Covalent Cross-Linking of Subunits of the Receptor for Immunoglobulin E Induced by Immunoprecipitation†

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ABSTRACT: The receptor on rat basophilic leukemia and related normal cells that binds monomeric immunoglobulin E (IgE) with high affinity contains four polypeptide chains: α (to which the IgE binds), β, and a disulfide-linked dimer of γ chains. In this study, we have analyzed a further component variably seen when the purified receptors are analyzed on polyacrylamide gels. This component has an apparent M_r of ~43 000 and, after treatment with reducing agents, yields one β and two γ chains. This complex is generated by immunoprecipitation of preparations totally lacking in it. This novel in vitro phenomenon has provided additional information about the structure of the receptor. Its possible relationship to in vivo aggregation that triggers degranulation of the cells is of interest.

Our group recently reported (Rivnay et al., 1982) that the interaction between the α and β subunits of the solubilized receptor for immunoglobulin E (IgE)[1] can be stabilized by maintaining an appropriate phospholipid to detergent ratio in the solvents used in purification. Under such conditions, two additional previously unobserved components were noted in the immune precipitates of the purified receptor–IgE complexes: a 43- and a 20-kDa component. The latter has been shown to be a disulfide-linked dimer of an additional subunit, γ (Perez-Montfort et al., 1983a). In this paper, we describe the composition of the 43-kDa component and the conditions under which this component is seen. Our results have permitted a better understanding of the interaction between the different subunits of the receptor for IgE.

Materials and Methods

Virtually all of the materials and methods used in this study have been described in the preceding paper (Perez-Montfort et al., 1983a). Where additional procedures and materials were used, the details are given under Results.

Results

Basic Observations. In a typical experiment, benzenearsonylated mouse anti-dinitrophenyl-IgE was incubated with cells grown in [^{3}H]leucine, the cells were washed and solubilized with 10 mM detergent at a detergent to phospholipid micellar ratio of $\rho \simeq 2$ (Rivnay & Metzger, 1982), and the supernatant was applied to an anti-benzenearsonate column. The column was washed with the detergent/lipid buffer over 48 h. The bound receptor–IgE complexes were then eluted with benzenearsonate hapten and immunoprecipitated with anti-IgE and *Staphylococcus aureus* (Cowan I). After being washed, the precipitates were extracted with $NaDodSO_4$ and analyzed without reduction by gel electrophoresis. The eluate was also analyzed directly, without prior immunoprecipitation. Figure 1 illustrates such an experiment—one in a series. It is an autoradiograph of a gel on which the eluate (left lane) is compared to the immunoprecipitate (right lane). In addition to the iodinated IgE near the origin, three prinicipal components are seen in the eluate: the broad band at 60 kDa, a band at ~32 kDa, and a band at ~20 kDa, representing the α chain, the β chain, and the dimer of γ chains, respectively. In the immunoprecipitated materials, the same bands are observed, but in addition, there is a band at ~43 kDa.

Labeling Properties of the 43-kDa Component. In order to determine the nature of the 43-kDa component, receptors intrinsically labeled with [^{3}H]leucine, [^{35}S]methionine, a mixture of tritiated amino acids, or [^{14}C]glucosamine were immunoprecipitated and compared by gel electrophoresis.

Figure 2 illustrates such an experiment in which the receptors were labeled with [^{35}S]methionine or [^{3}H]leucine. Figure 2A shows the pattern of counts for an unreduced immunoprecipitate. The pattern of tritium counts is in excellent agreement with the autoradiograph shown in lane 2 of Figure 1. By comparison with the pattern of tritium counts, the pattern of ^{35}S counts shows that the ~18-kDa dimer of γ chains did not incorporate the [^{35}S]methionine but that there was a substantial incorporation into the α chain, the 43-kDa component, and the β chain.

The results of other labeling studies are shown in Table I. In those experiments in which the 43-kDa component was not labeled, the same protocol was applied to receptors with a different labeling procedure (incorporation of [^{3}H]leucine or extrinsic iodination) in order to demonstrate that the 43-kDa component had not simply been lost. In each instance, labeling of the 43-kDa component was observed only where either the β chain or both the β and γ chains were modified.

