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Immunology

Tyrosine phosphorylation coupled to IgE receptor-mediated signal transduction and histamine release

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ABSTRACT **Antigen-induced cross-linking of IgE bound to its receptors at the surface of basophils or mast cells initiates a number of biochemical events culminating in the release of histamine-containing granules. In the present study, we investigated the possible involvement of tyrosine phosphorylation in signaling by the high-affinity IgE receptor (FcεRI). Cross-linking of FcεRI in rat basophilic leukemia cells (RBL-2H3) led to the phosphorylation of several proteins on tyrosine, the most prominent having a mass of 72 kDa. Tyrosine phosphorylation was rapid, detectable 1 min after stimulation, and correlated with both the time course and antigen dose for histamine release. Reversal of FcεRI cross-linking prevented continuation of the degranulation process and resulted in rapid loss of tyrosine phosphorylation. The receptor-mediated tyrosine phosphorylation was still induced in the absence of calcium in the medium. Depletion of protein kinase C with phorbol 12-myristate 13-acetate did not dramatically affect the tyrosine phosphorylation signal or the release of histamine. In contrast, the calcium ionophore A23187 induced histamine release in the absence of a perceptible increase in protein tyrosine phosphorylation. Thus, tyrosine phosphorylation is an early signal following FcεRI aggregation, independent of the exocytotic process itself. Taken together, our findings functionally link protein phosphorylation on tyrosine residues to FcεRI-mediated signal transduction leading to histamine release.**

Protein-tyrosine kinases of the receptor class mediate cell growth responses to environmental signals such as epidermal growth factor (1) and platelet-derived growth factor (2, 3). However, accumulating evidence has also implicated tyrosine phosphorylation in degranulation by fully differentiated cells such as platelets (4) and chromaffin cells (5, 6). Furthermore, $p55^{c\text{-}fgr}$, a protein-tyrosine kinase of the *src* family, has been recently shown to function in some aspect of neutrophil degranulation (7). Histamine release from mast cells and from the rat basophilic leukemia cells (RBL-2H3) provides a well-documented model for exocytosis (8–11). RBL-2H3 cells express high-affinity IgE receptors (FcεRI) and aggregation of these receptors results in a number of metabolic events culminating in the release of histamine-containing granules. Recent studies have shown that in immune complexes containing solubilized FcεRI proteins, both the β and γ components are phosphorylated *in vitro* exclusively on tyrosine residues by virtue of a protein-tyrosine kinase activity that coprecipitates with, but is distinct from, the IgE receptor complex (12, 13). These findings provided our rationale for investigating the possible role of protein tyrosine phosphorylation in the process of histamine release from basophils.

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EXPERIMENTAL PROCEDURES

Cell Stimulation and Histamine Release Assay. RBL-2H3 cells cultured as described (14) were plated at 1×10^6 cells per 35-mm^2 well in 2 ml of culture medium containing 0.3 μg of purified anti-2,4-dinitrophenyl (DNP) monoclonal mouse IgE per ml (DNP-48) (15). After overnight culture at 37°C and 5% CO_2/95% air, the cell monolayers were washed twice with Pipes-buffered saline (16) containing 1 mM $CaCl_2$ and were stimulated for 45 min at 37°C with the indicated DNP–human serum albumin concentrations or with 0.1 μg of the anti-FcεRI monoclonal antibody BC4 per ml (17). Histamine released into the supernatants was measured by the automated fluorometric technique (18). The percentage histamine release was calculated relative to the total histamine content of unstimulated cells lysed by 6% perchloric acid. Each experiment was done in triplicate. The corresponding cell pellets were analyzed for protein tyrosine phosphorylations. In some experiments, cells were stimulated in the absence of external calcium, in SMEM medium (Biofluids, Rockville, MD) supplemented with 0.1% bovine serum albumin instead of Pipes-buffered saline.

