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Signal transduction by integrins: Increased protein tyrosine phosphorylation caused by clustering of β_1 integrins

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ABSTRACT **The integrin family of cell adhesion receptors mediates many of the interactions between cells and the extracellular matrix. Because the extracellular matrix has profound influences on cell behavior, it seems likely that integrins transduce biochemical signals across the cell membrane. The nature of these putative signals has, thus far, remained elusive. Antibody-mediated clustering of integrin receptors was used to mimic the integrin clustering process that occurs during formation of adhesive contacts. Human epidermal carcinoma (KB) cells were incubated with an anti-β_1 integrin monoclonal antibody for 30 min on ice followed by incubation at 37°C with anti-rat IgG. This treatment, which induced integrin clustering, stimulated the phosphorylation on tyrosine residues of a 115- to 130-kDa complex of proteins termed pp130. When integrins were clustered in the presence of the phosphatase inhibitor sodium orthovanadate, pp130 showed a substantial increase in phosphorylation compared to the case in which integrins were clustered in the absence of vanadate. Maximal pp130 phosphorylation was observed 10–20 min after initiation of integrin clustering in the absence of vanadate or after 5–10 min in its presence. These time courses roughly parallel the formation of integrin clusters on the cell surface as observed by fluorescence microscopy. pp130 phosphorylation depended on the amount of anti-integrin antibody present. Additionally, the tyrosine phosphorylation of pp130 showed specificity since it was stimulated by antibodies to the integrin α_3 and β_1 subunits but not by antibodies to other integrin α subunits or to nonintegrin cell surface proteins. Immunoprecipitation experiments clearly demonstrated that pp130 is not itself a β_1 integrin. It is postulated, therefore, that the integrin-stimulated tyrosine phosphorylation of pp130 may reflect part of an important signal transduction process between the extracellular matrix and the cell interior.**

Cell interactions with the extracellular matrix are important determinants of cellular morphology, growth, and differentiation (1–4). Contacts between cells and the extracellular matrix are mediated in part by members of the integrin superfamily of adhesive receptors (5–10). Integrins are heterodimeric cell surface glycoproteins consisting of noncovalently linked α and β chains. The large extracellular amino-terminal domains of both chains associate to form an extracellular binding site for matrix proteins; each chain has a single α-helical transmembrane region and a short cytoplasmic domain (11, 12). The abbreviated intracellular domains of integrins interact with α-actinin, talin, and probably other as yet to be discovered proteins to link integrins to the actin-containing cytoskeleton (9, 13, 14). Interference reflection microscopy in concert with fluorescence microscopy has shown that integrins can be clustered on the ventral surfaces of adherent cells in structures known as focal contacts (14–17). These structures provide a link (mediated through integrins) between extracellular matrix proteins and the actin cytoskeleton.

In addition to mediating cell adhesion and motility (5, 11, 18), the interactions of integrins with their protein ligands have been reported to influence a number of other important cellular processes, including changes in cytoplasmic pH (19), activation of T lymphocytes (20–22), tumorigenicity (23, 24), and gene expression in fibroblasts (25) and monocytes (26). Because the ligation of integrins leads to profound changes in cell behavior, it seems reasonable to suggest that integrins provide more than a simple physical link between the extracellular matrix and the cytoskeleton; rather, integrins may transduce biochemical signals from the extracellular matrix to the cell interior. So far, however, integrin-mediated signaling pathways have remained elusive. In this report we provide evidence that protein phosphorylation on tyrosine may be involved in integrin signaling. We show that antibody-mediated clustering of integrins leads to enhanced phosphorylation on tyrosine residues of a complex of proteins in the 115- to 130-kDa range. This observation suggests at least one biochemical basis for integrin signal transduction.