Effect of Reduction. Immunoprecipitated receptors labeled with [^{3}H]leucine and [^{35}S]methionine were also analyzed after reduction. Figure 2B illustrates the radioactivity profiles of the two different isotopes. The ^{3}H pattern shows three prominent peaks due to the α chain, the β chain, and the γ chains, respectively. As noted previously, the apparent molecular weight of the α and β chains was not altered by reduction (Conrad & Froese, 1976; Holowka & Metzger, 1982) whereas the dimer of γ chains was completely cleaved (Perez-Montfort et al., 1983a). Notably, the 43-kDa component disappeared completely. Analysis of the ^{35}S pattern confirmed this result since only the peaks representing the α and β chains were observed at ~55 and ~32 kDa, respectively.

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[1] Abbreviations: IgE, immunoglobulin E; $NaDodSO_4$, sodium dodecyl sulfate.

Table I: Labeling Properties of 43-kDa Component

component	intrinsic labeling					extrinsic labeling	
	[^{3}H]-leucine	^{3}H-labeled amino acid mixture[a]	[^{35}S]-methionine	[^{14}C]-glucosamine	[^{32}P]P_i	surface labeling (intact cells)	detergent extract
45 kDa	+	+	+	−	+	−	+
α	+	+	+	+	−	+	+
β	+	+	+	−	+	−	+
γ	+	+	−	−	+	−	+

[a] Cells were biosynthetically labeled with a high specific activity (75–110 Ci/mmol) ^{3}H-labeled amino acid mixture containing L-leucine, L-lysine, L-phenylalanine, L-proline, and L-tyrosine (code TRK 550, Amersham Corp., Arlington, IL). Cells were harvested from stationary flasks, washed in depleted medium, and resuspended in the same medium (MEM Select-Amine Kit, Catalog No. 300-9050, GIBCO Laboratories, Grand Island, NY) at 1.0×10^6 cells/mL. After the amino acid mixture (5 mCi) was added, the suspension was incubated in a 250-mL spinner flask at 37 °C for 24 h.

Table II: Quantitative Analysis of Distribution of Counts in Labeled Receptors

component	unreduced sample (percent of total)		reduced sample (percent of total)	
	[^{35}S]-methionine[a]	[^{3}H]-leucine[b]	[^{35}S]-methionine[a]	[^{3}H]-leucine[c]
α	46	26.1 ± 4.4	42	25.5 ± 2.3
45 kDa	27	21.2 ± 7.1		6.7 ± 4.7
β	27	31.0 ± 3.9	58	45.3 ± 1.9
γ		17.3 ± 2.0		25.1 ± 2.2
β/γ		1.73 ± 0.1		1.80 ± 0.1

[a] Analysis of one experiment. [b] Analyses of six experiments. The mean ± 1 SD is given. [c] Analyses of four experiments. The mean ± 1 SD is given.

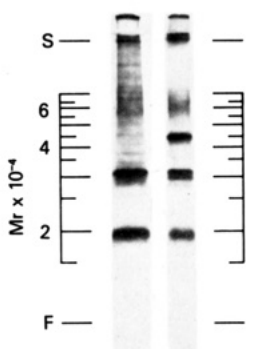

FIGURE 1: Radioautograph of a 12.5% polyacrylamide gel on which IgE–receptor complexes intrinsically labeled with [^{3}H]leucine were analyzed. Cells were first saturated with benzenearsonylated IgE and solubilized with detergent. The IgE–receptor complexes were bound to an antibenzenearsonate column and washed with buffer containing detergent and lipids. Half of the eluate was analyzed directly (left lane) and the other half immunoprecipitated with anti-IgE. The latter pellet was extracted with $NaDodSO_4$ and analyzed in the right lane. S marks the end of the stacking gel; F is the dye front.

