Aggregation of the High Affinity IgE Receptor Results in the Tyrosine Phosphorylation of the Surface Adhesion Protein PECAM-1 (CD31)*

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One of the earliest events after aggregation of the high affinity receptor for IgE (FceRI) on mast cells is the activation of protein tyrosine kinases resulting in tyrosine phosphorylation of numerous proteins. Using a monoclonal antibody raised against the rat basophilic leukemia RBL-2H3 cells, we identified that platelet/endothelial cell adhesion molecule 1 (PECAM-1 or CD31) was tyrosine phosphorylated in these cells. Aggregation of PECAM-1 did not induce a detectable increase in its tyrosine phosphorylation, nor did it result in degranulation. However, the minimal tyrosine phosphorylation of PECAM-1 in nonstimulated cells was dramatically increased after FceRI aggregation. This receptor-induced tyrosine phosphorylation of PECAM-1 was an early event, independent of Ca2+ influx or of the activation of protein kinase C and of cell adhesion. PECAM-1 is an adhesion molecule that is required for the transmigration of leukocytes across the endothelium into sites of inflammation. Therefore tyrosine phosphorylation of PECAM-1 may modulate its interaction with other molecules, thereby regulating the migration of basophils into inflammatory sites.

Mast cells and basophils accumulate at sites of inflammation and play pivotal roles in the initiation of the allergic response. Aggregation of the high affinity receptor for IgE (FceRI) on these cells initiates a biochemical cascade that results in degranulation and release of inflammatory mediators (1–3). The earliest event following aggregation of FceRI is the phosphorylation of proteins on tyrosine, an event that is critical for signal transduction in basophils or mast cells (4–9).

To understand the signaling cascade initiated by FceRI aggregation, we are attempting to identify molecules that become tyrosine phosphorylated after receptor activation. Rat basophilic leukemia RBL-2H3 cells provide a useful experimental model to study these signal transduction pathways for degranulation in mast cells/basophils (2, 3). Here we report that a monoclonal antibody raised to these cells recognized a 130-kDa tyrosine phosphorylated after receptor activation. Rat basophilic leukemia RBL-2H3 cells, we identified that platelet/endothelial cell adhesion molecule 1 (PECAM-1, also called CD31).

This was confirmed by immunochromic studies. PECAM-1 is a member of the Ig superfamily of cell adhesion molecules. It is an integral membrane glycoprotein that is expressed on platelets, endothelial cells, and cells of the myeloid lineage such as leukocytes and basophils (reviewed in Refs. 10 and 11). PECAM-1 localizes to intercellular junctions of endothelial cells or monolayer cultured cells in which it is expressed. It functions in interendothelial cell adhesion, leukocyte-endothelial interactions, transendothelial migration, and angiogenesis (reviewed in Ref. 10). PECAM-1 can mediate both homophilic (i.e. PECAM-1 on one cell interacting with PECAM-1 on another cell) and cation-dependent heterophilic binding. The heterophilic ligands for PECAM-1 include cell surface glycosaminoglycans (12) and α,β3 integrin (13).

PECAM-1 is a single chain molecule of 130 kDa containing six extracellular Ig-like domains of the C2 subclass, one transmembrane region, and a cytoplasmic tail (14–18). The cytoplasmic domain of PECAM-1 consists of 118 amino acids that include numerous serine, threonine, and tyrosine residues that could potentially become phosphorylated. In fact, the phosphorylation on serine residues of PECAM-1 has been observed after activation of endothelial cells, platelets and T lymphocytes (19–21). This suggests that the phosphorylation state of PECAM-1 could be important in regulating its function. However, there have been no previous reports of receptor-mediated tyrosine phosphorylation of PECAM-1.

Here we report that PECAM-1 is present on RBL-2H3 cells and becomes tyrosine phosphorylated following FceRI aggregation. Mast cells and/or basophils accumulate and play a critical role at sites of inflammation. This enhanced adhesion of activated cells to the endothelium of capillaries and their migration into sites of inflammation may be regulated by the tyrosine phosphorylation of the PECAM-1 molecule.