Analysis of Protein Tyrosine Phosphorylation. The cell pellets were lysed in the presence of protease and phosphatase inhibitors (7). Protein extracts were fractionated by electrophoresis on 8% acrylamide gels (40 μg per lane) and electrotransferred to nitrocellulose sheets as described (19). Relative molecular mass markers were the following: myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa). Phosphorylated proteins were detected with an affinity-purified rabbit anti-phosphotyrosine antibody or with anti-phosphotyrosine monoclonal antibody Py20 and iodinated protein A as described (19). Phenylphosphate (10 mM), but not the identical concentrations of phosphoserine or phosphothreonine, blocked the appearance of the bands on immunoblots. Filters were exposed to Kodak X-AR film for 3 days.

RESULTS

Aggregation of FcεRI Induces Tyrosine Phosphorylation. RBL-2H3 cells were sensitized with an anti-DNP mouse monoclonal IgE and stimulated with antigen. Histamine released into the supernatant was quantitated and corresponding cell lysates were analyzed by immunoblotting with anti-phosphotyrosine antibodies. Tyrosine phosphorylation of several proteins was detected in lysates from IgE-sensitized cells stimulated with antigen (Fig. 1*A*). The most prominent phosphoprotein had a relative molecular mass of 72 kDa (pp72). Additional but fainter bands were observed at

Abbreviations: FcεRI, high-affinity receptor for IgE; FcγR, IgG receptor; DNP, 2,4-dinitrophenyl; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, free intracellular calcium concentration.

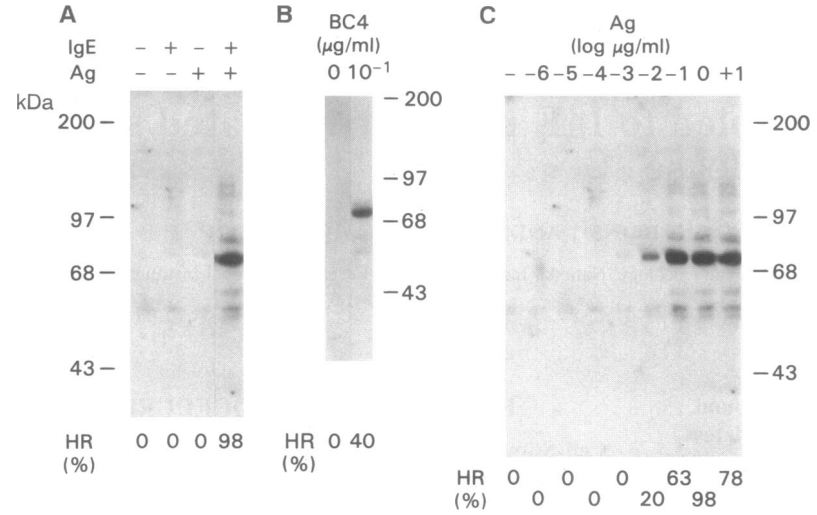

FIG. 1. IgE receptor-mediated histamine release and tyrosine phosphorylation of cellular proteins in RBL-2H3 cells. (*A*) RBL-2H3 cells were cultured overnight with or without anti-DNP IgE, washed, and challenged with antigen (1 μg/ml) or buffer alone. (*B*) Unsensitized cells were stimulated with the anti-FcεRI monoclonal antibody BC4 (17) for 45 min. (*C*) RBL-2H3 cells were cultured overnight with or without anti-DNP IgE, washed, and challenged with the indicated concentrations of antigen. Histamine released into the supernatants (HR) is indicated below.

57, 62, and 83 kDa. In contrast, IgE or antigen alone failed to induce tyrosine phosphorylation (Fig. 1*A*). As an independent means of activating the FcεRI, RBL-2H3 cells were incubated with the anti-FcεRI monoclonal antibody BC4 in the absence of IgE or antigen. This treatment resulted in histamine release and a pattern of tyrosine phosphorylation identical to that observed in sensitized cells stimulated with antigen (Fig. 1*B*). These findings demonstrated that tyrosine phosphorylation was coupled to FcεRI activation and suggested that phosphorylation on tyrosine residues was involved in signal transduction leading to histamine release.