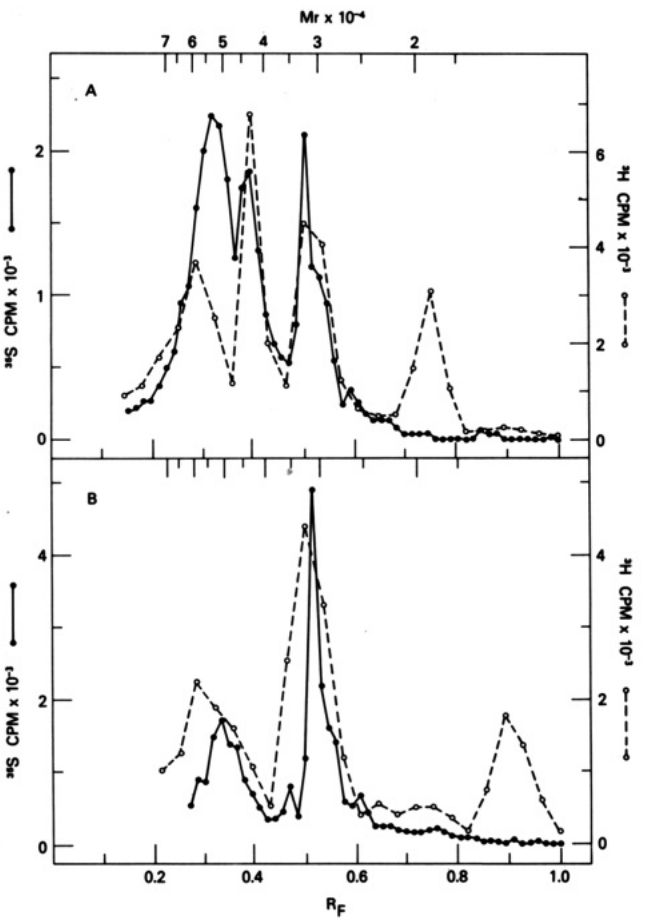

FIGURE 2: Analysis of IgE–receptor complexes isolated from cells grown in the presence of [^{3}H]leucine or [^{35}S]methionine. The complexes were purified on separate affinity columns. Immune precipitates of the IgE–receptor complexes were extracted with $NaDodSO_4$ and electrophoresed in the absence (A) or presence (B) of reducing agent on a 2.5% polyacrylamide gel. The gel was dried, cut into 2-mm slices ([^{35}S]methionine pattern) or 4-mm slices ([^{3}H]leucine pattern) and counted for ^{35}S or ^{3}H. The solid circles show the profile of radioactivity from the material labeled with [^{35}S]methionine and the open circles that of the material labeled with [^{3}H]leucine.

Quantitation of the radioactivity showed that all of the ^{35}S counts initially associated with the 43-kDa component before reduction were recovered in the β chains after reduction (Table II). These results demonstrated that the 43-kDa component contained an intact β chain.

The pattern of leucine counts suggested that one or more γ chains was also incorporated into the 43-kDa component. In order to verify this, we quantitatively analyzed the distribution of ^{3}H counts in several experiments. Immunoprecipitates of receptor were electrophoresed on $NaDodSO_4$ gels in the presence and absence of reducing agents and the bands quantitatively analyzed. Table II shows that the ^{3}H counts associated with the 43-kDa component were completely recovered in the β and the γ chains after reduction and that the percentage of the counts associated with the α chain was unchanged. This confirmed the involvement of the β and the γ chains in the generation of the 43-kDa component. The ratio of counts in β to counts in the dimer of γ chains from an unreduced pattern was not significantly different from the corresponding ratio of β to monomeric γ chains after cleavage of the 43-kDa component by reducing agents (bottom line of Table II).

