of two sets of proteins of about 65-75 and 120-125 kDa. In addition, integrin ligation leads to nuclear translocation of the p50 and p65 subunits of the NF-κB transcription factor, to activation of a reporter gene driven by a promoter containing NF-κB sites, and to increased levels of mRNAs for immediate-early genes, including the cytokine interleukin (IL)-1β. The tyrosine kinase inhibitors genistein and herbimycin A block both integrin-mediated tyrosine phosphorylation and increases in IL-1β message levels, indicating a causal relationship between the two events. The components tyrosine phosphorylated subsequent to cell adhesion include paxillin, pp125FAK, and the SH2 domain containing tyrosine kinase Syk. In contrast, integrin ligation with antibodies induces tyrosine phosphorylation of Syk but not of FAK or paxillin. In adhering cells, pre-treatment with cytochalasin D suppresses tyrosine phosphorylation of FAK and paxillin but not of Syk, while IL-1β message induction is unaffected. These observations indicate that the Syk tyrosine kinase may be an important component of an integrin signaling pathway in monocytic cells, leading to activation of NF-κB and to increased levels of cytokine messages.

Members of the integrin family of cell surface receptors are involved in many key biological processes, including cell to cell and cell to extracellular matrix adhesion, cell motility, hemostasis, lymphocyte trafficking, and inflammatory phenomena (1-3). Integrins are comprised of noncovalently linked α/β heterodimers (4). Different α and β subunits can associate in various combinations, which then determine the ligand-binding specificities of the intact integrin heterodimer complexes (2-4). Recently, it has become apparent that integrins function not only as adhesive proteins but can also transduce biochemical signals into the interior of the cell (5). One mode of integrin signal transduction involves the activation of cytoplasmic tyrosine kinases. In fibroblasts, platelets, endothelial cells, and cultured tumor cells, integrin-induced tyrosine phosphorylation involves a novel cytoplasmic tyrosine kinase termed pp125Fak (6-8). However, other aspects of integrin signaling have been observed, including calcium transients (9), changes in cytoplasmic pH (10), modulation of ion channels (11), activation of protein kinase C (12), as well as numerous other effects (13). The relationship between these later events and integrin-mediated tyrosine phosphorylation is currently unclear.

There have been observations in a variety of cell types showing that interactions with the extracellular matrix (ECM) can modulate gene expression (14-17). During inflammation, blood monocytes respond to chemotactic factors and subsequently extravasate into inflamed tissues. Monocytes migrate through the subendothelial basement membrane and underlying interstitial structures rich in extracellular matrix proteins and also interact with vascular endothelial cells and connective tissue cells. Partly as a consequence of cell-cell and cell-ECM interactions, monocytes are induced to secrete cytokines and to undergo maturation to macrophages. Integrins are prominent among the cell surface receptors that mediate many of the adhesive functions of monocytes (1, 2). In human peripheral blood monocytes, cell adherence to ECM components or ligation of β1 integrins with antibodies results in the rapid induction of multiple inflammatory mediator genes including several cytokines (18-20). The 5’-regulatory regions of many of the genes induced by integrin ligation contain binding motifs for the NF-κB transcription factor, suggesting a role for this factor in the gene induction process (5). In parallel to gene induction, a rapid and profound increase in protein tyrosine phosphorylation is observed, with the predominant phosphorylated component(s) having a molecular mass of about 76 kDa (21). Both of these responses are blocked by tyrosine kinase inhibitors, suggesting an important role for protein tyrosine phosphorylation in integrin signaling pathways, leading to inflammatory mediator gene induction. Aside from enhanced tyrosine phosphorylation, little is known of integrin-mediated signal transduction in monocytic cells. A significant reason for this is the difficulty involved in performing biochemical or molecular studies on peripheral blood monocytes as well as the donor to donor variability observed with these cells.

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** To whom correspondence should be addressed: Dept. of Pharmacology, University of North Carolina, CB 7365, Chapel Hill, NC 27599. Tel.: 919-966-4383; Fax: 919-966-5640.

The abbreviations are: FAK, focal adhesion kinase; BSA, bovine serum albumin; ECM, extracellular matrix; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.

