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Phosphorylation of the Receptor for Immunoglobulin E†

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ABSTRACT: Specific immune precipitation of immunoglobulin E (IgE)–receptor complexes from detergent extracts of ^{32}P-labeled rat basophilic leukemia cells yielded a phosphoprotein of M_r ~35 000 on gel electrophoresis in sodium dodecyl sulfate. This phosphoprotein was shown by several criteria to be the β chain of the receptor for IgE. Phosphorylation occurs at a serine residue (or residues) in a region (β_2) of the β chain that is thought to be exposed on the cytoplasmic face of the plasma membrane. Our results suggest that phosphorylation probably takes place after the insertion of the β chain into the membrane. The IgE-binding α chain of the receptor and the IgE associated with it are not phosphorylated. We have so far been unable to detect any changes in the state of phosphorylation of either chain of the receptor or of IgE itself after IgE-mediated triggering of the cells.

Mast cells and basophils have on their surfaces a receptor which binds immunoglobulin E (IgE)[1] with high affinity. This receptor is made up of two polypeptide chains denoted α and β (Metzger et al., 1982). The α chain of the receptor binds IgE and is a glycoprotein with a molecular weight of about 50 000. The recently identified β chain of the receptor has a molecular weight in the region of 35 000 and does not appear to be exposed on the outside of the plasma membrane.

When receptors for IgE are aggregated either indirectly by using specific antigen or anti-IgE which cross-link via IgE (Ishizaka & Ishizaka, 1971) or directly by using antibodies to the receptor itself (Ishizaka & Ishizaka, 1978; Isersky et al., 1978), the cells are stimulated to secrete histamine, serotonin, and other mediators of immediate hypersensitivity by a noncytoxic, calcium-dependent mechanism (Mongar & Schild, 1958). The sequence of biochemical events occurring between receptor activation and the final secretory response still remains to be elucidated, although a number of potential intermediate steps have been described. These include activation of a serine esterase (Becker & Austen, 1966), phospholipid methylation (Ishizaka et al., 1980), adenylate cyclase activation, changes in cyclic nucleotide levels and activation of phosphoprotein kinases (Holgate et al., 1980a,b), phosphatidylinositol turnover (Cockcroft & Gomperts, 1979), and an increase in membrane permeability to calcium (Foreman et al., 1973). In addition changes in protein phosphorylation have been observed during secretion from mast cells (Sieghart et al., 1978).

The role played by the receptor itself in any of these processes is entirely unknown. Furthermore, designing appropriate experiments to determine the way in which this receptor might function is not straightforward. One obvious approach is to search for structural changes in the receptor molecule which occur on stimulation, and the present study was one of several initiated for that purpose. The demonstration that the nicotinic acetylcholine receptor can be phosphorylated (Gordon et al., 1977a,b; Teichberg et al., 1977) and the important role of protein phosphorylation in a variety of regulatory mechanisms (Krebs & Beavo, 1979) encouraged us to examine the state of phosphorylation of the receptor for IgE in rat basophilic leukemia (RBL) cells. IgE-mediated secretion from these tumor cells is similar in all important respects to that from normal mast cells and basophils (Fewtrell & Metzger, 1981), and the availability of large numbers of pure cells from tissue culture makes this a particularly attractive system for such studies.

A preliminary account of some of these results has already been given (Fewtrell et al., 1981).

Materials and Methods

IgE, IgE Derivatives, and Anti-IgE Antibodies. Rat monoclonal IgE (IR162) and human monoclonal IgE (PS) were purified as described previously (Kulczycki & Metzger, 1974). A mouse hybridoma anti-dinitrophenyl (DNP) IgE selected and characterized by Liu et al. (1980) was isolated and purified as described previously (Holowka & Metzger, 1982). Iodinated and amidinated IgE's were prepared as described by Kulczycki & Metzger (1974) and Holowka et al. (1980), respectively. Iodination of mouse IgE by the Chloramine T

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[1] Abbreviations: RBL, rat basophilic leukemia; IgE, immunoglobulin E; $NaDodSO_4$, sodium dodecyl sulfate; DTBP, dimethyl 3,3′-dithiobis(propionimidate); DNP, dinitrophenyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

method labels the heavy chain almost exclusively (see Figure 1a). For this reason ^{125}I-labeled mouse IgE was used in preference to ^{125}I-labeled rat IgE (where both light and heavy chains are labeled), as this simplified the pattern of radioactivity seen after reduction. Azobenzenearsonate conjugation of IgE was carried out as described previously (Kanellopoulos et al., 1979). Rabbit anti-rat IgE, anti-human IgE, and goat anti-mouse IgE were affinity purified as previously described (Taurog et al., 1979; Holowka & Metzger, 1982).

^{32}P Labeling of RBL Cells. RBL cells of the secreting subline 2H3 (Morita et al., 1979) were maintained in monolayer culture and then harvested and passively sensitized with IgE as described previously (Taurog et al., 1979). Cell viability was always greater than 95% as assessed by trypan blue exclusion. Cells were washed twice with a phosphate-free medium of the following composition: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 0.25% gelatin, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, pH 7.4. The cells were then resuspended in 5 mL of the same medium but containing 0.05% gelatin and 5 or 10 mCi of carrier free [^{32}P]orthophosphoric acid (New England Nuclear, Boston, MA). The final cell concentration was between 3×10^7 and 6×10^7 cells/mL. The cells were incubated at 37 °C for 2 h with shaking during which time they took up virtually all the ^{32}P from the medium. In a few experiments sensitization with IgE was carried out at the same time or after ^{32}P loading rather than before.

