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Articles

Conformations of IgE Bound to Its Receptor $F_c\epsilon RI$ and in Solution†

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ABSTRACT: Previous resonance energy transfer studies suggested that murine immunoglobulin E (IgE) is bent near the junction of its F_c and F_{ab} segments when bound to its high-affinity receptor ($F_c\epsilon RI$) on RBL cells. To examine further the conformations of IgE, both bound to this receptor and in solution, a mutant recombinant IgE ($\epsilon/C\gamma3$*) was prepared that has a cysteine replacing a serine near the C-terminal ends of the heavy chain. The introduced cysteine residues provide a means for specific modification of IgE, and the sulfhydryl groups were selectively labeled with fluorescein-5-maleimide (FM-$\epsilon/C\gamma3$*). This IgE also binds a 5-(dimethylamino)napthalene-1-sulfonyl (DNS) group in the antigen-binding sites. Resonance energy transfer experiments carried out on receptor-bound FM-$\epsilon/C\gamma3$* yielded a distance of 53 Å between fluorescein near the C-terminal end of the F_c segment and amphipathic acceptor probes at the membrane surface. The average distance between this C-terminal fluorescein and acceptor eosin-DNS in the antigen-binding sites at the N-terminal ends of the F_{ab} segments was found to be 69 Å. These results combine with those from previous structural studies to provide an unprecedented detailed description of the bent geometry of IgE bound to its receptor on the membrane. Energy transfer measured for FM-$\epsilon/C\gamma3$* in solution between fluorescein near the C-terminal end of the F_c segment and eosin-DNS at the N-terminal ends of the F_{ab} segments indicates that the average distance between these probes is about 71 Å. This contrasts with the estimated value of 175 Å for a planar Y-shaped IgE molecule. This result points to the possibility that IgE in solution is also bent such that, on average, the termini of the F_{ab} and F_c segments are closer together than they would be in the planar form.

Immunoglobulin E (IgE),[1] like other classes of Ig, acts as an adaptor molecule linking antigen to effector systems. In particular, IgE binds to its high-affinity receptor, $F_c\epsilon RI$, in a 1:1 complex on the surface of mast cells and basophils. Cross-linking of these IgE–receptor complexes by multivalent antigens leads to cellular degranulation and release of mediators of the allergic response (Metzger et al., 1986). In previous resonance energy transfer studies of membrane receptor-bound IgE, fluorescent donor probes were placed at specific sites on IgE (Holowka & Baird, 1983b; Baird & Holowka 1985) and anti-IgE antibodies (Holowka et al., 1985). The distance of closest approach between these probes on receptor-bound IgE and amphipathic acceptor probes randomly distributed at the plasma membrane surface was measured. These previous results allowed us to put some limits on the possible orientations and positions of IgE when it is bound to its receptor. In particular, the antigen-binding sites must be quite far from the membrane surface (≥100 Å), while the interchain disulfide bonds in the Cε2 domain are as close as 45 Å to this surface.

Previous studies on the region of interaction between IgE and receptor revealed that a site of trypsin cleavage between the Cε2 and Cε3 domains of rat IgE is markedly protected from proteolysis when IgE is receptor-bound (Perez-Montfort & Metzger, 1982). More recently, genetically derived mutants and segments of human IgE were used to show that a 76 amino acid polypeptide containing the carboxy-terminal portion of Cε2 and the amino-terminal portion of Cε3 inhibits binding to IgE to the receptors (Helm et al., 1987). Recently, we reported on chimeric murine IgE molecules that have one or two F_c domains exchanged with the homologous domains of human immunoglobulin G (IgG) subclass I. Human IgG1 does not interact with IgE receptors. We found that one IgE

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[1] Abbreviations: Ig, immunoglobulin; DNS, 5-(dimethylamino)-naphthalene-1-sulfonyl; DTT, dithiothreitol; ED, 1-*N*-eosinthiocarbamyl-5-*N*-DNS-cadaverine; FM, fluorescein-5-maleimide; HAE, 5-(*N*-hexadecanoylamino)eosin; HAF, 5-(*N*-hexadecanoylamino)-fluorescein; ORB, octadecyl Rhodamine B chloride; RBL, rat basophilic leukemia.

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derivative, which has its C-terminal Cε4 domain switched to Cγ3 (ε/Cγ3), has the same receptor-binding properties as the wild-type IgE (Weetall et al., 1990). Binding was lost with all of the other IgE/IgG1 derivatives, indicating the importance of the Cε2 and Cε3 domains. All of these results are consistent with a structural model in which IgE bends somewhere between the Cε1 domain of the F_{ab} segments and the Cε3 domain in the F_c segment in order to bind to its receptor near the Cε2 and Cε3 junctional region (Holowka et al., 1985; Baird & Holowka, 1988).

Previous fluorescence anisotropy studies indicated that IgE has limited segmental motion, both in solution (Oi et al., 1984) and when bound to its receptor (Slattery et al., 1985; Holowka et al., 1990), but there is relatively little information about the three-dimensional structure of IgE in solution. In particular, it has not been determined to what extent the apparent bent conformation of receptor-bound IgE is different from the conformation of IgE in solution. In order to examine the conformation of IgE both when bound to its receptor and in solution, we have extended our studies with the chimeric IgE/IgG1 derivatives. Site-specific mutagenesis was carried out to replace a serine four residues from the C-terminus in the Cγ3 polypeptide (Ser^{444}, in Eu notation) with a cysteine.[2] This position is not resolved in the X-ray crystallographic structure of IgG1 due to local disorder, but it is located near the C-terminus of the folded Cγ3 domains (Deisenhofer, 1981). These mutated Cγ3 were exchanged into ε/Cγ3 to create ε/Cγ3*, which contains a new site for specific labeling by a sulfhydryl-specific probe.