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In this report, we describe a cell culture model using the human monocyte leukemia cell line, THP-1, which mimics important aspects of monocyte responses to ECM proteins. THP-1 cell adhesion to ECM proteins or ligation of β1 integrins with antibody induces protein tyrosine phosphorylation, increases inflammatory mediator gene message levels, and activates the NF-κB transcription factor. These responses are blocked by tyrosine kinase inhibitors such as herbimycin A and genistein. Among the proteins tyrosine phosphorylated in THP-1 cells in response to cell adhesion to ECM components are the pp125FAK (6, 22), the focal contact protein paxillin (23), and the nonreceptor tyrosine kinase Syk (24, 25). Ligation of β1 integrins with intact antibodies or with Fab′2 fragments results in the tyrosine phosphorylation of Syk but not of FAK or paxillin. The tyrosine phosphorylation of Syk is accompanied by an increase in its kinase activity. These results indicate that, like FAK, the Syk kinase is an integrin-responsive non-receptor tyrosine kinase. They also suggest that activation of Syk is closely correlated with the induction of inflammatory mediator gene messages, while there is no such correlation for FAK. Thus, Syk may be a vital part of an integrin signaling pathway in mononcic cells, leading to transcription factor activation, and to increased levels of cytokine messages.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies reactive with FAK, Raf-f, PTP1D (Syp), and paxillin, as well as goat anti-mouse IgG-peroxidase and goat anti-rabbit IgG-peroxidase conjugates, were purchased from Transduction Laboratories (Lexington, KY). The anti-β1 integrin antibody 4F10 was obtained from Life Technologies, Inc. Anti-Syk kinase polyclonal antibodies were raised in rabbits using fusion proteins as previously described (26). The mouse hybridoma Ts2/16 (anti-β1 integrin subunit) was a generous gift of Dr. M. Hemler (Dana Farber Cancer Research Institute, Boston, MA). Ts2/16 Fab′2 fragment was prepared by protein G affinity purification from the Ts2/16 immunoglobulin manufacturer's directions. Removal of intact antibody or Fc fragments was accomplished using a protein G affinity column. The purity of the Fab′2 was evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) prior to use in experiments. Herbimycin A, genestein, and calyculin A were purchased from Calbiochem. Protein G-Sepharose was from Pharmacia Biotech Inc. Human fibronectin, collagen type I, collagen type IV, and laminin, and the tissue culture reagents were from Life Technologies, Inc. Other reagents and chemicals were from Sigma.

Cell Culture—THP-1 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum (heat inactivated), 50 μg/ml streptomycin, and 50 units/ml penicillin. Substratum-coated dishes were prepared by incubating 10 μg/ml ECM proteins. The wells were blocked with 0.1% BSA in PBS and washed with PBS prior to use.

Adhesion Assay—Adhesion studies were performed using a modification of a previously described assay (27). Briefly, 48-well tissue culture plates were coated overnight at 4 °C with 0.2 ml of PBS containing 10 μg/ml ECM proteins. The wells were blocked with 0.1% BSA in PBS for 2 h at 4 °C and washed with PBS prior to use. THP-1 cells were washed, resuspended in RPMI 1640 medium, and added to substratum-coated wells (1 × 10⁵ cells/well) for 60 min at 37 °C. The plates were then washed twice with warm PBS and stained with a solution containing 0.1% crystal violet and 10% methanol in PBS for 15 min. Following three washes with PBS, the crystal violet stain was solubilized with 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 a bicinchoninic acid assay (Pierce).

Preparation of Cell Lysates—Cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 1 mM sodium vanadate, 0.2 μM calycin A, 5 mM NaF, 5 mM sodium pyrophosphate, 2 mM 3′- mercaptopropionic acid, 10 μg/ml aprotinin, 0.5% 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 a bicinchoninic acid assay (Pierce).

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 F, Millipore Corp.). The membranes were blocked with 1% BSA and 0.1% Tween 20 in PBS. The membranes were subsequently probed with primary antibody (1 μg/ml) in PBS containing 1% BSA and 0.1% Tween 20. The antibody-antigen complexes were detected by using goat anti-mouse IgG or goat anti-rabbit IgG peroxidase conjugates, followed by use of an enhanced chemiluminescence kit (Amersham Corp.) according to the manufacturer's instructions. In some cases, the blots were stripped of bound antibodies by incubating the membranes with stripping buffer containing 62.5 mM Tris, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS for 30 min at 50 °C. The stripped blots were reprobed with other antibodies.

Immunoprecipitation and Syk Kinase Autophosphorylation Assay—Cell lysates were precleared by incubation with protein G-Sepharose. The cleared lysates were first incubated with anti-FAK, anti-Raf-f, anti-paxillin, anti-PTP1D, or anti-Syk kinase antibody for 3 h at 4 °C, followed by the addition of protein G-Sepharose, and then incubated for an additional 3 h at 4 °C. The precipitates were washed extensively with lysis buffer. For Western blots, the precipitates were boiled in SDS-PAGE sample buffer to dissociate the proteins. For Syk kinase autophosphorylation assay, the anti-Syk immunocomplexes were further washed twice with kinase assay buffer (50 mM HEPES, pH 7.6, 10 mM MnCl₂, 2 mM MgCl₂, and 1 mM p-nitrophenyl phosphate) and resuspended in 50 μl of the same buffer containing 10 μg/ml of src (29) (3000 Ci/mmol) and 2 μl ATP. After 10 min at 30 °C, the reactions were stopped by addition of 20 μl SDS-PAGE sample buffer (×3) and boiling for 3 min.

