

Persistence of Tyrosine-phosphorylated FcεRI in Deactivated Cells*

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Engagement of the high affinity IgE receptor (FcεRI) with a multimeric antigen leads to immediate tyrosine phosphorylation of its β and γ subunits, recruitment, and activation of the tyrosine kinase Syk, and later to cell degranulation. Monovalent hapten treatment reverses these events, resulting in receptor dephosphorylation and an abrupt arrest of cell degranulation. Thus far, it has been assumed that there is a direct linkage between receptor tyrosine phosphorylation, Syk activation and phosphorylation, and cell degranulation. However, we show here that when FcεRI receptors are cross-linked for extended periods of time, hapten-mediated receptor dephosphorylation is delayed. These receptors, which remain tyrosine-phosphorylated despite the addition of hapten, are progressively targeted to a Triton X-100-insoluble fraction, suggesting their progressive association with the membrane skeleton. In contrast to FcεRI receptors, hapten-induced Syk dephosphorylation and the consequent arrest of degranulation are not affected by prolonged cross-linking. Thus, some tyrosine-phosphorylated receptors persist in deactivated cells. We propose that, with time, some tyrosine-phosphorylated receptors become inaccessible to phosphatases and, in addition, unable to activate Syk. This inactive status of tyrosine-phosphorylated FcεRI may be the result of membrane skeleton compartmentalization. However, another population of clustered receptors that includes the ones most recently formed is still immediately sensitive to hapten deactivation. This latter population is critical in maintaining Syk activity and cell degranulation. The shift from a transiently active state of phosphorylated receptors toward an inactive state could be a general mechanism of desensitization also utilized by other antigen receptors.

Activation of mast cells and basophils with allergens results in cell degranulation and the release of preformed and newly synthesized mediators of the allergic reaction (1, 2). This process is initiated by the binding of multivalent allergens or antigens to receptor-bound immunoglobulin E, which induces the aggregation of the tetrameric ($\alpha\beta\gamma_2$) high affinity IgE receptor (FcεRI) expressed on these cells. Receptor aggregation is the critical event in the initiation of the signaling cascade leading to cell degranulation (3). We and others have used a multivalent antigen and the corresponding antigen-specific IgE to study FcεRI-mediated activation of mast cells. The utilization of a multivalent antigen as triggering tool allows one to study

the reversibility of activation events because the addition of an excess of the corresponding monovalent hapten after multivalent antigen induces an immediate arrest of the ongoing early and late activation events (4–8). One possible explanation for this arrest is that the monovalent hapten disaggregates previously aggregated and active receptors. However, morphological studies by Oliver *et al.* (8) have shown that, when cells are triggered with a multivalent antigen for a long period of time (more than 10 min), the addition of monovalent hapten does not result in a rapid receptor disaggregation, even though cell signaling is halted abruptly. Thus, aggregated receptors remain on the cell surface of apparently inactive cells. These authors and others have proposed a model in which continuous formation of new aggregates is required to maintain cell activation, and hapten simply prevents the formation of new aggregates (8–12). Recently, Kent *et al.* (13, 14) challenged this interpretation. They have shown that when receptor aggregation is induced by small oligomers of chemically cross-linked IgE, the addition of excess monomeric IgE (which should prevent the formation of new aggregates) does not arrest ongoing signaling events. Therefore, under these conditions, small clusters of receptors remain active despite no new clusters being formed.

Interest in the question of the mechanism of hapten action has been renewed by recent findings about early events in FcεRI signaling. One of the earliest events following FcεRI aggregation is the activation of tyrosine kinases such as the Src family kinase Lyn and the Syk/ZAP70 family kinase Syk (15–19). This activation results in the tyrosine phosphorylation of various substrates, including the β and γ subunits of FcεRI (4, 5, 20–23). The tyrosine residues in the β and γ subunits that become phosphorylated are parts of conserved amino acid motifs called immunoreceptor tyrosine-based activation motifs (ITAM).¹ One ITAM is present in the β subunit and one in each γ subunit of FcεRI (24–27). Phosphorylation of these ITAMs is necessary to recruit and activate Syk and to propagate activation signals (18, 28–32). Cell treatment with monovalent hapten after multivalent antigen triggering (which halts mediator release) leads to a rapid receptor dephosphorylation (5). However, when cells are triggered with IgE dimers or trimers, the subsequent addition of monomeric IgE results in only a moderate decrease in the phosphorylation of the β and γ chains and of Syk, as well as in only a moderate decrease in mediator release (13, 14). With both modes of triggering, there seems to be a direct relationship between the tyrosine phosphorylation state of the receptors and the activation state of the cells, as defined by their capacity to release mediators, although the effects of adding a monovalent ligand (hapten or monomeric IgE) are dramatically different. In the present study, we have analyzed the effect of hapten disengagement after periods of

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¹ The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motifs; RBL, rat basophilic leukemia; DNP, 2,4-dinitrophenyl; HSA, human serum albumin; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

stimulation longer than in the past study (5), thereby creating conditions of cellular inactivation which should leave some receptors aggregated. We anticipated that the state of cellular activation would be correlated with the tyrosine phosphorylation status of the receptors. To our surprise, we found that in these conditions of inactivation, tyrosine-phosphorylated receptors persist in cells even though cell degranulation has ceased.