The relationship of FcεRI aggregation and tyrosine phosphorylation to histamine release was examined further in antigen dose–response and time course experiments. As shown in Fig. 1*C*, the degree of tyrosine phosphorylation correlated with the extent of histamine release over a wide dose range of antigen. In time course studies, tyrosine phosphorylation was observed 1 min after the addition of antigen to IgE-sensitized cells and persisted throughout the course of the experiment (Fig. 2). In a time-dependent manner, the extent of histamine release correlated with the degree of tyrosine phosphorylation. These findings demonstrated the early nature of the tyrosine phosphorylation event after FcεRI stimulation.

To determine whether tyrosine phosphorylation was dependent on continued receptor activation, RBL-2H3 cells were sensitized with anti-DNP IgE, washed, and activated for histamine release with DNP–human serum albumin. After 5 min of stimulation, antigen-dependent bridging of FcεRI was disrupted by the addition of excess hapten (DNP–lysine). Upon receptor disaggregation, further histamine release ceased (Fig. 2*A*), and tyrosine phosphorylation was rapidly lost (Fig. 2*B*). Therefore, tyrosine phosphorylation was dependent on continued receptor aggregation and was functionally linked to signal transduction leading to degranulation.

FcεRI-Induced Tyrosine Phosphorylation Is Independent of Protein Kinase C (PKC) Activity. Activation of FcεRI in RBL-2H3 cells results in the hydrolysis of inositol phospholipids with the release of inositol triphosphates, an increase in intracellular calcium, and activation of PKC. Direct activation of PKC by the addition of phorbol 12-myristate 13-acetate (PMA) to RBL-2H3 cultures does not induce exocytosis but potentiates the release of histamine induced by calcium ionophores (20, 21). To determine whether tyrosine phosphorylation was secondary to this cascade of biochemical events RBL-2H3 cells, either untreated or treated with PMA for 24 hr, were subjected to a 45-min pulse of PMA and examined for protein phosphorylation and histamine release. As shown in Fig. 3, neither histamine release nor tyrosine phosphorylation was induced by either PMA treatment, indicating that PKC does not directly activate tyrosine phosphorylation. As previously reported (22), 24-hr incubation of cultures with PMA depleted the cells of detectable PKC by immunoblot analysis (data not shown). In these cells depleted of PKC, tyrosine phosphorylation was still observed after FcεRI-mediated stimulation, although both tyrosine

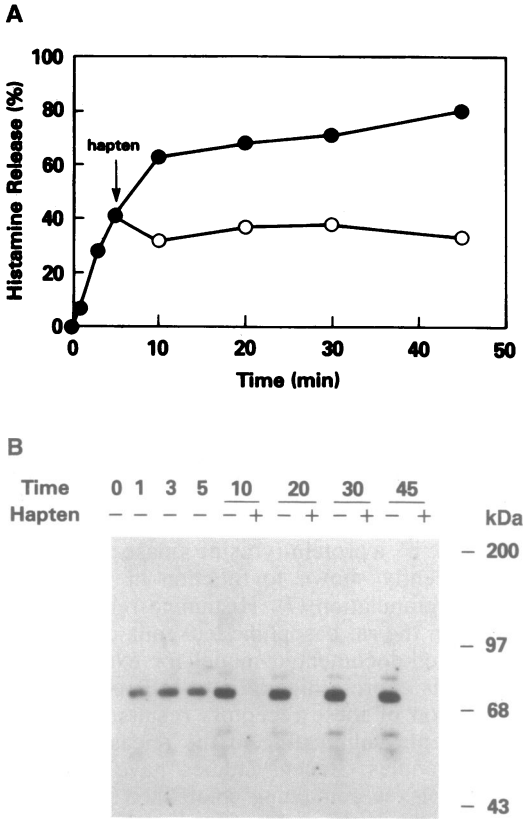

FIG. 2. Requirement of continued FcεRI cross-linking for histamine release and tyrosine phosphorylation. (*A*) Histamine release. Monolayers of RBL-2H3 cells were cultured with anti-DNP IgE, washed, and stimulated (●) for the indicated length of time with antigen (0.1 μg of DNP–human serum albumin per ml). After 5 min of stimulation, 50 μM DNP–lysine was added to some of the wells (arrow), which were further incubated for the times indicated (○). Histamine release was measured in supernatants and calculated as percentage total histamine. (*B*) Tyrosine-phosphorylated cellular proteins determined by immunoblotting. One representative of two experiments.

