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Characterization of Protein-tyrosine Phosphatases That Dephosphorylate the High Affinity IgE Receptor*

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An early event that follows aggregation of the high affinity receptor for IgE (FcεRI) is the phosphorylation of protein tyrosines, especially those on the β- and γ-subunits of the receptor. Disaggregation of the receptors leads to their rapid dephosphorylation, but even stably aggregated receptors undergo continual rounds of phosphorylation and dephosphorylation. We developed assays to study dephosphorylation of the receptors and other cellular proteins. Whole cell extracts dephosphorylated both subunits of the receptors rapidly and were as active against aggregated as against disaggregated FcεRI. Upon disaggregation, the *in vivo* dephosphorylation of the FcεRI and several other proteins followed first-order kinetics with closely similar rate constants despite substantial differences in the extent of phosphorylation. These results suggest that the level of phosphorylation of FcεRI is largely controlled by the aggregation-induced action of kinase(s) and not from changes in susceptibility to or activity of the phosphatases. Much of the total phosphatase is lost when the cells are permeabilized, but the rate of dephosphorylation of disaggregated FcεRI was comparable in intact and permeabilized cells. Thus, much of the activity utilized by the cell to dephosphorylate the FcεRI is likely to be associated with the plasma membrane.

The high affinity IgE receptor on mast cells and basophils (FcεRI) has a central role in mediating allergic responses (1, 2). Aggregation of the receptors results in phosphorylation of protein tyrosines as an early event that leads to a variety of later cellular phenomena (3, 4). The receptor itself lacks sequences typical for intrinsic protein-tyrosine kinase activity (5), and experimentally, receptors purified by affinity columns or well washed immunoprecipitates show virtually no kinase activity *in vitro* (6, 7). However, a variety of studies have implicated a kinase weakly associated with the receptor (6, 8, 9). In rats, the critical initial kinase appears to be Lyn (9). The receptor-associated Lyn from resting cells displays tyrosine kinase activity toward exogenous substrates, but little or no phosphorylation of the receptor itself is observed unless the receptors are aggregated (10–13). These observations and those made on the effect of inhibitors of phosphatases (12, 14) imply that protein-tyrosine phosphatases (PTPs)[1] are continuously modulating the resting system. The studies with inhibitors and other experimental approaches (15) show that the PTPs also continuously act on aggregated receptors. Finally, when individual receptors dissociate from the aggregate, *e.g.* by addition of monomeric hapten after stimulation by multivalent antigen, rapid dephosphorylation of the receptor and of other cellular proteins is observed (7, 10–13).

The PTPs involved in these processes remain undefined, and the purpose of the current study was to investigate some of their characteristics. We first developed an *in vitro* assay to test the PTP activity in total cell lysate toward receptors that had been phosphorylated in response to aggregation. This assay was also used to localize candidate PTP to subcellular fractions and to compare the susceptibility of aggregated *versus* disaggregated receptors. Finally, we examined the kinetics of dephosphorylation for FcεRI and other cellular proteins *in vivo* to gain insights about the underlying regulation.

EXPERIMENTAL PROCEDURES

Reagents—Preparation of the monoclonal anti-DNP murine IgE from the hybridoma H1 DNP-ε-26.82 (16) of the monoclonal antibody against the β-subunit of FcεRI (17) and of dinitrophenylated bovine serum albumin (DNP_{25}-BSA, 25 mol of DNP/mol of protein) have been described (17–19). ^{125}I-IgE was prepared by the chloramine-T method (20). DNP-caproate and the assay kit for lactic dehydrogenase (used to assess the efficiency of permeabilization) were from Sigma, an IgG fraction of goat anti-mouse IgE was from ICN Biochemicals (Costa Mesa, CA), the permeabilizing reagent streptolysin O was from either Life Technologies, Inc. (Gaithersburg, MD) or Murex Diagnostics (Dartford, UK), prestained molecular weight markers used in SDS-PAGE were from Novex (San Diego, CA), anti-SHP-1 was from Upstate Biotechnology, Inc. (Lake Placid, NY), anti-SHP-2 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), anti-phosphotyrosine was from Transduction Laboratories Inc. (Lexington, KY), and recombinant PTP, YOP34 (21), and the PTP assay kit against a standard tyrosine-phosphorylated peptide (22) were from Boehringer Mannheim.

Permeabilization and Activation of Cells—The 2H3 subline of rat basophilic leukemia (RBL) cells was grown adherent in stationary culture (23) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were harvested after exposure to 0.05% trypsin, 0.02% EDTA in Hanks' buffered salt solution (Biofluids). For permeabilizing sensitized adherent cells, 12 well plates (Costar Corp., Cambridge, MA) were seeded with 6×10^5 cells in the presence of 0.6 μg/ml IgE. After overnight culture, the cell monolayers were washed three times with phosphate-buffered saline (without Mg^{2+} or Ca^{2+}) and then incubated for 30 min at 37 °C in the presence of 300 units/ml streptolysin O (Life Technologies, Inc.). Cells were washed twice with phosphate-buffered saline, each time allowing the wash buffer to incubate for at least 1 min before removal. The efficiency of permeabilization was assessed by following the depletion of cytosolic proteins as described under "Results." Cells were activated by adding 1 μg/ml DNP_{25}-BSA in assay buffer (25 mM K^+ PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 mM ATP) at 37 °C.

Cells in suspension were sensitized with IgE, washed, and permeabilized at 1.25×10^7 cells/ml in assay buffer containing 1 unit/ml streptolysin O (Murex). The mixture was incubated for 3 min at 37 °C

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; DNP, 2,4-dinitrophenyl; DNP_{25}-BSA, dinitrophenylated bovine serum albumin; RBL, rat basophilic leukemia; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; ITAM, immune recognition tyrosine activation motif.