Reporter Gene Assays—3XMCWI56CAT, a construct containing NF-κB-responsive elements upstream of the chloramphenicol acetyltransferase (CAT) reporter gene, has been previously described (29, 30). These constructs were transfected into THP-1 cells using the DEAE-dextran method (50). Transfected cells were manipulated in various ways, as indicated in the legends, and CAT or luciferase enzyme activities measured using established protocols (51).

Electrophoretic Mobility Shift Assay (EMSA)—For EMSAs, THP-1 nuclear and cytoplasmic extracts were made using a modification of the procedure described by Cordie and co-workers (31). Each treatment group utilized 5–10 × 10⁶ cells. Following incubation, THP-1 cells were rinsed with PBS, and the nuclei were isolated. Nuclear extracts were sonicated in ice-cold citrate extraction buffer (250 μM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol). After equilibrium in CEB for 5 min, the cells were collected by centrifugation and lysed on ice in 50× the packed cell volume of Nonidet P-40/CB/PI (CEB contains 0.1% Nonidet P-40, 1 mg phenylmethylsulfonyl fluoride, 50 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml bestatin, 3 μg/ml E64, 1 mM 1,10-phenanthroline, and 100 μg/ml chymostatin). Adherent cells were equilibrated with 2 ml of ice-cold CEB buffer and subsequently recovered from the tissue culture dish by gently scraping the cells into 500 μl of Nonidet P-40/CB/PI. Nuclear pellets were pelleted by brief centrifugation, redissolved in protease inhibitors but no detergent, and then suspended and mixed in 25 μl of nuclear extraction buffer (NEB) (20 mM Tris-HCl, pH 8.0, 0.4 mM NaCl, 1.5 mM MgCl₂, 1.5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 40 μg/ml bestatin, 3 μg/ml E64, 1 mM 1,10-phenanthroline, 100 μg/ml chymostatin, and 25% glycerol). After 10 min of incubation on ice, the samples were clarified by centrifugation, and the supernatants (nuclear extracts) were collected and snap-frozen on dry ice before storing at −70 °C. Protein concentrations were determined using the bicinchoninic acid method (Pierce).

Electrophoresis of nuclear proteins on native PAGE was performed as previously reported (19). Briefly, the DNA-protein binding reactions were performed in 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5 mg of BSA, 0.3 μg of poly(dI-dC), and 4% Ficoll in a final volume of 20 μl. Each reaction contained 1 μg of THP-1 nuclear extract and 20–50,000 cpm of 5′-h-fos-luciferase reporter. For EMSAs, specific NF-κB substrates were identified in shifted complexes.
Tyrosine Phosphorylation and Cytokine Message Induction

RESULTS

THP-1 Cells Adhere to ECM Components via Integrins—We surveyed the expression of integrins on the THP-1 cell surface by flow cytometry. THP-1 cells maintained in suspension culture expressed several integrin subunits including β1, β2, α2, α5, α4, and α6 (data not shown). We also examined whether these cells could adhere to ECM component-coated substrata. As shown in Fig. 1A, THP-1 cells adhered to tissue culture plates coated with fibronectin, laminin, collagen type I, or collagen type IV. THP-1 cells exhibited the highest level of cell adhesion to the fibronectin substratum, a moderate level of adherence to laminin, and lower levels of cell adhesion to collagen type I or collagen type IV, while only a few cells adhered to albumin-coated control wells. To test whether cell adhesion to fibronectin was specifically mediated by integrins, THP-1 cells were treated with RGDS peptide or with a mouse antibody recognizing the β1 integrin subunit. Fig. 1B shows that both RGDS peptide and anti-β1 integrin antibody inhibited cell adhesion to fibronectin, whereas treatment with RGES peptide or normal mouse IgG had no effect. These results indicate that THP-1 cells use β1 integrins to interact with and adhere to ECM components such as fibronectin.

Engagement of THP-1 Cell Integrins Increases Protein Tyrosine Phosphorylation and IL-1β Message Levels—THP-1 cells were plated on fibronectin-coated dishes for different periods of time (7.5–60 min). Lysates from adherent cells were examined for protein phosphorylation on tyrosine residues by using anti-phosphotyrosine immunoblotting. As shown in Fig. 2A (top panel), cell adhesion to fibronectin gave rise to a marked increase in tyrosine phosphorylation of several proteins, including three broad bands centered around 67, 71, and 120 kDa. The increase in tyrosine phosphorylation was readily detected 7.5 min after cells were plated on to the fibronectin substratum, reached a maximal level after 15 min, and then declined, but was still significantly elevated after 1 h. Cell adhesion to fibronectin also induced increased message levels of the inflam-
Tyrosine Phosphorylation and Cytokine Message Induction