Analysis of the Isolated 43-kDa Component. As a further test, we isolated the 43-kDa component, β, and the dimer of

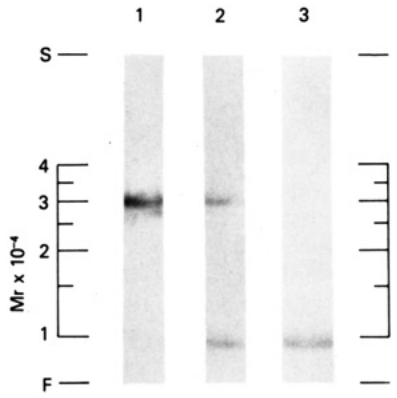

FIGURE 3: Radioautograph of a 10–30% polyacrylamide gradient gel on which [^{3}H]leucine-labeled components of the receptor of IgE were analyzed. Bands from a 12.5% polyacrylamide gel corresponding to the β chain (lane 1), the 43-kDa component (lane 2), and the γ chain (lane 3) were reelectrophoresed in the presence of reducing agent.

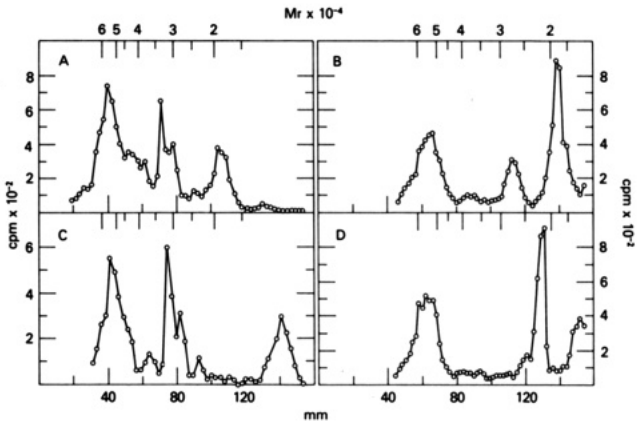

FIGURE 4: Analysis of IgE–receptor complexes isolated from cells grown in the presence of [^{3}H]leucine. The $NaDodSO_4$ extracts of the immune precipitates were analyzed before (A) and after reduction (C) on 12.5% polyacrylamide gels. 6 weeks later, another aliquot of the same preparation of IgE–receptor complexes was immunoprecipitated, extracted with $NaDodSO_4$, and electrophoresed in the absence (B) or in the presence (D) of reducing agent on a 12.5% polyacrylamide gel. The gels were dried, cut into 2-mm slices, and counted for tritium.

γ chains from a polyacrylamide gel on which an unreduced immunoprecipitate had been electrophoresed. The material from the dried gel slices was rehydrated in a $NaDodSO_4$ buffer containing 2-mercaptoethanol and individually reelectrophoresed. As can be seen in Figure 3, the reduction of the 43-kDa component (lane 2) yields two distinct components that correspond exactly to β and γ (lanes 1 and 3, respectively). An experiment similar to that shown in Figure 3 was performed with the 43-kDa component from receptors radioiodinated extrinsically. The results (data not shown) were in complete agreement with those shown in Figure 3. Quantitative analysis of the ^{3}H counts showed that the ratio of counts in β and γ was 1.34:1.

Analogy between the 43-kDa Component with the 30-kDa Component Observed under Conditions Where the β Chain Is Cleaved. During the course of this work, several experiments resulted in rather different patterns—patterns similar to those previously observed in the absence of protease inhibitors (Holowka & Metzger, 1982). Figure 4 shows the patterns derived from an unreduced preparation labeled with leucine and immunoprecipitated immediately after purification (A) and 6 weeks later (B). The pattern of the freshly immunoprecipitated material was similar to that regularly seen, showing the ~55-kDa α chain, the 40–43-kDa component, the β-chain doublet peak, and the disulfide-linked γ chains. At a later time, the same preparation gave a very different result. The 43-kDa material was virtually absent. The peak at 30 kDa was much diminished and was shifted to a slightly lower molecular weight, and finally, the component at ~20 kDa markedly increased in amount. That the latter peaks at 30 and 20 kDa in the "aged" material did not simply represent β chains and γ dimers was demonstrated by two results. First, upon reduction of the fresh preparation the β chain remained unchanged, and the 20-kDa γ dimer was converted to the 10-kDa monomer (Figure 4C). In the aged sample (Figure 4D), the 30-kDa component disappeared, and if anything, there was an increase in the 20-kDa component despite the fact that a 10-kDa component appeared in the expected amount (cf. panels D and B). Furthermore, a study performed around the same date with a similar preparation of protease inhibitors, using [^{35}S]methionine incorporation, showed in the unreduced immune precipitate no counts in the 43-kDa region, a small peak at ~30 kDa, and a prominent peak at ~22 kDa. The latter contained as many counts as in the β-chain peak (cf. Figure 2A).