The cells were washed with ice-cold phosphate-buffered saline, pH 7.4, containing 20% newborn calf serum (GIBCO, Grand Island, NY) and then once with buffered saline alone.

Triggering of Cells. In experiments where the effect of triggering on ^{32}P incorporation was to be examined, cells sensitized with mouse anti-DNP IgE were stimulated with bovine γ globulin to which an average of 15 DNP groups per molecule had been coupled (DNP antigen) as described (Farah et al., 1960). The exact details of the various triggering experiments are given under Results, but in all cases the concentrations of DNP antigen used were those that elicit a maximal secretory response in RBL cells, i.e., between 30 and 40% release of incorporated [^{3}H]serotonin (Fewtrell & Metzger, 1981).

Solubilization of Cells. Cells were solubilized by suspension at $(5–10) \times 10^7$ cells/mL for 20 min at 4 °C in 0.5% Triton X-100 in borate-buffered saline (0.2 M $NaBO_3$, 0.16 M NaCl) containing the protease inhibitors aprotinin (1 trypsin inhibitor unit per mL) and phenylmethanesulfonyl fluoride (1 mM) and in some experiments pepstatin (10 μg/mL) and leupeptin (10 μg/mL) (Holowka & Metzger, 1982). This buffer also contained the kinase and phosphatase inhibitors sodium fluoride (50 mM), sodium pyrophosphate (5 mM), and ethylenediaminetetraacetic acid (2 mM). These have been shown by Chaplin and his colleagues (1980) to inhibit further phosphorylation or dephosphorylation of lymphocyte proteins after solubilization of the cells. This buffer was adjusted to pH 8 except where cross-linking was planned when pH 8.5 was used.

After solubilization the suspension was diluted with borate-buffered saline containing the kinase and phosphatase inhibitors to give a final Triton X-100 concentration of 0.25%. The suspension was spun at 25000*g* for 1 h at 4 °C and the pellet discarded.

Chemical Cross-Linking. Cross-linking of soluble cell extracts with the cleavable cross-linking reagent dimethyl 3,3'-dithiobis(propionimidate) (DTBP; Pierce Chemical Co., Rockford, IL) was carried out as previously described (Holowka et al., 1980).

Purification of IgE–Receptor Complexes. In the majority of experiments, receptors for IgE complexed with rat or mouse IgE were purified as previously described (Holowka et al., 1980) by immunoprecipitation with anti-rat IgE or anti-mouse IgE and protein A containing *Staphylococcus aureus* (Pansorbin, Calbiochem, LaJolla, CA, or IgGSORB, The Enzyme Center Inc., Boston, MA). In most cases a "clearing" precipitation of human IgE—which does not bind to the rat receptor—and anti-human IgE plus protein A containing *Staph. aureus* was carried out prior to the immunoprecipitation with anti-rat IgE or anti-mouse IgE.

Staph. aureus immune precipitates were washed 3 times with 0.25% Triton X-100 in borate-buffered saline containing protease, kinase, and phosphatase inhibitors and once with borate-buffered saline containing kinase and phosphatase inhibitors alone. In a few later experiments the following washing procedure was used (M. Owen, personal communication): (first wash) 1% Nonidet P40, 1 mM EDTA, 0.5 M NaCl, and 1 mM phenylmethanesulfonyl fluoride in 10 mM Tris-HCl, pH 7.4; (second wash) as for first wash but 0.15 M NaCl + 0.1% $NaDodSO_4$; (third wash) 0.1% Nonidet P40 in 10 mM Tris-HCl, pH 7.4.

In a few experiments chemically cross-linked receptors labeled with amidinated, benzene arsonylated ^{125}I-labeled mouse anti-DNP IgE were purified by sequential affinity chromatography prior to the usual immunoprecipitation steps. Cell extracts containing cross-linked IgE–receptor complexes were mixed with agarose beads [derivatized with trinitrophenyllysine which had been prepared as previously described (Holowka & Metzger, 1982)]. The beads were then washed with 100 volumes of 0.25% Triton X-100 in borate-buffered saline, pH 8.5, containing kinase and phosphatase inhibitors and protease inhibitors at half the concentration used for cell solubilization. Millimolar amounts of N^{ϵ}-DNP-lysine were used to elute the bound material. The eluate was then exposed to anti-benzenearsonate antibodies coupled to agarose beads as described (Kanellopoulos et al., 1979). Elution was carried out with 10 mM (*p*-arsonophenyl)azotyrosine after the beads had been washed as described above.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Staph. aureus adsorbed immune precipitates were dissolved in 2% sodium dodecyl sulfate ($NaDodSO_4$) in 0.08 M Tris-HCl, pH 8, containing 10% glycerol and incubated in a boiling water bath for 2 min. When samples were to be reduced, 2-mercaptoethanol (10% final) was added prior to heating.