In the study described here, the newly introduced sulfhydryl groups were selectively labeled with fluorescein-5-maleimide (FM). These labeled IgE derivatives bind tightly to receptors in right-side-out oriented plasma membrane vesicles prepared from RBL cells. Resonance energy transfer measurements between the donor fluorescein at the C-terminal end of the ε/Cγ3* domain and acceptors at the N-terminal antigen-binding sites were carried out. The average end-to-end distances for IgE in solution and on membranes after forming the receptor–IgE complex were compared. The distance between the receptor-bound ε/Cγ3* C-terminal sites and the membrane surface was also determined, by using amphipathic probes inserted into the membrane bilayer as fluorescence acceptors. These distance measurements, together with our earlier studies, clearly demonstrate a bent conformation for IgE bound to its receptor on the cell membrane and allowed us to obtain evidence for a surprisingly similar conformation for IgE in solution.

Experimental Procedures

Reagents. FM, 5-(*N*-hexadecanoylamino)fluorescein (HAF), 5-(*N*-hexadecanoylamino)eosin (HAE), and octadecyl rhodamine B chloride (ORB) were obtained from Molecular Probes Inc. (Eugene, OR). Aprotinin, dithiothreitol (DTT), *N*-ε-[5-(dimethylamino)napthalene-1-sulfonyl]-L-lysine (DNS-Lys), trypsin treated with diphenylcarbamoyl chloride, ovomucoid trypsin inhibitor, and Sephadex G-50 were from Sigma Chemical Co. (St. Louis, MO). 1-*N*-Eosinthiocarbamoyl-5-*N*-DNS-cadaverine (ED), a gift from Drs. Linda Lee and Vernon Oi (Becton Dickinson Monoclonal Center, Mountain View, CA), was prepared by conjugating eosin-5-isothiocyanate to one terminal amino group of 1,5-diaminopentane (cadaverine) and DNS-chloride to the other terminal amino group. This compound was further purified by preparative thin-layer chromatography before use in these experiments; the recovered material gave a single spot on silica gel with an R_f of 0.25 in 9:1:0.25 chloroform/methanol/acetic acid solution. ED has a strong absorption peak at 524 nm due to eosin (ϵ_{524} = 85000 M^{-1} cm^{-1}) and a broad weak absorption peak at 340 nm due to DNS; its fluorescence emission is maximal at 548 nm in pH 7.4 PBS buffer.

IgE Preparations. The murine monoclonal anti-DNS IgE was purified from the supernatant of a switch-variant hybridoma cell line 27-74 (Dangl et al., 1988) by affinity chromatography (Weetall et al., 1990). The chimeric IgE ε/Cγ3* was genetically engineered in a similar manner to ε/Cγ3 (Shopes et al., 1990) for which the Cε4 exon from murine anti-DNS IgE was replaced with the Cγ3 exon from human IgG1. For ε/Cγ3*, the Cγ3 exon had the codon for a serine located near the C-terminus (Ser^{444} in Eu notation) mutagenized to code for cysteine 444.[2] Briefly, a Nsi I to Nae I fragment at the 3′ end of the coding region of Cγ3 was replaced by a synthetic fragment, which deleted the Nsi I site, inserted a Sma I site, and changed the codon for Ser^{444} to TGC so as to code for Cys^{444}. The mutations were verified by sequencing the resulting vector pUC18G1C444. The mutagenized Cγ3* exon was subcloned, along with wild-type Cε1, Cε2, and Cε3 exons, into a mammalian expression vector, pSVDNS, which contained the heavy-chain variable exon for anti-DNS specificity as previously described (Shopes et al., 1990). The resulting heavy-chain expression vector was transfected into the murine cell line A5C13, which constitutively secretes κ light chain that is specific for DNS. Intact chimeric anti-DNS ε/Cγ3 and ε/Cγ3* were secreted from A5C13 cells that had been transfected with the corresponding vectors and were affinity purified as previously described (Shopes et al., 1990; Weetall et al., 1990). The affinity-purified preparations were determined to be >95% pure by analyses with gel permeation chromatography in nondenaturing buffer on a Pharmacia Superose 6 column and with polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE). The concentrations of the IgE preparations were determined by protein absorbance measurement (ϵ_{280} = 1.62 mL/(mg·cm) and MW = 184000; Liu et al., 1980) or by radioimmunoassay with DNS-coated plates (Weetall et al., 1990). These methods gave virtually equivalent results. A monoclonal antibody (49/2C3) specific for human Cγ3 (Nik Jaafar et al., 1982) was a gift from Dr. Roy Jefferis (University of Birmingham, England).

Modification and Enzymatic Digestion. Native and chimeric IgE were labeled with ^{125}I by the chloramine T method as previously described (Holowka & Baird, 1983a). Sulfhydryl groups on the chimeric IgE ε/Cγ3* were selectively labeled after partial reduction in PBS buffer (20 mM phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7.4) containing 0.25 mM DTT for 50 min at room temperature (~22 °C). FM in a methanol stock solution was then added to a final concentration of 0.3–0.5 mM (2% methanol v/v), and the reaction was allowed to proceed for 10 min. The free FM was removed by first passing the samples through a 0.22-μM Spin X centrifuge filter (Costar, Inc.) to remove any insoluble dye, followed by dialysis against PBS and chromatography through a centrifuge column (Penefsky, 1977) containing Sephadex G-50. The stochiometry of labeling was determined by the absorbance of FM at 495 nm (ϵ_{495} = 72000; Snyder & Hammes, 1985). IgE does not absorb significantly at this wavelength. The IgE concentration was determined from the absorption at 280 nm after subtraction of the absorption due to fluorescein at that

[2] Shopes, B., *Structural Mutants of Chimeric Immunoglobulin* (submitted for publication).

wavelength (20% of fluorescein absorption at 495 nm; Holowka & Baird, 1983b).

FM-ϵ/Cγ3* and FM-ϵ/Cγ3 (modified in parallel with ϵ/Cγ3*) at 0.3 mg/mL were digested with trypsin (2% w/w) in PBS buffer at 37 °C as previously described (Holowka & Baird, 1983b). The reaction was quenched with ovomucoid at a 20-fold excess over trypsin (w/w), and the samples were cooled to 4 °C. Samples were analyzed by SDS–PAGE with or without reduction (10 mM DTT) using 12.5% polyacrylamide gels, a Bio-Rad Mini-Protean II system, and standard procedures (Holowka & Baird, 1983b). Gels containing fluorescent derivatives were examined and photographed immediately after electrophoresis by the Polaroid 57 film with illumination from a near-UV light box filtered by a Kodak Wratten 2E filter. Gels were subsequently stained with Coomassie blue. The relative fluorescence in individual bands in the gel was determined visually and in some cases by band excision and spectroscopic measurement of the extracted fluorescence (Holowka & Baird, 1983b).

