Cell Adherence to Fibronectin and the Aggregation of the High Affinity Immunoglobulin E Receptor Synergistically Regulate Tyrosine Phosphorylation of 105–115-kDa Proteins*

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Adherence of cells to extracellular matrix components modulates cellular responses. Here we compared the array of tyrosine phosphorylated proteins induced by the aggregation of the high affinity receptor for IgE (FceRI) in fibronectin-adherent and in nonadherent rat basophilic leukemia (RBL-2H3) cells. Adherence to fibronectin in the absence of FceRI aggregation induced tyrosine phosphorylation of 105–115-kDa proteins. This phosphorylation was reversed by EDTA and by a synthetic peptide containing the sequence Arg-Gly-Asp, demonstrating a requirement for fibronectin-integrin interaction. Aggregation of FceRI in fibronectin-adherent cells markedly enhanced the tyrosine phosphorylation of the same 105–115-kDa proteins. There were minimal differences in tyrosine phosphorylation of other proteins induced by the aggregation of FceRI in nonadherent and in fibronectin-adherent cells. Direct activation of protein kinase C and/or increase in calcium influx induced the phosphorylation of the 105–115-kDa proteins only in fibronectin-adherent cells. The magnitude of the phosphorylation of the 105–115-kDa proteins induced by the aggregation of FceRI in fibronectin-adherent cells was substantially greater than the sum of that due to adherence to fibronectin and the aggregation of FceRI in nonadherent cells. Therefore, cell adherence and the aggregation of FceRI synergistically regulate tyrosine phosphorylation of the 105–115 kDa proteins.

Integrins are the major cell surface receptors by which cells adhere to other cells or to the extracellular matrix (1–5). These interactions are important for many biological processes, including cell differentiation and proliferation, tumor metastasis, inflammation, and the immune response. Integrins are heterodimers of α and β subunits, each of which is a transmembrane protein. The extracellular domain of the α subunit has three or four tandem repeats of a putative Ca²⁺ binding motif. Many integrins bind the tripeptide Arg-Gly-Asp sequence present on various extracellular matrix glycoproteins including fibronectin, laminin, and collagen (6).

Integrins do not only establish a physical link between cells and the extracellular matrix, but they also transduce signals into the cell. For example, the activation of integrins induces protein tyrosine phosphorylation, increases in cytoplasmic pH, changes in intracellular Ca²⁺, or cAMP levels and initiates gene expression (7–16). Many effects of integrins are due to their capacity to modify cellular responses to various stimuli; for instance, in T cells the activation of integrins facilitates the CD3-mediated interleukin production and cell proliferation (17–21). Thus, adherence by integrins can activate several intracellular signaling pathways. Similarly, the function of integrins is modulated by intracellular signals induced by other receptors (22–26); hence, the activation of T cells through the CD3 receptor complex results in an increase in the integrin-mediated adhesion of these cells to specific substrates (22, 23). Likewise, phorbol myristate acetate (PMA) stimulates protein kinase C and results in an increase in cell adhesion (22, 24). These data suggest interactions between the intracellular signals transduced by integrins and other receptors.

There are high affinity receptors for IgE (FceRI) on the surface of mast cells, basophils, and related cultured cell lines such as the rat basophilic leukemia (RBL-2H3) cells (27, 28). Aggregation of the FceRI by antigen activates these cells to release inflammatory mediators and cytokines. The activation of these cells results in many intracellular biochemical reactions, including protein tyrosine phosphorylation (29–35), phospholipase C activation (36), stimulation of phospholipases A2 (37) and D (38), and an increase in Ca²⁺ influx (39).