Antibody-mediated ligation of integrins has been previously used to mimic the integrin clustering process that occurs during formation of adhesive contacts (35). Nonadherent THP-1 cells were treated with the anti-β1 integrin antibody TS2/16 and then analyzed for tyrosine phosphorylation and message levels. As shown in Fig. 2B (top panel), ligation of β1 integrins resulted in increased tyrosine phosphorylation (lane 3), although the pattern was somewhat different from that induced by cell adhesion to fibronectin (lane 2). With antibody ligation, bands at about 115 and 67 kDa were most prominent. Ligation of β1 integrins with antibody could also increase IL-1β message levels (Fig. 2B, middle panel, lane 3). The observed induction of tyrosine phosphorylation and message expression by anti-β1 antibody was not due to engagement of Fc receptors, since TS2/16 F(ab')2 induced tyrosine phosphorylation and IL-1β message as effectively as intact antibody (Fig. 2C).

Integrin Ligation Leads to Activation of NF-κB—To determine whether integrin ligation might lead to activation of transcription factors and the initiation of transcription, we employed gel mobility shift assays, as well as reporter gene assays. Since the IL-1β promoter, as well as the promoters for several other monocyte immediate-early genes, contains NF-κB motifs (5), we decided to focus on activation of the NF-κB transcriptional complex. THP-1 cells were transfected with a plasmid containing several copies of the NF-κB motif driving a CAT reporter gene, by a similar plasmid with mutated NF-κB sites, or by a luciferase reporter construct driven by the c-fos promoter. As seen in Fig. 3A, THP-1 cell adhesion to fibronectin resulted in a substantial induction of CAT activity in cells transfected with the construct containing NF-κB motifs but not in cells transfected with the mutated NF-κB construct. Expression of luciferase driven by the c-fos promoter was not affected by cell adhesion, indicating that the results observed with the NF-κB-driven vector were not due to generalized increases in transcriptional activity. As seen in Fig. 3B, activation of the NF-κB-driven reporter could be triggered by integrin ligation with intact anti-β1 antibody or with F(ab')2 fragments of the antibody, suggesting that integrin ligation, rather than cell adhesion, was sufficient for reporter activation.

Integrin ligation by antibodies or integrin-mediated adhesion to fibronectin also caused activation and nuclear translocation of the p50 and p65 subunits of the NF-κB complex. As seen in Fig. 3C, using a probe containing the MHC class I enhancer NF-κB site, within 1 h there was a strong increase in bands representing p50 and p65 complexes; an even more robust effect was observed after overnight stimulation. Use of a probe containing the IL-1β NF-κB motif gave similar results (data not shown). These observations, along with the reporter gene assays, suggest that integrin ligation can activate NF-κB, allowing this factor to contribute to the stimulation of transitory mediator gene IL-1β. Fig. 2A (middle panel) shows that maximal induction of IL-1β message was reached 1 h after cell adhesion to fibronectin began. By comparing the time courses, it can be seen that the induction of tyrosine phosphorylation occurred prior to IL-1β message expression. The adhesion-induced increase in IL-1β message over basal levels was approximately 4–5-fold. We have observed that certain other cytokine messages (e.g. IL-8) are also increased upon THP-1 adhesion to fibronectin (data not shown).

Tyrosine Phosphorylation and Cytokine Message Induction

Fig. 3. Activation of NF-κB by integrin ligation. THP-1 cells were transfected with an NF-κB-driven CAT reporter plasmid (panels A and B) or a c-fos-driven luciferase reporter plasmid (panel C). A: 24 h after transfection, cells (3 × 10⁶) were resuspended in 1% BSA in RPMI medium and left in suspension or plated on fibronectin-coated 60-mm dishes. NF-κB-transfected cells were also stimulated with 5 μg/ml anti-β1 integrin TS2/16 mAb IgG or TS2/16 F(ab')2 fragments followed by F(ab')2 goat anti-mouse Ab for 60 min at 4 °C and then incubated at 37 °C (panel B). Cells were collected 24 h later and lysed to measure CAT activity or 6 h later for luciferase activity. c-fos-transfected cells were also stimulated with 20 ng/ml phorbol 12-myristate 13-acetate, and luciferase activity was measured 6 h later. In A, solid bars represent non-adherent cells, hatched bars represent cells adhered to Fn, stippled bars represent cells treated with phorbol ester. Data are ±S.E. Panel C depicts the results of gel shift assays using a probe containing NF-κB motifs from the class I MHC enhancer; the experimental conditions are the same as Panels A and B. The identification of the NF-κB p50/p65 heterodimer was confirmed by supershifting the p65 band and blocking the p50 band with specific antibodies (32, 33). The faster moving band was similarly identified as p50/p50 homodimer. Cells were analyzed after 1 h (lanes 1–5) or after overnight stimulation (lanes 6–10) under the following conditions. Lanes 1 and 6, adherence to fibronectin; lanes 2 and 7, no stimulation (controls); lanes 3 and 8, TS2/16 antibody; lanes 4 and 9, TS2/16 F(ab')2 fragments; lanes 5 and 10, TS2/16 F(ab')2 followed by goat anti-mouse second antibody. Individual components were identified by interference with the mobility of p50 and p65; lane 11 is the same as lane 10 but is pretreated with anti-p65; lane 12, pretreated with anti-p50; lane 13, pretreated with anti-c-rel.
**Tyrosine Phosphorylation and Cytokine Message Induction**

