The cDNA encoding the rat equivalent of the human hematopoietic tyrosine phosphatase, also known as leukocyte phosphatase, was isolated from a rat basophilic leukemia mast cell cDNA library. By two-dimensional electrophoresis, the protein expressed in the mast cells was of a size (40 kDa) and pl (6.9) predicted from the deduced amino acid sequence. Thus, although previously shown to be preferentially expressed in T cells and B cells, the phosphatase is also found in mast cells. By immunofluorescence microscopy, rat hematopoietic tyrosine phosphatase localized to discrete, globular compartments within the cytoplasm and was not found either in the nucleus or associated with the cell surface membrane. Aggregation of high affinity IgE receptors in the mast cells induced tyrosine phosphorylation of the phosphatase. The tyrosine phosphorylation was mimicked by stimulation with calcium ionophore A23187 but not by direct activation of protein kinase C. Since phosphorylation of the phosphatase was dramatically reduced when the cells were activated in Ca²⁺-free media, it is dependent on a rise in intracellular Ca²⁺. These data strongly suggest that hematopoietic tyrosine phosphatase may be involved in the IgE receptor-mediated signaling cascade.

Mast cells and basophils play a central role in allergic and inflammatory reactions. They express high affinity IgE receptors (FcεRI) on their cell surfaces that, when aggregated, initiate biochemical events that lead to the release of inflammatory mediators. In the rat basophilic leukemia (RBL-2H3) mast cell line, aggregation of FcεRI induces activation of phospholipases A₂, C, and D, an increase in intracellular Ca²⁺ concentration, and activation and translocation of protein kinase C from the cytosol to the plasma membrane (1–5). In addition, numerous proteins become tyrosine phosphorylated upon aggregation of the FcεRI receptor (6–21).

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U28356. To whom correspondence should be addressed: Laboratory of Immunology, NIDR, National Institutes of Health, Bethesda, Maryland 20892. Tel.: 301-496-5105; Fax: 301-480-8328.

The abbreviations used are: FcεRI, high affinity IgE receptor; PCR, polymerase chain reaction; PTP, protein-tyrosine phosphatase(s); LC-PTP, leukocyte protein-tyrosine phosphatase; HePTP, hematopoietic tyrosine phosphatase; PAGE, polyacrylamide gel electrophoresis; RBL-2H3, rat basophilic leukemia 2H3 cells.

Regulation of the level of tyrosine phosphorylation of proteins through dephosphorylation is an important aspect of the signal transduction process. For example, the protein-tyrosine phosphatase (PTP) CD45 is essential for signaling from receptors on T cells, B cells, and mast cells (22–26). However, we found that CD45 is absent from several RBL-2H3 cell variants that have normal signaling through their FcεRI. Therefore, we used a molecular cloning approach to identify other PTPs that may be involved. The rat equivalent of human hematopoietic tyrosine phosphatase (HePTP), also known as leukocyte protein-tyrosine phosphatase (LC-PTP; Refs. 27 and 28) was isolated from an RBL-2H3 cell cDNA library. HePTP mRNA was present only in RBL-2H3 mast cells, the YAC-1 T cell line, and the thymus in Northern blots. Moreover, the protein became tyrosine phosphorylated upon aggregation of the FcεRI in RBL-2H3 cells. This phosphorylation was Ca²⁺-dependent and accordingly would be considered a “late” event in the activation process. Thus, HePTP may be involved in the signaling cascade initiated by IgE receptor aggregation.

EXPERIMENTAL PROCEDURES

Molecular Cloning—Two degenerate primers were designed based on common sequences in the catalytic domains of several PTP (His/Asp)-Phe-Trp-(Arg/Lys)-Leu-Glu-Met-(Val/Cys)-Ile-Trp-(Glu/Gly) to Val-His-Cys-Ser-Ala-Gly-(Val/Ile)-Gly (29). Forward 5′-GC GAA TTA TTC CCI A(T/C)I CCI GC(G/A) CT(G/A) TGI AC-3′ and reverse 5′-GC GAA TTA TTC CCI A(T/C)I CCI GC(G/A) CT(G/A) TGI AC-3′ digonucleotide primers were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer. RBL-2H3 cell cDNA, prepared as described previously (30), served as template in PCR reactions. The PCR products were subcloned into the EcoRI site of pBluescript SK (Stratagene, La Jolla, CA), and the sequences of both strands were determined by automated sequence analysis (Applied Biosystems Inc., Foster City, CA). The HePTP PCR product was used to screen a previously characterized RBL-2H3 cell cDNA.xgt11 library (30). Positive plaques were purified, their insert were subcloned in pBluescript SK, and the sequence of both strands was determined by automated sequencing.

The 5′-end was generated using the 5′ rapid amplification of cDNA ends of Life Technologies, Inc. Poly(A)⁺ RNA from RBL-2H3 cells and the PTP gene-specific primer 5′-TAGATCCAGCGTGTA-3′ corresponding to nucleotides 367–352 in the rat PTP were used. The rapid amplification of cDNA ends products were subcloned into pBluescript SK and sequenced on both strands by automated sequence analysis.