Recently, we reported that the mast cell analogue, RBL-2H3 cells, adhere strongly to fibronectin but weakly to other extracellular matrix components (40). The binding to fibronectin is Ca²⁺-dependent and can be inhibited by a synthetic peptide containing the sequence Arg-Gly-Asp, and as shown here is due to α1 integrins. RBL-2H3 cells are widely used as a model for studying FcεRI-induced signal transduction (41). In the present studies, RBL-2H3 cells were used to examine the effect of adherence to fibronectin on protein tyrosine phosphorylation induced by the aggregation of FcεRI. We show that the aggregation of FcεRI induced the strong tyrosine phosphorylation of 105–115-kDa proteins only in fibronectin-adherent cells.

**Experimental Procedures**

**Materials**—Eagle's minimum essential medium with Earle's balanced salt solution was from Microbiological Associates (Walkersville, MD). Glutamine, trypsin-EDTA, and fetal calf serum were purchased from Biofluids Inc. (Rockville, MD). Antibiotic/antimy-

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Adherence to Fibronectin Induces Tyrosine Phosphorylation of pp105-115 Proteins—As shown in Fig. 2, the adherence of RBL-2H3 cells to fibronectin induced the phosphorylation on tyrosine of proteins, the most prominent being in the range of 105–115 kDa (pp105–115). In time course experiments, the phosphorylation of the pp105–115 induced by interaction with fibronectin was first apparent within 5 min, reached a peak by 20 min and decreased slightly at 60 min (Fig. 3). It was also detectable in adherent cells that had attached to plastic plates by culture overnight in the fetal calf serum-containing medium (data not shown). Thus, tyrosine phosphorylation of pp105–115 continues as long as the cells are adherent. Previously we observed that the binding of RBL-2H3 cells to fibronectin was Ca"+-dependent and was inhibited by a pep-
Tyrosine Phosphorylation of pp105–115 in RBL-2H3

Fig. 1. Presence of $\beta_1$ integrins on RBL-2H3 cells. A. precipitation of $\beta_1$ integrins by antibodies and fibronectin. Affinity precipitations were with rabbit IgG (lane 1), rabbit anti-$\beta_1$ integrins (lane 2), BSA (lane 3); 120-kDa fragment of fibronectin (lane 4), or intact fibronectin (lane 5). B. immunoblotting with anti-$\beta_1$ integrins of the proteins precipitated with BSA (lane 1) or fibronectin (lane 2) coupled to Sepharose. Proteins from unlabeled RBL-2H3 cells were precipitated as above, separated by SDS-PAGE (10%), and then transferred to nitrocellulose. Immunoblotting was with anti-$\beta_1$ integrins (3 ng/ml) as described under “Experimental Procedures.” Molecular size standards are in kilodaltons.

Fig. 2. Adherence of RBL-2H3 cells to fibronectin-coated surfaces induces tyrosine phosphorylation of pp105–115. Cells were added to wells coated with only BSA or with fibronectin (FN). After 20 min at 37 °C, the cellular proteins were analyzed for tyrosine phosphorylation. The arrow shows the pp105–115.

Fig. 3. Time course for the adherence-induced tyrosine phosphorylation of pp105–115. Cells were added to wells coated with fibronectin for the indicated time at 37 °C. After incubation, the cellular proteins were analyzed for tyrosine phosphorylation. Only pp105–115 is shown.

containing the Arg-Gly-Asp sequence or Arg-Gly-Glu. The addition of a synthetic peptide containing the Arg-Gly-Asp sequence, but not Arg-Gly-Glu, reversed the phosphorylation of pp105–115 (Fig. 4A). Similarly, chelating Ca$^{2+}$ in the medium by adding EDTA rapidly reversed the tyrosine phosphorylation of pp105–115 (Fig. 4B). The action of EDTA is most likely to disrupt cell binding to fibronectin, because the 1-min incubation is too short to deplete intracellular Ca$^{2+}$. The rapid reversal of the phosphorylation of pp105–115 suggests the presence of active tyrosine phosphatase(s) in the cells. Taken together, these results indicate that tyrosine phosphorylation of pp105–115 is tightly coupled to adherence mediated by integrins.