MATERIALS AND METHODS

Cell Culture, Reagents, and Antibodies—The rat basophilic leukemia cell line, RBL-2H3 (RBL cells), media, and sera, dinitrophenyl(30)-human serum albumin (DNP-HSA), ϵ -dinitrophenyl-lysine (DNP-lysine), phenyl phosphate, trypsin, [^{32} P]orthophosphate, [γ - ^{32} P]ATP, the chemiluminescent substrate for Western blotting AMPPD, the anti-phosphotyrosine antibody 4G10, goat anti-mouse IgG, and goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase, anti-DNP monoclonal mouse IgE, rabbit anti-mouse IgE, anti-rat FcεRI β subunit monoclonal antibody JRK, rabbit anti-FcεRI γ subunit antibody, and rabbit anti-Syk antibody have been described (5, 28, 32, 33).

In Vivo Phosphorylation, Immunoprecipitation, SDS-PAGE Electrophoresis, and Western Blotting—RBL cells were starved of phosphate by replacing the usual culture medium with phosphate-free Eagle's minimal essential medium (Life Technologies, Inc.) and 10% dialyzed fetal bovine serum (Biofluids) for 4 h at 37 °C before the addition of 5 μ g/ml monomeric monoclonal anti-DNP mouse IgE for 1 h at 37 °C and then labeled at 3×10^7 cells/ml with 37 MBq/ml [^{32} P]orthophosphate for 2 h at 37 °C. The cells (1×10^7 /ml) have been described (5, 28, 32, 33). The cells were incubated with medium alone or with 0.1 or 1 μ g/ml DNP-HSA for the time indicated for individual experiments. The stimulation was followed or not by the addition of 50 μ M DNP-lysine for 30 s. The cells were then immediately washed and lysed (5×10^7 cells/ml) in a lysis buffer containing 200 mM boric acid, pH 8, 160 mM NaCl, 0.5% Triton X-100, 1% BSA, protease inhibitors, and phosphatase inhibitors as described previously (Triton buffer) (5). The lysates were spun down at 4 °C for 20 min at $12,000 \times g$. The supernatants were precleared with protein A-Sepharose beads (Pharmacia Biotech Inc.) and then sequentially immunoprecipitated with control antibodies and rabbit polyclonal anti-IgE or mouse monoclonal anti-phosphotyrosine, all bound to protein A-Sepharose beads. In some experiments, the anti-phosphotyrosine immunoprecipitates were eluted from the beads with 10 mM phenyl phosphate (Sigma) in Triton buffer without BSA at 4 °C for 15 min, and the tyrosine-phosphorylated proteins were reprecipitated with rabbit anti-Syk antiserum. All the samples were analyzed by SDS-PAGE under reducing conditions and autoradiography. In some experiments, the radioactivity associated with some bands was quantified using the Ambis analytical system (28). For Western blotting, the postnuclear supernatants were immunoprecipitated and analyzed by SDS-PAGE as above, and the proteins were transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA) for 2 h at 80 V. Membranes were then incubated overnight in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 5% BSA before a 2-h incubation in the same buffer containing 1 μ g/ml polyclonal anti-Syk antibody. After two washes in TTBS (TBS containing 0.1% Triton X-100 and 2.5% BSA), the membranes were incubated with 0.2 μ g/ml goat anti-rabbit antibody conjugated to alkaline phosphatase and developed with the chemiluminescence substrate AMPPD according to the manufacturer (Tropix). In some experiments the pellets resulting from the lysis of unlabeled cells in Triton X-100 were resububilized in 100 μ l of a buffer containing 1% SDS, 20 mM Hepes, pH 7.4, 150 mM NaCl, 0.1 μ M sodium vanadate and boiled for 5 min. The supernatants were recovered, diluted with 900 μ l of the Triton buffer, immunoprecipitated with the anti- β antibody or the rabbit anti- γ antiserum, and analyzed by Western blotting.

Phosphoamino Acid Analysis and Two-dimensional Tryptic Phosphopeptide Mapping—Two-dimensional thin layer electrophoresis of phosphoamino acids was performed by a modification of the method of Hunter and Sefton as described previously (34).