Binding Measurements. IgE and IgE derivatives, ϵ/Cγ3* and FM-ϵ/Cγ3*, were tested for competitive binding with ^{125}I-labeled IgE to receptors on RBL cells as previously described (Baird et al., 1989).

Preparation of Vesicles from RBL Cells. Adherent RBL cells (subline 2H3), maintained in stationary cell culture (Taurog et al., 1979) were chemically induced to vesiculate by using a previously described procedure (Baird & Holowka, 1985). The resulting plasma membrane vesicles have been shown to contain the receptor for IgE and to be mostly unilamellar and right-side-out with no internal organelles (Holowka & Baird, 1983a). These vesicles were used for energy transfer measurements after extensive dialysis against PBS containing 0.02 unit/mL aprotinin. For use in some experiments, receptors on vesicles were saturated by incubation with a 3–5-fold excess of unlabeled IgE in PBS at room temperature for 2 h, followed by 10-fold dilution in PBS, centrifugation at 25000g for 40 min, and resuspension of the pelleted vesicles in PBS. Final suspensions of vesicles contained about (5–7) $\times 10^6$ cell equivalents of vesicles per milliliter as determined by relative binding of ^{125}I-labeled IgE (Holowka & Baird, 1983a).

Spectroscopic Measurements. Absorption spectra were recorded with a Hewlett-Packard 8451A UV–visible spectrophotometer. Fluorescence measurements were carried out at controlled temperature (22 °C) in a SLM 8000 fluorescence spectrophotometer operated in ratio mode. Samples (2 mL) were contained in 10 × 10 × 48 mm acrylic cuvettes (Sarstedt Co.) with constant stirring. Fluorescein fluorescence of FM and HAF was excited at 480 nm, and the emission was monitored at 510 nm. Eosin fluorescence of ED was monitored with excitation and emission wavelengths of 520 and 550 nm, respectively. Steady-state anisotropy of the fluorescent probes was measured as previously described (Holowka & Baird, 1983a).

For donor–acceptor pairs used in the energy transfer experiments, the critical transfer distance R_o was calculated from (Förster, 1959)

$$R_o = (9.79 \times 10^3)(J \kappa^2 Q_d n^{-4})^{1/6} \text{ Å} \quad (1)$$

The overlap integral (J) was calculated as previously described (Cantley & Hammes, 1975); the orientation factor (κ^2) was taken as $^2/_3$ [random orientation, Holowka and Baird (1983b)]; the refractive index of the medium (n) was taken as 1.4. The quantum yield value (Q_d) for FM was determined with sodium fluorescein in 0.1 N NaOH [Q_d = 0.92, Weber and Teale (1957)] as a standard. The R_o values for donor–acceptor pairs HAF–HAE and HAF–ORB were determined previously (Holowka & Baird, 1983a).

The intramolecular energy transfer distance, R between the FM (donor) label on FM-ϵ/Cγ3* and the ED (acceptor) added to the antigen-binding sites was determined for FM-ϵ/Cγ3* in solution (6–10 nM) and bound to receptors on the vesicles (6 nM). An equivalent amount of the vesicle preparation (but with vesicle receptors saturated with unlabeled IgE) was added to samples for the measurements of FM-ϵ/Cγ3* in solution. This addition eliminated nonspecific association of ED with FM-ϵ/Cγ3* and nonspecific adsorption of FM-ϵ/Cγ3* to the cuvette walls. Under these conditions, we found that >95% of the FM-ϵ/Cγ3* fluorescence transfers to a new cuvette. Parallel samples containing FM-ϵ/Cγ3 were used to correct for the FM label not located at the sulfhydryls at the C-terminal end of FM-ϵ/Cγ3*. The fluorescein fluorescence of FM-ϵ/Cγ3* was monitored as ED was added in microliter aliquots to saturate the binding sites (>50 nM) and then as excess DNS-Lys (>1 μM) was added subsequently to displace the ED. There are two antigen-binding sites per IgE molecule (one per F_{ab} segment), and the distance R between the donor and acceptors was determined from the efficiency of energy transfer, E, with a model describing a donor equidistant from two acceptors (Hammes, 1981):

$$E = 1 - Q_{da}/Q_d = 2(R_o/R)^6/[1 + 2(R_o/R)^6] \quad (2)$$

Q_d was assumed to be proportional to the fluorescein fluorescence of FM-ϵ/Cγ3* after the sequential additions of ED and DNS-Lys minus the fluorescein fluorescence of the same concentration of FM-ϵ/Cγ3 after the same two additions. Q_{da} was taken as fluorescein fluorescence of ϵ/Cγ3* after the ED addition and was corrected by subtraction of the fluorescence of the control sample of FM-ϵ/Cγ3 as for Q_d. The fluorescence corrections of the FM-ϵ/Cγ3 control samples are typically 9–15% of the FM-ϵ/Cγ3* fluorescence, and they account for any labeling at sites other than the C-terminal sulfhydryls and for dilution due to ED and DNS-Lys additions.

Measurement of the energy transfer distance between FM located at the C-terminus of FM-ϵ/Cγ3 and amphipathic acceptor probes HAE or ORB at the membrane surface was carried out as follows. Varying concentrations of acceptor probes were added to identical 2-mL samples of membrane vesicles containing ~(5–7) × 10^6 cell equivalents of receptors and incubated at 22 °C until probe partitioning into the membrane reached equilibrium (within an hour for HAE; several hours for ORB). One vesicle sample in each set contained no acceptor. A set of parallel samples containing the different amounts of acceptors also contained the donor amphipathic probe HAF, and these samples were used to determine the surface densities of acceptors as previously described (Holowka & Baird, 1983a). Briefly, curves representing the degree of HAF fluorescence quenching as a function of acceptor concentration were fit by the double-exponential equations of Wolber and Hudson (1979) to yield the surface densities corresponding to the bulk concentrations. The fluorescence quenching of membrane-bound HAF that occurs as the acceptor probes partition into the membrane allows this process to be directly monitored (Holowka & Baird, 1983a).

