Enzymatic Cleavage Products of the α Subunit of the Receptor for Immunoglobulin E†

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ABSTRACT: The high-affinity membrane receptor for immunoglobulin E on mast cells and on a tumor analogue, rat basophilic leukemia cells, consists of two polypeptide chains: an α chain of $M_r \simeq 50\,000$ and a β chain of $M_r \simeq 30\,000$. In this study we reacted α chains purified from tumor cells with proteolytic and glycolytic enzymes and compared the products by using differential labeling procedures. A variety of proteolytic enzymes cleave the chain into two similar-sized fragments, α_1 and α_2. The α_1 fragment behaves as if it were slightly larger on electrophoresis through polyacrylamide gels and is rich in carbohydrate as determined by incorporation of [^{14}C]glucosamine. Less incorporation is observed into α_2 but it, like α_1, binds to concanavalin A. Labeling of the surface proteins on intact cells by lactoperoxidase-catalyzed iodination leads to preferential, perhaps exclusive, labeling of the α_2 fragment. The relative proportion of incorporated ^{3}H-labeled amino acids and radioactive Bolton–Hunter reagent suggests that the polypeptide portions of α_1 and α_2 are similar in size. From these and other data we propose that the α chain may be U-shaped. Results from endoglycosidase digestions show that the receptor as isolated is heterogeneous because of variable glycosylation.

Mast cells, basophils, and a tumor analogue, rat basophilic leukemia (RBL)[1] cells, contain a cell surface receptor for immunoglobulin E (IgE) that mediates their degranulation by a mechanism that requires bridging of at least two receptor moieties (Segal et al., 1977). We are currently investigating the structure of this receptor in an attempt to understand the molecular consequences of receptor bridging.

Our laboratory has presented evidence that the functional IgE receptor consists of two noncovalently linked subunits. The larger subunit (α), which travels as an unusually broad band of M_r = 50–60K on electrophoresis through polyacrylamide gels in $NaDodSO_4$, is an integral membrane glycoprotein and contains the IgE binding site (Conrad & Froese, 1976; Kulczycki et al., 1976). It can be purified from RBL tumors in submilligram amounts (Kanellopoulos et al., 1979), shows a moderately high content of hydrophobic amino acids, and contains about 30% carbohydrate (Kanellopoulos et al., 1980). The smaller subunit (β), first identified by its ability to cross-link to α both on intact cells and in solution (Holowka et al., 1980), has a M_r of 30–35K, cannot be labeled by lactoperoxidase-catalyzed cell surface iodination, and appears not to be glycosylated (Holowka & Metzger, 1981). β, but not α, reacts with the photolyzable hydrophobic reagent 5-iodonaphthyl 1-azide in situ (Holowka et al., 1981). Although the interaction between α and β, prior to purification, seems to be extremely strong (Holowka & Metzger, 1981), the β subunit is variably lost during affinity purification of α and has not yet been preparatively isolated.

In this study we dissected the structure of the α subunit by using protease treatment combined with differential labeling techniques. The results indicate that α can be cleaved into well-defined fragments which differ in the degree of incorporation of various labels. These results allow preliminary discussions concerning the topological organization of various domains of α and provide a framework for more detailed structural analysis.

Materials and Methods

Reagents. Enzymes were obtained from the following sources: trypsin (232 units/mg), α-chymotrypsin (66 units/mg), and papain (25 units/mg) from Worthington (Freehold, NJ); Pronase and thermolysin (7420 Plough units/mg) from Calbiochem-Behring Corp. (La Jolla, CA); bromelain (3185 units/g of protein), subtilisin BPN′ (Type VII, 11 units/mg of solid), ficin (1.9 units/mg of protein), and lactoperoxidase from Sigma (St. Louis, MO); protease V-8, endo-β-*N*-acetylglucosaminidase D (endoglycosidase D), endo-β-*N*-acetylglucosaminidase H (endoglycosidase H), and mixed glycosidases (from *Thermophilus cornutus*) from Miles (Elkhart, IN); neuraminidase and α-D-*N*-acetylgalactosaminyloligosaccharidase from Bethesda Research Laboratories (Rockville, MD).