Tryptic phosphopeptide mapping of the β and γ chains was performed as described (35). Tryptic phosphopeptide mapping of Syk was performed as described (36) with the following modifications. ^{32} P-Labeled Syk was transferred to nitrocellulose and excised after autoradiography. Digestion was performed with 10 μ g/ml trypsin in 50 mM NH_4HCO_3 . Extracted proteins were vacuum-dried and dissolved in 5 μ l of thin layer buffer at pH 4.72 (5% 1-butanol, 2.5% pyridine, 2.5% glacial acetic acid in water). The samples were applied to thin layer chromatography cellulose plates (100-mm DC-cellulose, EM Separ-

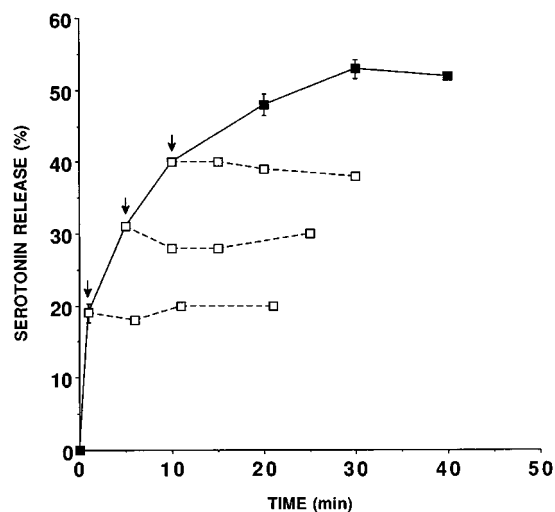


FIG. 1. **Inhibition of antigen-induced serotonin release by monovalent hapten.** RBL cells labeled with [^{3} H]serotonin and saturated with anti-DNP IgE were stimulated with 100 ng/ml DNP-HSA for different periods of time (filled squares). The hapten DNP-lysine (50 μ M) was added (open squares) after the time of triggering indicated by the arrows (1, 5, and 10 min). The amount of [^{3} H]serotonin released into the medium is displayed as a percentage of total [^{3} H]serotonin content.

tions, Gibbstown, NJ) and electrophoresed in the first dimension for 30 min at 1000 V. The plates were air-dried and the second dimension, consisting of ascending chromatography in isobutyric acid buffer (62.5% isobutyric acid, 1.9% 1-butanol, 4.8% pyridine, 2.9% glacial acetic acid in water), was performed for 6 h at room temperature. The ^{32} P-labeled phosphopeptides were detected by autoradiography.

Serotonin Release—The assay was performed as described (37).

In Vitro Kinase Assay—Assay was performed as described with the following modifications (34). Following immunoprecipitation with rabbit anti-Syk antibody, the beads were washed five times with lysis buffer and once with the kinase buffer (30 mM Hepes, pH 7.4, 5 mM MgCl_2 , 5 mM MnCl_2 , and 100 mM sodium metavanadate). To perform the *in vitro* kinase reaction, the beads were resuspended in kinase buffer containing 10 μ Ci of [γ - ^{32} P]ATP and 1 μ M cold ATP. After 3 min of incubation at 30 °C, the beads were washed twice with lysis buffer and the immunoprecipitates analyzed by SDS-PAGE and autoradiography.

RESULTS

Delay in Hapten-induced Dephosphorylation of FcεRI β And γ Chains after 10 Min of Stimulation Contrasts with the Immediate Cessation of Exocytosis—As reported previously (6–8), triggering of rat basophilic leukemia cells (RBL-2H3) saturated with an anti-DNP IgE with the multivalent antigen DNP-HSA induces a serotonin release which reaches its maximum (55% of the total cellular serotonin) after 30 min of stimulation. Addition of monovalent hapten halts exocytosis immediately, regardless of the length of stimulation (Fig. 1). Receptor engagement also results in the rapid phosphorylation of the β and γ chains, and the addition of hapten after 1 min of stimulation induces dephosphorylation of the β and γ chains (Fig. 2 and Ref. 5). However, after 10 or 30 min of stimulation, the addition of hapten for 30 s does not induce any substantial receptor dephosphorylation (Fig. 2A, compare lane 6 with lane 5 and lane 8 with lane 7).