MATERIALS AND METHODS

Antibodies. PIH5 (anti-human integrin α_2 subunit, mouse monoclonal) and PIB5 (anti-human integrin α_3 subunit, mouse monoclonal) were generous gifts of W. Carter (44). A. Sonnenberg (45) provided GoH3 (anti-human integrin α_6 subunit, rat monoclonal). C. Damsky (25) kindly supplied BIE5 (anti-human integrin α_5 subunit, rat monoclonal) and AIIB2 (anti-human integrin β_1 subunit, rat monoclonal). The anti-human β_2 microglobulin antibody (mouse monoclonal) was obtained commercially from Sera-Lab (Crawley Down, Sussex, U.K.). An anti-phosphotyrosine mouse monoclonal antibody (PY20) was obtained from ICN. All monoclonal antibodies were used as hybridoma supernatants. The polyclonal anti-phosphotyrosine antibody was prepared and affinity purified as described (27). Goat anti-rat IgG and rabbit anti-mouse IgG as well as fluorescein-labeled goat anti-rat IgG and sheep anti-mouse IgG were obtained commercially from Cappel Laboratories. Protein A-Sepharose was purchased from Pharmacia; ^{125}I-labeled protein A (^{125}I-protein A) (2–10 μCi/μg; 1 Ci = 37 GBq) was obtained from New England Nuclear.

Cell Culture and Integrin Clustering. KB cells were obtained from the American Type Culture Collection and were routinely cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 50 μg of streptomycin per ml, and 50 units of penicillin per ml. The cells were transferred to 60-mm

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Abbreviation: EGF, epidermal growth factor.

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dishes (2–2.5 × 10^6 cells per dish) 18–24 hr prior to an integrin-clustering experiment.

Immediately prior to integrin-clustering, KB monolayers were washed with ice-cold Eagle's minimal essential medium (EMEM) supplemented with 20 mM Hepes (pH 7.3). The cells were incubated on ice for 30 min in the presence of the appropriate dilution (1:20 unless otherwise indicated) of the anti-integrin subunit antibody. The cells were washed in ice-cold EMEM and then incubated for the indicated times at 37°C in the presence of a 1:100 dilution of either goat anti-rat IgG or rabbit anti-mouse IgG or in the absence of second antibody as a control. This procedure was sufficient to induce clustering of cell surface β_1 integrins, as indicated by immunofluorescence microscopy (see below). Some experiments were performed in the presence of sodium orthovanadate (100 μM).

Following the incubation with the second antibody, the cells were immediately placed on ice and the medium was removed from the monolayer. The cells were then lysed in a buffer containing detergents and inhibitors of proteases and phosphatases (27). The lysates were mixed with SDS/PAGE sample buffer, heated for 5 min at 100°C, and stored at −80°C until electrophoresis.

SDS/PAGE and Anti-Phosphotyrosine Western Blotting. Samples (75 μl) were electrophoresed on 8% polyacrylamide gels under reducing conditions as described by Laemmli (28). The resolved proteins were electrophoretically transferred to nitrocellulose and the phosphotyrosyl-containing proteins were detected using anti-phosphotyrosine antibodies and ^{125}I-protein A as described (27, 29, 30).

Surface Labeling and Immunoprecipitations. Monoclonal antibodies were used to precipitate integrins from control cells or from cells in which integrins had been clustered (5, 18). In parallel experiments integrins were immunoprecipitated from cells that were surface labeled using lactoperoxidase (Sigma) and ^{125}I as described (5). Material from equal numbers of cells was subjected to SDS/PAGE (28). Phosphotyrosyl-containing proteins were visualized by Western blotting with anti-phosphotyrosine antibody and ^{125}I-protein A (27); surface-labeled samples were visualized by autoradiography (5).

Flow Cytometry. The expression of cell surface sites for anti-integrin and anti-β_2-microglobulin antibodies was ascertained by flow cytometry as described (18).