To determine if cell adherence and spreading on other biological surfaces induces tyrosine phosphorylation of pp105–115, RBL-2H3 cells were plated on surfaces coated with 30 ng/ml of antibodies to different RBL-2H3 surface molecules. Although this resulted in cell adherence and spreading (Fig. 5), there was no increase in the tyrosine phosphorylation of pp105–115 (Fig. 6). Therefore, tyrosine phosphorylation of pp105–115 resulted from the integrin-mediated adherence of cells to fibronectin.

In transformed chicken fibroblasts the $\beta_1$ integrins are phosphorylated on tyrosine (48–50). However, our experiments suggest that pp105–115 is not the $\beta_1$ integrins. RBL-2H3 cell lysates from cells that had been adherent on fibronectin were affinity-purified with anti-phosphotyrosine or with anti-$\beta_1$ integrins adsorbents. The precipitated phosphorylated proteins (eluted with phenyl phosphate) or integrins were transferred to nitrocellulose paper and probed with either anti-phosphotyrosine or anti-$\beta_1$ integrin antibodies. The $\beta_1$ integrins was not precipitated by the anti-phosphotyrosine affinity column, although the 105–115-kDa phosphorylated proteins could be detected in these immunoblots (data not shown). Similarly, the antibodies to $\beta_1$ integrin did not precipitate tyrosine-phosphorylated pp105–115 (data not shown). These results, together with the difference in the apparent molecular mass between $\beta_1$ and pp105–115, demonstrate that pp105–115 is not $\beta_1$ integrin.

FccRI Aggregation Induces Strong Tyrosine Phosphorylation of pp105–115 in Fibronectin-adherent but Not in Nonadherent RBL-2H3 Cells—Recently, we and others (29–35) have reported the rapid tyrosine phosphorylation of several proteins coupled to FccRI-mediated signal transduction in RBL-2H3 cells. Here, we compared the array of tyrosine phosphorylated proteins induced by the aggregation of the FccRI in fibronectin-adherent to those in nonadherent RBL-2H3 cells. FccRI aggregation of adherent and nonadherent RBL-2H3 cells led

Fig. 4. The peptide Arg-Gly-Asp (RGD) and EDTA reverse adherence-induced tyrosine phosphorylation of pp105–115. A, adherence-induced tyrosine phosphorylation requires continuous cell adherence to fibronectin (FN). Cells were allowed to adhere to fibronectin for 20 min at 37 °C and then incubated with 1 mM of the peptide Gly-Arg-Gly-Asp-Ser-Pro or Gly-Arg-Gly-Glu-Ser-Pro for 30 min at 37 °C. B, protein tyrosine phosphorylation induced by cell adherence to fibronectin is dependent on Ca$^{2+}$. Cells were allowed to adhere to fibronectin for 20 min at 37 °C; 3 mM EDTA was then added for the indicated times.
tyrosine phosphorylation of 38-, 72-, 80-, 97-, 105-115-, and 140-kDa proteins (Fig. 7, lanes 3 and 4). The extent of the phosphorylation of the 38-, 72-, 80-, 97-, and 140-kDa proteins was slightly increased in adherent cells when compared with nonadherent cells. For example, by densitometry there was a 24% increase in tyrosine phosphorylation of the 72-kDa protein in adherent cells. However, the intensity of phosphorylation of the proteins in the molecular mass 105-115 kDa range was markedly enhanced in cells attached to fibronectin. The extent of the phosphorylation of the 105-115-kDa proteins induced by FccRI in fibronectin-adherent cells was substantially greater than that induced by both adherence to fibronectin and by the aggregation of FccRI in nonadherent cells (Fig. 7, lanes 2 and 4). Aggregating FccRI on cells adherent to antibody-coated surfaces strongly phosphorylated the 38-, 72-, 80-, 97-, and 140-kDa proteins (Fig. 8, lane 1 versus lanes 4-6); however, the phosphorylation of the 105-115-kDa proteins was strikingly less than that induced by FccRI aggregation on cells adherent to fibronectin (Fig. 8, lane 3 versus lanes 4-6). Hence, adherence to fibronectin by integrin is essential for optimal tyrosine phosphorylation of the 105-115-kDa proteins induced by the aggregation of FccRI. Two-dimensional electrophoresis was used to confirm that in fibronectin-adherent cells the 105-115-kDa proteins phosphorylated by adherence and by FccRI aggregation are identical (Fig. 9). Adherence to fibronectin resulted in the tyrosine phosphorylation of several proteins that correspond to the pp105-115 seen in the previous experiments (Fig. 9, A versus B). Tyrosine phosphorylation of the same pp105-115 was enhanced in adherent cells in which FccRI were aggregated (Fig. 9, B versus C); no additional proteins of this size range were phosphorylated on tyrosine. Taken together, the data indicate that adherence to fibronectin by integrin and the aggregation of FccRI synergistically regulated tyrosine phosphorylation of pp105-115.