Two-dimensional thin layer electrophoresis shows that the pattern of amino acid phosphorylation of the β and γ chains after 10 min of stimulation (a and c, respectively) does not change after the addition of hapten for 30 s (b and d). The tyrosine phosphorylation status of β and γ was specifically examined after alkaline treatment of the gel, which removes serine/threonine phosphorylation. Hapten-induced tyrosine dephosphorylation of both β and γ chains is substantial after 1 min of stimulation but much reduced after 10 or 30 min of

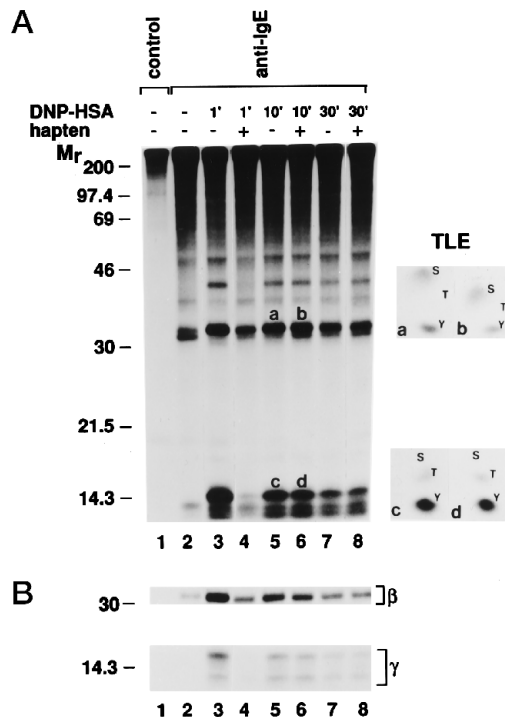


FIG. 2. Addition of monovalent hapten for 30 s does not reverse FcεRI β and γ tyrosine phosphorylation after long times of stimulation. A, immunoprecipitates from lysates of ^{32}P -labeled cells using a control antibody (lane 1) or an anti-IgE antibody (lanes 2–8) were analyzed by SDS-PAGE and autoradiography. RBL cells saturated with anti-DNP IgE were incubated with medium alone (lanes 1 and 2) or with 100 ng/ml DNP-HSA (lanes 3–8) for the time indicated for each lane. Cells were then chilled on ice or incubated for a further 30 s with 50 μM DNP-lysine (lanes 4, 6, and 8). M_r calibration is shown at left (all figures). The β (a and b) and γ (c and d) species extracted from the bands cut out from lanes 5 and 6 of the gel were hydrolyzed and analyzed by two-dimensional thin layer electrophoresis. B, the gel shown in A was re-exposed after treatment with 1 M KOH for 1 h at 60 $^{\circ}\text{C}$.

stimulation (Fig. 2B). The phosphopeptides generated by treatment of β and γ with trypsin were also compared. Two-dimensional phosphopeptide mapping does not reveal any qualitative difference between the pre- and post-hapten phosphorylated β and γ chains (Fig. 3, A and B, respectively).

A time course reveals that after 10 min of triggering, hapten-induced dephosphorylation is not absent but delayed (Fig. 4). Although some receptor dephosphorylation is apparent after 30 s or 1 min of hapten treatment, receptor phosphorylation remains substantial (lanes 6 and 7). However, after 5 min of hapten treatment, receptor phosphorylation returns essentially to base-line levels (compare lanes 8, 4, and 2). Thus, there is a delay in the hapten-induced receptor dephosphorylation after 10 min of stimulation when compared with the arrest of cell degranulation which is immediate.

Persistently Tyrosine-phosphorylated Receptors Are Associated with a Relatively Insoluble Fraction—It has been previously reported that receptors cross-linked for long periods of time or with doses of multivalent antigen higher than optimal become relatively insoluble (9–11). This insolubility has been interpreted as resulting from interactions with the membrane skeleton. This cellular redistribution of some phosphorylated receptors could explain their relative insensitivity to the hapten-induced dephosphorylation after prolonged triggering. We therefore investigated whether receptors that are persistently tyrosine-phosphorylated after hapten addition were progressively targeted to the insoluble fraction. RBL cells were triggered for various periods of time and solubilized in the usual