RESULTS

Integrin Clustering Leads to Enhanced Tyrosine Phosphorylation of a 130-kDa Protein Complex. A key aspect of the development of adhesive contacts is the appearance of clusters of integrin molecules (14–17). The possibility that clustering of integrins leads to changes in the phosphorylation state of tyrosine residues was assessed in KB cells. This is a human epidermoid carcinoma cell line that expresses α_2, α_3, α_5, α_6, and β_1 integrin subunits on the cell surface, as determined with monoclonal anti-subunit antibodies and flow cytometry (data not shown). Integrins were clustered using monoclonal anti-β_1 as primary antibody, followed by exposure to a secondary anti-IgG reagent at 37°C (see *Materials and Methods*).

Fluorescence microscopy confirmed that the conditions used did induce the clustering of integrins. Thus, as seen in Fig. 1, in cells that were treated with primary anti-β_1 subunit antibody and then maintained on ice during the addition of fluoresceinated second antibody, the distribution of β_1 was diffuse, with some concentration at the cell margins. However, when cells were incubated with second antibody at 37°C, surface clusters of integrins formed in a time-dependent manner. Some redistribution of integrins was apparent by 5 min, distinct clusters were readily visible at 10 min, and large clusters were seen at 30 min. Some cell rounding was also observed during the clustering process.

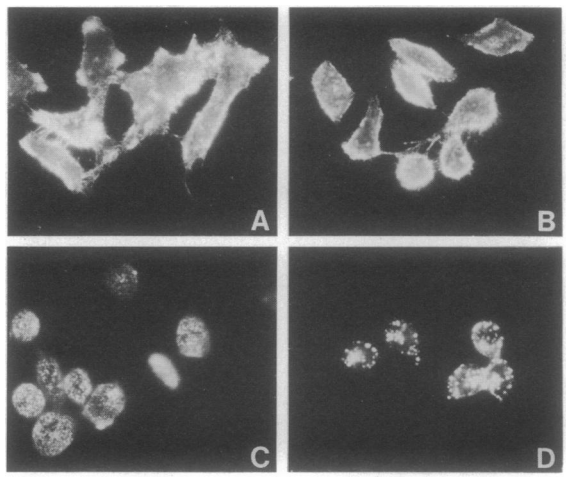

FIG. 1. Clustering of integrins. Adherent KB cells were exposed to rat anti-β_1 at 4°C and then integrins were clustered at 37°C for various times with fluoresceinated goat anti-rat IgG. Control cells were incubated on ice during exposure to primary antibody and exposure to second antibody. Fluorescein images were obtained on an inverted-phase fluorescence photomicroscope. (*A*) Zero minutes at 37°C. (*B*) Five minutes at 37°C. (*C*) Ten minutes at 37°C. (*D*) Thirty minutes at 37°C.

Phosphorylation of tyrosine residues was assessed by electrophoresis of cell extracts followed by Western blotting using anti-phosphotyrosine antibodies. Inspection of the blots showed that about 12 phosphotyrosine-containing proteins (Fig. 2*A*) were detected in extracts from KB cells. When KB cells were treated with anti-integrin β_1 and goat anti-rat IgG antibodies, a complex of proteins of 130 kDa (Fig. 2*A*, lanes 3 and 4) showed enhanced tyrosine phosphorylation over basal levels. It is not known if this group of proteins consists of multiple isoforms of a single protein or different proteins. However, this protein complex will be referred to as pp130 for convenience.

Incubation of KB cells with anti-integrin β_1 antibody alone or with goat anti-rat IgG alone had no effect on pp130 phosphorylation (Fig. 2*A*, lanes 2 and 6), indicating that integrin clustering was probably needed for the enhancement of phosphorylation. When KB cells were incubated with EGF, proteins of 170, 110, and 52 kDa showed enhanced tyrosine phosphorylation (Fig. 2*A*, lane 5). The 170-kDa protein is the EGF receptor itself, as determined by immunoprecipitation with specific antibodies (data not shown). Clearly, pp130 is not the EGF receptor nor is it a substrate for the EGF receptor kinase (Fig. 2*A*, compare lane 4 with lane 5). The 110-kDa protein phosphorylated by the EGF receptor kinase is clearly distinct from the pp130 complex.

