Reconstitution of the Receptor for Immunoglobulin E into Liposomes

REINCORPORATION OF PURIFIED RECEPTORS*

Benjamin Rivnay‡§, Guido Rossi‡§, Maryanne Henkart¶, and Henry Metzger‡

From the §Section on Chemical Immunology, Arthritis and Rheumatism Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases and ¶Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Mast cells and related cells have on their surface receptors that bind immunoglobulin E (IgE) with high affinity and which, when aggregated, trigger exocytosis. We recently demonstrated that when these receptors are solubilized with mild detergents, their subunits dissociate unless an appropriate lipid:detergent ratio is maintained. The conditions required to maintain the receptors’ integrity appeared to parallel those previously determined as necessary to obtain adequate incorporation of unpurified IgE-receptor complexes from detergent extracts into liposomes. We now show that purified IgE-receptor complexes having the full complement of subunits become preferentially inserted into liposomes. If the receptor subunits are chemically cross-linked to each other, at least some of such receptor complexes can be incorporated, even though lipid is omitted during their purification. The findings suggest that the IgE-binding α subunit of the receptor is anchored to the bilayer by means of one or both of the other subunits.

The initial event in the degranulation of mast cells and basophils that is mediated by IgE\(^1\) involves aggregation of the receptors for IgE on the plasma membrane of such cells (1). In order to study the early reactions associated with activation under well defined conditions, we are attempting to prepare liposomes that individually contain multiple copies of native, purified receptors. We have employed a tumor analog of normal cells, an RBL cell line, as the source for the receptors (2).

Our previous studies (3) using unfractionated detergent extracts of RBL cells showed that the efficiency of incorporation of receptors into liposomes varied when different detergents and lipids were used. Furthermore, the extent of incorporation was sensitive to the micellar ratio of detergent to phospholipid, designated \(\rho\) (3). In parallel studies, we found that in order to preserve the subunit structure of the receptor, conditions similar to those found to be effective for its incorporation were required (4). In that study, we observed two new components of 45 and 20 kDa, respectively, in addition to the previously described \(\alpha\) and \(\beta\) subunits. Subsequent analysis has shown that the 20-kDa component is a disulfide-linked dimer of a 10-kDa polypeptide that has the properties of an additional subunit (\(\gamma\)) of the receptor (5). The 45-kDa component proved to be a complex that upon reduction yielded intact \(\beta\) and \(\gamma\) chains and that appears to be variably generated by aggregation of the receptors (6).

In the present work, we have attempted to incorporate purified receptors into liposomes and have examined the subunit composition of the material that became inserted.

MATERIALS AND METHODS

Proteins—Rat IgE and mouse anti-dinitrophenyl IgE were purified from ascitic fluids of the tumor IR 162 (7) and the hybridoma H1.2.A.4 (8). Rabbit anti-mouse IgE and anti-rat IgE were purified as previously described (11). Amidi- nated IgE, used in the experiments with cross-linking reagents, and IgE conjugated with azobenzene-sorosaneate groups were prepared as described previously (10, 12).

Lipids—Phospholipids were prepared from frozen RBL tumors or rabbit livers and quantitated as reported previously (3), \(\alpha\)-I-Phos-photidylcholine (lecithin) type IV-S from soybean was purchased from Sigma and was used without further purification.

Preparation of IgE-Receptor Complexes—Receptors were purified from the 2H3 subline (13) of RBL cells. Tumors were grown for 6–8 days in WKY-Nweanling rats by injecting \(10^7\) washed, tissue cultured cells intraperitoneally (12), and a membrane preparation was obtained as follows.\(^1\) The minced tumors were homogenized in the presence of 1 \(\mu\)g/ml of leupeptin (Boehringer-Mannheim), 3 \(\mu\)g/ml of pepstatin A (Sigma), and 0.3–0.7 trypsin inhibitor-units/ml of apro tinin (Sigma) in a Dounce homogenizer (A pestle) by three to four strokes in isotonic sucrose/Tris solution (0.25 M sucrose and 10 mM Tris-HCl, pH 7.4) followed by three more strokes with a B pestle. The homogenate was centrifuged for 5 min at 12,000 \(\times g\), and the supernatant microsomal fraction was then pelleted by centrifugation for 70 min at 48,000 \(\times g\). The pellet was resuspended, washed, and used within 24 h. The receptors were solubilized with IgE at 4°C and the microsomes were washed by three 1-h centrifugations in an SW 50.1 rotor, through 5% Ficoll in sucrose/Tris at 45,000 rpm in an L5-50 or L8-70 ultracentrifuge (Beckman). The pellets were homogenized briefly between and after the centrifugations.