Carrier-free $Na^{131}I$, D-[U-^{14}C]glucosamine, and the high specific activity tritiated amino acid mixture (code TRK 550) were obtained from Amersham Corp. (Arlington Heights, IL), and Bolton–Hunter reagent [*N*-succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate] was from New England Nuclear (Boston, MA). Triton X-100 (scintillation grade) was from Research Products International Corp. (Elk Grove, IL). Sodium dodecyl sulfate ($NaDodSO_4$) and other reagents for $NaDodSO_4$–polyacrylamide gel electrophoresis were electrophoretic purity grade from Bio-Rad (Richmond, CA). The hapten *p*-azobenzenearsonate–L-tyrosine was from Bioresearch (San Rafael, CA) and concanavalin A–Sepharose from Pharmacia (Piscataway, NJ).

Purification of α Chain. The pure IgE α-chain complex was isolated from rat basophilic leukemia tumors maintained in young WKY/N rats and labeled with [^{125}I]Bolton–Hunter reagent (Bolton & Hunter, 1973) as previously described (Kanellopoulos et al., 1979). Separation of α from IgE was achieved by gel filtration on a Sephacryl S-300 column in 0.1%

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[1] Abbreviations used: RBL, rat basophilic leukemia; IgE, immunoglobulin E; $NaDodSO_4$, sodium dodecyl sulfate; BH, Bolton–Hunter reagent; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

$NaDodSO_4$ in borate-buffered saline. Fractions containing α chains were pooled, diluted 7-fold with 0.05% $NaDodSO_4$ in H_2O (to achieve submicellar concentrations of $NaDodSO_4$), and concentrated in an Amicon ultrafiltration cell using a YM-10 membrane. The material was then equilibrated with a buffer consisting of 0.125 M Tris-HCl, 0.05% $NaDodSO_4$, and 10% glycerol, pH 6.8 ("$NaDodSO_4$-digest buffer"), by dialysis.

^{131}I-Labeled α chain was isolated from cells subjected to lactoperoxidase-catalyzed surface radioiodination (Cone & Marchalonis, 1974; Conrad & Froese, 1976) and reaction with IgE prior to immune precipitation with anti-IgE and *Staphylococcus aureus* cells (Pansorbin, Calbiochem-Behring Corp.) (Holowka et al., 1980). For experiments involving digestion of a mixture of surface-labeled and Bolton–Hunter-labeled α chains, the immune precipitate from surface-iodinated cells was dissolved in $NaDodSO_4$, mixed with Bolton–Hunter-labeled purified α chain, and then diluted with 50 mM Tris-HCl, pH 7, by an amount sufficient to lower the $NaDodSO_4$ concentration below its critical micellar concentration (Helenius & Simons, 1975). The mixture was then concentrated by ultrafiltration and dialyzed against $NaDodSO_4$-digest buffer as described above.