To characterize the potential role of phosphatases, KB cells were incubated in the presence of sodium orthovanadate, a potent inhibitor of tyrosine phosphatases (31). Sodium orthovanadate (100 μM) alone had little or no effect on tyrosine phosphorylation in untreated cells (Fig. 2*B*, compare lane 1 with lane 4). However, when integrins were clustered in the presence of vanadate, there was a marked increase in phosphorylation of pp130 compared to integrin-clustered cells incubated without vanadate (Fig. 2*B*, compare lane 2 with lane 3). pp130 was the only protein that showed increased phosphorylation in the presence of vanadate. This suggests that pp130 phosphorylation subsequent to integrin clustering is not due to inhibition of a vanadate-sensitive tyrosine phosphatase activity.

Time Course of pp130 Tyrosine Phosphorylation. When KB cells were incubated in the presence of anti-integrin β_1 rat

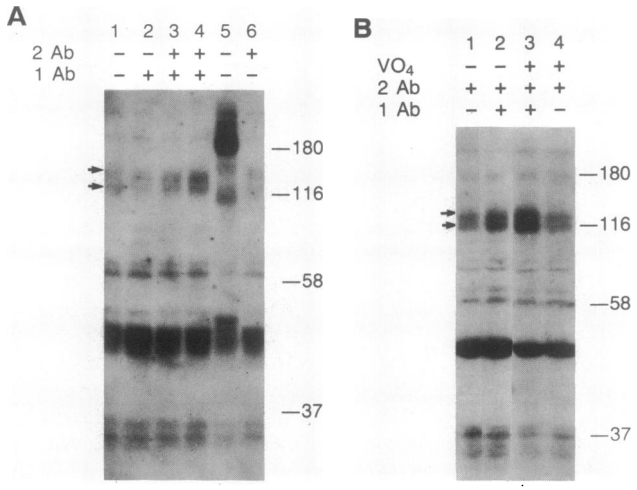

FIG. 2. (*A*) Effect of integrin clustering on tyrosine phosphorylation. KB cells were incubated on ice for 30 min in the presence of a 1:20 dilution of anti-integrin β_1 subunit antibody (1 Ab). The cells were washed and then incubated in the presence of a 1:100 dilution of goat anti-rat IgG (2 Ab) at 37°C for 2 min (lane 3) or 10 min (lane 4). The cells in lane 5 were incubated for 2 min at 37°C in the presence of 300 ng of epidermal growth factor (EGF) per ml. Following the incubations, the cells were analyzed for phosphotyrosyl-containing proteins by Western blot as described (27). + or −, Presence or absence, respectively, of the indicated antibody. Positions of molecular mass markers (in kDa) and the pp130 complex (double arrows) are indicated. (*B*) Effect of vanadate on tyrosine phosphorylation. KB cells were incubated with or without anti-integrin β_1 antibody, as in *A*, and were then incubated at 37°C for 10 min with a 1:100 dilution of goat anti-rat IgG. Some dishes were incubated in the presence of 100 μM sodium vanadate. Analysis as in *A*. The presence (+) or absence (−) of anti-integrin β_1 antibody (1 Ab), goat anti-rat IgG (2 Ab), and sodium vanadate (VO_4) is as indicated. Positions of molecular mass markers (in kDa) and the pp130 complex (double arrows) are indicated.

monoclonal antibody and goat anti-rat IgG, pp130 phosphorylation increased as a function of integrin-clustering time. A small increase in pp130 phosphorylation was apparent after 5 min (Fig. 3*A*), while maximal pp130 phosphorylation was seen after 10–20 min (Fig. 3*A*). When integrin clustering was conducted in the presence of vanadate (Fig. 3*B*), the time course of pp130 phosphorylation appeared more rapid than in cells incubated in the absence of vanadate. With vanadate, increased pp130 phosphorylation was clearly observed after 2 min of exposure to anti-rat IgG, with maximal pp130 phosphorylation occurring after 5–10 min of clustering; pp130 phosphorylation appeared to decline thereafter.