**Fig. 4.** Inhibition of adhesion- and integrin ligation-induced tyrosine phosphorylation and IL-1β mRNA expression by herbimycin A or genistein. THP-1 cells were pretreated at 37°C with the concentrations (indicated in the figure) of herbimycin A for 4 h or of genistein for 1 h. The cells were then incubated nonadherently (NAD) or plated on fibronectin-coated dishes (ADH) (panels A and B), or they were ligated with anti-β1 integrin antibody at 4°C (β1), washed, and incubated nonadherently at 37°C (panel C). For Western blotting (middle and bottom panels), total cellular RNA isolated after 1 h of incubation was probed with IL-1β or β-actin probe (6 μg total RNA/lane).

**Fig. 5.** Tyrosine phosphorylation of FAK, paxillin, and/or Syk kinase by cell adhesion to fibronectin or by β1 integrin ligation. THP-1 cells were plated on fibronectin-coated dishes for 30 min at 37°C or ligated at 4°C with the anti-β1 integrin antibody TS2/16 or its Fab′/2 fragment, washed, and then incubated in suspension for 30 min at 37°C. Following the incubations, cell lysates were immunoprecipitated with antibodies to PTP1D, Raf-1, FAK, or paxillin as indicated in the figure. The precipitated immunocomplexes were analyzed for phosphotyrosyl-containing proteins by anti-phosphotyrosine immunoblotting. The blots were stripped and reprobed with the respective antibody used for immunoprecipitation. NAD, nonadherent; Fn, adherent to fibronectin; β1, ligated with intact TS2/16; IgG, ligated with intact TS2/16; Fab′/2, ligated with this fragment of TS2/16.

Herbimycin A and Genistein Inhibit Tyrosine Phosphorylation and IL-1β Message Expression Induced by Cell Adhesion or by Ligation of β1 Integrins—To evaluate whether tyrosine phosphorylation plays a critical role in the signaling pathways leading to increased message levels, THP-1 cells were treated with protein-tyrosine kinase inhibitors and examined for effects on tyrosine phosphorylation and IL-1β message induction. Herbimycin A and genistein are selective inhibitors of tyrosine kinases with distinct mechanisms of action; herbimycin A blocks tyrosine kinases by attacking critical sulfhydryl groups (36), while genistein inhibits these enzymes by binding to the ATP binding sites (37). Fig. 4A (top panel) demonstrates that treatment of THP-1 cells with herbimycin A resulted in an inhibition of the tyrosine phosphorylation induced by cell adhesion to fibronectin. The inhibition was dose dependent; 10 μM herbimycin A strongly suppressed the induction of tyrosine phosphorylation, while 2 μM was less effective. Expression of IL-1β mRNA was inhibited by herbimycin A in a manner similar to the effect on tyrosine phosphorylation (Fig. 4A, middle panel). Another tyrosine kinase inhibitor, genistein, also exhibited similar inhibitory effects on tyrosine phosphorylation and IL-1β expression (Fig. 4B); the inhibitory effects of genistein were noticeable at 20 μM and virtually complete at 100 μM. As in the case of adhesion-induced events, the responses induced by anti-β1 antibody could be blocked with herbimycin A or genistein. As shown in Fig. 4C, TS2/16-induced tyrosine phosphorylation and IL-1β expression were also inhibited by herbimycin A or genistein in a dose-dependent manner. Thus, the responses induced by integrin ligation or by adhesion could be inhibited by the tyrosine kinase inhibitors herbimycin A or genistein, suggesting that integrin-mediated increases in IL-1β expression of certain genes.