α chain biosynthetically labeled with ^{3}H-labeled amino acids was purified by a modification of the method of Holowka et al. (1980) in order to remove labeled β chains completely. Briefly, biosynthetically labeled cells were saturated with azobenzenearsonate-conjugated IgE (Kanellopoulos et al., 1979) and solubilized in nonionic detergent, and the extract was subjected to affinity chromatography on anti-benzenearsonate–Sepharose 6B. This column was washed 3 times (once every 24 h) with 100 column volumes of the buffer previously described by Holowka et al. (1980) before eluting with 1 mM *p*-azobenzenearsonate–tyrosine. This eluate, when analyzed directly by $NaDodSO_4$–polyacrylamide gel electrophoresis, contained predominantly labeled α chain as well as some high molecular weight material but was essentially free of contaminating β chain. For more complete purification, the affinity column eluates were concentrated and electrophoresed on a 3 mm thick 10% acrylamide slab without reducing agents. The α-chain band was located by counting a portion of the gel, and the remainder was extracted by agitation of the gel with buffer containing 0.05 M Tris-HCl, 0.4 M glycine, and 0.1% $NaDodSO_4$, pH 7, for two 12-h periods. The extracted material was then diluted 5-fold with 0.05% $NaDodSO_4$ in H_2O, concentrated, and dialyzed vs. $NaDodSO_4$-digest buffer. Labeling of cells with [^{14}C]glucosamine was carried out as described previously (Kulczycki et al., 1976; Holowka & Metzger, 1981). The α–IgE complexes were isolated by affinity chromatography as described above for the ^{3}H-labeled amino acid labeled receptor. The eluates were pooled and BH-labeled complexes of α chain and IgE were added to them prior to their application on a Sephacryl S-300 column equilibrated with 0.05% $NaDodSO_4$ in borate-buffered saline. The α-chain peak was next concentrated on hydroxylapatite (Kanellopoulos et al., 1980), and the eluate was dialyzed against the $NaDodSO_4$-digestion buffer.

Protease Digestion of α Chain. Proteolysis was carried out at 37 °C in $NaDodSO_4$-digest buffer according to the general method of Cleveland et al. (1977). Samples were boiled prior to addition of enzyme to destroy any endogenous protease activity. For trypsin or chymotrypsin digestion the buffer contained additionally 5 mM Ca^{2+}. For experiments involving SH-dependent proteases (bromelain, papain, ficin) both α-chain and enzyme solutions were separately incubated at 37 °C in $NaDodSO_4$-digest buffer containing 1 mM EDTA and 5 mM cysteine for 15 min prior to mixing. In all cases digestion was stopped by the addition of 20% $NaDodSO_4$ and 2-mercaptoethanol to final concentrations of 2% and 10%, respectively, followed immediately by boiling for 2 min.

Glycosidase Digestion of α Chain. [^{125}I]Bolton–Hunter-labeled α chain purified by chromatography in $NaDodSO_4$ was equilibrated with the appropriate buffer containing Triton X-100 prior to glycosidase digestion. The concentration of α was $\simeq$0.5 μg/mL and digestion protocols were as follows. *Neuraminidase*: digestion was carried out in 50 mM phosphate and 0.06% Triton X-100, pH 6.0, at an enzyme concentration of 4.8 units/mL for 24 h. *Endoglycosidase D*: digestion was performed in 50 mM citrate/phosphate and 0.1% Triton X-100, pH 6, at an enzyme concentration of 0.1 unit/mL for 24 h. *Endoglycosidase H*: digestion was carried out in 100 mM citrate/phosphate and 0.1% Triton X-100, pH 5.4, at an enzyme concentration of 0.17 unit/mL for 24 h. *Mixed glycosidases*: digestion was carried out in 50 mM citrate/phosphate and 0.1% Triton X-100, pH 4, containing 0.5 mg/mL bovine serum albumin for 24 h; the crude enzyme concentration was 0.5 mg/mL. *α-D-N-Acetylgalactosaminyloligosaccharidase*: digestion was carried out in 75 mM phosphate and 0.06% Triton X-100, pH 6.5, for 48 h at an enzyme concentration of 0.17 unit/mL. All digestions were performed at 37 °C.

Miscellaneous. Slab gels containing acrylamide concentrations of 10%, 12.5%, or 17% were prepared as described (Laemmli, 1970; Ames, 1974). Alternatively, samples were analyzed on 4–30% acrylamide gradient gels (PAA 4/30, Pharmacia). Reduced low molecular weight standards from Bio-Rad were used to calibrate the gels. These consisted of phosphorylase *b* (M_r = 94K), bovine serum albumin (M_r = 68K), ovalbumin (M_r = 43K), carbonic anhydrase (M_r = 30K), soybean trypsin inhibitor (M_r = 21K), and lysozyme (M_r = 14.3K).