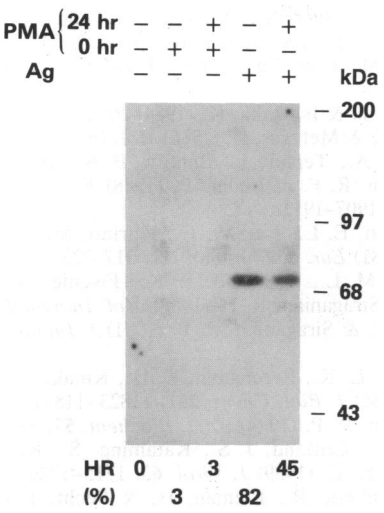

FIG. 3. Effect of phorbol esters on histamine release and tyrosine phosphorylation. RBL-2H3 cells were plated at 1×10^6 cells in 2 ml of culture medium containing anti-DNP IgE. After 2 hr, 400 nM PMA was added to some of the wells (PMA 24 hr). Twenty-four hours later, the cells were washed twice and stimulated for 45 min with either 40 nM PMA (PMA 0 hr) or antigen (Ag) in calcium-containing Pipes-buffered saline, and histamine release (HR) was determined. Corresponding cell pellets were used for immunoblotting. Extracts from cells treated for 24 hr with 400 nM PMA contained no detectable PKC as judged by immunoblotting with monoclonal antibodies to PKC types II and III. One representative of four experiments.

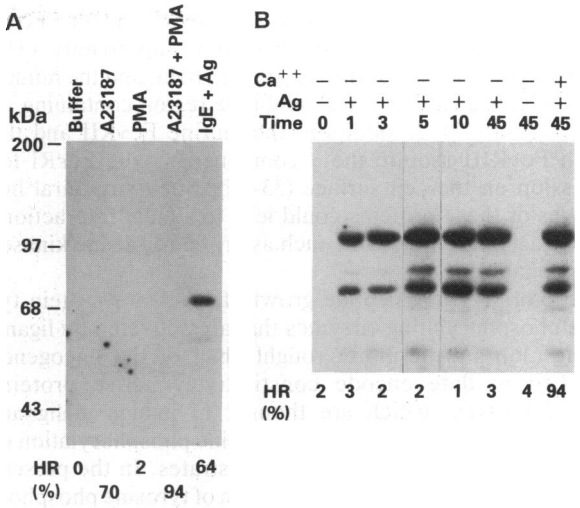

FIG. 4. Calcium requirement for histamine release but not tyrosine phosphorylation. (*A*) The effect of the calcium ionophore A23187. RBL-2H3 cells were cultured for 16 hr with or without anti-DNP IgE. Monolayers were washed and incubated for 45 min as indicated. Concentrations: PMA, 40 nM; A23187, 1 μM; antigen, 1 μg/ml. (*B*) Stimulation in the absence of extracellular calcium. IgE-sensitized monolayers were washed twice in SMEM calcium-free medium containing 4 mM EDTA and were incubated for the indicated length of time (min) with or without antigen in the presence of 50 μM EDTA. Control cells were washed and stimulated with antigen in the presence of 1 mM $CaCl_2$. One representative of four experiments.

phosphorylation and histamine release were reduced by ≈50% (Fig. 3). Therefore, the FcεRI-induced phosphorylation on tyrosine residues was still present even when there was no detectable PKC, although our data may suggest a role for PKC in modulating the tyrosine phosphorylation and histamine release responses.

Effect of Calcium on FcεRI-Induced Tyrosine Phosphorylation. The calcium ionophore A23187 induces an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) and degranulation in RBL-2H3 cells by a mechanism that circumvents the IgE receptor complex. This provided an opportunity to determine whether an increase in $[Ca^{2+}]_i$ could result in tyrosine phosphorylation. Cells were stimulated with the calcium ionophore A23187 at a concentration that induces high levels of $[Ca^{2+}]_i$ and significant histamine release. Under these conditions, no tyrosine phosphorylation was observed (Fig. 4*A*). Furthermore, stimulation with A23187 together with activation of PKC with PMA also failed to induce tyrosine phosphorylation (Fig. 4*A*). These findings demonstrate that FcεRI-mediated tyrosine phosphorylation is not induced by a major increase in free cytosolic calcium in the presence or absence of PKC activation. Moreover, we conclude that tyrosine phosphorylation is coupled to functional FcεRI signaling but is independent of the exocytotic process itself.