Slab gels prepared by using the method of Laemmli (1970) were run and processed as described previously (Holowka et al., 1980). Autoradiography of dried gel slabs was done at −80 °C by using intensifying screens (Du Pont, Wilmington, DE) and Kodak X-Omatic AR film. Autoradiographs were scanned by using a Zeineh soft laser scanning densitometer (LKB Instruments, Inc., Rockville, MD). For liquid scintillation counting, dry gel slices 2 mm wide were rehydrated in 120 μL of water and incubated overnight in 1 mL of NCS tissue solubilizer (Amersham, Arlington Heights, IL) before 18 mL of complete counting cocktail 3a20 was added (Research Products International Corp., Elk Grove Village, IL). ^{32}P was counted in the ^{32}P channel of a Tracor Analytic (Elk Grove Village, IL) Mark III liquid scintillation counter and [^{125}I]IgE in a Beckman (Fullerton, CA) γ 8000 counter. Counts were corrected for double crossover and isotope decay during counting.

Identification of the Phosphorylated Amino Acid. Appropriate regions of the dried slab gels were excised and extracted with 0.1% $NaDodSO_4$ in Tris–glycine buffer, pH 7,

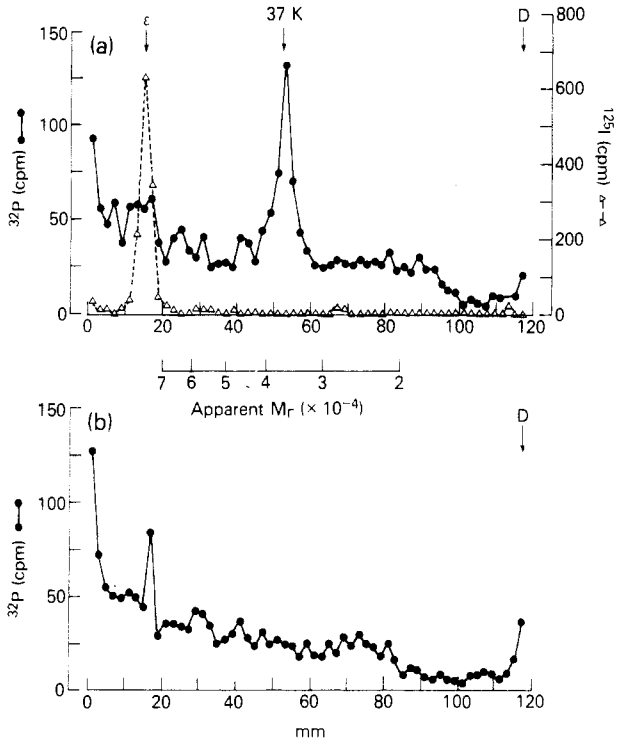

FIGURE 1: $NaDodSO_4$ gel electrophoresis on a 12.5% gel of material immunoprecipitated with anti-mouse IgE from extracts of ^{32}P-labeled RBL cells. Samples were reduced prior to electrophoresis. (a) Cells sensitized with ^{125}I-labeled mouse IgE. The position of the heavy chain of IgE is indicated (ϵ); the light chain was not labeled significantly with ^{125}I (see Materials and Methods). (b) Unsensitized cells. In this and subsequent figures the apparent molecular weight calibrations were determined by using standard proteins run on the same gel. The abscissa also shows the distance in millimeters (mm) migrated in the resolving gel, and D indicates the migration of the tracking dye, bromophenol blue.

over 2 days. The extract was made 0.025% in deoxycholate, 6 μg/mL in carrier human IgG, and 6% in trichloroacetic acid. The solutions were centrifuged at 800g for 20 min, the supernatants removed, and the dried pellets hydrolyzed with 0.1 mL of 6 N HCl under vacuum at 106 °C for 2 h. After removal of the HCl by lyophilization, the samples were dissolved in a standard pH 3.5 pyridine–acetate buffer containing approximately 50 nmol each of *O*-phosphoserine, *O*-phosphothreonine, and *O*-phosphotyrosine; the latter was a gift from Dr. S. Cohen (Vanderbilt University, Nashville, TN). Samples were spotted on 20-cm silica gel 1B flexible sheets (J. T. Baker Chemical Co., Phillipsburg, NJ) and electrophoresed with a standard pH 3.5 pyridine–acetate buffer for ~40 min at 1000 V (Ushiro & Cohen, 1980). The strips were stained with ninhydrin, the positions of the standards were circled, and the strip was autoradiographed.

Results

Phosphorylation of the β Chain of the Receptor for IgE. When immune precipitates from ^{32}P-labeled RBL cells were run on $NaDodSO_4$ gel electrophoresis, a phosphoprotein with a molecular weight in the region of 35 000–37 000 was reproducibly seen (Figure 1a). The same labeled component was observed whether the cells had been sensitized with mouse or with rat IgE and the immune precipitates prepared with the homologous anti-IgE. It was not precipitated from cells that had not been sensitized with IgE (Figure 1b) nor was it seen in control precipitates in which human IgE (which does not bind to rat receptors for IgE) was added to the cell extract and precipitated with anti-human IgE (data not shown). This clearly suggests that the M_r ~35 000 phosphoprotein is closely associated with bound IgE on RBL cells and its molecular weight is consistent with its being the β chain of the receptor for IgE (Holowka & Metzger, 1982). In no experiment were we able to detect any phosphorylation of cell-bound IgE or of the M_r ~50 000 α chain of the receptor which is responsible for binding IgE (Figure 1a).

A number of experiments provided further evidence that the M_r ~35 000 phosphoprotein is the β chain of the receptor for IgE. Figure 2 shows that this phosphoprotein comigrates on $NaDodSO_4$ gels with the purified β chain of the receptor which had been labeled in situ in the RBL cell membrane with the photolyzable hydrophobic reagent 5-[^{125}I]iodonaphthyl 1-azide (Holowka et al., 1981).