Autoradiography of dried slab gels was performed at −90 °C (Swanstrom & Shank, 1978) with Kodak SB5 film and various Du Pont intensifying screens (Detail, Par Speed, or Quanta III). For the Pharmacia gradient gels autoradiography was carried out by using the stained, frozen gels as described above.

For quantitation of ^{125}I or ^{131}I, the dried gels were cut and counted directly in a γ counter. For ^{3}H and ^{14}C counting, the dried gel slices (2 mm) were rehydrated with 120 μL of water, extracted overnight with 1 mL of NCS tissue solubilizer, and counted in 18-mL 3a20 scintillation cocktail (Research Products International Corp., Elk Grove, IL).

Binding assays with concanavalin A–Sepharose were carried out in a buffer containing 0.1 M Tris, 0.2 M $NaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.2% Triton X-100, pH 7.5.

Results and Discussion

Effect of Proteases on α Chains Labeled with Bolton–Hunter Reagent. Incorporation of radioiodine via the Bolton–Hunter reagent (BH) into purified IgE–α complexes in Triton X-100 solution (Kanellopoulos et al., 1979) currently represents the most convenient method of obtaining α radiolabeled to high specific activity. Such material was routinely used in the initial experiments.

The upper panels in Figure 1 show typical results from proteolytic digestions of BH-labeled α. Representative results of treatment with papain and subtilisin BPN′ are shown. Other proteases tested (Pronase, protease V-8, elastase, ficin, thermolysin, and trypsin) gave strikingly similar results. Each