For the energy transfer measurements, equal amounts of FM-ϵ/Cγ3* were added to each of the samples containing vesicles with different acceptor densities, and the binding process was monitored by the time-dependent quenching of the FM fluorescence. The fraction of receptor-bound FM-ϵ/Cγ3* was determined for each experiment by measurement of fluorescence before and after sedimentation of the vesicles

in a separate sample (45% bound was typical). The fluorescence of the FM-ϵ/Cγ3* samples was corrected by subtracting the component due to nonbound FM-ϵ/Cγ3*. A correction was also derived from parallel samples containing FM-ϵ/Cγ3 to account for FM not conjugated to the sulfhydryl introduced into the Cγ3 domain as described above. These measurements provided a curve of Q_d/Q_{da} vs acceptor surface density where Q_d was taken as the fluorescein fluorescence of bound FM-ϵ/Cγ3* in the sample containing no acceptors, and Q_{da} was taken as the fluorescein fluorescence in samples containing a particular density of acceptor.

The distance of closest approach, L, between the fluorescence label at the C-terminus of FM-ϵ/Cγ3* and Rhodamine or eosin acceptors probes at the membrane surface was determined as previously described (Holowka & Baird, 1983b) by fitting Q_d/Q_{da} vs acceptor density plots with a linear least-squares algorithm. The slope P of the best fit was related to L according to Shaklai et al. (1977):

$$P = (\pi R_o^6/2)(1/L^4) \quad (3)$$

Alternatively, the data were fit with theoretical curves derived from Monte Carlo calculations (Snyder & Friere, 1982; Holowka & Baird, 1983b). Consistent results were obtained from both methods.

Results

*Fluorescent Modification of ϵ/Cγ3**. In initial experiments, we found that very little FM is incorporated into ϵ/Cγ3* in the absence of reduction. After mild reduction with 0.25 mM DTT, the sulfhydryl groups in the C-terminal domain of the IgE derivative ϵ/Cγ3* were selectively labeled with FM. In four different preparations, the stoichiometry of labeling for FM-ϵ/Cγ3* was 1.2–2.1 mol of FM/mol of Ig. To quantify the labeling in locations other than the sulfhydryls introduced into the C-terminal domain of ϵ/Cγ3*, a control sample of ϵ/Cγ3 was incubated with FM under identical conditions. Preparations of FM-ϵ/Cγ3 had FM/Ig stoichiometries of 0.2–0.6, which were 10–28% of those for FM-ϵ/Cγ3* prepared in parallel. This background labeling is probably due largely to partial reduction and alkylation by FM of the interheavy disulfides located in Cϵ2 (Holowka & Baird, 1983b).

The location of the FM label introduced into ϵ/Cγ3* was analyzed by SDS–PAGE before and after digestion with trypsin. Under nonreducing conditions, SDS–PAGE of the unlabeled ϵ/Cγ3* preparation shows discrete bands corresponding to monomers, dimers, and trimers of intact Ig-$[(H_2L_2)_n]$. The presence of the oligomers is probably due to intermolecular disulfide bonds between the cysteines introduced into the C-terminal domains.[2] The percentage of the protein mass in the form of monomers varied from 40% to 70% in different preparations. No oligomers larger than trimers were ever observed within or at the top of the gel (data not shown). After the partial reduction and FM alkylation procedure all of the FM-ϵ/Cγ3* and FM-ϵ/Cγ3 appear as H_2L_2 monomers (>90% from visual observation; data not shown). Under reducing conditions, SDS–PAGE shows all detectable FM fluorescence in FM-ϵ/Cγ3* to be associated with the ϵ heavy chain (Figure 1, panels A and B, lanes 1). Very little fluorescence can be detected for the FM-ϵ/Cγ3 sample for the same amount of protein loaded on to the gel (Figure 1, panels A and B, lanes 2). In more heavily loaded gels, most fluorescence was located with the ϵ-chain with some at the electrophoretic front and none detectable in the light chain.

Trypsin digestion of FM-ϵ/Cγ3* results in the appearance of a nonfluorescent band with apparent molecular mass of 52 kDa and a fluorescent band with apparent molecular mass of 14 kDa (Figure 1, panels A and B, lanes 3–6). A 52-kDa fragment of IgE was previously characterized as the first three domains of the mouse ϵ-chain (V_h–Cϵ1–Cϵ2) (Holowka & Baird, 1983b). For native IgE, a 14-kDa fragment arises from cleavage near the C-terminal end of the Cϵ3 domain and contains Cϵ4 (Holowka & Baird, 1983b). For FM-ϵ/Cγ3* the 14-kDa fragment probably arises from cleavage at the same site and contains Cγ3. More than 80% of the FM fluorescence is associated with this fragment after complete trypsin digestion (Figure 1, lane 6) as determined by spectroscopic analysis of excised gel bands. Further support for the identity of the 14-kDa band was provided by the anti-Cγ3 antibody 49/2C3 (Nik Jaafar & Jefferis, 1982). Incubation of the trypsin-digested FM-ϵ/Cγ3* with this antibody resulted in selective immunoprecipitation of the fluorescent 14-kDa fragment as revealed by SDS–PAGE. The same procedure carried out with FM-ϵ/Cγ3 yielded no immunoprecipitated fluorescence with this antibody (data not shown).