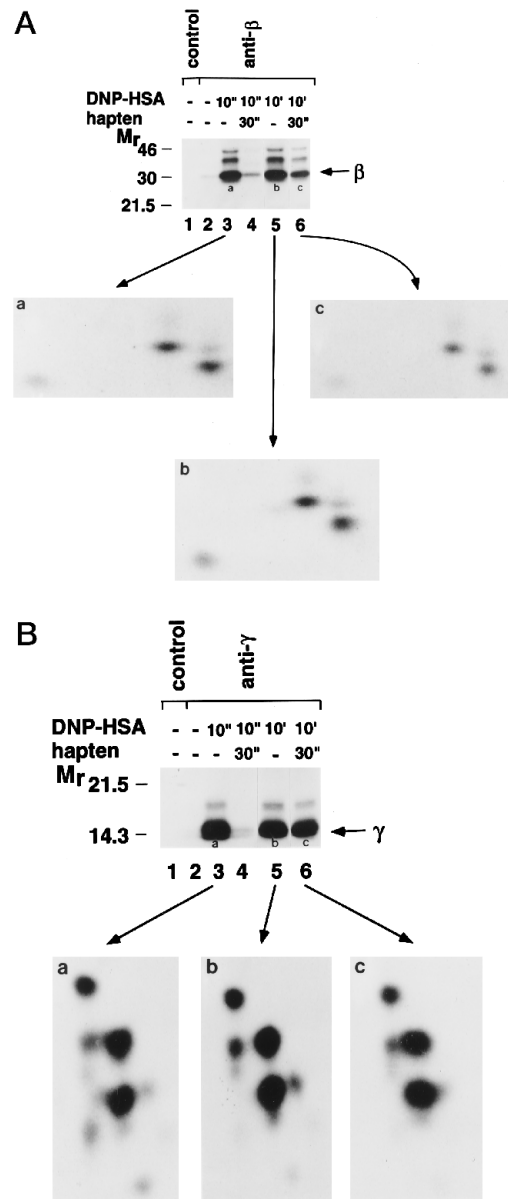


FIG. 3. Two-dimensional tryptic phosphopeptide analysis of β and γ chains. RBL cells loaded with anti-DNP IgE and labeled with [^{32}P]orthophosphate were incubated for 10 s (lanes 1–4) or 10 min (lanes 5 and 6) with medium alone (lanes 1 and 2) or DNP-HSA (lanes 3–6). Cells were chilled on ice or incubated for a further 30 s with DNP-lysine (lanes 4 and 6). Cell lysates were immunoprecipitated with anti-phosphotyrosine monoclonal antibody, and the phosphoproteins were eluted from the beads with phenyl phosphate, reprecipitated with anti- β (A) or anti- γ (B) antibody, and analyzed by SDS-PAGE and autoradiography. Phosphoproteins corresponding to the β and γ chains (a–c) were extracted from slices cut out of lanes 3, 5, and 6 of the gels. The phosphopeptides obtained after trypsin digestion were resolved by two-dimensional phosphopeptide analysis. Samples were electrophoresed from right (cathode) to left (anode); chromatography was performed from bottom to top. The three panels were exposed for the same period of time. Analogous results were obtained when comparing peptide maps of β and γ recovered after an anti-IgE precipitation.

mild conditions (0.5% Triton X-100). The Triton-insoluble fraction was then lysed in an SDS-containing buffer, and after dilution of SDS, this lysate was immunoprecipitated with anti- β or anti- γ antibodies (Fig. 5). In unstimulated cells as well as in cells stimulated for 10 s and 1 min, few receptors are recovered from the Triton-insoluble fraction as shown by an anti- β (Fig. 5A, upper panel) and an anti- γ (Fig. 5B, upper panel) blot. However, after 10 and 30 min of stimulation large amounts of both the FcεRI β and γ chains are recovered in this

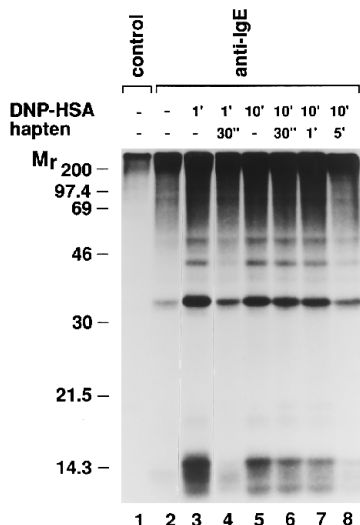


FIG. 4. The hapten-induced FcεRI β and γ dephosphorylation is delayed after 10 min of stimulation. RBL cells saturated with anti-DNP IgE and labeled with [^{32} P]orthophosphate were incubated at 37 °C for 1 min (lanes 1–4) or 10 min (lanes 5–8) with medium alone (lanes 1 and 2) or with multivalent antigen (lanes 3–8). Cells were then chilled on ice (lanes 1–3 and 5) or incubated with DNP-lysine (lanes 4 and 6–8) for the additional time indicated in each lane. Immunoprecipitates with a control antibody (lane 1) or an anti-IgE antibody (lanes 2–8) were resolved by SDS-PAGE and autoradiography.