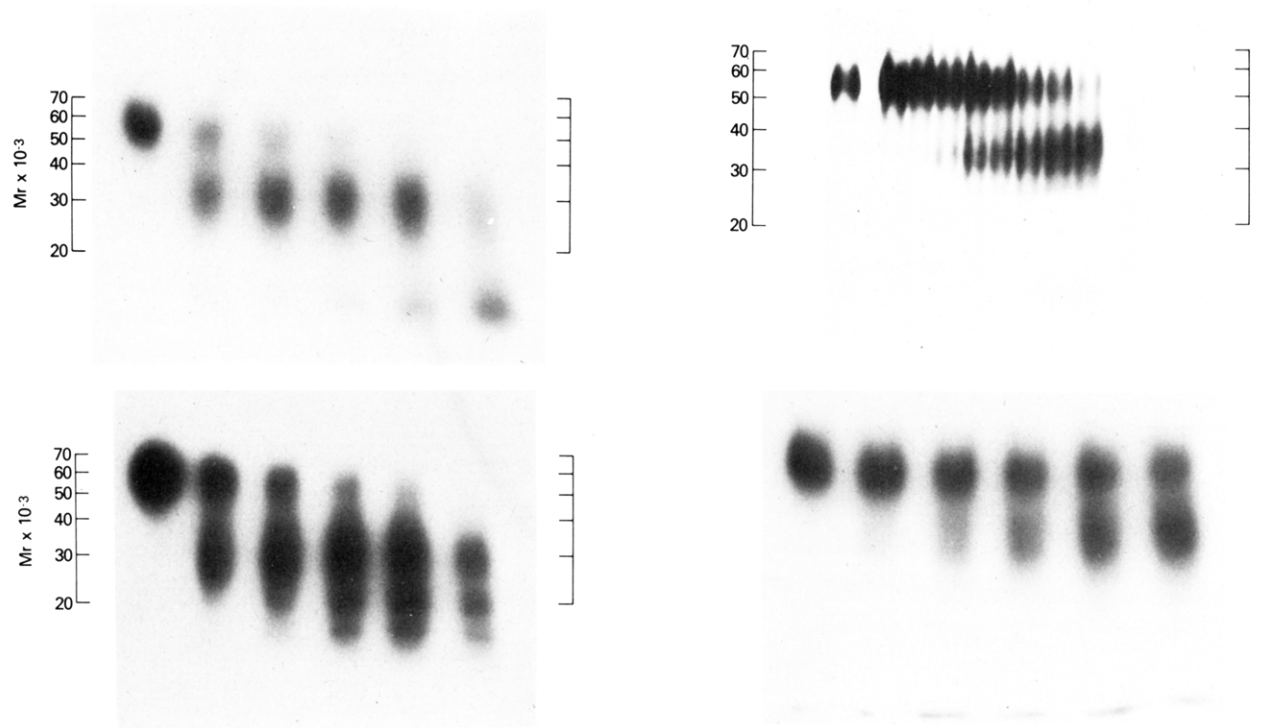

FIGURE 1: Autoradiographs of protease-treated, BH-labeled α analyzed on $NaDodSO_4$–polyacrylamide gel electrophoresis. Unless otherwise indicated digestion was carried out in a 0.125 M Tris-HCl, pH 6.8, buffer containing 10% glycerol and 0.05% $NaDodSO_4$. All gels in this and subsequent figures were run under reducing conditions. (Upper left) 4–30% acrylamide gradient gel of α treated with 0.5 μg/mL preactivated papain in the presence of 5 mM cysteine and 1 mM EDTA for 0, 2, 4, 10, 30, and 120 min. (Upper right) 17% acrylamide gradient gel of α treated with 0.2 μg/mL subtilisin BPN′ in the presence of 100 μg/mL ovalbumin carrier for 0, 1, 2, 5, 10, 20, 30, 50, and 90 min. (Lower left) 4–30% acrylamide gradient gel of α treated with 1 μg/mL preactivated bromelain in the presence of 5 mM cysteine, 1 mM EDTA, and 0.1 mg/mL ovalbumin carrier for 0, 5, 15, 30, 60, and 125 min; an additional band of $M_r \simeq$ 10K, usually produced under these conditions, is not clearly visible in this figure. (Lower right) 4–30% acrylamide gradient gel of α treated with 1.2 μg/mL preactivated bromelain in the presence of 5 mM cysteine, 1 mM EDTA, and 0.5% $NaDodSO_4$ for 0, 5, 15, 30, 60, and 120 min; the gel was destroyed before the standards were stained so no molecular weight scale is indicated.

of these enzymes generally produced two diffuse bands of $M_r \simeq$ 32K and $M_r \simeq$ 10K when the gels were analyzed by autoradiography. The kinetics of digestion indicated that digestion of α initially produced the M_r = 32K band exclusively, and this was followed by generation of the M_r = 10K band. After very extensive digestion little radioactivity was recovered on the gel, indicating breakdown to very small peptides which were eluted from the gel during staining and destaining. Chymotrypsin digestion gave a somewhat different result in that a noticeably broader $M_r \simeq$ 32K band was produced (not shown). Occasionally, instead of a single diffuse band of $M_r \simeq$ 32K, the 17% acrylamide slab gels showed a series of sharp bands in the same region of the gel. This was most noticeable for digestions with chymotrypsin or protease V-8. Purified preparations of α stored in $NaDodSO_4$ solution at 4 °C for several months generated small amounts of breakdown products similar to those in Figure 1 (upper panels).

Initially, in order to control accurately the enzyme:substrate ratio, we included carrier protein (usually ovalbumin) along with the much smaller amounts of radiolabeled α in the digestion mixture. The enzyme:substrate (carrier + α) ratio was commonly kept constant at 1:100 (w/w). The α chain was notably more resistant to proteolysis than the carrier protein. The digestion of the latter was followed by Coomassie blue staining of the gels and showed rapid breakdown into $M_r \leq$ 20000 fragments long before digestion of the α chain was complete and before the M_r = 32000 fragments of α were cleaved further. When the carrier concentration was increased from 0.1 to 1 mg/mL (with corresponding increases in enzyme), the effect with most enzymes was simply an increase in the rate of digestion. However, with bromelain, an enzyme