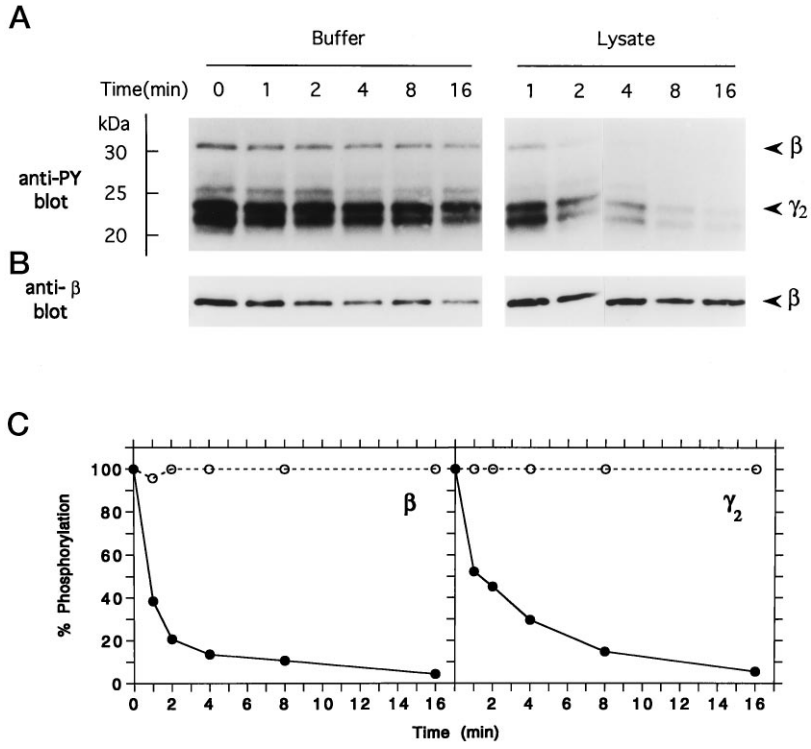

FIG. 1. **Dephosphorylation of β- and γ_2-subunits of FcεRI *in vitro*.** FcεRI were immunoprecipitated with anti-IgE from a detergent extract of 8.3×10^5 activated cells. The precipitates were washed and incubated with either buffer or total cell lysate from 2.1×10^5 cells at 30 °C. At the indicated times (see "Experimental Procedures") the reaction was quenched, the precipitates were washed, and the components were separated by SDS-PAGE and then analyzed by immunoblotting. *Panel A,* autophotograph after blotting with anti-phosphotyrosine antibody. The positions of the subunits β and γ_2 are shown on the *right*. *Panel B*, autophotograph after stripping the first blot and then reblotting with anti-β antibody. *Panel C*, densitometric analysis of phosphorylation levels of β (*left panel*) and γ_2 (*right panel*). The values are shown relative to those at time 0 that were taken as 100%. *Open circles*, data from specimens incubated with buffer only, corrected for recovery; *filled circles*, data from specimens incubated with lysate, corrected for recovery. The data shown are representative of two such experiments.

with gentle agitation. This protocol resulted in the permeabilization of >99% of the cells, as assessed by trypan blue uptake. Such permeabilized cells (10^7 cells/ml) were then stimulated for 2 min at 37 °C with 1 μg/ml DNP_{25}-BSA in assay buffer.

Solubilization and Immunoprecipitation—Cells were solubilized in lysis buffer containing 0.5% Triton X-100, 50 mM Tris, pH 7.6, 50 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM iodoacetate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each aprotinin, leupeptin, and pepstatin A for 30 min at 4 °C. The lysates were clarified by centrifugation (16,000 × *g* for 10 min). The supernatants were first precleared with 50 μl of protein A-Sepharose (Pharmacia Biotech Inc.), and selected proteins were reacted with appropriate antibodies for 1 h at 4 °C. The solutions were then incubated with 60 μl of 50% protein A-Sepharose overnight at 4 °C. The beads were washed three times with 500 μl of lysis buffer and extracted with hot sample buffer (25 mM Tris, pH 6.8, 2% SDS, and 10% glycerol) by boiling for 5 min. The eluted proteins were separated by SDS-PAGE on precast gels (Novex).

Receptors aggregated with a multivalent antigen solubilize more slowly than monomeric receptors and for this reason may be subject to dephosphorylation during solubilization due to slow delivery of PTP inhibitors. Therefore, except where otherwise noted, the monovalent hapten DNP-ε-NH_2 caproate was included in the solubilization buffer to disaggregate the receptors. When adherent cells were solubilized by this protocol, FcεRI but not other cellular proteins showed enhanced levels of tyrosine phosphorylation. With cells in suspension, where the solutions can be votexed to promote the action of detergent (13), addition of hapten did not further enhance the recovery of phosphotyrosine.

Immunoblotting—Analysis of protein tyrosine phosphorylation by Western blotting with anti-phosphotyrosine antibody was performed as described previously (15). Western blotting by various antibodies was carried out using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence for detection (Amersham Corp.). Autophotographs were quantitatively analyzed by computerized densitometry (Molecular Dynamics, Inc., Sunnyvale, CA). In separate experiments, we checked that under the conditions we used here, the chemiluminescence generated by the two critical antibodies, anti-β and anti-phosphotyrosine, was linearly proportional to the amount of subunit and phosphotyrosine in the precipitate (data not shown).