Concentration Dependence of pp130 Phosphorylation. The increase in pp130 tyrosine phosphorylation depended on the concentration of anti-integrin β_1 antibody as well as on the clustering time. When KB cells were incubated with increasing concentrations of the anti-integrin β_1 antibody, the amount of pp130 phosphorylation was enhanced (Fig. 4). Phosphorylation of pp130 was robust in the presence of a 1:20 dilution of antibody. This dilution is sufficient to saturate nearly 100% of the β_1 subunit available at the cell surface (data not shown). The phosphorylation response of pp130 was decreased in the presence of a 1:200 dilution of anti-integrin β_1 and declined to near control levels in the presence of a 1:2000 dilution of antibody.

Scanning of autoradiograms with a laser densitometer was used to estimate the increase in pp130 tyrosine phosphorylation. Previous studies have demonstrated a good correlation between the Western blot signal with the anti-phosphotyrosyl antibody and the level of protein tyrosine phosphorylation measured by chemical means (29, 30). At 1:20 dilutions of β_1 antibody and at optimal times of clustering

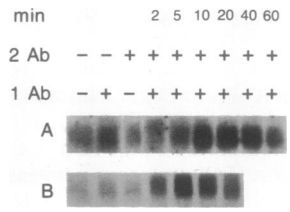

FIG. 3. Time course of pp130 phosphorylation. KB cells were incubated for 30 min on ice in the presence or absence of a 1:20 dilution of anti-integrin β_1 antibody, followed by goat anti-rat IgG (2 Ab) at 37°C. The controls were incubated at 37°C for 60 min in *A* and 20 min in *B*. Analysis as in Fig. 1. The presence (+) or absence (−) of anti-integrin β_1 (1 Ab) or goat anti-rat IgG (2 Ab) is as shown. Time of exposure (min) to second antibody is indicated. Note: The darker background in *A* is due to a longer autoradiographic exposure time. (*A*) Without vanadate. (*B*) With 100 μM vanadate.

with second antibody, the tyrosine phosphorylation of pp130 was ≈3.5-fold higher than control levels.

Integrin Specificity in Stimulating pp130 Tyrosine Phosphorylation. The specificity of pp130 phosphorylation for individual integrins was ascertained by incubating KB cells with monoclonal antibodies to the integrin α_2, α_3, α_5, α_6, and β_1 subunits as well as with antibody to β_2-microglobulin, a nonintegrin component of the histocompatibility complex. As demonstrated by flow cytometry (data not shown), KB cells have abundant surface receptors for all of the above antibodies. Fig. 5 shows that incubation of KB cells with antibodies to β_2-microglobulin or to the integrin subunit α_2, α_5, or α_6, followed by clustering with the appropriate anti-IgGs, had little effect on pp130 phosphorylation. However, anti-integrin α_3 antibody enhanced pp130 phosphorylation to the same extent as the anti-integrin β_1 antibody (Fig. 5, lanes 3 and 8). This suggests that enhanced pp130 phosphorylation is a consequence of the clustering of specific integrins and does not result when other KB cell surface antigens are clustered. The α_3 subunit associates with β_1 (12), suggesting that the α_3/β_1 heterodimer may be particularly effective in inducing pp130 tyrosine phosphorylation.

pp130 Is Not a β_1 Integrin. Because several integrin subunits have molecular masses of 120–140 kDa, these were possible candidates for pp130; therefore we examined the possibility that pp130 was itself an integrin. KB cell β_1 integrins were clustered to stimulate pp130 tyrosine phosphorylation, the cells were lysed, and the β_1 integrins were immunoprecipitated. The immunoprecipitates and residual supernatants were then subjected to Western blotting using the anti-phosphotyrosine antibodies. Fig. 6 shows that the