Cultured RBL cells were prepared as described (14). Cells were routinely used 10 days after transfer, with one change of medium 2–3 days prior to harvesting. Cells were harvested with trypsin (M. A. Bioproducts, Walkersville, MD), washed twice in Tris-saline (15 mM Tris-HCl, pH 7.4, 124 mM NaCl, and 5 mM KCl), saturated with IgE, and used after washing as such or after preparation of a crude membrane fraction following sonication under hypotonic conditions (3).

Membranes or cells were solubilized at a micellar ratio of detergent: phospholipids of 1.6 \(\leq \rho \leq 2.5\) by mixing a preparation containing 2–8 mM phospholipid with appropriate amounts of the zwitterionic detergent CHAPS and incubating the mixture 1 h on ice. Soluble receptors were recovered from the supernatants after centrifugation for 1 h at 31,000 \(\times g\). The parameter \(\rho\) was calculated using 4.7 \(\mu\)M for the molar concentration of the detergent (3) i.e. \(\rho = ([\text{detergent}] - 4.7)/(3.7)\).

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‡ Present address, Weizmann Institute of Science, Rehovot, Israel.
§ Present address, Laboratorio di Patologia Generale, Universita di Napoli, 2° Facolta di Medicina E Chirurgia, Naples, Italy.
¶ The abbreviations used are: IgE, immunoglobulin E; RBL, rat basophilic leukemia; CHAPS, 3-[(3-cholamidopropyl)dimethylam monio]-1-propanesulfonate.

B. Kanner, personal communication.
[phospholipid] where the concentrations are millimolar. Phospholipid phosphate was measured in membranes prepared from tumors or broken cells; an approximation of 2 mM phospholipid for 5 x 10^7 cells/ml was used for suspensions of intact cells (3).

Purification of receptors to which arsonylated IgE was bound was performed on affinity columns conjugated with purified anti-benzensearsonate antibodies (12). Partial purification of receptors bearing arsonylated anti-dinitrophenyl mouse IgE was performed on columns of Sepharose conjugated with trinitrophenyllysine (10).

**Incorporation of Receptors into Liposomes—** Incorporation of receptors was achieved by raising the lipid concentration in the preparation of purified receptors with presonicated liposomes to 8-10 mM phospholipid, adding detergent to reach the desired value of ρ, and incubating for 1-2 h on ice. Mixtures were then dialyzed against Tris buffer containing 1% Triton X-100.

**RESULTS**

Receptors complexed with arsonylated IgE were partially purified maintaining a detergent-phospholipid micellar ratio of ~2. They were iodinated and then divided into two portions. Both were further purified on columns conjugated with anti-benzensearsonate antibodies, one in the presence of phospholipids, the other with a solvent containing detergent only.

Fig. 1 shows an autoradiogram of a polyacrylamide gel on which the purified receptors were analyzed. The material isolated in the absence of lipids (lane 1) shows the IgE near the origin (as well as a high molecular weight breakdown product of IgE) and a weakly iodinated α chain appearing as a diffuse band at ~60 kDa. Except for some material running ahead of the buffer front (likely representing labeled lipids), only traces of additional bands were seen. On the other hand, the material isolated in the presence of phospholipids (lane 2) shows a prominent doublet at 33 kDa and 34 kDa, representing the β subunit, and the disulfide-linked dimer of γ chains at 20 kDa (5).

The patterns obtained by immunoprecipitating the eluates with anti-IgE (lanes 3 and 4) are virtually identical with those seen by examining the eluates directly. The immunoprecipitates from both eluates contain the α chains along with the IgE, indicating that the former are still bound to the latter. With the material containing the β and γ chains, these are also precipitated, demonstrating that they are still associated with the α chains. A quantitative analysis of the material in lanes 3 and 4 is given in Table I. We have described elsewhere that upon immunoprecipitation, a 45-kDa component representing a disulfide-linked complex of β and γ chains is variably generated (6). As can be seen in Fig. 1, none of this component appeared in these immunoprecipitates although a subsequent immunoprecipitate of the same material did (below).

**TABLE I**

| Specimen | Preparation | IgE α chain | 45-kDa component | β chain | γ chain dimer |
|----------|-------------|-------------|-----------------|---------|--------------|
| 1        | Column eluate (- lipid) | 31 | 0.38 | 0.34 |
| 2        | Column eluate (+ lipid) | 26 | 2.9 | 3.3 |
| 3        | Gradient fraction (heavy) | 42 | 0.13 | 0.005 | 0.04 |
| 4        | Gradient fraction (light) | 19 | 2.4 | 3.1 |

*Appropriate sections of the gel were excised and the ^125^I counts compared relative to those obtained in the band formed by the α chains.