In Vitro PTP Assay Using Phosphorylated FcεRI as Substrate—Phosphorylated FcεRI to be used as substrate for an *in vitro* PTP assay was prepared from intact cells in suspension. Stimulated cells were solubilized, and FcεRI was immunoprecipitated. The immunoprecipitates were first washed with lysis buffer and then further with buffer containing 25 mM HEPES, pH 7.2, and 5 mM EDTA.

The amount of phosphotyrosine on the receptor subunits used in the *in vitro* PTP assay can be estimated as follows. On average, about 10% of the receptors that become phosphorylated in our routine stimulations can be precipitated by anti-phosphotyrosine (data not shown). Since we routinely use 8.3×10^5 cells that have approximately 3×10^5 receptors per cell, the minimum number of phosphotyrosines would be 0.04 pmol. The phosphorylation of the β- and γ-subunits has recently been assessed directly, and the amount of phosphotyrosine associated with the ITAMs of β:γ_2 were found to be in the ratio of ≈1:3.3 (24). Then, if all phosphorylated receptors are always fully phosphorylated, the maximal number of phosphotyrosines would be 4.3×0.04, or 0.17 pmol.

The total cell lysate for *in vitro* PTP assay was prepared by solubilizing resting cells (4.2×10^6 cells/ml) in 0.05% Triton X-100, 25 mM HEPES, pH 7.2, 100 mM NaCl, 5 mM EDTA, 10 mM glutathione, and 10 μg/ml each aprotinin, leupeptin, and pepstatin A for 30 min at 4 °C. The lysates were clarified by centrifugation (16,000 × *g* for 10 min). Two hundred μl of the supernatant was added to the washed immunoprecipitates of FcεRI and incubated at 30 °C. The reaction was quenched by 1 mM Na_3VO_4 at 4 °C. The immunoprecipitates were washed once with buffer containing 25 mM HEPES, pH 7.2, 5 mM EDTA, and 1 mM Na_3VO_4, extracted with hot sample buffer, and analyzed by SDS-PAGE.

The procedures used to fractionate cells into total membranes and cytosol have been described (12). When compared at equal cell equivalents, >96% of the β-subunit of FcεRI was recovered in the membrane pellet ($n = 6$), whereas approximately 95% of the PTPs SHP-1 and SHP-2 were in the cytosolic fraction ($n = 3$). Before the PTP assay, the fractions were adjusted to have the same buffer composition as in the lysate.

During the *in vitro* PTP assay, some dissociation of the β- and γ-subunits from the IgE-binding α-subunit was observed. Therefore, to normalize the level of phosphorylation/receptor we corrected for the amount of β. After probing with anti-phosphotyrosine antibody, the nitrocellulose membranes were stripped of bound antibody by incubation with a buffer containing 62.5 mM Tris, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 50 °C. The membranes were then washed and reprobed with anti-β antibody.

In Vitro PTP Assay Using Phosphorylated Peptide as Substrate—The preparation of substrate (corresponding to residues 53–65 in the COOH-terminal region of hirudin (22)) for PTP assay and detection of residual phosphotyrosine after the assay were performed according to the manufacturer's instruction. Fifty μl of the cell lysate, prepared as above, was added to 4.8 pmol of peptide and incubated at 30 °C.

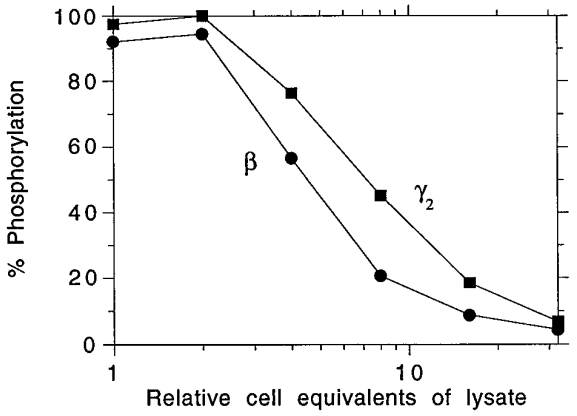

FIG. 2. **Dephosphorylation in response to varying doses of lysate.** Varied amounts of cell lysate were added to immunoprecipitated FcεRI for 2 min. The assays and analyses were as in Fig. 1. On the *abscissa*, *1* represents lysate from 2.6×10^4 cells. The data shown are representative of two such experiments.

RESULTS

Methodological Aspects—Our objective was to characterize the phosphatases that with the kinase(s) regulate the degree to which the tyrosines on the β- and γ-subunits of FcεRI are phosphorylated. We chose to examine first the phosphatase activity in total cell lysate that could dephosphorylate receptors that had been aggregated. RBL-2H3 cells sensitized with DNP-specific IgE were reacted with antigen, and the FcεRI was extracted with detergent. The receptors were precipitated by reacting the receptor-bound IgE with anti-IgE and protein A, and the precipitates were washed and subjected to PTP assays. A detergent extract of resting RBL cells was used as the source of total PTP, and aliquots were incubated with the immunoprecipitates at 30 °C. At the end of the assay, the precipitates were extracted with SDS, and the subunits were resolved electrophoretically on polyacrylamide gels. The proteins were transferred to nitrocellulose membranes, and the latter were analyzed for phosphotyrosine by Western blotting (Fig. 1*A*, *anti-PY blot*). The conditions of the assay cause some of the β- and γ-subunits of FcεRI to dissociate in unison from the IgE-bound α-subunit in the immune complex (25). Therefore, Western blotting with anti-β was used to quantitate the amount of β (and γ) remaining in order to normalize the phosphorylation data to a constant number of subunits (Fig. 1*B*, *anti-β blot*). After such correction, the data show no evidence for dephosphorylation of either the β- or γ-subunits of the receptor in the presence of assay buffer only (Fig. 1*C*, *open circles*). Therefore, the immunoprecipitates of the receptor prepared in our experiments had no detectable associated PTP activity (*cf.* Ref. 26).