Antibody Preparation—A rat PTP-specific peptide comprising amino acids 121–134 (SKDRYKTILPNPQS) with a cysteine residue at the carboxyl end was synthesized by Chiron Mimotopes (Emeryville, CA). Antibodies were generated in rabbits as described previously (13). Some affinity-purified anti-PTP antibodies were biotinylated using NHS-LC-biotin (Pierce) according to the manufacturer’s recommendations.

Two-dimensional Gel Electrophoresis—Whole cell lysates from both
amplification of cDNA ends technique.

**RESULTS**

Isolation and Analysis of Rat HePTP cDNA—Part of the catalytic domain of rat HePTP was amplified from RBL-2H3 cell cDNA using reverse transcriptase-based PCR and degenerate oligonucleotide primers. The amplified product was used as described previously (31). For activation, 150-mm diameter Petri dishes were seeded with 12.2 × 10^5 cells. After overnight culture, the cells were washed twice with 30 ml of phosphate-buffered saline at room temperature. The cells were then incubated at 37°C for 10 min unless otherwise indicated in 10 ml of Eagle’s minimum essential medium with Earle’s salts containing 0.1% bovine serum albumin, 10 mM Tris (pH 7.4), and 0.05% anti-FcRl monodonal antibody BC4; 0.5 mM calcium ionophore A23187; or 40 mM phorbol 12-myristate 13-acetate. The supernatants were removed and assayed for histamine by automated analysis (32).

**Immunoprecipitations—** After activation, the monolayers were washed once with 10 ml of ice-cold phosphate-buffered saline containing protease inhibitors (concentrations as in lysis buffer), solubilized in 1 ml of lysis buffer (3% Brij 96, 20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 90 milliunits/ml aprotinin, 1 μg/ml soybean trypsin inhibitor, 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml pepstatin). Postnuclear lysates were precleared 1 h at 4°C with protein A-Sepharose beads. The beads were washed 7 times with ice-cold lysis buffer, and proteins were eluted by boiling 5 min in SDS-PAGE sample buffer. Brij 96, protease inhibitors, and Protein A-Sepharose agarose were then stripped of antibodies according to the protocol of the American Chemical Society (33). Blots were probed with 100 ng/ml biotinylated anti-rat HePTP followed by 10 ng/ml horseradish peroxidase-conjugated streptavidin (Pierce); 0.5 mM calcium ionophore A23187; or 40 mM phorbol 12-myristate 13-acetate. The supernatants were removed and assayed for histamine by automated analysis (32).

**Immunoblotting—** Whole cell lysates and immunoprecipitated proteins were separated by SDS-PAGE using 4%–20% linear gradient gels (Novex) under reducing conditions. Separated proteins were electrophoresed to nitrocellulose membranes and blocked by overnight incubation in 4% bovine serum albumin serum albumin (5%)/Tween 20 (pH 7.4). For detection of tyrosine-phosphorylated proteins, membranes were probed with 40 ng/ml anti-phosphotyrosine mAb PY-20 (Pierce) coupled to horseradish peroxidase (I.CN). Signals were detected by chemiluminescence using a Renaissance kit (DuPont NEN) and Kodak X-Omat radiographic film (Eastman Kodak Co.). Blots were stripped of antibodies according to the protocol of the American ECL kit and reprobed for PTP with 100 ng/ml biotinylated anti-rat HePTP IgG followed by 10 ng/ml horseradish peroxidase-conjugated streptavidin (Pierce).

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Tyrosine Phosphorylation of HePTP

Characteristics of the Tyrosine Phosphorylation of HePTP—Some proteins are phosphorylated on tyrosine very early after IgE receptor aggregation, and others are phosphorylated only after a rise in intracellular Ca\(^{2+}\) and/or after activation of protein kinase C (9, 33, 34). Experiments therefore examined the relationship between tyrosine phosphorylation of the HePTP, Ca\(^{2+}\) influx, and activation of protein kinase C. Stimulation with the Ca\(^{2+}\) ionophore A23187 was as effective as Fc\(\gamma R\) cross-linking in inducing tyrosine phosphorylation of HePTP (Fig. 6). In contrast, direct activation of protein kinase C by the addition of phorbol 12-myristate 13-acetate failed to do so (Fig. 6). Therefore, an increase in intracellular calcium but not PKC activation can directly stimulate tyrosine phosphorylation of HePTP.

To more fully explore the role of extracellular calcium in the Fc\(\gamma R\)- and ionophore-induced tyrosine phosphorylation of HePTP, RBL-2H3 cells were first rinsed and then activated in Ca\(^{2+}\)-free media containing EDTA (Fig. 7, lanes 4–6). In the absence of extracellular Ca\(^{2+}\), tyrosine phosphorylation of HePTP was substantially diminished when compared with controls (lanes 6 and 3). However, because some tyrosine-phosphorylated HePTP was still detected after receptor aggregation or after ionophore stimulation in Ca\(^{2+}\)-free media (lanes 5 and 6), the small rise in intracellular Ca\(^{2+}\) due to release from intracellular compartments probably contributed the requisite divalent ions. Identical results were obtained when the concentration of EDTA in the wash and incubation media was 40 \(\mu\)M or 4 mM. Thus, Fc\(\gamma R\)-mediated tyrosine phosphorylation of HePTP is a Ca\(^{2+}\)-dependent process and is one of the late signaling events.