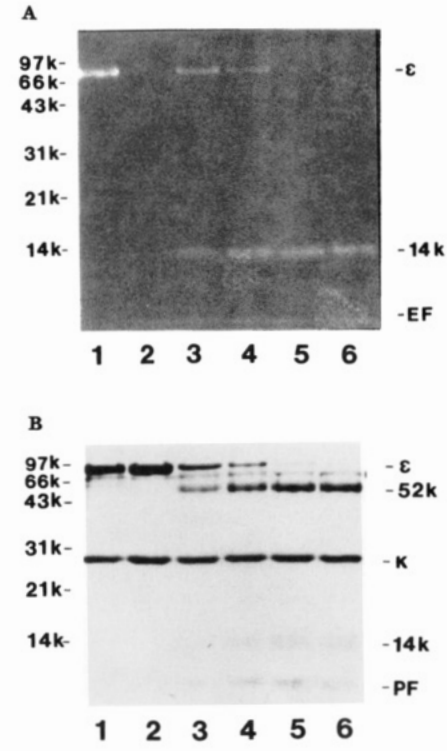

FIGURE 1: SDS–PAGE analysis of IgE derivatives. Fluorescence (A) or Coomassie blue staining (B) is shown for same gel run under reducing conditions. Lanes contain equal amounts (5 μg) of FM-ϵ/Cγ3* (lanes 1) or FM-ϵ/Cγ3 (lanes 2). Trypsin digestion of FM-ϵ/Cγ3* (lanes 3–6) was carried out for 20, 40, 80, and 120 min, respectively. Stoichiometry of labeling for these preparations are 1.6 mol of FM/mol of ϵ/Cγ3* and 0.3 mol of FM/mol of ϵ/Cγ3. Number labels indicate the apparent molecular weight, and ϵ and K indicate the positions of IgE heavy and light chains, respectively. EF indicates the electrophoretic front of the fluorescent gel, and PF indicates the protein front of the Coomassie stained gel.

Fluorescence parameters for the FM derivatives of ϵ/Cγ3* and ϵ/Cγ3 when these IgE derivatives are in solution and bound to receptors on the plasma membrane are summarized in Table I. The quantum yield for fluorescein in FM-ϵ/Cγ3*, 0.28, is somewhat less than that observed for fluorescein isothiocyanate conjugated to IgE (0.41) (Holowka & Baird, 1983b). This could reflect some self-quenching of FM at the C-terminal sulfhydryls due to possible proximity of those sites in the two Cγ3 in the paired heavy chains. The quantum yield for FM-ϵ/Cγ3 is quite low, <0.1. This is consistent with a high degree of stacking interactions occurring after reduction of disulfide bonds in Cϵ2 and modification with FM and other

Table I: Spectroscopic Parameters for FM-Labeled Derivatives of IgE

derivative	state[a]	Q_d[b]	A_d[d]
FM-ε/Cγ3*	solution	0.28	0.14
	receptor	0.28[c]	0.21
FM-ε/Cγ3	solution	<0.1	0.15
	receptor	<0.1[c]	0.33

[a] IgE derivatives free in solution or bound to receptors on plasma membrane vesicles derived from RBL cells. [b] Quantum yield of the FM probe. [c] Q_d not measured directly but inferred from no observed change in fluorescence upon IgE binding to receptors on vesicles. [d] Steady-state anisotropy of the FM probe at 22 °C (excitation at 490 nm; emission at 520 nm).

fluorescent alkylating agents (D. Holowka and B. Baird, unpublished observation). The FM fluorescence does not change when FM-ε/Cγ3 and FM-ε/Cγ3* bind to receptors (Table I).

The steady-state fluorescence anisotropy values for fluorescein in FM-ε/Cγ3 and FM-ε/Cγ3* (Table I) are less than those expected for such probes rigidly attached to IgE in solution, and this indicates some freedom of movement of these probes during their excited-state lifetimes (Holowka & Baird, 1983b). The anisotropy increases more for FM-ε/Cγ3 than for FM-ε/Cγ3* when these IgE derivatives bind to receptors on the plasma membrane. This result is consistent with the view that the label in FM-ε/Cγ3 is primarily located in Cε2, which is probably near the site of the binding of IgE to receptor (Helm et al., 1987). Cε2 is expected to be more rigidly constrained in the receptor complex than the C-terminal domain, which is primarily labeled in FM-ε/Cγ3* and which is not involved in the binding interaction (Weetall et al., 1990). Low steady-state anisotropy values are also found for the acceptor probes used in the energy transfer experiments described below: 0.19 for HAE and ORB at the surface of the plasma membrane (Holowka & Baird, 1983a) and 0.28 for ED in the antigen-binding sites (data not shown). These results indicating rotational freedom of both donors and acceptors support the approximation that the donor and acceptor dipole moments are randomly distributed with respect to each other.

As demonstrated previously, ε/Cγ3 binds as well as native IgE to receptors on RBL cells in a competition assay with ^{125}I-labeled IgE (Baird et al., 1989) and in direct binding assays (Weetall et al., 1990). The IgE derivatives ε/Cγ3* and FM-ε/Cγ3* were also tested in a competition assay and were found equivalent to native IgE in binding to receptor (data not shown). These results show that neither the replacement of Cε4 by Cγ3 containing a sulfhydryl nor its FM modification affects binding of IgE to its high-affinity receptor.

Distance from FM in the C-Terminal Domain of Receptor-Bound ε/Cγ3 to the Membrane Surface.* Resonance energy transfer was measured between the FM donors that are predominately on the C-terminal domain of receptor-bound FM-ε/Cγ3* and amphipathic acceptor probes, either HAE or ORB, inserted into the plasma membrane bilayer. Time-dependent quenching of the FM fluorescence was monitored upon addition of FM-ε/Cγ3* to RBL cell membrane vesicles containing different surface densities of acceptors (Figure 2A). Values for the acceptor densities were determined directly by parallel measurements of energy transfer between membrane probes HAF (donor) and HAE or ORB (acceptors) (data not shown). The FM fluorescence measured in the presence (Q_{da}) and absence (Q_d) of acceptors was corrected for unbound FM-ε/Cγ3* and for the FM fluorescence of FM-ε/Cγ3 measured in a paralle sample (Figure 2A). If FM-ε/Cγ3* is added to vesicles that contain acceptor probes but have receptors occupied with unlabeled IgE, there is no time-dependent quenching of donor fluorescence, indicating that the quenching observed in Figure 2A is dependent on FM-ε/Cγ3* binding to receptors.