This gel also highlights a problem that we encountered in many of our initial experiments and to a lesser extent in later experiments. This was that while $NaDodSO_4$ gels of the specific immune precipitates invariably showed a M_r ~35 000 phosphoprotein, it was often accompanied by other ^{32}P-labeled

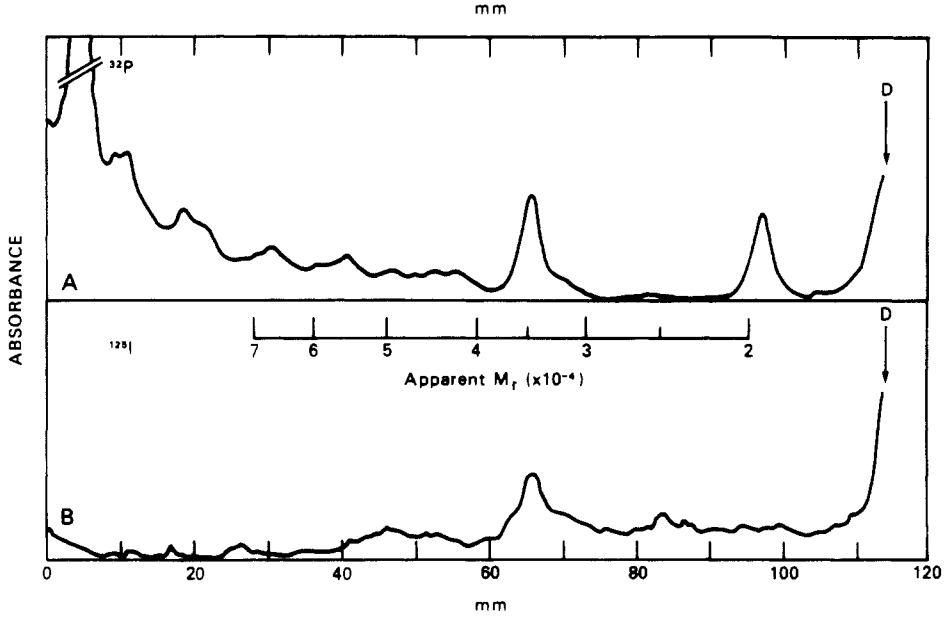

FIGURE 2: $NaDodSO_4$ gel electrophoresis (12.5% gel) of anti-IgE immune precipitates from detergent extracts of sensitized RBL cells. (A) ^{32}P-Labeled cells; (B) [^{125}I]iodonaphthyl azide labeled cells. Samples were reduced. Densitometric scans of the autoradiographs are shown.

bands. The amounts and molecular weights of these additional components varied considerably from experiment to experiment. One exception was a component with an M_r in the region of 20000 which was frequently seen in specific immune precipitates (e.g., Figures 2 and 5a,b). It seems likely that this represents contamination by a phosphoprotein that is particularly abundant in RBL cells since this component was also observed in the clearing precipitates (human IgE + anti-human IgE) and it appeared to be the major phosphoprotein band seen when the whole cell extract was run on $NaDodSO_4$ gels (data not shown). These additional components were largely eliminated (see Figure 1a) by increasing the length of time and the amount of *Staph. aureus* used in the clearing precipitation, without affecting the amount of phosphorylated β chain subsequently precipitated.

The M_r ~35000 component was the only phosphorylated species seen when a Triton X-100 extract of ^{32}P-labeled RBL cells sensitized with azobenzenearsonate-conjugated rat IgE was exposed to anti-benzenearsonate antibodies coupled to agarose beads (see Materials and Methods). After being extensively washed, the material bound to the beads was eluted with 10 mM (*p*-arsonophenyl)azotyrosine. An anti-rat IgE immune precipitate from this eluate gave a single phosphorylated species of M_r ~35000, but the yield was very small. This is consistent with the finding (Holowka & Metzger, 1982; J. M. Kanellopoulos, A. Goetze, S. Wank, and H. Metzger, unpublished observations) that in the absence of cross-linking (see below) the β chain of the receptor for IgE becomes dissociated from the α chain when affinity columns to which the IgE–receptor complexes are bound are washed extensively.

Chemical Cross-Linking Experiments. A final unambiguous demonstration that the M_r ~35000 phosphoprotein was indeed the β chain of the receptor for IgE came from chemical cross-linking experiments similar to those first used to demonstrate the existence of the β chain (Holowka et al., 1980). Holowka and his colleagues showed that when RBL cells or Triton X-100 extracts from these cells were exposed to the cleavable cross-linking reagent 3,3′-dithiobis(propionimidate) (DTBP) the M_r 50000–55000 IgE binding component of the receptor for IgE became cross-linked to a protein with an M_r of ~30000–35000. Subsequent reduction of the M_r ~90000 cross-linked species led to the recovery of the two original proteins. The tight association and 1:1 stoichiometry between this M_r 30000–35000 protein [originally termed $X_{\epsilon R}$ (Holowka et al., 1980) and subsequently β (Holowka & Metzger, 1982)] and the IgE binding component (α) led to the suggestion that β was a subunit of the receptor for IgE and not simply a copurified contaminant (Holowka et al., 1980, 1981; Kanellopoulos et al., 1980; Holowka & Metzger, 1982).