**DISCUSSION**

The cDNA we isolated is the rat equivalent of the human PTP HePTP or LC-PTP (27, 28). While the two human sequences are nearly identical, they do differ in one major respect: the location of the presumptive translation initiation
functions in a receptor-dependent manner in each of these cell types. The cytochemical distribution of rat HePTP is intriguing; it localizes to globular or elongated cytoplasmic elements. This suggests that the enzyme is compartmentalized to an organelle or to some subcellular specializations. We speculate that the NH₂-terminal noncatalytic domain of HePTP may play a role in targeting the enzyme to intracellular locales. PTP1B (36) and DPTP61F (37) are nontransmembrane phosphatases that contain carboxyl-terminal sequences involved in directing these proteins to the endoplasmic reticulum. PTP-MEG1 (38), PTPH1 (39), and PTPD1 (40) are other cytosolic PTP that contain amino-terminal sequences with homology to proteins that associate with the cytoskeleton. This has lead some to conjecture that these or other such PTP may be involved in focal adhesions (41). Although the amino terminus of HePTP does not share homology with cytoskeleton-associated proteins, we nonetheless examined adherent RBL-2H3 cells for colocalization of the enzyme to sites of cellular attachment to the substratum. By laser confocal microscopy, HePTP did not accumulate along the basal (adherent) surface of RBL-2H3 cells. Thus, it is unlikely that HePTP is associated with focal adhesion sites in these cells.

The signaling process initiated by FcαRI aggregation involves tyrosine phosphorylation of several proteins. Here we report that FcαRI aggregation induces tyrosine phosphorylation of the cytosolic protein-tyrosine phosphatase, HePTP. The results suggest that the FcαRI-induced tyrosine phosphorylation of HePTP is dependent on an elevation in the intracellular Ca²⁺ concentration. First, cells activated through the IgE receptors in media lacking Ca²⁺ showed a dramatic diminution in the level of HePTP tyrosine phosphorylation. The residual low level of HePTP phosphorylation seen under these conditions may be attributed to the relatively small amount of calcium stored within intracellular compartments and released upon cell stimulation (42). Second, triggering of the cells with Ca²⁺ ionophore in either calcium containing or calcium-free media mimicked the results obtained by aggregating the FcαRI.

4 M. Swietek, E. H. Berenstein, W. D. Swaim, and R. P. Siraganian, unpublished results.
Thus, elevated intracellular Ca\(^{2+}\) concentrations are needed for the tyrosine phosphorylation of HePTP.

The rise in intracellular Ca\(^{2+}\) concentration that results from receptor engagement in many cell types is accompanied by activation of protein kinase C (42). Optimal tyrosine phosphorylation of some mast cell proteins, such as the focal adhesion kinase (p125 \(F{\kappa}k\)) and the cytoskeletal protein Paxillin, require both protein kinase C activation and influx of extracellular calcium (16, 18). However, in the present experiments, direct activation of protein kinase C with 40 nM phorbol 12-myristate 13-acetate failed to elicit HePTP phosphorylation. Thus, tyrosine phosphorylation of HePTP can occur independent of protein kinase C activation.

The requirement for calcium mobilization in the tyrosine phosphorylation of HePTP indicates that it occurs late in the signaling cascade (43). That is, it is preceded by other events including tyrosine phosphorylation and activation of phospholipase C\(\gamma\)1, Lyn, and Syk. The time course experiments showing that HePTP became tyrosine phosphorylated between 1 and 5 min after Fc\(\varepsilon\)RI aggregation also bear this out.

The SH2 domain-containing protein-tyrosine phosphatase, Syk (also referred to as PTP1-D or SH-PTP2), was shown to be tyrosine phosphorylated in response to epidermal growth factor and platelet-derived growth factor receptor activation (44, 45). It was also constitutively tyrosine-phosphorylated in cells transformed with v-Src (44), suggesting that the Src family of kinases may be involved in phosphorylating the PTP. Lyn is a Src family tyrosine kinase found in abundance in RBL-2H3 cells; it coimmunoprecipitates with the Fc\(\varepsilon\) kinases may be involved in phosphorylating the PTP. Lyn is a transformed with v-Src (44), suggesting that the Src family of tyrosine phosphorylated in response to epidermal growth factor receptor activation (44, 45). That is, it is preceded by other events including tyrosine phosphorylation and activation of phospholipase C\(\gamma\)1, Lyn, and Syk. The time course experiments showing that HePTP became tyrosine phosphorylated between 1 and 5 min after Fc\(\varepsilon\)RI aggregation also bear this out.

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