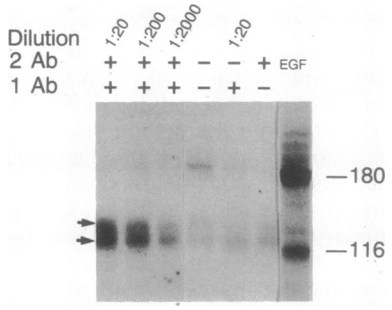

FIG. 4. Effect of anti-integrin β_1 antibody dilution on pp130 phosphorylation. KB cells were incubated on ice for 30 min in the absence or presence of a 1:20 dilution, 1:200 dilution, or 1:2000 dilution of anti-integrin β_1 antibody (1 Ab). Treatment with 2 Ab and analysis as in Fig. 1. Sodium vanadate (100 μM) was present with 2 Ab. Some cells were incubated with 300 ng of EGF per ml at 37°C for 2 min. Positions of molecular mass markers (in kDa) and the pp130 complex (double arrow) are indicated. The dilution of the anti-integrin β_1 antibody is indicated.

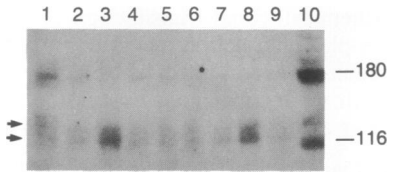

FIG. 5. Specificity of pp130 phosphorylation. KB cells were incubated without antibodies (lane 1) or with a 1:20 dilution of primary antibody (anti-α_2, lane 2), (anti-α_3, lane 3), (anti-β_2-microglobulin, lane 4), (anti-α_5, lane 6), or (anti-α_6, lane 7), (anti-β_1, lane 8). Cells were washed and then incubated for 10 min at 37°C with the appropriate second antibody (goat anti-rat IgG or sheep anti-mouse IgG) and sodium vanadate (100 μM). Lanes 5 and 9, incubations with rabbit anti-mouse IgG alone or anti-rat IgG alone, respectively. Lane 10, incubation with EGF. Analysis as in Fig. 1. Positions of the molecular mass markers (in kDa) and the pp130 complex (double arrows) are indicated.

anti-β_1 integrin immunoprecipitates did not seem to contain any detectable amounts of phosphotyrosyl-containing proteins. Instead, pp130 was found in the supernatants (Fig. 6, lanes 2, 4, and 6). This cannot be attributed to a failure to precipitate integrins, because Fig. 6, lanes 9 and 10, clearly shows that integrins from ^{125}I surface-labeled cells were precipitated under similar conditions in a parallel experiment. Conversely, the lack of phosphotyrosyl-containing proteins in the immune complexes was not due to dephosphorylation during immunoprecipitation, since immunoprecipitation using the PY20 anti-phosphotyrosine monoclonal antibody clearly showed significant amounts of phosphotyrosyl-containing proteins in the pp130 complex (data not shown). Thus it seems clear that the phosphotyrosyl-containing proteins of the pp130 complex are not β_1 integrin subunits.

DISCUSSION

As cells adhere to a substratum coated with extracellular matrix proteins, integrins become localized on the ventral surface of the cell in structures known as focal contacts (14–17). These structures provide a link between the extracellular matrix and the actin-containing cytoskeleton. In this investigation antibodies against integrins were used to mimic the early events of focal contact formation, particularly the clustering of integrins in discrete patches. The use of antibodies allowed integrin-mediated signaling events to be studied in a controlled manner. This report demonstrates that antibody-mediated integrin clustering led to enhanced tyrosine phosphorylation of a complex of proteins of ≈115–130 kDa, termed pp130. Although the identities of the proteins of the pp130 complex are currently unknown, they are clearly not integrin subunits (see Fig. 6). Thus, integrin-stimulated phosphorylation of pp130 may reflect a biochemical signal transduction process, although the exact nature of that process awaits elucidation.