When detergent extracts of ^{32}P-labeled RBL cells were exposed to the cleavable cross-linking reagent DTBP, the M_r 35000 phosphoprotein behaved exactly like the β chain of the receptor. Immune precipitates from cross-linked extracts showed a new band with M_r ~95000 (Figure 3, lane b) in place of the M_r ~35000 band seen in the absence of DTBP (Figure 3, lane a). The M_r ~95000 band disappeared after reduction, and the M_r ~35000 component reappeared (Figure 3, lane f; cf. lanes e and f). We again had problems with additional phosphoproteins appearing in our specific immune precipitates, but these were also seen in the corresponding controls (lanes c, d, g, and h) and are therefore most unlikely to be related to the receptor for IgE.

Extensive purification of chemically cross-linked IgE–receptor complexes using two affinity purifications followed by a clearing precipitation and finally immune precipitation with

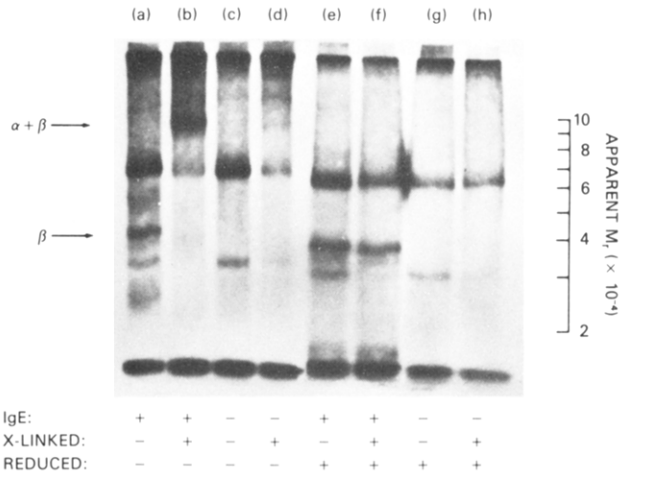

FIGURE 3: Effect of the cleavable cross-linking reagent DTBP on the ^{32}P-labeled component of the receptor for IgE. The autoradiograph shows the distribution of ^{32}P after $NaDodSO_4$ gel electrophoresis of immune precipitates from ^{32}P-labeled RBL cells. (a) Un-cross-linked soluble extract from cells sensitized with amidinated IgE; (b) cross-linked extract from sensitized cells; (c) control precipitate from an un-cross-linked extract of cells that had not been sensitized with IgE; (d) control precipitate from a cross-linked extract of unsensitized cells; (e–h) as for (a–d) but after reduction with β-mercaptoethanol. The unreduced (a–d) and reduced (e–h) samples were run at the same time but on separate 10% gels.

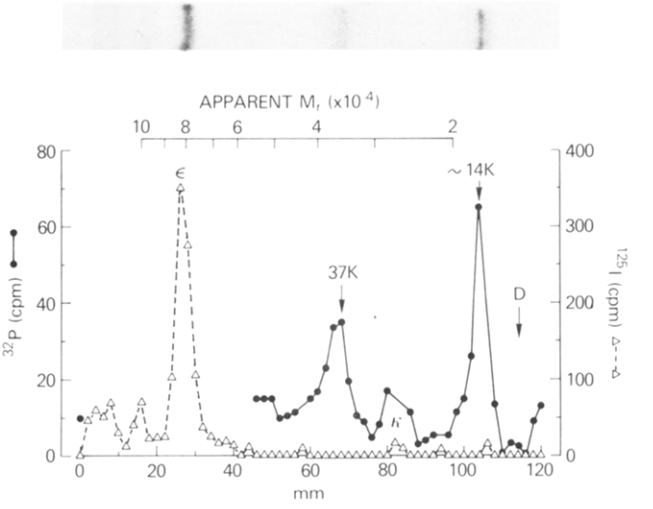

FIGURE 4: $NaDodSO_4$ gel electrophoresis (12.5% gel) under reducing conditions of affinity purified and immune precipitated receptors for IgE which had been exposed to the cross-linking reagent DTBP after solubilization. Soluble extracts were from ^{32}P-labeled cells sensitized with ^{125}I-labeled mouse anti-DNP IgE. The latter had been benzene arsonylated and amidinated. The positions of the light (κ) and heavy (ϵ) chains of IgE are indicated. We were unable to determine the ^{32}P counts in the regions of the gel where the [^{125}I]IgE migrated because the ^{125}I crossover was too large. However, it is clear from the autoradiograph (which detects ^{32}P much more efficiently than ^{125}I) that there are no ^{32}P-labeled bands in the first 45 mm of the gel.

anti-IgE (see Materials and Methods) gave much cleaner gel patterns. As expected reduction of the cross-linked material generated the M_r 37000 β chain, but in addition a much smaller phosphoprotein was seen (Figure 4) which had an apparent M_r identical with that of the molecular weight marker lysozyme (M_r 14300). This component did not appear to be a contaminant since it was also seen in other experiments in which cross-linked IgE–receptor complexes were extensively purified.