EXPERIMENTAL PROCEDURES

Materials—Pipes, Triton X-100, Nonidet P-40, protease inhibitors, and protein A-agarose beads were from Sigma. CNBr-activated Sepharose 4B beads were from Pharmacia Biotech Inc. The materials for electrophoresis were purchased from Novex (San Diego, CA), polyvinylidene difluoride transfer membrane and Ultrafree PFL (low binding cellulose) were from Millipore (Bedford, MA), and the sources of other materials were as described previously (22).

Antibodies—The monoclonal antibody (mAb) R23 was generated from the spleen of mice immunized with multiple injections of RBL-2H3 cells emulsified in adjuvant using methods described previously (23–25). For these experiments mAb R23 was purified from ascites fluid by ammonium sulfate precipitation followed by ion exchange chromatography on DE52. By immunodiffusion it was found to be of the IgG1 isotype. For immunoprecipitation experiments it was coupled to cyanogen bromide-activated Sepharose 4B beads. For immunoblotting it was coupled to CNBr-activated Sepharose 4B beads.

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‡ The abbreviation used are: PECAM-1, platelet/endothelial cell adhesion molecule 1 (also called CD31); PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; mAb, monoclonal antibody; PBS, phosphate-buffered saline.
FIG. 1. The mAb R23 immunoprecipitates a 130-kDa protein that is tyrosine phosphorylated after FcεRI aggregation. A. Immunoblot analysis of the proteins recognized by R23. Cell lysates were prepared from RBL-2H3 cells under nonreducing or reducing conditions and analyzed by immunoblotting with mAb R23 (3 μg/ml). B, fluorescence-activated cell sorter analysis of mAb R23 binding to RBL-2H3 cells. C, cells (5 × 10⁶) were either nonstimulated (BC4−) or stimulated for 30 min with 0.03 μg/ml of anti-FcεRI and mAb BC4 (BC4+). Proteins were immunoprecipitated with mAb R23 coupled to Sepharose 4B beads. The immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine antibodies and mAb R23. The arrow indicates the protein detected with mAb R23. Molecular mass markers (in kDa) represent migration of preblabeled standards.

ogen bromide-activated Sepharose 4B. Anti-phosphotyrosine monoclonal antibody PY-20 was from ICN Immunobiologicals (Lisle, IL). Mouse monoclonal anti-FcεRI (mAb BC4) and anti-trinitrophenol-specific IgE have been described previously (22, 26, 27). Polyclonal rabbit anti-human PECAM-1 and anti-rat PECAM-1 were kindly provided by Dr. Kim Piotrowski (Blood Center of Southeastern Wisconsin, Milwaukee, WI). Polyclonal rabbit anti-mouse PECAM-1 was generously provided by Dr. Beat A. Imhof (Basel Institute for Immunology, Basel, Switzerland). All other antibodies have been described previously (24, 25).

Cell Culture, Stimulation, and Microscopy—The RBL-2H3 cells were maintained as monolayer cultures in Eagle’s minimum essential medium supplemented with 15% heat-inactivated fetal calf serum, penicillin, streptomycin, and amphotericin (28). The Syk-deficient TB1A2 variant cells and the 3A5 cells that have stably transfected Syk have been described previously (29). Cells were activated with antigen (di-nitrophenol-coupled human serum albumin), anti-FcεRI antibodies (mAb BC4), calcium ionophore A23187 (0.5 μM), or phorbol 12-myristate 13-acetate (40 μM) as described previously (4). Briefly, 10⁶ cells were seeded in Petri plates (10-cm diameter), and after overnight culture, the cell monolayers were washed once with 3 ml of Eagle’s minimum essential medium containing 0.1% bovine serum albumin and 10 mM Tris, pH 7.5. The cells were then stimulated in the same medium. For stimulation with antigen, the cells were cultured overnight with antigen-specific IgE. After stimulation for the indicated times, the medium was removed for histamine analysis. In experiments to deplete extracellular Ca²⁺, the monolayers were washed with calcium-free Eagle’s minimal essential medium containing 10 μM EDTA and stimulated in this medium.