In the presence of total lysate, both subunits were rapidly and completely dephosphorylated during the *in vitro* PTP assay (Fig. 1, *A* and *C*). By varying the dose of lysate and the time of incubation, we developed conditions such that the dephosphorylation of the β- and γ-subunits over a fixed time period was proportional to the amount of cell lysate added (Fig. 2). These data showed that the lysate from 2.1×10^5 RBL cells hydrolyzed approximately 50% of the phosphotyrosine on the β- and γ-subunits of the receptors derived from 8.3×10^5 cell eq/min. Thus, each cell equivalent of lysate had adequate activity in the *in vitro* assay to dephosphorylate a cell equivalent of phosphorylated FcεRI in approximately 30 s.

The whole cell lysate may well contain a variety of phosphatases with varying activities against the phosphorylated subunits of the receptor. Therefore, to assure that the assay was at least in principle capable of providing quantitative estimates of phosphatase activity in such a potentially complex system, we utilized the recombinant PTP, YOP34, and compared the results with it to those obtained with the RBL lysate against a phosphorylated peptide (22). The complex mixture and the recombinant phosphatase gave similarly proportional responses in a dose-response assay (data not shown).

TABLE I
Distribution of phosphatase activity in subcellular fractions of RBL-2H3 cells

Substrate[a]	Fraction[b]	% Dephosphorylation		Relative PTP[d]
		Absolute[c]	Relative	
β	Lysate	90	100	100
	Cytosol	56	62	42
	Crude membrane	66	73	54
	1 × washed membrane	67	74	56
	2 × washed membrane	32	36	23
γ_2	Lysate	82	100	100
	Cytosol	28	34	22
	Crude membrane	60	73	54
	1 × washed membrane	62	76	57
	2 × washed membrane	30	37	23
Peptide	Lysate	68	100	100
	Cytosol	54	79	62
	Crude membrane	37	55	35
	1 × washed membrane	22	32	21
	2 × washed membrane	ND[e]	ND	ND

[a] The phosphorylated receptors were prepared from 8.3×10^5 cell eq as described under "Experimental Procedures." The phosphorylated peptide was 4.8 pmol/sample.

[b] The subcellular fractions were prepared as described under "Experimental Procedures." In the assay using receptors, they were reacted for 4 min with 2.1×10^5 cell eq; in the assay using peptide, they were reacted for 2 min with 5.2×10^4 cell eq.

[c] The data represent the averages from two separate experiments; a total of three specimens were assayed against receptors whereas four were assayed against the phosphopeptide. S.E. of the data ranged from 2 to 11% for the β-subunits and from 4 to 15% for the γ-subunits.

[d] The relative amounts of phosphatases were calculated by the approximation that the extent of dephosphorylation was proportional to the log of cell equivalents (and thus the amount of PTP).

[e] ND, not determined.

PTPs against FcεRI Distribute Equally between Membrane and Cytosol—We were interested in determining the intracellular distribution of the PTP(s) that could dephosphorylate the receptor subunits. Unactivated RBL cells were sonicated, and a cell-free supernatant was prepared by centrifugation at low speed. This supernatant was re-centrifuged at higher speed to sediment the membranes. The latter were either washed or resuspended by sonication in lysis buffer. The various fractions derived from equivalent numbers of cells as well as unfractionated lysate were then assayed for PTP activity against immunoprecipitates of phosphorylated receptor or a tyrosine-phosphorylated peptide (Table I). Relative amounts of PTP against the receptor subunits present in each preparation were then calculated from the extent of dephosphorylation. These data showed that the PTP activity against the β- and γ-subunits was about equally distributed between cytosol and membrane fractions. However, when sedimented and resuspended in buffer twice, the washed membrane preparations retained only half of their initial PTP activity. When such preparations were assayed against the phosphorylated peptide, a somewhat greater fraction of the total PTP activity was found in the cytosolic fraction.

Effect of Preclearing Lysate with Anti-SHP-1 or Anti-SHP-2—We next determined whether the cytosolic PTP dephosphorylating FcεRI was one of two PTPs that have been implicated in signal transduction through cell surface receptors (27, 28). These are the SH2 domain-containing cytosolic enzymes, SHP-1 and SHP-2. By immunoblotting, both SHP-1 and SHP-2 were present in RBL cell lysate (data not shown; see Ref. 29). RBL lysate from resting cells was first precipitated with anti-