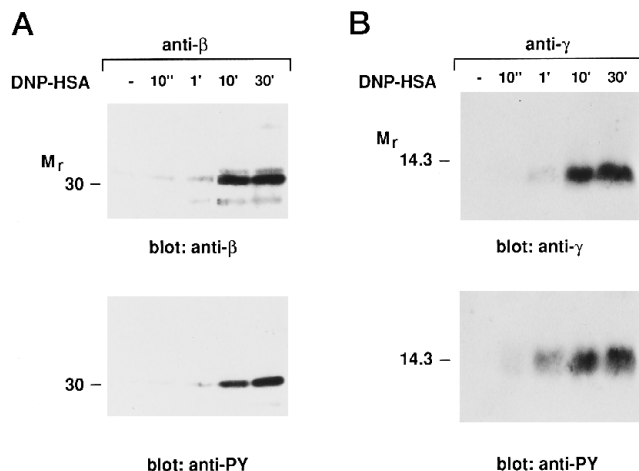


FIG. 5. Tyrosine-phosphorylated FcεRI β and γ chains associate with a Triton-insoluble fraction after prolonged stimulation. RBL cells saturated with anti-DNP IgE were incubated with medium alone or with 1 μ g/ml DNP-HSA for the times indicated. Cells were lysed in Triton X-100 buffer. The insoluble fraction was then lysed in a buffer containing 1% SDS. Immunoprecipitation was performed with the anti- β (A) or anti- γ (B, upper panel) antibodies; the proteins were separated by SDS-PAGE and subjected to Western blotting with the anti- β (A, upper panel), anti- γ (B, upper panel), or anti-phosphotyrosine (lower panels) antibodies.

fraction, and these receptors are heavily tyrosine-phosphorylated (Fig. 5, lower panels). This result indicates that, in contrast to receptors phosphorylated after a short triggering, persistently tyrosine-phosphorylated receptors are found in a Triton-insoluble cellular fraction.

Syk Dephosphorylation Does Not Correlate with Receptor Dephosphorylation—To understand better the mechanisms by which hapten arrests cell degranulation immediately while leaving some receptors phosphorylated, we examined the phosphorylation of various substrates including the tyrosine kinase Syk, which is located just downstream of receptor phosphorylation in the signaling pathway (30). Anti-phosphotyrosine immunoprecipitates from ^{32} P-labeled RBL cells that have been

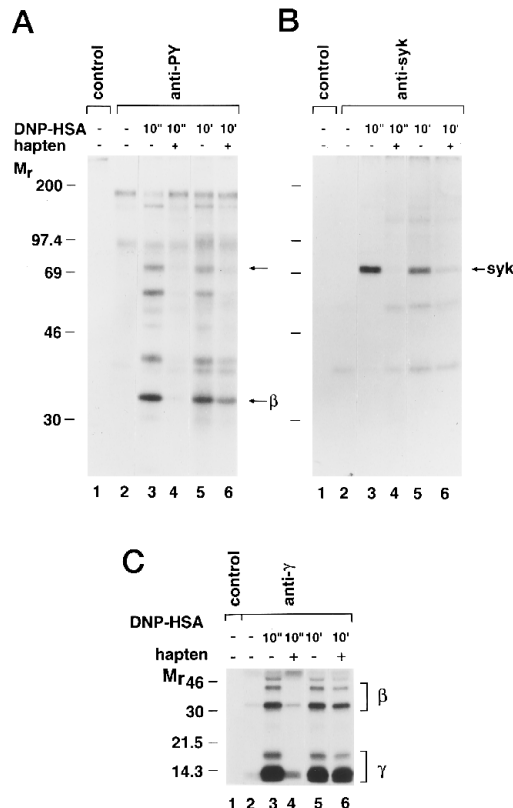


FIG. 6. Effect of monovalent hapten on Syk phosphorylation. RBL cells saturated with anti-DNP IgE and labeled with [^{32} P]orthophosphate were incubated at 37 °C for the time indicated in each lane in the absence (lanes 1 and 2) or in the presence (lanes 3–6) of DNP-HSA. The cells were then chilled on ice (lanes 1–3 and 5) or incubated for an additional 30 s with DNP-lysine (lanes 4 and 6). Lysates were immunoprecipitated with anti-phosphotyrosine antibody. The phosphorylated proteins were either analyzed by SDS-PAGE and autoradiography (A) or eluted from the beads with phenyl phosphate and reimmunoprecipitated with anti-Syk (B) or anti- γ (C) antibodies.

stimulated with antigen and treated with or without hapten were analyzed by SDS-PAGE and autoradiography. As expected, phosphoproteins are present after 10 s of stimulation (Fig. 6A, lane 3). A similar pattern of bands is seen after 10 min of stimulation (lane 5). The 72 and the 33 kDa bands (arrows) were identified as Syk and β , respectively, by immunoblotting (not shown). Hapten treatment, following a 10-s stimulation, results in the disappearance of all the bands (compare lanes 4 and 3). Hapten treatment following a 10-min stimulation results in the dephosphorylation of some bands only, including those migrating to the positions corresponding to β and Syk. However Syk dephosphorylation appears much greater than β dephosphorylation (compare lanes 6 and 2). To confirm the identity of these two bands, the tyrosine-phosphorylated proteins were specifically eluted from the anti-phosphotyrosine immunoprecipitates with phenyl phosphate and reprecipitated with anti-Syk (Fig. 6B) or anti- γ antibodies (Fig. 6C). Also under those conditions Syk dephosphorylation following a 10-min stimulation is greater than β and γ dephosphorylation. The radioactivity associated with the bands corresponding to Syk, β , and γ before and after hapten treatment from three independent experiments was counted by gel scanning. The hapten-induced decrease in Syk-associated radioactivity was significantly greater than that for β or γ ($p = 0.026$).