For fluorescent microscopy, cells were cultured on coverslips and then stimulated as described above. After rinsing with PBS, cells were fixed with 2% paraformaldehyde (EM grade, Electron Microscopy Sciences) for 10 min. The coverslips were rinsed with PBS, incubated with 0.1 M glycine, and then permeabilized at −20 °C for 6 min with cold methanol. After rinsing with PBS and blocking with 0.5% bovine serum albumin/PBS, the cells were incubated with rabbit anti-PECAM-1 (10 μg/ml) for 1 h at room temperature. The secondary antibody was fluorescein isothiocyanate–Fab’₂ donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories). The coverslips were mounted onto microscope slides and viewed under a Leica 4D TCS confocal microscope.

Immunoprecipitation—After stimulation for the indicated times, the monolayers were rinsed once with 12 ml of ice-cold PBS containing 1 mM Na₃VO₄ and solubilized in ice-cold lysis buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 100 mM NaCl, 50 mM NaF, 1 mM Na₂VO₄, 2 mM phenylmethylsulfonyl fluoride, 21 μg/ml aprotinin). After incubating on ice for 30 min, the cells were scraped off the plates, and the lysates were centrifuged for 30 min at 16,000 g at 4 °C. The post-nuclear supernatants were preclarified by incubation for 1 h at 4 °C with Sepharose 4B and then immunoprecipitated with antibodies coupled to the same beads. After rotation at 4 °C for 90 min, the beads were washed five times with ice-cold lysis buffer, and the proteins were eluted by boiling for 5 min with sample buffer as described previously (30).

Immunoblotting—Cell lysates and immunoprecipitated proteins were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Immobilon P). The membranes were blocked for a minimum of 4 h with 4% protease-free bovine serum albumin in blotting buffer (10 mM Tris pH 7.4, 0.9% NaCl, 0.05% Tween 20) and probed with 40 ng/ml anti-phosphotyrosine mAb PY-20 conjugated to horseradish peroxidase. For immunoblotting with mAb R23, proteins were separated under nonreducing conditions, and the secondary antibody was horseradish peroxidase-conjugated donkey anti-mouse IgG, whereas for immunoblotting with the polyclonal anti-PECAM-1, proteins were separated under reducing conditions, and the secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG. In some experiments antibodies were stripped from the membranes, and the membranes were reprobed with other antibodies as recommended by the manufacturer. In all blots, proteins were visualized by enhanced chemiluminescence (ECL Kit, Amersham Corp.) as described previously (5).

Adhesion Studies—Flat bottom Immunolon-2 assay wells (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 30 μg/ml of fibronectin or 30 μg/ml of mAb R23 by incubating at 37 °C overnight. Wells were washed and then blocked with PBS containing 4% bovine serum albumin at 37 °C. After 3 h, the wells were washed three times with Pipes buffer (25 mM Pipes, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM CaCl₂, and 0.01% bovine serum albumin, pH 7.4). RBL-2H3 cells grown overnight were trypsinized from flasks, allowed to recover by incubation at 37 °C in culture medium for 30 min. The cells were then washed three times with the Pipes buffer and suspended in Pipes buffer at 2 × 10⁶ cells/ml. 40 μl of this preparation was added to the wells and incubated at 37 °C for 45 min. Then 20 μl of mAb BC4 solution (0.09 μg/ml) was added, and the cells were incubated at 37 °C for another 45 min. The cells were solubilized by adding 60 μl of 2% lysis buffer.

Large Scale Isolation of Proteins for Amino Acid Sequencing—The RBL-2H3 cells were grown as tumors in newborn rats as described previously (28), and single cells were isolated (31). Lysates prepared from 20 × 10⁶ cells were affinity purified with 12 mg of mAb R23 coupled to beads and eluted with 0.5% SDS, and the sample was concentrated to 150 μl. The purified proteins were separated on 8% SDS-PAGE and electrophoretically transferred to membranes. The major band stained with Ponceau S was excised and subjected to N-terminal amino acid sequencing on a model 494 protein sequencer.
RESULTS