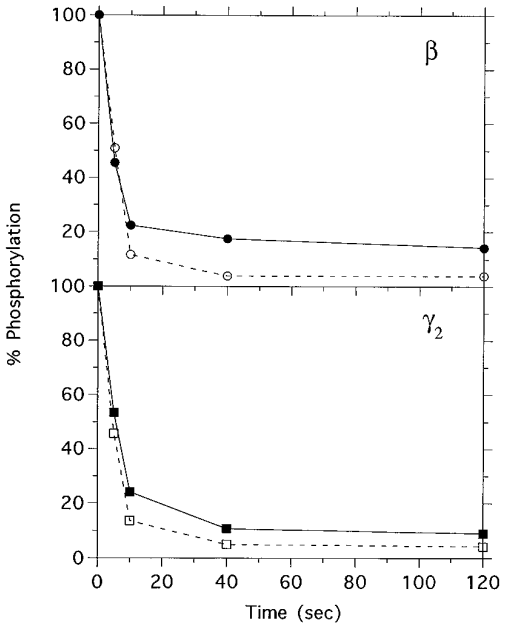

FIG. 3. **Rates of dephosphorylation of FcεRI *in vivo*.** IgE-sensitized, adherent cells were either left intact (*filled symbols*) or permeabilized (*open symbols*), washed, and incubated with 1 μg/ml DNP_{25}-BSA at 37 °C for 2 min followed by 100 μM DNP-caproate at 37 °C. At the indicated times the reactions were quenched, and the phosphorylation of the immunoprecipitated FcεRI was analyzed. *Top panel,* dephosphorylation of β; *lower panel,* dephosphorylation of γ_2. The data points are the averages of duplicate samples from two separate experiments.

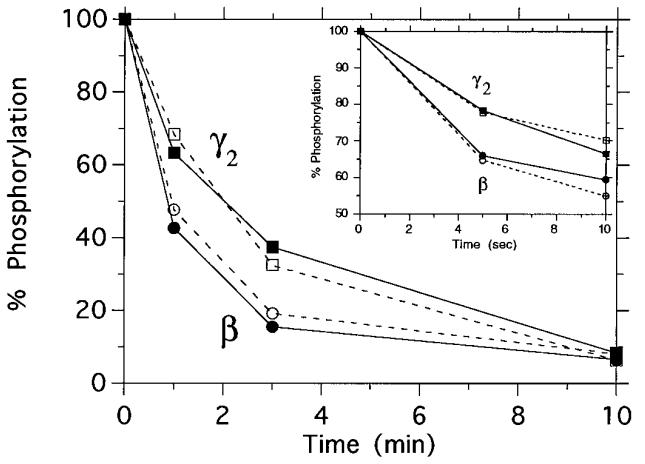

FIG. 4. **Rates of dephosphorylation of aggregated and disaggregated FcεRI *in vitro*.** IgE-sensitized cells were activated with DNP_{25}-BSA at 37 °C for 2 min. The receptors were solubilized in the presence of phosphatase inhibitors, and monovalent DNP hapten was added to one-half of the solution. The receptors were then immunoprecipitated with anti-IgE and subjected to an *in vitro* PTP assay as described in Fig. 1. *Inset,* data from a separate experiment used to collect data at early time points. *Circles,* β-subunit; *squares,* γ_2-subunit; *filled symbols,* aggregated receptors; *open symbols,* disaggregated receptors. The data points in the *inset* represent the averages of triplicate samples; those for the time points at 1, 3, and 10 min are the averages of duplicate samples from one of two such experiments. A first-order plot of the combined data (ln % phosphorylation *versus* sec) gave slopes of −0.00059 and −0.00066 for the aggregated and disaggreggated receptors. The corresponding correlation coefficients were 0.99, 0.98, 0.96, and 0.98.

SHP-1 or anti-SHP-2, and the supernatant was then allowed to dephosphorylate FcεRI *in vitro*. The antibodies removed about 99% of the respective PTP, as assessed by Western blotting (data not shown). Nevertheless, the precleared supernatants dephosphorylated both β- and γ-subunits as rapidly as the control sample (data not shown), indicating that neither SHP-1 nor SHP-2 was significantly involved in dephosphorylation of FcεRI *in vitro*.

Permeabilized Cells Retain PTP That Dephosphorylates FcεRI—The results of the fractionation showed that about equal amounts of PTP activity against the receptor subunits were present in the cytosol and the membranes. An alternative method was used to probe the same question, *i.e.* the localization of the PTP used by the cell to regulate the phosphorylation of receptor tyrosines. Sensitized adherent RBL cells permeabilized with streptolysin O were briefly stimulated with antigen, and the receptors were then disaggregated by the addition of hapten. The cells were then solubilized, and immunoprecipitates of the receptor were analyzed for residual phosphotyrosines.

Several proteins were examined to assess the loss of cytosolic components from the permeabilized cells. The average loss of lactic dehydrogenase was 88%; the corresponding losses for the two cytosolic phosphatases, SHP-1 and SHP-2, were 71 and 66%, respectively. On the other hand, the membrane-bound Lyn kinase was fully retained.

As with the intact cells, aggregating the receptors on IgE-sensitized permeabilized cells with antigen led to a dramatic increase in phosphotyrosine, whereas in the absence of antigen there was no signal above background (data not shown). After addition of hapten, the rates of dephosphorylation for the intact and permeabilized cells were indistinguishable (Fig. 3).

PTP Dephosphorylates Aggregated or Disaggregated FcεRI in Vitro Equally Well—It was of interest to compare the dephosphorylation of aggregated *versus* disaggregated receptors in the *in vitro* PTP assay. Cells were activated as before, and their receptors were solubilized (with lysis buffer that did not contain hapten (see "Experimental Procedures")). One aliquot was immunoprecipitated directly with anti-IgE; another was first reacted with hapten to disaggregate the receptors before immunoprecipitation. Fig. 4 shows that the rates of dephosphorylation of the receptor subunits from aggregated and disaggregated receptors were equivalent.

In this experiment it is of course important to verify that the aggregated receptors remained aggregated during the course of the dephosphorylation assay. Because the state of phosphorylation of only those subunits that remained associated with the immunoprecipitates was measured, effective disaggregation could only have occurred if there had been substantial dissociation of the β- and γ-subunits during the course of the assay. Use of anti-β blots verified that at the earliest points (*inset*) >70% of the β-subunit remained with both the aggregated and hapten-disaggregated receptors.