Effect of Hapten Addition on Syk Activity—We next investigated how Syk activity was affected by hapten treatment. Anti-Syk immunoprecipitates from unlabeled RBL cells treated with antigen and with or without hapten were subjected to an *in*

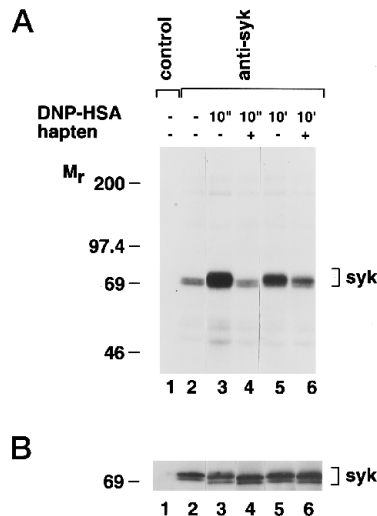


FIG. 7. Monovalent hapten halts the antigen-induced autophosphorylation of Syk regardless of the time of stimulation. RBL cells saturated with anti-DNP IgE were incubated for 10 s (lanes 1–4) or 10 min (lanes 5 and 6) with medium alone (lanes 1 and 2) or 100 ng/ml DNP-HSA (lanes 3–6). Cells were then chilled on ice (lanes 3 and 5) or incubated for a further 30 s with 50 μ M DNP-lysine (lanes 4 and 6). Immunoprecipitates with control antibody (lane 1) or anti-IgE (lanes 2–6) were subjected to an *in vitro* kinase assay and analyzed by SDS-PAGE and autoradiography (A) or subjected to Western blotting with an anti-Syk antibody (B).

vitro kinase assay and analyzed by SDS-PAGE and autoradiography. Under resting conditions, a basal level of autophosphorylation is observed (Fig. 7A, lane 2). Receptor engagement increases the autophosphorylation of Syk (compare lanes 5, 3, and 2), which, upon hapten addition after 10 s of stimulation, returns essentially to base-line levels (compare lanes 4 and 2). After 10 min of stimulation, hapten addition still induces a substantial decrease in Syk kinase activity (compare lanes 6 and 2). The amounts of Syk kinase precipitated in each condition are comparable as shown by the anti-Syk immunoblotting (Fig. 7B).

Phosphopeptide Mapping of Phosphorylated and Dephosphorylated Syk—To analyze qualitatively the changes in Syk phosphorylation following receptor aggregation and hapten treatment, we performed a two-dimensional peptide map from anti-Syk immunoprecipitates (Fig. 8). We could not analyze the basal level of Syk phosphorylation because the counts recovered after extraction and digestion of the sample were too low. The pattern of phosphopeptide spots is different between the pre- (a and c) and post- (b and d) hapten Syk. At least four phosphopeptides are present after antigen stimulation. All the spots except one, marked with an arrow, are substantially dephosphorylated following the addition of hapten, regardless of the length of antigen stimulation. This result shows that the same sites in Syk become dephosphorylated by hapten treatment after 10 s or 10 min of antigen stimulation.

DISCUSSION

Experimental data from many laboratories have contributed to a model of FcεRI-mediated cell activation in which FcεRI aggregation results in both receptor and cellular protein tyrosine phosphorylation which leads to cell activation and degranulation. Subsequent hapten-mediated cell deactivation results in receptor and protein dephosphorylation and cessation of degranulation (Fig. 1 and Refs. 1–3). In this model, cellular tyrosine phosphorylation is tightly linked to the tyrosine phosphorylation state of the receptor through activation of Syk, as Syk is thought to require an interaction with a phosphorylated ITAM to become activated (19, 28–32). However, this model