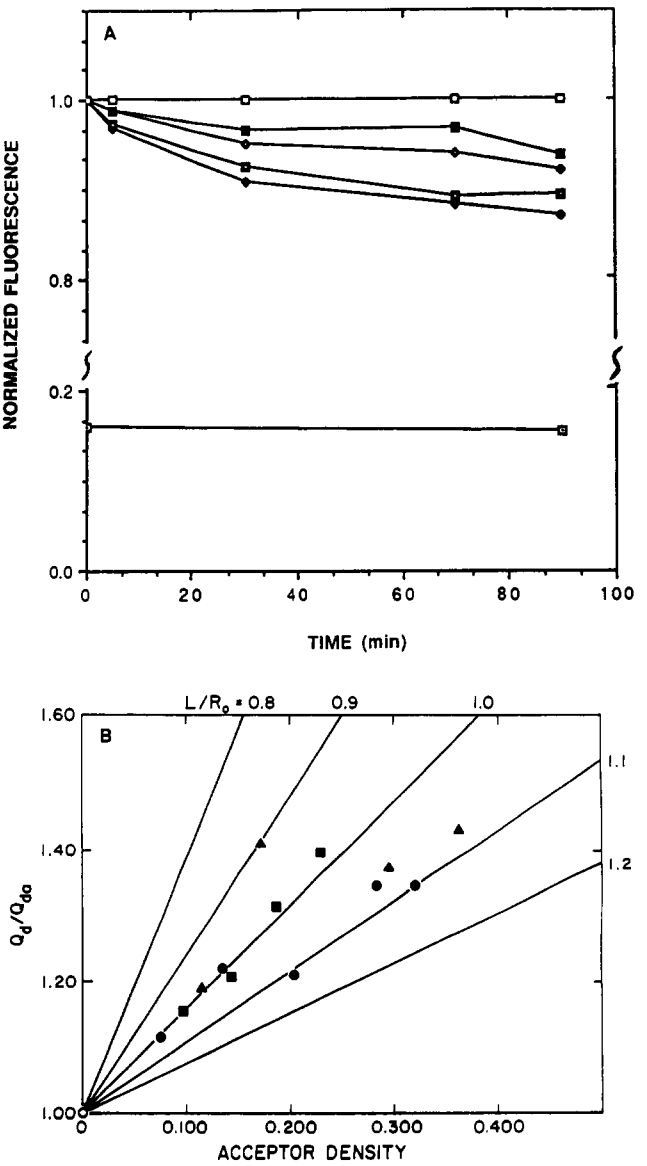

FIGURE 2: Energy transfer between donor FM at the C-terminus of receptor-bound FM-ε/Cγ3* and acceptor HAE at the membrane surface. (A) Fluorescence quenching of FM-ε/Cγ3* (5 nM) accompanies its binding to receptors on vesicles containing the acceptor probe at densities 0 (□), 1.0×10^{-3} (■), 1.4×10^{-3} (◇), 1.9×10^{-3} (⧈), and 2.3×10^{-3} (◆) acceptors/Å^2, respectively. The fluorescence intensities at time = 0 of the FM-ε/Cγ3* samples containing HAE are normalized to the sample with no HAE. The control sample containing FM-ε/Cγ3 (⊡) has no acceptor in the membrane. (B) The ratio of corrected donor fluorescence for FM-ε/Cγ3* in the absence (Q_d) and presence (Q_{da}) of acceptors is plotted as a function of acceptor density (units of acceptors/R_o^2; R_o = 50 Å). The three different symbols (●, ▲, ■) are from independent experiments. The solid lines are calculated from eq 3 for the indicated values of L/R_o.

Figure 2B shows the efficiency of energy transfer (as Q_d/Q_{da}) for FM-ε/Cγ3* and several densities of HAE in three different experiments. The data are compared with several theoretical curves (eq 3; Shaklai et al., 1977) corresponding to different values of L/R_o, where L is the distance of closest approach between the donor probe and the membrane surface. The best fit to the data is obtained with $L/R_o = 1.08$, corresponding to L = 54 Å. Fitting the data with the Monte Carlo method (Snyder & Freire, 1982) yields L = 52 Å. Similar measurements with ORB as acceptor yielded similar values for L: 56 Å as determined with eq 3 and 53 Å as determined

Table II: Summary of Energy Transfer Measurements with FM-ϵ/Cγ3*[a]

state	acceptor[b]	R_0 (Å)	E[c]	L (Å)[d]	R (Å)[e]
receptor-bound	HAE	50	na[f]	53	na
receptor-bound	ORB	52	na	54	na
solution	ED	50	0.20	na	71
receptor-bound	ED	50	0.22	na	69

[a] The donor probe FM is located at the C-terminus of ϵ-Cγ3*. Corrections for nonspecific label have been made as described in text. [b] The acceptor probe ED is located in antigen-binding sites; HAE and ORB are located at the membrane surface. [c] Efficiency of energy transfer calculated with eq 2. [d] L calculated with eq 3; the value is the average of four experiments (standard deviations SD = ±2.8 for HAE as acceptors and ±3.5 for ORB as acceptors). [e] R calculated with eq 2; the value is the average from four experiments (SD = ±0.9 for solution form and ±1.2 for receptor-bound form). [f] Not applicable.

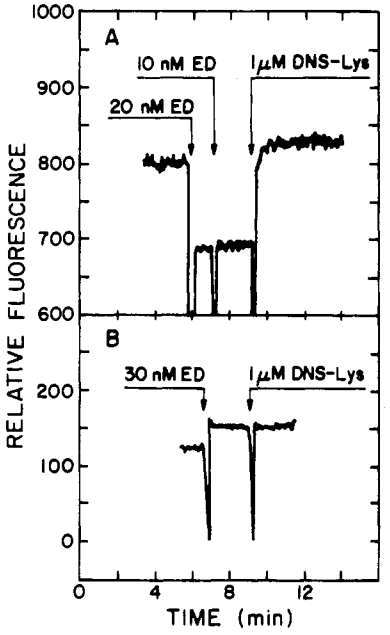

FIGURE 3: Energy transfer between donor FM at the C-terminus and acceptor ED in the antigen-binding sites of receptor-bound IgE derivatives. Data are shown for changes in fluorescein fluorescence of FM-ϵ/Cγ3* (A) or FM-ϵ/Cγ3 (B) upon ED addition and upon addition of a large excess of DNS-Lys to displace the ED. Excitation was at 480 nm, and emission was at 510 nm.

with the Monte Carlo method (Table II).