**Herbimycin A and Genistein Inhibit Tyrosine Phosphorylation and IL-1β Message Expression Induced by Cell Adhesion or by Ligation of β1 Integrins—**To evaluate whether tyrosine phosphorylation plays a critical role in the signaling pathways leading to increased message levels, THP-1 cells were treated with protein-tyrosine kinase inhibitors and examined for effects on tyrosine phosphorylation and IL-1β message induction. Herbimycin A and genistein are selective inhibitors of tyrosine kinases with distinct mechanisms of action; herbimycin A blocks tyrosine kinases by attacking critical sulfhydryl groups (36), while genistein inhibits these enzymes by binding to the ATP binding sites (37). Fig. 4A (top panel) demonstrates that treatment of THP-1 cells with herbimycin A resulted in an inhibition of the tyrosine phosphorylation induced by cell adhesion to fibronectin. The inhibition was dose dependent; 10 μM herbimycin A strongly suppressed the induction of tyrosine phosphorylation, while 2 μM was less effective. Expression of IL-1β mRNA was inhibited by herbimycin A in a manner similar to the effect on tyrosine phosphorylation (Fig. 4A, middle panel). Another tyrosine kinase inhibitor, genistein, also exhibited similar inhibitory effects on tyrosine phosphorylation and IL-1β expression (Fig. 4B); the inhibitory effects of genistein were noticeable at 20 μM and virtually complete at 100 μM. As in the case of adhesion-induced events, the responses induced by anti-β1 antibody could be blocked with herbimycin A or genistein. As shown in Fig. 4C, TS2/16-induced tyrosine phosphorylation and IL-1β expression were also inhibited by herbimycin A or genistein in a dose-dependent manner. Thus, the responses induced by integrin ligation or by adhesion could be inhibited by the tyrosine kinase inhibitors herbimycin A or genistein, suggesting that integrin-mediated increases in IL-1β...
message levels require protein-tyrosine kinase activity.

**Identification of Proteins Tyrosine Phosphorylated in Response to Cell Adhesion to a Fibronectin Substratum or to Ligation of β1 Integrins with Antibodies**—We sought to identify proteins that were tyrosine phosphorylated in THP-1 cells subsequent to engagement of integrins, which might be important in integrin-mediated message induction. We obtained antibodies to several proteins known to be involved in signal transduction cascades or in cytoskeletal organization and examined their tyrosine phosphorylation status. We examined FAK (125 kDa) as a possible component of the 120-kDa complex and Raf-1 (74 kDa), PTP1D (72 kDa), paxillin (68 kDa), and Syk kinase (72 kDa) as possible components of the 65-kDa-75-kDa complexes. Specific antibodies were used to immunoprecipitate these potential substrates, followed by immunoblotting with anti-phosphotyrosine antibody. As shown in Fig. 5A, it is clear that FAK, paxillin, and Syk kinase were tyrosine phosphorylated upon cell adhesion to fibronectin; there was no evidence for tyrosine phosphorylation of PTP1D or Raf. Only Syk kinase, but not FAK or paxillin, was found to be tyrosine phosphorylated when cells were stimulated by treatment with anti-β1 integrin antibody. The induction tyrosine phosphorylation of Syk kinase was not due to engagement of Fc receptors, since the F(ab′)2 fragment of TS2/16 was also effective (Fig. 5B). Thus, increased tyrosine phosphorylation of Syk occurs during integrin-mediated cell adhesion or subsequent to integrin ligation by antibodies. Immunodepletion experiments revealed that Syk, paxillin, and FAK contribute to the overall patterns of tyrosine phosphorylation observed in response to adhesion or integrin ligation with antibody, but they do not fully account for the patterns (data not shown); thus, additional unidentified proteins are also tyrosine phosphorylated in response to integrin ligation.

**Activation of Syk Kinase by Engagement of Integrins**—Following stimulation of the cells through integrins, there were significant increases in the activity of the Syk kinase, as indicated by immunocomplex kinase assays (Fig. 6). In nonadherent THP-1 cells, there was detectable kinase activity, but the activity increased 4-, 3.5-, and 3-fold, respectively, upon cell adhesion to a fibronectin substratum, ligation of β1 integrins with TS2/16, or with TS2/16 F(ab′)2; the radioactivity in each band was quantitated using a PhosphorImager. As shown in Fig. 7, herbimycin A and genistein suppressed the kinase activity of Syk. The concentrations of inhibitors required to block Syk activation were similar to those required to inhibit the overall pattern of tyrosine phosphorylation and the induction of...
IL-1β message. Thus, in monocytes, Syk is an integrin-responsive tyrosine kinase whose activation parallels, and may impinge on, the induction of cytokine messages.

**Role of the Cytoskeleton**—We have explored the role of the cytoskeleton in the induction of tyrosine phosphorylation and IL-1β message expression by use of the drug cytochalasin D, an inhibitor of actin filament assembly (38). As seen in Fig. 8A, treatment with cytochalasin D inhibits spreading of THP-1 cells on fibronectin but does not affect adhesion. The tyrosine phosphorylation of FAK, and particularly of paxillin, that is induced in THP-1 cells by interaction with a fibronectin substrate is strongly inhibited by cytochalasin D (Fig. 8B). This suggests that some degree of microfilament assembly is required for these phosphorylation events. By contrast, adhesion-induced tyrosine phosphorylation of Syk is not blocked by cytochalasin D treatment; likewise, IL-1β message induction is not affected. These observations clearly distinguish between presumptive integrin signaling events that require actin filament assembly and events such as Syk activation that do not have this requirement. They also indicate that extensive cytoskeletal reorganization is not required for integrin-mediated increases in IL-1β message levels.