We interpret these results as follows: The β chain has been cleaved and a 23-kDa "β_1" fragment is produced (Holowka & Metzger, 1982). This fragment is not reducible, has methionine, and has virtually the same mobility as the γ-chain dimer; the latter, however, is reducible. The 30-kDa component appears to be an analogue of the 45-kDa component, being in this case a disulfide-linked complex of β_1 with either one or two γ chains. Upon reduction it yields a β_1 fragment and γ chain(s).

Formation of the 43-kDa Component. The complexity of the analysis required to demonstrate the composition of the 43-kDa component, as well as the relatively poor incorporation of counts into the receptor by a variety of labeling procedures, limited our ability to test a large number of factors that might influence the generation of this component. The following variables were checked.

(1) The phenomenon described here was not dependent upon a specific type of affinity column. The extract from cells grown in [^{3}H]leucine was applied to beads conjugated with (trinitrophenyl)lysine. The bound receptor–IgE complexes were eluted with 10 mM dinitrophenylcaproate after extensive washing. The eluate was immunoprecipitated, analyzed by gel electrophoresis, and compared with the immunoprecipitated eluate from an antibenzenearsonate column. The results of both immunoprecipitates were identical with those described in Figure 1, lane 2.

(2) In order to study the effect of alkylating agents, cells grown in the presence of [^{3}H]leucine were incubated with IgE. Half of the preparation was solubilized as usual and the other half in the presence of 20 mM iodoacetamide. After separate purification, the receptor–IgE complexes were respectively immunoprecipitated and analyzed by gel electrophoresis. A quantitative analysis of the counts associated with the 43-kDa component showed no difference between the samples. In other experiments, only the immunoprecipitation was performed in the presence of iodoacetamide, and again, there was no effect on the generation of the 43-kDa component.

(3) The supernatant of the control human IgE anti-human IgE precipitate performed on mouse IgE–receptor complexes purified from cells grown in [^{3}H]leucine was analyzed by gel electrophoresis without further additions. The $NaDodSO_4$ extract of the precipitated material was also analyzed. Neither the pellet nor the supernatant of this precipitation yielded a 43-kDa component. On the other hand, a part of the same supernatant was incubated with anti-mouse IgE. The prep-

aration was then split. Half was centrifuged and the pellet was extracted with $NaDodSO_4$ and analyzed by gel electrophoresis. The other half was not centrifuged, and the total material was analyzed. The 43-kDa component was seen only in the precipitated material. The results demonstrated that the 43-kDa component was not induced by a nonrelevant antigen–antibody precipitation and was generated only by the precipitation of the receptor–IgE complexes with specific antibodies.

With regard to the possible role of protein A containing *Staphylococcus aureus* (Pansorbin) used in the process of the immunoprecipitation, it should be emphasized that the Pansorbin had already been used in the control precipitation without apparent effect on the generation of the 43-kDa component.

Discussion

The curious finding examined in this study was that an eluate from an affinity column that contained only α, β, and the dimer of γ chains of the receptor for IgE yielded a 43-kDa component after immunoprecipitation. Three possibilities had to be considered: the 43-kDa component could be a degradation product of the 55-kDa α chain, a complex of the labeled β (33-kDa) or the labeled γ (9-kDa) chain with an unlabeled contaminant, or, finally, a complex between the labeled β and the labeled γ chains. That the 43-kDa component incorporated phosphorus whereas the α chain did not (Perez-Montfort et al., 1983a; Table I) ruled out the first hypothesis.