**PMA and Ca²⁺ Ionophore Induce Tyrosine Phosphorylation of pp105-115 Only in Fibronectin-adherent Cells—Ca²⁺ influx and protein kinase C activation can induce protein tyrosine phosphorylation and are implicated in the signal transduction of various receptors (34, 51, 52). We therefore investigated whether Ca²⁺ influx and protein kinase C activation by themselves are sufficient to induce pp105-115 tyrosine phosphorylation. In nonadherent cells, treatment with the Ca²⁺ ionophore A23187 to increase intracellular Ca²⁺ and/or with PMA to activate protein kinase C did not induce tyrosine phosphorylation of the 105–115-kDa proteins (Fig. 10). In contrast, in fibronectin-adherent cells both the Ca²⁺ ionophore and PMA led to a significant increase in the tyrosine phosphoryl-
cells were preincubated with antigen-specific IgE. They were then phosphorylated in cells adherent to plastic coated with antibodies to RBL-2H3 surface proteins. Following incubation for 20 min at 37 °C, antigen was added to lanes 2-6 for a further 25 min at 37 °C. %HR, percent histamine release. The arrow indicates the pp105-115.

**FIG. 8.** FcRRI aggregation induces low level tyrosine phosphorylation of pp105–115 in cells adherent to plastic coated with antibodies to RBL-2H3 surface proteins. The RBL-2H3 cells were preincubated with antigen-specific IgE. They were then added to wells coated with only BSA (lanes 1 and 2), fibronectin (lane 3), mAb BGD6 (lane 4), mAb BB3 (lane 5), or mAb BD6 (lane 6). Following incubation for 20 min at 37 °C, antigen was added to lanes 2-6 for a further 25 min at 37 °C. %HR, percent histamine release. The arrow indicates the pp105-115.

**FIG. 9.** FcRRI aggregation enhances tyrosine phosphorylation of the same pp105–115 induced by adherence to fibronectin. Two-dimensional electrophoresis and immunoblotting of the tyrosine phosphorylated proteins in nonadherent (A), fibronectin-adherent (B), and fibronectin-adherent FcRRI-activated cells (C). RBL-2H3 cells were added to wells coated with only BSA (A) or fibronectin (B and C). After 20 min at 37 °C, they received either PIPES buffer (A and B) or antigen (C) for 25 min at 37 °C. Cell lysates were prepared as above, transferred to urea buffer (9.5 M urea, 8% Nonidet P-40), boiled for 15 min, centrifuged, and the supernatant subjected to isoelectrofocusing (pH 3-10) followed by SDS-PAGE (4-20%). The proteins were then transferred to nitrocellulose and analyzed for tyrosine phosphorylation. Arrowheads mark the pp105-115.