**DISCUSSION**

Ligation of β1 integrins in peripheral blood monocytes results in a prompt and robust induction of a number of inflammatory mediator genes including IL-1β, IL-8, IL-6, and tumor necrosis factor, as well as genes for a number of transcription factors (18–20). Recently, we have demonstrated that β1 integrin ligation in monocytes also results in a burst of tyrosine phosphorylation and that this is necessary for subsequent integrin-mediated gene induction (21). Here, we have shown that similar events, including integrin-mediated tyrosine phosphorylation and increases in inflammatory gene message levels, also occur in THP-1 cells, a monocytic cell line that is more amenable to study of biochemical and molecular events than are primary monocytes. We also show that the transcription factor NF-κB is activated in response to integrin ligation. Further, we have identified some of the proteins that are tyrosine phosphorylated in THP-1 cells subsequent to integrin ligation; these include the focal contact protein paxillin and the FAK and Syk non-receptor tyrosine kinases.

The patterns of tyrosine phosphorylation induced in THP-1 cells by integrin-mediated cell adhesion or by antibody ligation of β1 integrins are similar but distinct. Both types of stimuli result in prominent tyrosine phosphorylation of several components in the 65–75- and the 115–125-kDa ranges. However, as observed in whole cell lysates, integrin-mediated adhesion to fibronectin produces a strong doublet at 65–75 kDa, while antibody ligation results primarily in phosphorylation of the lower molecular mass component of the doublet; likewise, among the tyrosine-phosphorylated proteins at 115–125 kDa, adhesion results in strong phosphorylation of a slowly migrating component (120 kDa), while antibody ligation primarily affects a more rapidly migrating component (115 kDa). The basis for the differences in tyrosine phosphorylation patterns in THP-1 cells stimulated by anti-integrin antibodies and those stimulated by integrin-mediated cell adhesion is unclear at this time. One reasonable possibility is that cell adhesion results in a more complete engagement of the cytoskeleton than does the formation of integrin dimers or multimers triggered by antibodies and the degree of cytoskeletal organization can affect the recruitment of proteins subject to tyrosine phosphorylation. Despite differences in the overall patterns of tyrosine phosphorylation induced by cell adhesion or by antibody ligation of integrins, it is clear that both of these stimuli can activate NF-κB and can give rise to increased levels of cytokine messengers. Furthermore, as we have demonstrated here for THP-1 cells and previously for monocytes (21), inhibitors of tyrosine kinases can block message induction mediated by cell adhesion or by ligation of β1 integrins. These inhibitors also blocked activation of an NF-κB driven promoter-reporter construct in transient transfection assays (data not shown). Thus, it seems highly probable that integrin engagement can trigger activation of tyrosine kinases and protein tyrosine phosphorylation events that are critical for the control of inflammatory mediator genes in monocytic cells. Therefore, it is important to carefully analyze integrin-mediated tyrosine phosphorylation to try to define those events most closely related to the message induction process.

We have examined THP-1 lysates for evidence of integrin-mediated tyrosine phosphorylation of several proteins previously identified as being involved in signal transduction cascades or linked to integrin-dependent cytoskeletal reorganization. In THP-1 cells, the focal contact protein paxillin and the cytoplasmic tyrosine kinases FAK and Syk became tyrosine phosphorylated in response to integrin-mediated cell adhesion. The activation of FAK by integrin ligation, as well as by other stimuli, has been observed in a number of cell types (35, 39); paxillin is a substrate for FAK, and its tyrosine phosphorylation seems to parallel the activation of the FAK kinase (23, 41). That FAK and paxillin are tyrosine phosphorylated during THP-1 cell adhesion suggests that these molecules may be involved in cytoskeletal reorganization and formation of focal adhesive sites in these cells; a similar role for these proteins has been postulated in fibroblasts (23, 42). Since cytochalasin D effectively blocks the tyrosine phosphorylation of FAK and paxillin, some degree of cytoskeletal organization and cell spreading seem to be required for these events.