The experiments performed with a preparation of receptors labeled with [^{35}S]methionine (an isotope that does not incorporate into the γ chain) showed that all the counts associated with the 43-kDa component before reduction were recovered in the β chain after reduction. These results demonstrated the presence of an intact β chain in the 43-kDa component. When the receptors were labeled with [^{3}H]leucine, which incorporated into both the β and the γ chains, all the counts associated with the 43-kDa component before reduction were redistributed after reduction in the β and the γ chains. Reelectrophoresis under reducing conditions of a 43-kDa component isolated from a nonreduced gel confirmed that the β and the γ chains were the only labeled constituents of the 43-kDa component, virtually ruling out the presence of a major contaminant. The apparent molecular weight of the 43-kDa component initially suggested that one β chain ($\sim$32 kDa) became associated with one γ chain ($\sim$10 kDa). However, after reduction, the ratio of [^{3}H]leucine in the β chain to that in the two γ chains is $\sim$1.8:1 (Table II). Then, if the 43-kDa component consisted of β and only *one* γ chain, one would expect that reduction of the isolated 43-kDa component would yield a ratio of [^{3}H]leucine in the resultant β and γ components of 3.6:1. The value observed, 1.34:1, together with the apparent molecular weight of the complex make it much more likely that the 43-kDa component contains one β and two γ chains. We have discussed in the preceding paper why the receptor for IgE must now be considered as a protein consisting of four subunits: one α, one β, and two γ chains (Perez-Montfort et al., 1983a). The parallel increase of counts in the β and the γ chains under conditions where the 43-kDa component was cleaved (bottom line of Table II) also supports the postulate that one β and two γ chains are complexed in the 43-kDa component.

The amount of 43-kDa component that was generated was irregular. It seemed remarkably constant in some experiments such as that shown in Figure 4 of the preceding paper (Perez-Montfort et al., 1983a) but in other experiments was quite variable, though never corresponding to a complete transformation of β and γ chains into the 43-kDa component. The reasons for the variability of this reaction are still unclear.

Some experiments were performed under conditions where a degradation product of β was observed. This portion of the β chain had been observed previously and was called β_1, (Holowka & Metzger, 1982; Metzger et al., 1982). The same phenomenon of cross-linking between the β_1 and the γ chains occurred during the immunoprecipitation and yielded a 30-kDa component. This finding suggests that it is the β_1 region of the β chain that interacts with the γ chains (see also below).

With regard to the nature of the bonding of β to γ, two experimental results are notable. (1) The complex was dissociated by $NaDodSO_4$ only after reduction. (2) The formation of the 43-kDa component was not prevented by pretreatment with alkylating reagents. These results indicate that the reaction may be occurring by disulfide interchange. Whatever the nature of the bonding, it will be interesting to determine whether the complex results from an intra- or an interreceptor reaction. Further information on this point will be required in order to interprete unambiguously the cross-linking of the β_1 fragment with the γ chains in terms of the structure of the native receptor.

The generation of the 43-kDa component by immunoprecipitation is a phenomenon for which we know of no precedent. Since immunoprecipitation is now widely used for purification of cellular components, it is possible that other examples of such an occurrence will be observed. The interesting question arises whether this phenomenon has any physiological counterpart. The critical initiating event in IgE-mediated degranulation of mast cells is the aggregation of the surface receptors of IgE (Metzger & Ishizaka, 1982). Since the 43-kDa component is generated by aggregation in vitro, it is conceivable that a like event occurs in vivo. We are attempting to test this experimentally.

References

Conrad, D. H., & Froese, A. (1976) *J. Immunol. 116*, 319.
Holowka, D., & Metzger, H. (1982) *Mol. Immunol. 19*, 219.
Metzger, H., & Ishizaka, T. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 7.
Metzger, H., Goetze, A., Kanellopoulos, J., Holowka, D., & Fewtrell, C. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 8.
Perez-Montfort, R., Kinet, J.-P., & Metzger, H. (1983a) *Biochemistry* (preceding paper in this issue).
Perez-Montfort, R., Kinet, J.-P., Fewtrell, C., & Metzger, H. (1983b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1083.
Rivnay, B., & Metzger, H. (1982) *J. Biol. Chem. 257*, 12800.
Rivnay, B., Wank, S. A., & Metzger, H. (1982) *Biochemistry 21*, 6922.