More significantly this M_r 14000 component was recovered together with the M_r 37000 phosphoprotein when the M_r

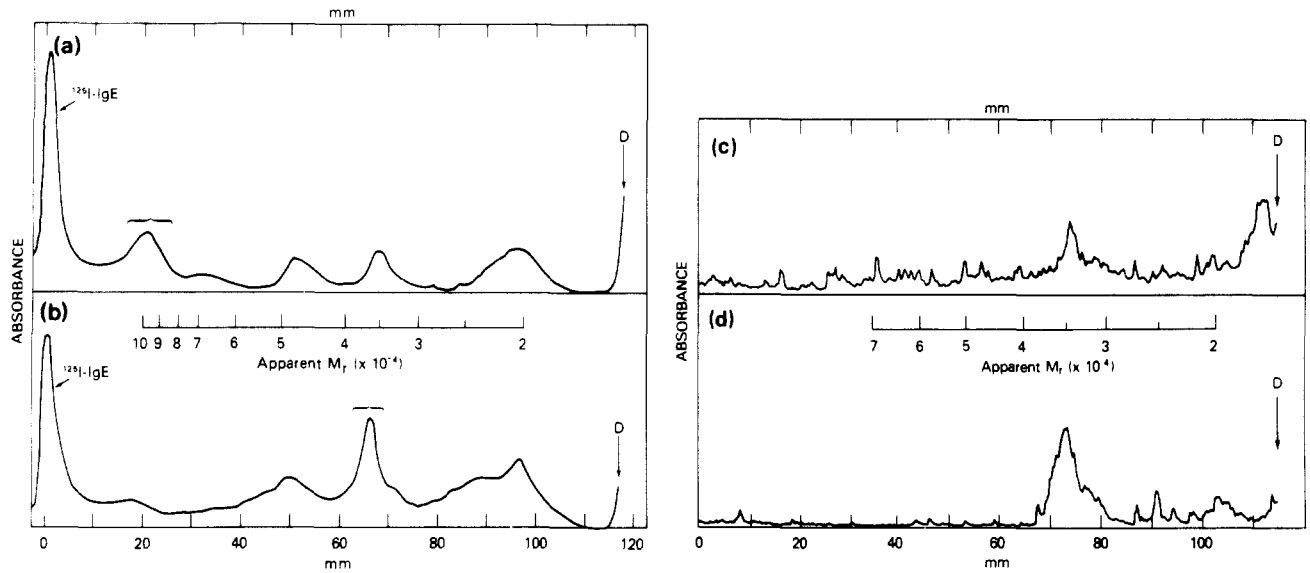

FIGURE 5: $NaDodSO_4$ gel electrophoresis (10% gels) of immune precipitates from DTBP cross-linked (a) and un-cross-linked (b) extracts of ^{32}P-labeled RBL cells which had been sensitized with amidinated ^{125}I-labeled mouse IgE. Samples were unreduced. (c) Second-dimensional electrophoresis of the material eluted from the M_r ~95 000 region (indicated by the bracket) of gel a after reduction and (d) the material eluted from the M_r 37 000 region of gel b after reduction. Densitometric scans of the autoradiographs are shown.

95 000 cross-linked complex (Figure 5a) was eluted from a gel and rerun after reduction (Figure 5c). This figure also shows that the M_r 37 000 component obtained after reduction of the cross-linked species comigrates with un-cross-linked ^{32}P-labeled β chain (cf. Figure 5c,d) similarly eluted from a gel (Figure 5b).

These results conclusively showed that the two phosphorylated proteins were derived from a cross-linked species whose size is consistent with a 1:1 complex between the α and β chains of the receptor for IgE. This raised the interesting possibility that the M_r 14 000 phosphoprotein is the proteolytic fragment or domain of the β chain termed β_2 (Holowka & Metzger, 1982; Metzger et al., 1982).

Identification of the Phosphorylated Amino Acid Residue. In order to determine the nature of the amino acid residue that had been phosphorylated, we isolated the M_r ~35 000 phosphoprotein band from a $NaDodSO_4$ slab gel analysis of the immune precipitate derived from an extract of ^{32}P-labeled cells. On high-voltage electrophoresis of the 2-h acid hydrolysate of this material, the ^{32}P traveled with the *O*-phosphoserine standard and was well separated from the *O*-phosphothreonine and *O*-phosphotyrosine standards which migrate more slowly under these conditions.

Effect of IgE on Receptor Phosphorylation. When RBL cells were incubated with ^{32}P before or after sensitization with IgE, any effect of cell-bound IgE on the pattern of phosphorylation could be assessed. In addition such an experiment can potentially clarify whether phosphorylation occurs prior to or after the insertion of the receptor into the plasma membrane. Figure 1a shows that only a single phosphorylated species was precipitated by anti-IgE from cells whose receptors had been saturated with ^{125}I-labeled IgE before labeling with ^{32}P. An identical pattern was observed when [^{125}I]IgE labeling was carried out after the cells had been exposed to ^{32}P (data not shown), and the ratio of ^{32}P counts recovered in the M_r ~35 000 band to ^{125}I counts in the ϵ chain of IgE seen on $NaDodSO_4$ gels was not significantly different in the two cases (Table I).

Effect of Cell Activation. We tried, in a number of different experiments, to detect changes in the state of phosphorylation of the β chain of the receptor for IgE which might occur on stimulation. Cells were sensitized with [^{125}I]IgE so that we would have an estimate of receptor recovery in our immune precipitates. In initial experiments in which the cells were triggered with anti-IgE, we failed to solubilize the majority of the receptors (as measured by the recovery of [^{125}I]IgE) from cells that had been stimulated. This was presumably due to the formation of large immune complexes which sedimented with the nuclear debris after solubilization of the cells. When cells were sensitized with ^{125}I-labeled mouse anti-DNP IgE and then stimulated with the multivalent DNP antigen, almost complete receptor solubilization was obtained over a wide range of antigen concentrations (0.02–10 μg/mL).