Ligation of β1 integrins with antibodies did not result in tyrosine phosphorylation of FAK or paxillin but did cause increased tyrosine phosphorylation of Syk, a cytoplasmic tyrosine kinase that contains SH2 domains (24, 25). The Syk kinase has been reported to associate with several receptors, including the antigen receptors on B-cells (43), the Fc receptors for IgE (FcεRI) (44) and IgG (FcγRI, FcγRII) (45, 46), and granulocyte colony-stimulating factor receptor (26). Syk kinase becomes activated and tyrosine phosphorylated when those receptors bind their respective ligands. A consensus motif has been defined in the cytoplasmic domains of receptors that bind Syk or the related kinase ZAP70 (26). Impaired signal transduction processes have been found in Syk kinase negative cells or in cells that express a Syk mutant lacking kinase activity (43). Since Syk can be activated via Fc receptors, we used F(ab')₂ fractions of anti-integrin antibodies to demonstrate that integrin ligation, rather than Fc receptor engagement, was responsible for the increase in Syk enzymatic activity and tyrosine phosphorylation that we observed. In addition, the fact that Syk can be activated by integrin-mediated adhesion to fibronectin substrata also indicates that integrins rather than Fc receptors are involved. Since integrin cytoplasmic domains do not contain any obvious homologies to the Syk binding motif found in other cognate receptors (26), it seems likely that the interaction between integrins and Syk is indirect. In support of this, we have not been able to co-immunoprecipitate Syk and integrins from cell lysates (data not shown). Recent studies in platelets have shown that the Syk kinase can be activated via engagement of the αIIbβ3 integrin by fibrinogen (48). Thus, in both mononuclear cells and platelets, Syk is an integrin-responsive tyrosine kinase. The activation of Syk, in contrast to the activation of FAK, is not completely dependent on cytoskeletal assembly as observed here as well as in previous studies on platelets (48). Interestingly, recent investigations in rat baso-
philic leukemia cells have shown that cross-linking of the FcεRI receptor can trigger tyrosine phosphorylation of paxillin, while receptor cross-linking in adherent cells can induce tyrosine phosphorylation of both FAK and a stronger phosphorylation of paxillin (47, 49). These results may have similar underlying mechanisms to the results presented here, since multi-valent cross-linking of FcεRI receptors may cause engagement of the cytoskeleton, setting the stage for FAK and paxillin phosphorylation.

Either integrin-mediated cell adhesion or antibody ligation of integrins can effectively increase IL-1β message levels, as well as causing changes in protein tyrosine phosphorylation. However, antibody ligation affected the Syk tyrosine kinase but not FAK or its substrate paxillin. Furthermore, treatment with cytochalasin D, which effectively blocked cell spreading and FAK and paxillin tyrosine phosphorylation, failed to block Syk tyrosine phosphorylation or increases in IL-1β message levels. Thus, it seems that FAK is not required for integrin-mediated IL-1β message induction in THP-1 cells, consistent with our previous observations on primary monocytes (21). Conversely, our results suggest that Syk may play an important role in the signal transduction process leading from integrin engagement to the induction of inflammatory mediator genes. Thus, the activation of Syk uniformly precedes and accompanies integrin-mediated gene induction, while similar concentrations of tyrosine kinase inhibitors block both Syk autophosphorylation and IL-1β message induction. The integrin-mediated changes in tyrosine phosphorylation and Syk activation are transient, even after continued cell adhesion or antibody ligation. This suggests that these processes are regulated, perhaps through the agency of protein phosphatases that are induced or activated subsequent to integrin stimulation. Similar events have been associated with the regulation of kinase activities stimulated by mitogens (40).

Current evidence indicates that Syk is an integrin-responsive tyrosine kinase. It also suggests the existence of a signal transduction pathway in monocytic cells that involves the ligation of integrins, activation of the Syk tyrosine kinase, the triggering of downstream events, activation of the NF-κB transcription complex, and the eventual induction of messages for inflammatory mediator genes. Extensive cytoskeletal reorganization does not seem to be required in this pathway, since cytochalasin D treatment had little effect on IL-1β message levels. In those types of monocytic cells that do express FAK, such as THP-1, there is a second integrin-mediated signaling pathway that involves FAK activation, and that seems to impact primarily on cytoskeletal organization rather than gene induction. At this point, a number of issues require further study. It is not certain that activation of the Syk tyrosine kinase is essential for integrin-mediated gene induction, as opposed to simply accompanying the induction process, nor can the participation of additional tyrosine kinases be ruled out. Further experiments will be required to establish a causal role for Syk in integrin signaling. In addition, the downstream events linking changes in tyrosine phosphorylation to alterations of inflammatory gene message levels remain to be defined. In monocytic cells, it is clear that integrin ligation induces transcriptional activation of the IL-1β gene but does not affect message stability. In the THP-1 cell system, integrin ligation leads to activation of NF-κB, increased transcription from NF-κB-driven reporter constructs, and increases in IL-1β message levels. However, it is not clear at present if integrin-mediated changes in IL-1β message levels in THP-1 cells are primarily controlled at the transcriptional level or by other means, such as changes in message stability or RNA splicing. If transcriptional activation does occur, the precise role of NF-κB and of other transcription factors remains to be defined. Despite these questions, current observations provide important insights into integrin signal transduction processes in monocytic cells and emphasize a key role for specific tyrosine phosphorylation events.

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