Kinetics of Phosphorylation and Dephosphorylation of FcεRI and Other Cellular Proteins in Adherent Cells—It was of interest to compare the *in vivo* dephosphorylation of the receptor with other cellular proteins after the addition of hapten to antigen-activated cells. We first examined the kinetics of phosphorylation for various cellular proteins. IgE-sensitized adherent RBL cells were stimulated with antigen, the cells were solubilized, and the whole cell lysate (as well as immunoprecipitates of the receptor) was assessed for phosphotyrosines. As shown in the *top panels* of Fig. 5, the phosphorylation of the β- and γ-subunits occurred at similar rates, reaching a maximum at 4–8 min after stimulation. These rates were about 4-fold slower than those obtained for cells in suspension (data not shown; see Refs. 12 and 26); on the latter, using the same dose of antigen, maximum phosphorylation of the receptors was generally seen by about 1–2 min after the addition of antigen, and by 4 min it declined to about 40–75% of the maximum. The phosphorylation of at least some of the major phosphotyrosine-

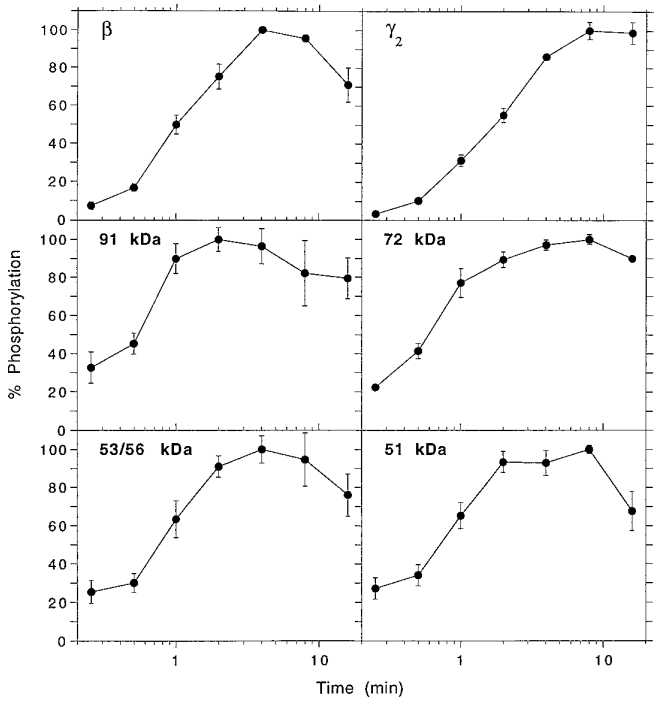

FIG. 5. **Rates of phosphorylation of β- and γ_2-subunits of FcεRI and other cellular proteins *in vivo*.** ^{125}I-IgE-sensitized adherent cells were incubated with 1 μg/ml DNP_{25}-BSA at 37 °C for the indicated times. Proteins in the total cell lysate as well as in immunoprecipitates of FcεRI were analyzed by immunoblotting with anti-phosphotyrosine antibody. The ^{125}I-IgE counts present in the sample were used to correct the densitometric values for recovery and then plotted as percentage of the maximum level of phosphorylation. (The relative phosphorylation levels at 4 min after stimulation for these proteins are indicated as *PY* in each of the *panels* in Fig. 6.) Data points represent averages of duplicate samples from four separate experiments, and the S.E. is shown.

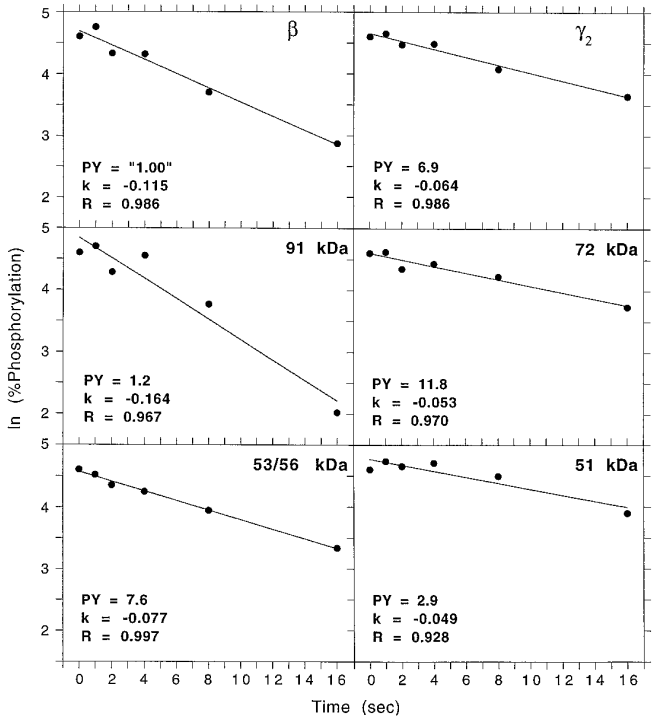

FIG. 6. **Disaggregation-induced dephosphorylation of FcεRI and other cellular proteins.** IgE-sensitized cells were incubated with 1 μg/ml DNP_{25}-BSA at 37 °C for 4 min followed by 100 μM DNP-caproate at 37 °C. At the indicated times the reactions were quenched, and the proteins in the total cell lysate as well as in immunoprecipitates of FcεRI were analyzed. Data points represent averages of duplicate samples from two separate experiments. The phosphorylation relative to the β-subunit (*PY*), the slope (*k*), and correlation coefficient (*R*) are given in each *panel*.

containing proteins appeared to reach a plateau somewhat earlier (Fig. 5, *middle panels*).