*That this value is somewhat lower than the value for IgE in specimen 2 is not surprising since the eluate might be expected to contain some IgE from which the receptor had dissociated. Such IgE would not be appreciably incorporated into liposomes.

A sample of each of the eluates was mixed with additional detergent and liposomes made of tumor lipids at a final ρ of 2.5 (27.4 mM detergent, 8 mM lipids), incubated for 2 h at 4 °C, dialyzed to remove the detergent, and then analyzed on a continuous 3-36% sucrose gradient. The results are shown in Fig. 2. Only the IgE is labeled with ^125^I so that the position of these counts (*filled circles*) indicates the distribution of the
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**FIG. 2.** Sucrose gradient centrifugation patterns of reincorporated IgE-receptor complexes. A, pattern obtained with the material analyzed in lane 2 of Fig. 1; B, pattern analyzed in lane 1 of the same figure. The specimens contained 3000 and 2600 cpm of $^{125}$I-IgE, respectively. Open circles, $^{125}$I; filled circles, $^{131}$I. The scales on the right indicate the refractive index of those fractions assessed as shown by the small filled circles.

IgE. Both the IgE and the receptors are labeled with $^{125}$I, the IgE much more so than the receptor (Table I), so that the distribution of these counts (open circles) gives little additional information. A shows the results with the preparation containing intact receptors. Relatively good incorporation of the material into liposomes (banding at a concentration of sucrose giving a refractive index of 1.347) was observed. On the other hand, little of the IgE bound to $\alpha$ chains only (B) was associated with the band of liposomes.

In order to examine the incorporated material more completely, some of the material analyzed in lane 2 of Fig. 1, i.e. intact receptors, was reconstituted and the liposomes applied to a sucrose gradient in amounts sufficient for subsequent analysis on gels. The gradient pattern showed $^{131}$I counts (on IgE) at both the position where free proteins travel ($\eta = 1.38$) and at the position of the liposomes ($\eta = 1.347$) in about equal amounts. It is likely that during the week that intervened between the first and second study, some dissociation of the IgE from the receptor, or of the subunits of the receptor from each other, or both, had occurred. Material from the top and bottom of the gradient was solubilized, immunoprecipitated, and analyzed on a polyacrylamide gel. An autoradiograph of the gel is shown in Fig. 3. The material from the lower portion of the gradient (lane 1) showed only the band of IgE and a very faint band at the position of the $\alpha$ chains (not seen in the reproduction). The material incorporated into liposomes, on the other hand, showed the $\beta$ and $\gamma$ components as well (Fig. 3, lane 2). In addition, a prominent band at 43 kDa is seen. As already mentioned, this material represents disulfide-linked $\beta$-$\gamma$ complexes that form variably (cf. Fig. 1, lane 4) after immunoprecipitation (6).

A quantitative analysis of this gel is also presented in Table I. Together, the results confirm that the receptors incorporated into the liposomes (specimen 4) have the same subunit composition as those freshly isolated (specimen 2). On the other hand, the IgE- $\alpha$ chain complexes that are not incorporated into liposomes (specimen 3) are largely free of $\beta$ and $\gamma$ chains.

**Cross-linked Receptors**—In order to obtain further information on the conditions required for incorporation of the receptors into liposomes, we performed experiments on receptors that had been stabilized by chemically cross-linking them prior to purification. The following experiment is one of several performed, each of which gave similar results.

IgE-receptor complexes were partially purified from membrane preparations of cultured cells and extrinsically iodinated. An aliquot was chemically cross-linked with a reducible bifunctional reagent (see "Materials and Methods"). Both the cross-linked and uncross-linked preparations were then further purified on anti-benzazearsonate columns in the presence or absence of lipids. Fig. 4 shows an analysis on polyacrylamide gels of the four eluates.

**FIG. 3.** Analysis of IgE-receptor complexes from sucrose gradient fractions. Some of the material previously analyzed (Fig. 1, lane 2, and Fig. 2A) was mixed with liposomes (composed of a 2:1 mixture of tumor/soybean lecithin phospholipids) at $\rho = 2.5$ (24.7 mM detergent, 8 mM lipid) incubated for 1 h at 4°C and then dialyzed. The material was applied to a 3–36% sucrose gradient, and after centrifugation samples from the top fractions (containing the liposomes) and from the bottom fractions (containing unincorporated material) were dissolved and immunoprecipitated with anti-IgE. The dissolved precipitates, containing 750 ± 50 cpm of $^{125}$I-IgE, were analyzed on a 12.5% gel of which the autoradiogram is shown. Lane 1, unincorporated material; lane 2, material in liposomal peak.
Fig. 4. Analysis of purified IgE-receptor complexes on polyacrylamide gels. A, 14% gel; B, 8% gel. 125I-IgE-receptor complexes were partially purified in the presence of lipids, extrinsically iodinated with 125I, and then reacted with cross-linking reagent or not. The two samples were then divided in two aliquots again; one-half was purified further in the presence and the other half in the absence of added lipids in the washing and eluting buffers. The phospholipids in this experiment were derived from rabbit liver. The samples were analyzed directly of gels, the autoradiographs of which are shown. Lane 1, cross-linked, purified without lipids; lane 2, cross-linked, purified with lipids; lane 3, uncross-linked, purified without lipid; lane 4, uncross-linked, purified with lipid. Each lane contained 1500 cpm of 125I-IgE.