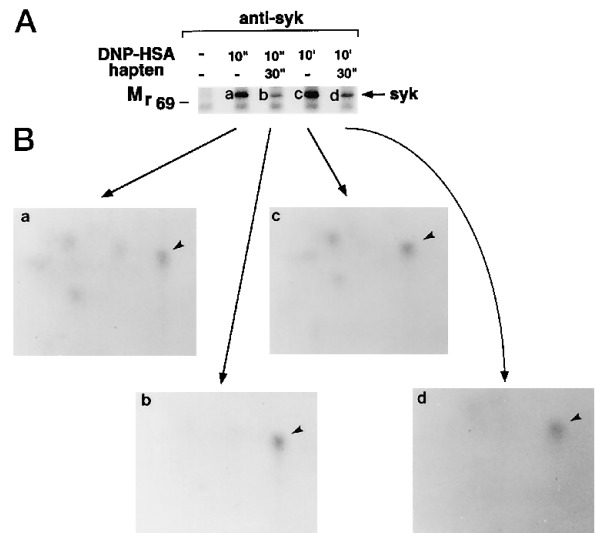


FIG. 8. Two-dimensional tryptic phosphopeptide analysis of Syk. A, RBL cells loaded with anti-DNP IgE and labeled with [^{32} P]orthophosphate were stimulated with DNP-HSA at 37 °C for the time indicated in each lane. They were then chilled on ice or incubated for an additional 30 s with DNP-lysine. Lysates were immunoprecipitated with the anti-Syk antibody and analyzed by SDS-PAGE and autoradiography. B, proteins corresponding to Syk (a–d) were recovered from the gel slices as shown in A, digested with trypsin, and resolved by two-dimensional phosphopeptide analysis. Samples were treated as described under “Material and Methods.”

fails to explain the inhibition of degranulation that occurs when too strong a cross-linking stimulus is applied (reviewed in Ref. 38).

Here, we have been able to create experimental conditions where phosphorylated receptors persist despite a cessation of degranulation induced by hapten treatment. One explanation for the inactive yet persistently phosphorylated receptors could be that their phosphorylation state is qualitatively different from those of active receptors. For example, other non-ITAM phosphorylation could result in tyrosine-phosphorylated ITAMs that are unable to activate Syk. However, phosphopeptide mapping and phosphoamino acid analyses of the receptor subunits show identical qualitative phosphorylation patterns for both short and prolonged cross-linkings and no difference in qualitative dephosphorylation patterns following haptenization (Figs. 2 and 3). These results suggest that after prolonged stimulation the phosphorylated receptors are somehow unable to access the cellular activation machinery.

Results from other studies suggest that the tyrosine kinase Syk could be the signaling molecule that these phosphorylated receptors cannot access. Treatment of RBL cells with piceatannol or with *N*-acetyl-L-cysteine inhibits antigen-mediated tyrosine phosphorylation of Syk (and its kinase activity) and that of most other cellular proteins, whereas Lyn-induced receptor phosphorylation is maintained (39, 40). At the same time, receptor-mediated downstream events including secretion are abrogated. These experiments demonstrate that Syk activation is essential to FcεRI-mediated cellular responses and, together with our more recent experiments using overexpression of active and dominant negative Syk (32), show that receptor phosphorylation is upstream of Syk activation. In our current experiments, the reduction in tyrosine phosphorylation of Syk and the abrupt cessation of Syk activation and cellular degranulation despite the persistence of receptor phosphorylation are compatible with the hypothesis that these receptors are unable to access Syk (Fig. 7). Furthermore, the similarity in tryptic phosphopeptide maps of Syk after short and long periods of triggering suggests that the inability of persistently phospho-

rylated receptors to activate Syk is not due to a different pattern of Syk phosphorylation (Fig. 8).

Our results imply that access of aggregated receptors to some components of the cell activation machinery, particularly Syk and the tyrosine phosphatase(s) responsible for dephosphorylating β and γ , is controlled. This could be mediated by a number of different mechanisms that may rely in particular on the size and the configuration of receptor aggregates, as outlined in the following model. Receptor engagement with antigen initially induces the formation of small and relatively mobile aggregates that are capable of mediating intracellular signaling and are sensitive to disaggregation and inactivation by monovalent hapten. However, with time, or with higher concentrations of antigen, large aggregate formation is superimposed on the continuous formation of small aggregates. These large aggregates are immobile, detergent-insoluble and are not immediately disaggregated by hapten. Existing data on detergent solubility and FcεRI aggregate size are consistent with this interpretation (12). Furthermore, we show here that the persistently tyrosine-phosphorylated receptors present after long periods of stimulation are associated with a Triton-insoluble cellular fraction (Fig. 5). These data support our model according to which only small aggregates are able to recruit and activate Syk. Large aggregates are not able to access Syk, possibly because of their known interaction with the membrane skeleton. For the same reason, phosphorylated receptors present in large aggregates cannot access the phosphatases responsible for FcεRI dephosphorylation and remain phosphorylated despite hapten treatment. However, hapten disaggregates the small proportion of small aggregates present, induces receptor dephosphorylation, and thereby arrests the activation of Syk and of downstream events. This model would also explain the results of Kent *et al.* (13, 14). IgE oligomers induce formation of small aggregates of a defined size that are capable of activating Syk. Addition of monomeric IgE, while preventing the formation of new aggregates, does not influence the size of the already formed aggregates. Therefore, these small aggregates remain active, as observed. Recently developed microscopy and molecular techniques, like confocal microscopy and chimeric green fluorescent proteins (41), should provide the tools required to test this model further.

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