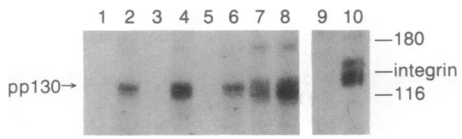

FIG. 6. pp130 is not a β_1 integrin. KB cell surface integrins were clustered as described in the legend to Fig. 1. The cells were lysed with detergent and centrifuged to remove debris. Integrins were immunoprecipitated by addition of a 1:20 dilution of anti-integrin β_1 subunit antibody followed by addition of anti-rat IgG and protein A-Sepharose. Control immunoprecipitates were performed by omitting the anti-integrin β_1 subunit antibody from the reaction mixture. The immunocomplex was centrifuged and the supernatants (integrin antibody-nonreactive material) and pellets (integrin antibody-reactive material) were saved. Equal cell equivalents of supernatant and pellet material were assayed for phosphotyrosyl-containing proteins. Lane 1, pellet from control cells; lane 2, supernatant from the same cells as lane 1; lane 3, pellet from integrin-clustered cells; lane 4, supernatant from the same cells as lane 3; lane 5, control for nonspecific binding to the protein A pellet; lane 6, control supernatant from the same cells as lane 5; lane 7, whole cell extract from control cells; lane 8, whole cell extract from integrin-clustered cells; lanes 9 and 10, control and anti-integrin immunoprecipitates, respectively, from ^{125}I surface-labeled cells that were integrin clustered. Positions of molecular mass markers (in kDa), pp130, and the ^{125}I-labeled integrins are shown.

The time course of pp130 phosphorylation was approximately parallel to the formation of integrin clusters at the cell surface (see Figs. 1 and 3). In the absence of vanadate, maximal phosphorylation was observed at 10–20 min after initiation of integrin clustering; the response declines thereafter. The reason for the decline in pp130 phosphorylation is not yet known. Perhaps this process resembles the desensitization seen in many receptor–effector systems (32). An additional possibility is that the clustered integrins are slowly internalized into the cell, thus terminating the signal transduction process; the kinetics of the process are consistent with the previously observed kinetics of integrin internalization (33). Clustering of cell surface β_1 integrins is also accompanied by changes in cell shape. However, it seems unlikely that the shape changes *per se* are responsible for inducing tyrosine phosphorylation since we have observed similar increases in tyrosine phosphorylation of pp130 in round cells in suspension subsequent to treatment with first plus second antibody (data not shown).

The time course of pp130 phosphorylation appeared to be more rapid in the presence of vanadate, suggesting that tyrosine phosphatases can readily dephosphorylate pp130. When these phosphatases are inhibited by vanadate, a net increase in pp130 phosphorylation is observed to occur earlier than in the absence of vanadate. Even when vanadate is present, the time course of pp130 phosphorylation seems somewhat slow compared to growth factor-induced tyrosine phosphorylation events, which are often detectable within seconds (27, 29, 30, 34). The difference in the time courses probably reflects the nature of the receptor–ligand systems themselves. Receptors for growth factors often contain intrinsic tyrosine kinase domains, whereas integrins have short cytoplasmic segments that are clearly not tyrosine kinases (11, 12, 34). Additionally, there are fundamental differences in the ligands for these receptors: growth factors are soluble, of relatively low molecular mass, and can thus diffuse readily in three dimensions, whereas the extracellular matrix molecules, which are the ligands for integrins, are often extremely large and are organized into immobile supramolecular complexes. These two distinct types of ligands may require very different kinetics of signal transduction.

pp130 tyrosine phosphorylation depended on the concentration of anti-integrin β_1 antibody as well as the clustering time. In addition, antibody stimulation of pp130 tyrosine phosphorylation showed specificity. Thus when KB cells were incubated with a panel of anti-integrin α-subunit antibodies, only the anti-integrin α_3 antibody had an effect comparable to the anti-integrin β_1 antibody. This may indicate that pp130 tyrosine phosphorylation is most readily coupled to clustering of α_3/β_1, an integrin that can act as a fibronectin, collagen, or laminin receptor (12), whereas other integrins may transduce different signals or may only be weakly linked to the tyrosine phosphorylation process. However, it is probably premature to rule out protein tyrosine phosphorylation as a potential signaling pathway for other integrins.