of γ chains, respectively. In addition, a band at ~10 kDa is seen, likely representing reduced γ chains. The uncross-linked preparation that had been exposed to lipid-free detergent solutions shows, as expected, little of the β and γ components (lane 3). Lanes 1 and 2 show the material that had been cross-linked and subsequently purified in the absence (lane 1) or presence (lane 2) of lipids. In addition to the IgE at the origin, the principal component is a band at 90–95 kDa in both specimens. Traces of material with an apparent molecular weight between that of the cross-linked material and the α chains are also seen. Fig. 4B shows an autoradiogram of an 8% acrylamide gel on which the same specimens were analyzed. The intermediate sized material can be better appreciated on this gel, and the apparent molecular weight of the major cross-linked product more precisely assessed.

The cross-linked product from Fig. 4A, lane 1, was excised and re-electrophoresed on a gel, along with material excised from the bands at 30, 20, and 10 kDa from the specimen shown in lane 4 of the same gel. All the specimens were reduced. The cross-linked material yielded bands at ~30 and 10 kDa and a faint band at 60 kDa; the band at 30 kDa isolated from the specimen in lane 4 remained unchanged and the 20- and 10-kDa components from this specimen both ran as 10-kDa components (Fig. 5). These results demonstrate that the cross-linked material contains α, β, and γ chains.

The ability of the IgE-receptor complexes to incorporate into liposomes was assessed, the samples being analyzed by centrifugation in an Airfuge (15). Fig. 6 shows an analysis on polyacrylamide gels of the fractions from the top of the stepwise gradients (7) representing liposomes and from the unincorporated material at the bottom (B). The material that had not been cross-linked and that had been exposed to lipid-free detergents incorporated poorly. On the other hand, the uncross-linked material that had not been exposed to lipid-free detergents and both of the cross-linked preparations showed more substantial and roughly equivalent incorporation into the liposomes.

DISCUSSION

Several years ago, we observed that when labeled in situ with the hydrophobic probe iodonaphthylazide (16), the β subunit but not the α chain of the receptor for IgE became modified. We suggested that possibly the α chains were anchored into the plasma membrane via the β subunit (17). We subsequently observed that in order to incorporate IgE-receptor complexes into liposomes efficiently, low ratios of lipid:detergent had to be maintained. This in turn suggested that similar conditions would be useful in overcoming the unusual instability of the subunit interactions of the receptor (9), and this proved to be the case (4). Coincidently, an
that postulates changes in the conformation of the \( \alpha \) chains rather than the dissociation of the \( \beta \) and \( \gamma \) chains \textit{per se} to explain the ineffective incorporation of IgE-\( \alpha \) chain complexes. In such a model, the protective effect of chemical cross-linking would result from stabilization of the conformation of \( \alpha \) chains rather than from maintenance of the interactions between the \( \alpha \), \( \beta \), and \( \gamma \) subunits. If this is not the case and the other subunits are required for anchoring \( \alpha \), which of the former play the primary role? Since like the \( \beta \) chains, the \( \gamma \) chains are modified by the iodonaphthyl reagent (5) and by other criteria show similar hydrophobic properties,\(^3\) we cannot yet determine the relative contributions of the different subunits to the effectiveness of incorporation.

These questions will be difficult to resolve and seem to us of second order importance. More significant questions are those related to the functioning of the receptor. The results presented in this paper will, we think, provide a useful step in exploring this aspect. However, further modifications of our procedures will be necessary in order to incorporate multiple copies of receptors into individual liposomes (or bilayers). In some cases, substantially nonrandom (i.e. skewed) distributions of incorporation into liposomes have been observed (19, 20). If a similar phenomenon were to pertain with respect to the receptor for IgE, we would be even closer to our goal than the present results suggest.

Recent studies on receptors for IgG on macrophages (21, 22) and on the receptors for IgE (23, 24) have implicated the formation of ion channels as a direct consequence of the aggregation of the receptors. Our progress in maintaining the native structure of the receptor and in reincorporating the purified material into liposomes will allow us to probe this mechanism.

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J. Biol. Chem. 1984, 259:1212-1217.