Table I: Effect of IgE Binding on the Phosphorylation of the β Chain of the Receptor

^{32}P labeling	recovery (cpm)[a] [^{125}I]IgE	^{32}P-labeled β chain	ratio ^{32}P:^{125}I
after sensitization	1247	230	0.18
before sensitization	1110	156	0.14

[a] Cells were labeled with ^{32}P before or after sensitization with [^{125}I]IgE and solubilized as usual, and the anti-IgE immune precipitates were analyzed on $NaDodSO_4$-polyacrylamide gel electrophoresis. The counts shown are those recovered in the regions of the gel (see Figure 1a) corresponding to the ϵ chain of IgE ([^{125}I]IgE) and the M_r ~35 000 phosphoprotein (^{32}P-labeled β chain).

A number of different conditions for triggering were explored. Antigen concentrations of 1 and 10 μg/mL were tested; both of these cause maximal secretion of [^{3}H]serotonin (unpublished observations). These concentrations of antigen represent respectively an approximately 5- and 50-fold excess of hapten groups over IgE antigen combining sites on the cells and a molar concentration approximating the K_D of the antibody for the monovalent ligand (Liu et al., 1980), so a substantial fraction of the receptors should have been triggered under these conditions. The length of preincubation of the cells with ^{32}P prior to triggering was varied (5–60 min), as was the period of triggering (10–120 min), and in one experiment the cells were triggered 10 min before they were exposed to ^{32}P.

The effects of triggering were assessed by comparing the ratio of ^{32}P in the M_r ~35 000 band to ^{125}I in the ϵ chain of

IgE seen on $NaDodSO_4$ gels of immune precipitates from extracts of stimulated and unstimulated cells labeled with ^{32}P. This was done qualitatively after autoradiography of the gels or quantitatively by scintillation and γ counting of gel slices. In no case did we see a significant change in the phosphorylation of the β chain on stimulation. However, because of the complexity of the experiments and the very low levels of ^{32}P associated with the M_r ~35000 band, we would probably not have detected a change of 15% or less in the amount of ^{32}P associated with this protein after triggering. These constraints also set severe practical limits on the number of different protocols that could be tested.

Although we failed to observe changes in the state of phosphorylation of the β chain on triggering, we regularly saw a substantial increase in ^{32}P associated with the M_r ~20000 contaminant that was often present in our control and specific immune precipitates (see Figures 2 and 5a,b).

Discussion

Our results demonstrate that the M_r ~35000 β chain of the receptor for IgE is a phosphoprotein. The evidence for this is as follows: (a) specific precipitation with the corresponding anti-IgE of a M_r ~35000 phosphoprotein from ^{32}P-labeled RBL cells sensitized with rat or mouse IgE; (b) absence of this protein in anti-human IgE immune precipitates; (c) failure to precipitate this protein with anti-IgE using cells which had not been sensitized with IgE; (d) comigration on $NaDodSO_4$ gel electrophoresis of the M_r ~35000 phosphoprotein with [^{125}I]iodonaphthyl azide labeled β chain of the receptor for IgE; (e) conversion of this phosphoprotein to a M_r ~95000 species after cross-linking, consistent with a 1:1 complex between the α and β chains of the receptor for IgE; (f) recovery of a M_r ~35000 phosphoprotein after reduction of the cross-linked M_r 95000 species.

An interesting finding was that reduction of the M_r ~95000 cross-linked complex also led to the recovery of a small phosphoprotein in addition to the intact β chain of the receptor. This protein, which migrated with lysozyme (M_r 14300) on $NaDodSO_4$ gel electrophoresis, was not seen in the absence of cross-linking. Although it is difficult to formally exclude the possibility that this protein is nonspecifically cross-linked to the α–β complex, the fact that it was always seen (in varying amounts) after reduction of this species makes this unlikely. The apparent M_r of the cross-linked complex (95000) is smaller than would be expected if it contains three separate proteins with molecular weights in the region of 55000, 35000, and 14000. A more attractive possibility is that the M_r ~14000 phosphoprotein represents a fragment or domain of the β chain. Previous studies suggested that the β chain consists of two regions (Holowka & Metzger, 1982) denoted β_1 and β_2 (Metzger et al., 1982). This was based on the finding that in the absence of suitable protease inhibitors, the polypeptide which was found to be labeled with iodonaphthyl azide and cross-linked to the α chain had an M_r of ~20000 rather than 35000 (Holowka & Metzger, 1982). This 20000-dalton fragment was dubbed β_1. It was inferred that the remaining 15000 daltons (called β_2) must be exposed to cellular proteases on the inner aspect of the plasma membrane since there was no evidence for exposure of any portion of the β chain on the external face of the plasma membrane (Holowka et al., 1980; Holowka & Metzger, 1982).