In the absence of extracellular calcium, no degranulation occurs from RBL-2H3 cells in response to FcεRI-mediated triggering, but minimal increases in $[Ca^{2+}]_i$ have been observed (23, 24). To determine whether the phosphorylation signal was present under these conditions, RBL-2H3 cells were first sensitized with IgE and then stimulated with antigen in calcium-free medium containing EDTA. As shown in Fig. 4*B*, tyrosine phosphorylation was readily observed. Furthermore, both the kinetics and the extent of tyrosine phosphorylation were similar in the presence and absence of extracellular calcium. Taken together, our findings demonstrate that increased intracellular calcium is neither sufficient nor required for FcεRI-dependent tyrosine phosphorylation.

DISCUSSION

Basophils are terminally differentiated myeloid cells that function physiologically by releasing granules containing inflammatory mediators (8–11). FcεRI-mediated signaling is thought to result in guanine nucleotide-binding regulatory protein-independent phospholipase C activation (25, 26), degradation of inositol phospholipids, activation of PKC, and an increase in intracellular free calcium resulting from both the release of calcium from internal stores and calcium influx (8–11). However, none of these events can totally account for the degranulation signal (22), suggesting the involvement of additional and so far undescribed metabolic events. Our present findings demonstrated increased tyrosine phosphorylation as an early metabolic event coupled to FcεRI aggregation. Furthermore, tyrosine phosphorylation was independent of PKC activity, of calcium influx, and of the major increase in $[Ca^{2+}]_i$. It is also likely to be independent of phospholipase C activation since FcεRI-mediated stimulation of inositol phospholipid breakdown is dramatically reduced in the absence of external calcium (27, 28). Therefore, phosphorylation of tyrosine residues must be considered in hypotheses linking FcεRI aggregation to subsequent secretory events.

The mechanism involved in increased steady-state tyrosine phosphorylation in response to FcεRI cross-linking is not yet identified. One model would implicate an inhibition of protein-tyrosine phosphatase activity. Alternatively, protein-tyrosine kinases may act as intracellular effectors of the FcεRI signal, as has been suggested for activation of the T-cell receptor through its association with CD4 and $p56^{lck}$, a protein-tyrosine kinase (29, 30). In this regard, we have identified $p60^{c\text{-}src}$ and $p59^{fyn}$ protein-tyrosine kinases in extracts of RBL-2H3 cells (unpublished data).

The observation that the FcεRI can trigger tyrosine phosphorylation raises the possibility that this could be a general property of Fc receptor-induced signaling. Indeed there are structural homologies between the FcεRI and the IgG recep-

tor (FcγR) (31). The α chain of the FcεRI and the FcγR proteins belong to the immunoglobulin superfamily (31). Interestingly, the FcεRI, the murine FcγRIIa, and the human FcγRIII have a similar transmembrane region containing an aspartic acid (32). In addition, the murine FcγRII and the human FcγRIII require the γ component of the FcεRI for expression on the cell surface (33–35). Such structural homologies of these proteins could lead to similar interactions with intracellular molecules such as protein-tyrosine kinases or phosphatases.

Receptors for polypeptide growth factors are protein tyrosine phosphorylating enzymes that are activated by ligand binding (36). Furthermore, roughly half of the oncogenes described to date encode constitutively active protein-tyrosine kinases, which are thought to induce malignant transformation by increasing the tyrosine phosphorylation of certain critical growth regulatory substrates. In the present study, we have demonstrated induction of tyrosine phosphorylation during signal transduction mediated by the FcεRI complex. However, it is well known that the FcεRI stimulation induces histamine release by a secretory process. Thus, our present findings suggest that tyrosine phosphorylation is also involved in normal cellular processes that are not proliferative in nature.

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