The nature of the pp130 tyrosine kinase-phosphatase system is not yet known. The results with vanadate suggest that regulation of pp130 phosphorylation occurs at the level of the tyrosine kinase in that sodium vanadate, which is a known

tyrosine phosphatase inhibitor (31), had little or no effect on phosphorylation of pp130 in control cells. This suggests that clustering of integrins may increase a kinase activity directed toward pp130 rather than decreasing a pp130 phosphatase activity; however, the involvement of a transmembrane phosphatase (35), which might be regulated during integrin clustering, cannot be excluded. There seems always to be a basal level of phosphorylation of pp130. This may be due to the fact that the KB cells were usually studied while attached to a substratum coated with serum, which contains integrin-binding ligands such as fibronectin and vitronectin. Alternatively, the low basal level of phosphorylation may be due to tyrosine kinases that are constitutively active in this highly transformed cell line. Preliminary studies with KB cells in suspension culture showed some degree of basal tyrosine phosphorylation of pp130 (data not shown), thus favoring the second interpretation.

Integrins are not themselves tyrosine kinases, yet this report demonstrates that integrin clustering increases pp130 tyrosine phosphorylation. Studies in other systems offer a possible explanation for this paradox. The most likely interpretation is that the clustering process causes the integrin to interact with another protein, which is itself a kinase or a phosphatase. This may result in activation of the putative kinase, inhibition of a phosphatase, or a change in intracellular organization causing the kinase or phosphatase to have access to additional substrates. The data we have obtained thus far do not discriminate between activation and altered access to substrates. In lymphoid cells, certain surface receptors can physically interact with and activate members of the membrane-associated *src* family of tyrosine kinases. For example, the relatively short cytoplasmic tails of the CD4 and CD8 surface glycoproteins interact with and activate the *lck* kinase (36). Additionally, the T-cell receptor can physically associate with the *fyn* kinase (37), whereas the B-cell antigen receptor associates with the *lyn* kinase (38). In each of these cases, receptor clustering seems to be important to the activation of the *src*-type kinases. Another example of this sort may be represented by the recently described modulation of $pp60^{src}$ in nerve growth cones (39). In the KB cell system, preliminary data (L.J.K., H.S.E., J. T. Parsons, and R.L.J., unpublished observations) indicate that one component of the pp130 complex is recognized by anti-pp125, a monoclonal antibody to a v-*src* substrate purified by anti-phosphotyrosine affinity procedures (40).

Immunoprecipitation with anti-pp125 demonstrated a basal level of phosphotyrosine in this protein in control or EGF-stimulated cells and an increase in pp125 phosphotyrosine in integrin clustered cells. Although the concept of an association between the cytoplasmic domains of integrin clusters and *src* family kinases represents an appealing model, and is consistent with our preliminary data, we cannot yet exclude other mechanisms. Further, given the rather slow response, it is conceivable that tyrosine phosphorylation of pp130 is an event that is "downstream" from the events initially activated by integrin clustering. Various forms of "cross-talk" between tyrosine kinases or phosphatases and other signaling cascades have been observed in other situations (34, 41).

The results presented here seem to have some resemblance to events previously described in platelets (42, 43). In these specialized cells, activation with thrombin (or with other agonists of platelet aggregation) leads to a wave of tyrosine phosphorylation involving several platelet proteins. Treatments that interfere with the function of glycoprotein IIb/IIIa, a major platelet integrin, suppress the phosphorylation of some of these proteins, suggesting that IIb/IIIa somehow regulates this process.

In summary, we have provided evidence that the clustering of β_1 integrins leads to enhanced tyrosine phosphorylation of a nonintegrin protein complex of 130 kDa. This may represent an important biochemical signal transduction pathway for integrins. Tyrosine phosphorylation mediated by integrins might conceivably regulate several important phenomena, including extracellular matrix effects on gene expression and cell differention, as well as anchorage dependence of cell growth.

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