Characterization of mAb R23—To investigate the role of tyrosine phosphorylated proteins in FceRI-mediated signaling, different monoclonal antibodies raised against rat basophilic leukemia RBL-2H3 cells were tested to determine whether they immunoprecipitated tyrosine phosphorylated proteins. One of these antibodies, mAb R23, immunoprecipitated a 130-kDa protein that was weakly tyrosine phosphorylated in non-stimulated cells but whose phosphorylation was dramatically enhanced after FceRI aggregation (Fig. 1, A and C). By immunoblotting, mAb R23 identified a 130-kDa protein in RBL-2H3 cells only under nonreducing conditions, suggesting that binding of this antibody depends on the secondary structure of the molecule (Fig. 1A). Immunofluorescence and fluorescence-activated cell sorter analysis demonstrated binding of mAb R23 to intact cells, suggesting that the antibody recognized the extracellular domain of a transmembrane protein (Fig. 1B). These experiments suggested that this 130-kDa protein was tyrosine phosphorylated after FceRI aggregation.

Although the relative migrations of the tyrosine phosphorylated protein and the protein recognized by immunoblotting were similar, it was still possible that these were two different proteins of similar size. To directly demonstrate that the membrane protein was tyrosine phosphorylated, proteins were immunoprecipitated from lysates of cells that had first been stimulated by FceRI aggregation and then surface labeled by biotinylation. By both one- and two-dimensional analysis the tyrosine phosphorylated and the surface-labeled proteins were identical (data not shown). Therefore, mAb R23 binds to the extracellular domain of a 130-kDa membrane protein that is tyrosine phosphorylated after receptor aggregation.

Identification of Protein Recognized by mAb R23 as PECAM-1

SRPQHQVLFY p110–120

SRSQHRVLFY Mouse PECAM-1 (40–49)

VKPQHYMLFY Human PECAM-1 (40–49)

**Fig. 2.** N-terminal amino acid sequence analysis of the purified protein and comparison with the sequence of human and mouse PECAM-1 (CD31).
cellular tyrosine phosphorylation (data not shown). There was also no change in the tyrosine phosphorylation of PECAM-1 itself. Similarly, although RBL-2H3 cells attached to surfaces coated with mAb R23, this did not induce tyrosine phosphorylation of PECAM-1.

Some proteins are tyrosine phosphorylated early after FcεRI aggregation, whereas others are phosphorylated only after influx of extracellular Ca\(^{2+}\) and/or activation of protein kinase C (7, 9). Stimulation of cells to degranulate with either IgE and antigen or the calcium ionophore A23187 resulted in a slight increase in PECAM-1 tyrosine phosphorylation (Fig. 5). However, there was no increase in PECAM-1 tyrosine phosphorylation after direct activation of protein kinase C by the addition of phorbol 12-myristate 13-acetate. To further define the role of Ca\(^{2+}\) in FcεRI-mediated tyrosine phosphorylation of PECAM-1, cells were stimulated in the presence or the absence of extracellular Ca\(^{2+}\) (Fig. 6). The absence of extracellular Ca\(^{2+}\) did not affect FcεRI-mediated tyrosine phosphorylation of PECAM-1. Therefore, unlike other adhesion related molecules such as pp125\(^{FAK}\), the tyrosine phosphorylation of PECAM-1 is an early event that is upstream of the influx of calcium and/or the activation of protein kinase C. The adherence of RBL-2H3 cells to extracellular matrix proteins, mediated at least in part by integrins, regulates the FcεRI-induced tyrosine phosphorylation of the focal adhesion kinase, pp125\(^{FAK}\) (32, 33). Thus, cell stimulation results in minimal if any tyrosine phosphorylation of pp125\(^{FAK}\), unless the RBL-2H3 are adherent (32). However, the FcεRI-mediated tyrosine phosphorylation of PECAM-1 was equally strong in nonadherent and adherent cells (Fig. 7). Thus, unlike pp125\(^{FAK}\), the FcεRI-mediated tyrosine phosphorylation of PECAM-1 is independent of cell adhesion. Activation of mast cells also results in enhanced adherence (33). The increased tyrosine phosphorylation of PECAM-1 in stimulated cells could therefore mediate the enhanced adherence of activated mast cells. Both nonactivated and activated RBL-2H3 cells adhered to surfaces coated with mAb R23 equally well but did not adhere to purified recombinant PECAM-1 (data not shown). Therefore, adherence as measured by these gross parameters of adherence through binding to PECAM-1 was not significantly modified after FcεRI aggregation.