We then investigated the kinetics of hapten-induced dephosphorylation of these proteins. As shown previously, disaggregation of FcεRI induced rapid and complete dephosphorylation of the receptor subunits (10–12). The principal other phosphotyrosine-containing proteins were also rapidly dephosphorylated. In each case, the rate of dephosphorylation was consistent with first-order kinetics (Fig. 6). The rate constant, *k*, for the dephosphorylation of these proteins as well as their relative extent of phosphorylation before disaggregation of FcεRI is shown in each *panel*. It is striking that the differences in the rate constants are much smaller than the differences in the absolute levels of phosphotyrosine.

Inhibition of Kinases Causes Dephosphorylation—We had previously shown that receptors stably aggregated by trimeric IgE are rapidly dephosphorylated when the action of kinase is inhibited (15). In the current experiments we investigated if larger aggregates of receptors formed by multivalent antigen would behave similarly. There is evidence that at least such larger aggregates become rapidly associated with specialized domains that may be critically involved in the initial signal transduction (30).

Sensitized RBL cells were permeabilized with streptolysin O and stimulated with antigen, and EDTA was then added at various times after stimulation to halt kinase activity. The cells were solubilized, and immunoprecipitates of FcεRI as well as the whole cell lysates were assessed for phosphotyrosines. As previously noted for permeabilized cells (29), the phosphorylation of the receptor was preserved, but phosphorylation of some of the other cellular proteins was somewhat diminished (data not shown). Addition of EDTA led to a rapid decline of protein-bound phosphotyrosine to basal levels (Fig. 7).

DISCUSSION

Phosphorylation of tyrosines in the so-called immune recognition tyrosine activation motifs (ITAMs) (31, 32) is the initial event triggered by antigens when they aggregate one of the family of plasma membrane proteins collectively referred to as the multichain immune recognition receptors (33). With respect to FcεRI, the role of the aggregation has been intensively and quantitatively examined, but the molecular details are still not fully elucidated. Our group has presented direct evidence favoring a transphosphorylation mechanism (13); others have suggested that the association of the aggregates with specialized membrane domains (30) or immobilization of the aggregates *per se* (34) also play important roles.

The phosphorylation of the ITAMs on FcεRI (and likely on other multichain immune recognition receptors) is a rapid dynamic process in which the levels of phosphorylation are controlled by the opposing action of kinases and phosphatases. The kinase(s) involved in these initial events triggered by multichain immune recognition receptors are being elucidated, but much less is known about the corresponding phosphatase(s) (4, 35). The objectives of the present studies were to develop a quantitative assay for the dephosphorylation of FcεRI and to use it to answer some fundamental questions about the responsible tyrosine phosphatase(s).

Other than their amino acid sequences, there is virtually no structural information on the cytoplasmic domains that contain the ITAMs. Therefore, using arbitrary surrogates such as a peptide or a simple chimeric construct containing one or another ITAM as substrate could yield results that would be

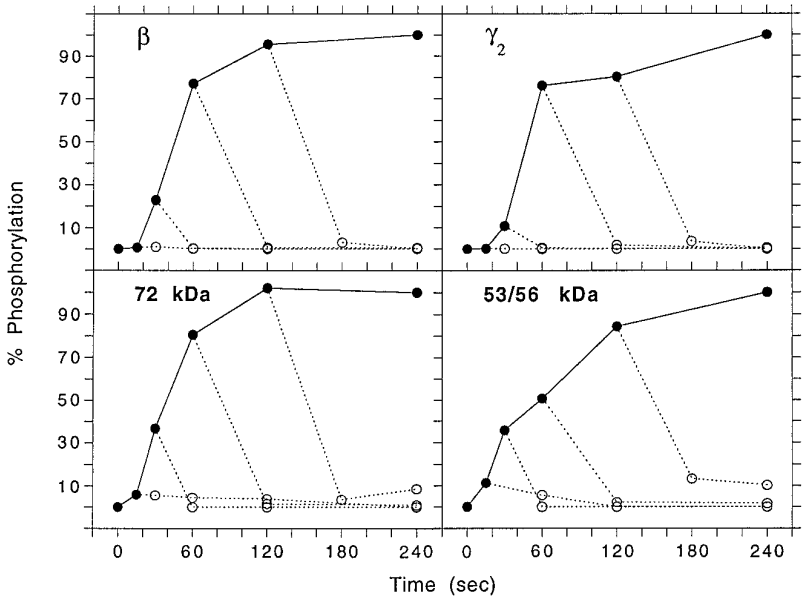

FIG. 7. **Effect of inhibiting kinases on protein-bound phosphotyrosine in permeabilized cells stimulated with DNP_{25}-BSA.** IgE-sensitized cells were permeabilized with streptolysin O and incubated with 1 μg/ml DNP_{25}-BSA at 37 °C (*filled circles*). At the times indicated, EDTA was added to a final concentration of 7.4 mM (*open circles*). Samples were analyzed as before. The data points represent the averages of duplicate samples.

qualitatively or at least quantitatively misleading. We therefore chose to utilize intact FcεRI as the substrate even though this was experimentally more demanding.