The finding of an M_r ~14000 phosphoprotein after reduction of cross-linked complexes of the receptor for IgE provides the first direct evidence for this β_2 domain of the receptor as a discrete entity. In a few experiments where the purification procedure was particularly lengthy (>1 week), very little intact β chain was seen after reduction of the M_r ~95000 cross-linked receptor complex. In these experiments the major species after reduction was the M_r ~14000 phosphoprotein. This is consistent with the gradual cleavage of the β chain into its constituent domains β_1 (which is not phosphorylated and is therefore not visible) and β_2, the M_r ~14000 phosphoprotein.

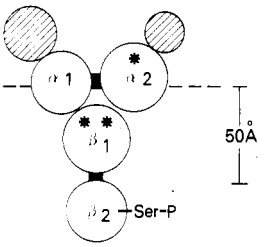

FIGURE 6: Schematic representation of the receptor for IgE. This figure, a modified version of the preferred model (A) shown in Figure 9 of Goetze et al. (1981), shows the presumptive location of the *O*-phosphoserine residue(s). As in the original figure, the horizontal line represents the surface of the outer leaflet of the plasma membrane bilayer; the hatched areas represent carbohydrate; the black areas represent sites of high susceptibility to proteolysis; the single asterisk represents the principal site of surface labeling; the double asterisk represents the site of labeling by iodonaphthyl azide; the circles represent spheres whose volumes are proportional to their masses.

This phosphoprotein was not seen in the absence of cross-linking: after cleavage of the β chain the β_2 fragment appears to be rapidly lost unless its association with the IgE–receptor complex is stabilized by cross-linking. This suggests that β_2 interacts poorly, if at all, with the α chain of the receptor and does not interact closely with the β_1 domain after cleavage. This is consistent with the previously proposed model (Holowka & Metzger, 1982; Goetze et al., 1981; Metzger et al., 1982) in which β_1 is a hydrophobic domain residing in the plasma membrane and interacting with the α chain of the receptor while β_2 is a hydrophilic region which is thought to be exposed on the cytoplasmic face of the plasma membrane (Figure 6).

The role played by β-chain phosphorylation is not clear. Our finding that ^{32}P-labeled β chains can be immune precipitated from extracts of cells which had been labeled with IgE before they were exposed to ^{32}P suggests that phosphorylation can occur after the receptor has been inserted into the plasma membrane and has bound IgE. In addition, since the yield of ^{32}P-labeled β chain was no greater when cells were labeled with ^{32}P before they were exposed to IgE, it is unlikely that a substantial amount of this phosphorylation occurs during receptor synthesis and processing. It also appears that the binding of IgE to the α chain of the receptor has no effect on β chain phosphorylation. These conclusions are based on the assumption that IgE and the α and β chains of the receptor form a tight complex and are not free to exchange within the membrane. This assumption seems reasonable in view of the very high affinity of IgE for the α chain (Rossi et al., 1977) and in turn that of α for β (Holowka & Metzger, 1982). Amino acid analysis showed that phosphorylation occurs at a serine residue (or residues) in the β chain, and since this residue appears to be located in the β_2 region of the molecule, it should be readily accessible to cytoplasmic protein kinases.

A role for receptor phosphorylation in cell activation is obviously an attractive possibility, and some preliminary evidence for this has been obtained with the acetylcholine receptor (Gordon et al., 1977a,b; Teichberg et al., 1977). However, we have so far been unable to detect a change in the state of phosphorylation of either chain of the receptor for IgE after antigen-induced activation of RBL cells. On the other hand, we regularly saw an increase in ^{32}P associated with

an $M_r \sim 20000$ protein in our control precipitates, so clearly changes in ^{32}P incorporation do occur on stimulation. It is interesting to note that this $M_r \sim 20000$ protein is similar in size to myosin light chain which is one of several platelet proteins whose state of phosphorylation is increased during platelet activation (Daniel et al., 1977, 1981).

It has been reported that the α chain of the receptor for IgE in normal rat mast cells is a phosphoprotein, and a marked increase in its state of phosphorylation was observed after the cells had been exposed to the divalent cation ionophore A23187 (Hempstead et al., 1981). However, the role of receptor phosphorylation in ionophore-induced cell activation is not obvious, and it is possible that an unusually high cytoplasmic Ca^{2+} concentration in ionophore-treated cells could result in nonphysiological Ca^{2+}-activated protein phosphorylation. In contrast to these findings with normal mast cells, we have consistently failed to detect any phosphorylation of the α chain of the receptor in either resting or antigen-stimulated RBL cells, but we have not looked at the effects of A23187.

In the studies with normal mast cells (Hempstead et al., 1981), no phosphorylated β chain of the receptor for IgE was seen. However, it is clear that under the conditions used in those experiments, which involved repeated affinity purifications (Hempstead et al., 1981), the β chain would almost certainly have been lost (Holowka & Metzger, 1982).

Clearly, further experiments are required to resolve these apparent differences and to determine whether changes in the state of phosphorylation of the receptor for IgE play a role in the initiation of secretion from both normal mast cells, basophils, and the tumor cells. However, until the yields of ^{32}P-labeled components of the receptor for IgE can be improved, such studies will be difficult given the practical limitations on the amount of ^{32}P and the number of cells that can be used in a single experiment.

Acknowledgments

We thank Dr. David Holowka for providing us with a sample of [^{125}I]iodonaphthyl azide labeled β chain. We also thank him for many stimulating discussions and much useful advice. George Poy provided excellent technical assistance. Dr. S. Cohen (Vanderbilt University, Nashville, TN) kindly provided a sample of *O*-phosphotyrosine.

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