**FIG. 10.** Ca2+ ionophore and PMA induce tyrosine phosphorylation of 105–115-kDa proteins only in fibronectin-adherent cells. Cells were added to wells coated with only BSA (lanes 1, 3, and 5) or fibronectin (lanes 2, 4, and 6) and allowed to adhere for 20 min at 37 °C. PIPES buffer (lanes 1 and 2), PMA (40 nM final concentration, lanes 3 and 4), or Ca2+ ionophore A23187 (final concentration 0.5 μM, lanes 5 and 6) was then added for an additional 25 min at 37 °C. The densitometry of the 105-115-kDa proteins in lanes 2-6 were as follows: 88, 5, 158, 16, and 154 respectively (arbitrary units, with lane 1 used as background). %HR, percent histamine release. The arrow indicates the pp105-115.

Tyrosine phosphorylation of pp105–115 in RBL-2H3

Tyrosine phosphorylation of pp105–115 induced by adherence to fibronectin. First, cell adherence and spreading on biological surfaces other than fibronectin did not induce tyrosine phosphorylation of these proteins. Second, a synthetic peptide containing the Arg-Gly-Asp sequence reversed the adherence-induced phosphorylation of these proteins. Third, protein tyrosine phosphorylation induced by adherence to fibronectin was dependent on the presence of extracellular Ca2+. These data strongly suggest that the pp105-115 phosphorylation is due to an interaction of β1 integrins on RBL-2H3 cells with fibronectin. However, in the immunoprecipitation studies proteins other than the β1 also bound to intact fibronectin (Fig. 1). Thus, these other surface molecules could play a role in the tyrosine phosphorylation induced by adherence.

Although adherence of RBL-2H3 cells to fibronectin resulted in cell spreading, tyrosine phosphorylation of pp105-115 was not due to the morphological changes. The induction of tyrosine phosphorylation of pp105-115 by adherence to fibronectin occurred before there was significant cell spreading (within 5 min), reached a peak before the cells had completely spread (within 20 min), and was present long after the cells had fully spread. Furthermore, cell adherence and spreading on other biological surfaces did not induce the tyrosine phosphorylation of pp105-115. These data imply that tyrosine phosphorylation of pp105-115 induced by cell adherence to fibronectin is independent of cell spreading.

Protein tyrosine phosphorylation is a common signaling pathway for many different receptors (7-9, 29, 53-55). Integrins by activating protein tyrosine kinase(s) or inactivating a tyrosine phosphatase could result in enhanced tyrosine phosphorylation of pp105-115. The activation of integrins in other cells induced tyrosine phosphorylation of proteins with molecular mass similar to the ones reported here. For example, adherence of NIH 3T3 cells to fibronectin-coated surfaces induced tyrosine phosphorylation of 120-kDa proteins (7). Clustering of β1 integrins on adenocarcinoma cells led to...
tyrosine phosphorylation of 115–130-kDa proteins (8) and aggregation of the very late antigen-4 (VLA-4) on T cells stimulated tyrosine phosphorylation of a 105-kDa protein (9). Integrin activation of chicken embryo cells induced tyrosine phosphorylation of the novel protein tyrosine kinase pp125FAK (10).

As we and others have reported previously (29–35), FcRI aggregation induced the tyrosine phosphorylation of several proteins. Some of these proteins, e.g. pp72, were phosphorylated only by the aggregation of FcRI. In contrast, either cell adherence or aggregation of the FcRI in nonadherent cells induced low level tyrosine phosphorylation of pp105–115 proteins. However, FcRI aggregation of fibronectin-adherent cells strongly phosphorylated pp105–115. Thus, although the stimulation of either integrins or FcRI alone induced some tyrosine phosphorylation of 105–115-kDa proteins, maximal phosphorylation was observed only after the aggregation of FcRI in fibronectin-adherent cells. The magnitude of phosphorylation of pp105–115 by FcRI aggregation in fibronectin-adherent cells was not simply an additive effect of tyrosine phosphorylation induced by adherence to fibronectin and by the aggregation of FcRI in nonadherent cells. Therefore, integrins and FcRI are synergistically regulating the extent of the tyrosine phosphorylation of pp105–115.