*Distance from the F_{ab} Binding Sites to the F_c C-Terminus of FM-ϵ/Cγ3**. The DNS specificity of ϵ/Cγ3* allows placement of a suitable acceptor probe (ED) in the antigen-binding sites. The distance between these sites and the FM probe, predominately at the C-terminus of FM-ϵ/Cγ3*, can be measured by energy transfer between fluorescein and eosin. Figure 3A shows a typical fluorescence trace for this measurement for ϵ/Cγ3* bound to receptors on the vesicles. Upon addition of ED to the suspension, the FM fluorescence is immediately decreased, but the shape of the emission spectra is not significantly altered (not shown). Eosin fluorescence contributes to the signal at the wavelengths used to monitor the FM fluorescence. Therefore, a more accurate measurement of energy transfer is obtained by comparing the fluorescence after the addition of ED (Q_{da}) to that after the subsequent addition of an excess of DNS-Lys, which effectively competes for the DNS-binding sites but does not contribute to the fluorescence signal (Q_d). A similar measurement for the control sample of ED and FM-ϵ/Cγ3 bound to receptors on the vesicles shows no energy transfer and no change in fluorescence due to the addition of DNS-Lys (Figure 3B). After subtraction of the fluorescence of the control sample (FM-ϵ/Cγ3), the efficiency of energy transfer between FM at the C-terminus and ED at the antigen-binding sites of FM-ϵ/Cγ3* was determined to be 0.22. For energy transfer between a single donor site and two equidistant acceptor sites this efficiency corresponds to a distance of 69 Å (eq 2; Table II).

Energy transfer was also measured for ED bound to the antigen-binding sites of FM-ϵ/Cγ3* in solution, and the data obtained look very similar to those shown in Figure 3A. Comparison of the FM fluorescence after addition of ED to that after the subsequent addition of excess DNS-Lys yields an efficiency of energy transfer of 0.20 after correction with the control sample (data not shown). This value corresponds to a distance of 71 Å between the FM probes at the C-terminus and the ED in the two F_{ab} binding sites for FM-ϵ/Cγ3* (eq 2; Table II). The control sample containing ED and FM-ϵ/Cγ3 in solution had similar fluorescence signals to those shown in Figure 3B and showed no detectable energy transfer (data not shown).

Discussion

The energy transfer experiments described in this report provide direct measurements on receptor-bound IgE, locating the position of the F_c segment C-terminus in relation to the membrane surface and to the N-termini of the F_{ab} segments. The distance between the F_c and F_{ab} sites was also determined for IgE in solution. These studies were made possible by the preparation of a genetically engineered monoclonal Ig, ϵ/Cγ3*, with antigen-binding sites specific for DNS at its N-terminal ends and two cysteine residues introduced near the C-terminal ends of the heavy chains. Mild reduction of ϵ/Cγ3* followed by reaction with FM results in selective modification of the C-terminus with this fluorescent donor probe. The high degree of specific labeling within FM-ϵ/Cγ3* is demonstrated by the location of the FM fluorescence in trypsin fragments of FM-ϵ/Cγ3* (Figure 1) and by comparison to FM-ϵ/Cγ3, which does not contain the C-terminal cysteine residues and is labeled with a much lower stoichiometry. As expected from previous studies with the ϵ/Cγ3 derivative (Weetall et al., 1990), both ϵ/Cγ3* and FM-ϵ/Cγ3* bind as well as native IgE to high-affinity receptors on RBL cells.

The energy transfer distances reported here for receptor-bound IgE are consistent with our previous model of this structure (Holowka et al., 1985) and, moreover, allow further refinement. Our current model is shown in Figure 4. The distance of 53 Å measured between the end of the C-terminal domain (Cϵ4 in native IgE) and acceptor probes at the membrane surface (Table II) is incorporated with the distances to the surface previously measured: 45 Å for probes conjugated to sulfhydryls in Cϵ2 (Holowka & Baird, 1983b) and ≥100 Å for probes in the antigen-binding sites at the two N-terminal ends (Baird & Holowka, 1985). The results strongly support the hypothesis that receptor-bound IgE has a compact structure with the C-terminal domain bending away from the membrane surface. This bent conformation is further supported by the distance measured between probes at the C-terminal end and in the antigen-binding sites (Table II). This distance of 69 Å is considerably less than that expected for an extended Y-shaped IgE. If we assume each Ig domain is ~40 Å long (Amsel & Pojak, 1979), we roughly estimate the extended end-to-end length to be in the range of ~140–~200 Å, corresponding to angles ranging from 180° to 0° between the major axes of the two F_{ab} segments. The measured energy transfer distances are unlikely to have large errors due to fixed dipole–dipole orientations (as expressed by κ^2 in eq 1) because

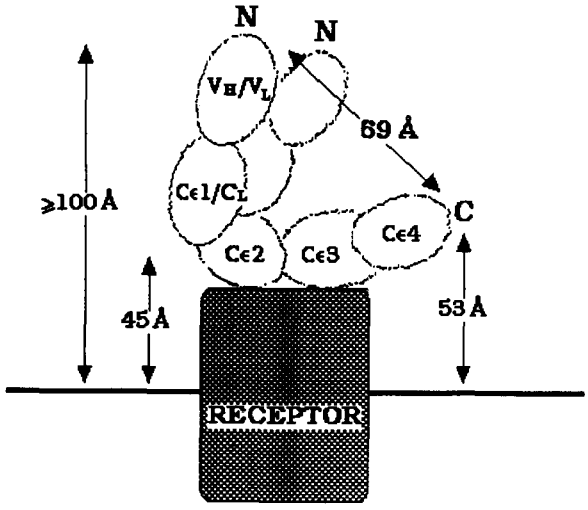

FIGURE 4: Model of IgE bound to receptor on the plasma membrane. Pairs of domains within the ϵ heavy chains are labeled, and the amino-, and carboxy-terminal ends are indicated as N and C, respectively. The solid horizontal line represents the outer surface of the plasma membrane.

both donors and acceptors show some freedom of rotation on the nanosecond time scale as indicated by their measured steady-state anisotropies.