In our assays, the isolated receptors had no phosphatase associated with them (Fig. 1*A*). Recently Swieter *et al.* (26) reported such an association, but our results are not necessarily in conflict. First, Swieter *et al.* (26) isolated their receptors by procedures designed to minimize the dissociation of weakly interacting phosphatase, and indeed they found that the activity they measured was relatively easily dissociable. On the other hand, we deliberately used procedures that would tend to dissociate weakly interacting phosphatases, in part because it is not possible to distinguish in a straightforward way between contaminants, spurious associations, or physiological associations. Finally, we deliberately wanted to avoid assaying trace amounts of activity. Our assays were conducted at a lower temperature (30 °C *versus* 37 °C) and generally for much shorter times than those employed by Swieter *et al.* (26). Indeed, the activity we have measured appears in some instances 100-fold greater than can be estimated from their paper (see also below).

The studies employing cell fractions suggest that there are cytosolic phosphatases that in principle might participate in the dephosphorylation of receptors (Table I). However, much of the activity we observed was localized in the membrane fraction (Table I), and permeabilized cells, which had lost much of their cytosolic proteins, were as able to dephosphorylate receptors as intact cells (Fig. 3). Therefore, it appears that membrane-bound phosphatases are importantly involved. (This finding is of course consistent with a receptor-associated phosphatase such as described by Swieter *et al.* (26).) *In vivo*, a membrane-bound phosphatase could be vastly more effective than *in vitro* assays might reveal because of proximity and other effects (36).

That the relevant phosphatase is likely to be membrane-bound raises the question of whether it might be CD45. Although CD45 was found to be necessary for activating a T lymphocyte line through the FcεRI with which it had been transfected (37), the evidence that CD45 is required for initiating IgE-mediated activation of mast cells is contradictory (38–40). With respect to CD45, there is another consideration. As already noted, there are interesting data related to the association of aggregated receptors with specialized membrane domains. The latter are resistant to solubilization by certain

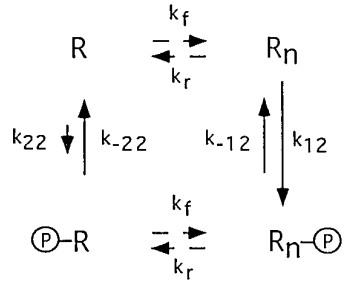

FIG. 8. **Kinetic scheme for phosphorylation of FcεRI.** *R*, monomeric FcεRI·IgE complexes; R_n, aggregated *R*; and *circled P*, phosphotyrosine. k_f results from the interaction of discrete epitopes on a polyvalent antigen with the receptor-bound IgE, and k_r results from the dissociation of individual epitopes from the IgE. No assumptions about the direction of this equilibrium are implied by the equivalent size of the *dashed arrows*.

detergents and are enriched in sphingolipids, glycophosphatidylinositol-anchored proteins, and membrane-anchored kinases (30, 41). One could propose a model in which such domains are deficient in phosphatases. Such paucity coupled with an enrichment in kinase(s) could promote the phosphorylation of the aggregated receptors that become associated with these specialized regions. Indeed, CD45 is thought to be excluded from these specialized domains (42), and the experimental data[2] suggest that this exclusion may be involved in regulating the activation of the kinase Lck in T lymphocytes.

We tested the possibility that in RBL cells the specialized membrane regions might be deficient in PTP(s) necessary to dephosphorylate FcεRI but found no evidence for such a lack. We aggregated receptors under conditions shown by the Cornell investigators to promote the association of the receptors with such domains (30, 41) and then blocked continued kinase action with EDTA. The results showed that there was no failure of prompt dephosphorylation of the receptors (Fig. 7). These observations parallel our previous findings on the dynamic phosphorylation/dephosphorylation to which smaller aggregates of the receptor are subject (15).

We also examined this latter aspect quantitatively *in vitro* by comparing the susceptibility of aggregated *versus* disaggregated receptors with dephosphorylation by PTP. The results

[2] Rodgers, W., and Rose, J. K. (1996) *J. Cell Biol* **135,** 1515–1523.

showed that aggregation did not protect the phosphorylated tyrosines from hydrolysis (Fig. 4). Our group is attempting to analyze quantitatively the initial response to aggregation of FcεRI. A simple scheme was proposed as a model on which to base future experiments (Fig. 8) (43). Earlier data showed that k_{-22} is substantial and effectively overwhelms k_{22}. The current data indicate that $k_{-12} \approx k_{-22}$. It is possible that by promoting interactions with the cytoskeleton, phosphorylation might stabilize receptors in their aggregated state, thereby enhancing the ratio k_f/k_r by decreasing k_r. It follows that the concentration of the aggregated phosphorylated species, which appears to be the critical component that initiates the cascade of events, will be independently determined by the ratio k_f/k_r for both the unphosphorylated and the phosphorylated receptors and by k_{12}/k_{-12}.

The latter ratio will of course reflect not only the intrinsic properties of the enzymes involved but also their concentrations. We have previously proposed that in the cell line we are studying, the failure of phosphorylation to correlate with aggregation under some experimental conditions could be explained if the kinase responsible for k_{12} is limiting (43). Recent experiments have provided experimental support for this proposal (44).

Using a peptide substrate, one group has proposed that stimulation of the RBL cells leads to an activation of a membrane-bound PTP (45). Our studies appear to rule out any major activation of PTP that might limit the level of phosphorylation of FcεRI and some of the earliest substrates that become tyrosine-phosphorylated. We recognize that the data presented in Fig. 6 probably reflect the actions of multiple phosphatases and that the individual "bands" whose phosphorylation (Fig. 5) and dephosphorylation (Fig. 6) we assessed may represent multiple substrates. Nevertheless, earlier data as well as all of the results presented here suggest that the cell maintains a constant brake on this system through the constitutive action of phosphatases. Aggregation moves the system, principally by enhancing the effectiveness of kinases.

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