In previous studies the aggregation of FcRI in RBL-2H3 cell monolayers attached by culture in the presence of fetal calf serum induced the phosphorylation of a 110-kDa protein (34). This phosphorylation was Ca2+-dependent and was also induced by PMA and by Ca2+ ionophore. These similarities suggest that pp110 reported previously may be one of the 105–115-kDa proteins. However, the pp110 was characterized in cells grown as monolayers in the presence of fetal calf serum; these conditions were different from those in the present experiments. The similarities in these proteins could be fortuitous or a result of a true molecular relationship.

Recently, we have shown that adherence of RBL-2H3 cells to fibronectin-coated surfaces caused cell spreading, reorganization of the cytoskeleton, and redistribution of the secretory granules (40). As we have reported previously and confirm here, these changes are accompanied by a dramatic enhancement of histamine release induced by FcRI stimulation (Fig. 7). In the present experiments, adherence of the cells to surfaces coated with different antibodies also enhanced FcRI-mediated histamine release (Fig. 8); however, there was little increase in the phosphorylation of pp105–115. Thus, FcRI-mediated tyrosine phosphorylation of pp105–115 was greater in cells adherent to fibronectin compared with cells plated on antibody-coated surfaces, although both enhanced histamine release. These results suggest that marked increase in tyrosine phosphorylation of pp105–115 is not essential for enhanced histamine release.

The phosphorylation of pp105–115 in adherent cells was enhanced not only by the aggregation of FcRI, but also by stimulation with PMA and Ca2+ ionophore. In some experimental models, cell activation either induced or enhanced adherence to matrix proteins (22–26). Thus, cell stimulation could enhance the adherence of the RBL-2H3 cells that in turn may result in enhanced tyrosine phosphorylation of pp105–115. However, this probably does not explain the present observations. Stimulation of the cells by FcRI aggregation or with PMA did not increase the number of cells bound to fibronectin. Furthermore, aggregating FcRI on cells adherent by culture to tissue culture surface, i.e. all cells were adherent to the culture surface, still enhanced pp105–115 tyrosine phosphorylation (34). Another possible mechanism of the increased pp105–115 phosphorylation could be the redistribution of cellular proteins. Adherence of RBL-2H3 cells to fibronectin-coated surfaces induced massive reorganization of the cytoskeleton (40). Besides integrins, several tyrosine kinases and/or substrates, including talin, tensin, paxillin, and pp125FAK, accumulate at the sites of cell-substratum contact (10, 48–50, 56–58). Several of these proteins are also hyperphosphorylated on tyrosine. Thus, in fibronectin-adherent cells, tyrosine kinases and/or substrates clustered at points of cell contact could be more accessible to signal transduction pathways. Similarly, FcRI aggregation, PMA, and Ca2+ ionophore induced reorganization of the cytoskeleton (59, 60). Furthermore, PMA induced the serine/threonine phosphorylation of cytoskeletal proteins and affects their interaction with integrins (61, 62). Thus, it is possible that in RBL-2H3 cells, FcRI aggregation, PMA, and Ca2+ ionophore affected the interaction of integrins with other proteins and as a result modulated the integrin-induced tyrosine phosphorylation of pp105–115.

It has been suggested that the signals transduced by integrins and those of other receptors may converge (4). Here we show that the signals induced by integrins and by FcRI are actually regulated by different pathways of the phosphorylation of the same proteins. This suggests the convergence of the signals induced by integrins and by FcRI. Identifying the 105–115-kDa proteins and their cellular functions should shed light on their role in integrins and FcRI signal transduction pathways.

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Note Added in Proof—Further experiments indicate that pp125FAK is one of the 105–115-kDa proteins (M. M. Hamawy, S. E. Mergen- hagen, and R. P. Siraganian, manuscript in preparation).

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Tyrosine Phosphorylation of pp105–115 in RBL-2H3

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