The markedly bent structure determined for receptor-bound IgE contrasts with the commonly accepted description of the IgE molecule. The latter model is based on the X-ray crystallographic structure of IgG, which appears roughly Y-shaped with the major axes of the F_{ab} segments lying in the same plane as the major axis for the F_c segment (Rajan et al., 1983). IgE is similar to IgG in polypeptide structure except that IgE has a full-size domain ($C\epsilon2$) in place of the polypeptide hinge region of IgG. In this regard, it should be noted that the only X-ray structures of entire IgG molecules are those of the hinge-deleted proteins (Silverton et al., 1977; Rajan et al., 1983). Assuming that the average conformation for IgE in solution is planar, we previously hypothesized that IgE undergoes a significant structural change and bends somewhere between the $C\epsilon1$ domain of the F_{ab} segments and the $C\epsilon3$ domain in the F_c segment upon binding to its receptor on the cell surface. An unexpected result from the present studies is the energy transfer distance measured between the N- and C-terminal ends for FM-ϵ/Cγ3* in solution (Table II). This distance of 71 Å indicates that IgE in solution has an average bent structure and appears, by this criterion, to be similar in conformation to receptor-bound IgE (Figure 4).

The results suggesting a bent structure for IgE require further consideration because of the averages involved: what structure(s) is (are) represented by a single energy transfer measurement that yields an average distance? Like other large proteins, the IgE molecule has a distribution of conformations as allowed by its internal rotational modes (i.e., its segmental flexibility). Accordingly, each energy transfer distance measured on our experiments represents an average over the corresponding distribution of distances between the donor and acceptor probes. We consider two limiting cases consistent with an average bent structure for IgE.

(Case 1) IgE has a single conformation that is rigidly bent with the each antigen-binding site equidistant from the C-terminus. Then there is a single end-to-end distance, and this is the distance measured in the energy transfer experiment (71 Å corresponding to $E = 0.20$ for IgE in solution). If IgE is rigidly bent "side-ways" such that the F_c segment lies in the same plane as the F_{ab} segments and the C-terminus is close to a single antigen-binding site, then this distance is 63 Å for $E = 0.20$.

(Case 2) IgE is very flexible, bending in all directions from a planar structure. In this case there is a distribution of end-to-end distances, and the energy transfer distance is an average that is weighted more heavily by the shorter distances according to the R^{-6} dependence (eq 2). As an illustration of this weighted averaging, consider the simple case of IgE with 90° between the major axes of the two F_{ab} segments, which define a plane. Assume that the domains in F_c segment ($C\epsilon2$–$C\epsilon2$–$C\epsilon4$) are aligned rigidly and that the major axis of this segment flexes and spends an equal amount of time in each of three discrete positions: (1) in the same plane as the F_{ab} segments (i.e., no bending; the distance from the C-terminus to each of the two N-termini is 175 Å when the same approximations as above are used); (2) bending forward from this plane such that the C-terminus is a distance d from each of the N-termini; (3) bending backward from this plane such the C-terminus is a distance d from the N-termini (symmetric with position 2). In this case $E = 0.20$ corresponds to d = 65 Å for positions 2 and 3, and the angle through which the F_c segment flexes on each side of the plane is 165°. A variation of this case is the IgE F_c segment flexing from side to side and occupying three discrete positions of the same plane as the F_{ab} segments such that the C-terminus gets close (distance d') to a single antigen-binding site on each side. In this latter case $E = 0.20$ corresponds to $d' = 58$ Å, and the angular excursion is ~155°. The angles indicated in these simple examples are extreme because no bending is allowed between the domains within the F_c segment.

Previous fluorescence anisotropy measurements have partially characterized the segmental flexibility of IgE. IgE in solution appears to be less flexible than the IgG subclasses (Oi et al., 1984), although some internal motion is evident for both free and receptor-bound IgE (Slattery et al., 1985). Nanosecond depolarization measurements of IgE bound to receptors on membrane vesicles show only limited internal motion that includes twisting about the long axis of the F_{ab}, as well as possible contributions from elbow-bending at the V–$C\epsilon1$ junction and limited amounts of F_{ab} wagging or scissoring (Holowka et al., 1990). These anisotropy data and the other experimental information summarized in Figure 4 together with consideration of the steric constraints imposed by proximity of the membrane surface suggest that receptor-bound IgE is held in a relatively rigid conformation. The case is less clear for IgE in solution because the receptor and membrane constraints are not present and interpretation of the anisotropy data is more uncertain. However the anisotropy data do not appear to support rapid flexing as included in case 2 described above.

The possibility that Ig's, including IgE, are bent in solution has been considered previously (Burton, 1990). However, direct physical measurements providing information about these structures are very limited. Recent experiments measuring sedimentation and small-angle X-ray scattering for IgE in solution yielded data that can only be reasonably fitted by a model in which this molecule is bent (Burton, 1990; K. G. Davis and D. R. Burton, manuscript in preparation). For example, the radius of gyration for IgE is found to be similar to that for IgG, suggesting a compact structure for IgE because it has about 20–30% greater mass than IgG due to additional glycosylation sites and additional domains ($C\epsilon2$). These data by themselves cannot distinguish between a rigid bent structure and a very flexible structure, but possible conformations of IgE might be revealed in highly resolved electron micrographs.

Initial efforts with negative staining and rotary shadowing electron microscopy are consistent with a more compact structure for IgE, but so far the data are not adequate to provide a conclusive picture (D. Holowka and K. L. Mercer, unpublished data).

In summary, the energy transfer results described here provide strong support for our previously proposed model of a relatively rigid bent structure for IgE bound to its membrane receptor. The C-terminal end of IgE is about as far from the membrane surface as the interchain disulfides in the Cε2 domain (~50 Å), and the antigen-binding sites are remarkably close to the C-terminal end (~70 Å). Our finding that this same average end-to-end distance is maintained for IgE in solution suggests a new model for this molecule as a bent structure rather than the commonly depicted planar Y-shape structure. Experiments in progress are aimed at addressing whether the energy transfer distance obtained for IgE in solution is due to a rather rigid compact conformation or to a dynamic average over a wide range of conformations.

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