FcεRI aggregation induced a redistribution of PECAM-1 in cells (Fig. 8). As expected, in nonstimulated cells PECAM-1 was membrane-associated. After 20 min of stimulation it had redistributed to the ruffles near the apical surface of the cells. There was also an increase in punctate staining just below the mem-
brane at the apical surface. Therefore, FcεRI aggregation results in tyrosine phosphorylation of PECAM-1 and in its redistribution on the cell surface.

Cells deficient in Syk protein tyrosine kinase were used to further define at what point in the activation cascade the PECAM-1 is tyrosine phosphorylated (Fig. 9). FcεRI aggregation with anti-receptor antibodies induced minimal tyrosine phosphorylation of PECAM-1 in Syk-deficient cells. This was increased in the cells that had been reconstituted by the stable transfection of Syk. Therefore, although some tyrosine phosphorylation of PECAM-1 occurs upstream or independent of Syk, it is predominantly dependent on the presence of Syk in the cells. However, this does not necessarily mean that Syk tyrosine phosphorylates PECAM-1.

**DISCUSSION**

Using a monoclonal antibody to RBL-2H3 cells we identified the adhesion molecule PECAM-1 as one of the substrates that is tyrosine phosphorylated after FcεRI aggregation. The phosphorylation of PECAM-1 was an early event after receptor activation and was not seen when cells were stimulated with phorbol myristate acetate and did not require cell adherence. The FcεRI-induced tyrosine phosphorylation of PECAM-1 was much stronger and persistent with anti-receptor antibodies than with IgE antigen. However, there was still variation in the extent of PECAM-1 tyrosine phosphorylation with different anti-FcεRI antibodies (data not shown). The varying efficiency of different FcεRI aggregation signals in inducing tyrosine phosphorylation of PECAM-1 may be due to differences in the way these signals orient and aggregate the receptor (34).

The present model for signaling by FcεRI suggests the cooperation between Lyn and Syk protein tyrosine kinases (29). Receptor aggregation induces activation of a protein tyrosine kinase, probably Lyn, which results in tyrosine phosphorylation of the receptor subunits. Syk is then recruited by the tyrosine phosphorylated receptor subunits; its activation then propagates downstream signals including the tyrosine phosphorylation of phospholipase C-γ and the rise in intracellular calcium. Tyrosine phosphorylation of PECAM-1 in this cascade of events was partly independent of Syk, although it was enhanced by the presence of Syk. Tyrosine phosphorylation was also independent of the presence of calcium in the medium. Although FcεRI, Lyn, and Syk are all either membrane proteins or associated with the membrane at different stages of receptor activation, we could not detect any association by immunoprecipitation of these molecules with PECAM-1, another membrane protein (data not shown). Moreover, there was no kinase activity in the PECAM-1 immunoprecipitates. Nevertheless, tyrosine phosphorylation of PECAM-1 may be due to Lyn, Syk, or other tyrosine kinases activated by receptor aggregation.
PECAM-1 may also be involved in regulating the function of other cellular proteins. For example, aggregation of PECAM-1 regulates the adhesive properties of β1 and β2 integrins on neutrophils, monocytes, and T cells (50–52). The interaction of endothelial PECAM-1 with leukocyte or basophil PECAM-1 could result in up-regulation of the activity of integrins, which may provide the interaction necessary for transmigration through the endothelial cell junctions. Similarly, tyrosine phosphorylation of PECAM-1 could also influence its capacity to regulate the adhesive activity of integrins.

In summary, we have found that PECAM-1 is present on RBL-2H3 cells and is tyrosine phosphorylated after receptor aggregation. Such modification of the molecule may be important for its physiological function. This is supported by evidence that the cytoplasmic domain of PECAM-1 plays an important role. First, transfectants of PECAM-1 lacking the cytoplasmic domain are defective in aggregation (42). Second, alternatively spliced forms of PECAM-1 lacking the potential tyrosine phosphorylation site exhibit different aggregation properties (53). Third, phosphorylation of PECAM-1 on serines regulates its down-regulation (19). Therefore, tyrosine phosphorylation of PECAM-1 may be crucial not only for the transmigration of basophils into inflammatory sites but also for the regulation of degranulation.

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