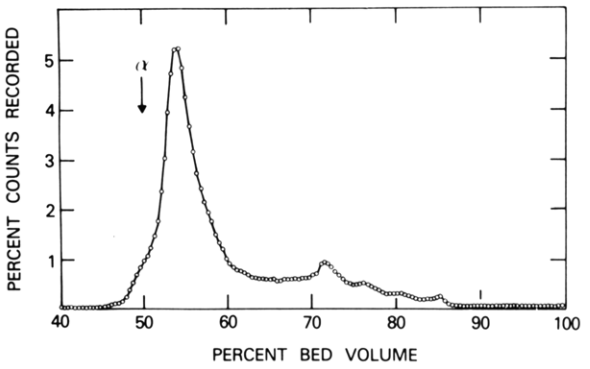

FIGURE 2: Chromatography of bromelain-digested, BH-labeled α on Sephacryl S-300 in 0.1% $NaDodSO_4$ in borate-buffered saline. α was treated with 3 μg/mL preactivated bromelain in the presence of 0.3 mg/mL ovalbumin for 2 h. The arrow marks the position where undigested α is eluted.

subsequently investigated in some detail, different products were stabilized when the digestion was performed at differing carrier concentrations. Digestion at very low carrier concentration stabilized primarily the $M_r \simeq$32K species; at higher carrier concentrations additional bands of M_r 20–24K, 15K, and 10K were observed (Figure 1, lower left). The M_r 20–24K and 15K bands were not normally produced by any other enzyme tested. In earlier studies, using another lot of bromelain, digestion at 0.1–0.3 mg/mL carrier produced the $M_r \simeq$ 32K species as the only major digestion product irrespective of length of time of digestion (up to 2 h). This allowed a clean isolation of the $M_r \simeq$ 32K species by gel filtration of the digestion mixture (Figure 2). The nonspecific role of the

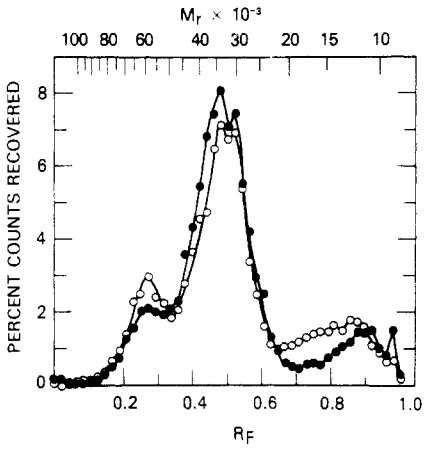

FIGURE 3: Radioactivity profiles from $NaDodSO_4$–polyacrylamide gel electrophoresis on 12.5% acrylamide gels of 20-min papain digest: (●) BH-labeled α; (○) α intrinsically labeled with ^3H-labeled amino acids. Each sample was digested separately and then run on the same slab gel after reduction.

carrier protein was demonstrated in other experiments where equal concentrations of ovalbumin or hemocyanin carrier gave identical digestion patterns of the BH-labeled α.

The effect of varying the $NaDodSO_4$ concentration on the digestion of α by bromelain was investigated in the absence of carrier protein. With increasing $NaDodSO_4$ concentration generation of bands smaller than $M_r \simeq 32K$ was progressively inhibited. In 0.5% $NaDodSO_4$, bromelain generated only the $M_r \simeq 32K$ species (as well as a very low molecular weight band) (Figure 1, lower right). This therefore represents an alternative procedure for conversion of α to the $M_r \simeq 32K$ species only.

Effect of Proteases on α Chains Intrinsically Labeled with ^3H-Labeled Amino Acids. As shown above, under appropriate conditions, complete digestion of BH-labeled α produces only a single $M_r \simeq 32K$ band. Quantitation of the recovered radioactivity showed that all of the counts initially associated with the α chains were recovered in the $M_r \simeq 32K$ region on both slab gels and gel filtration columns. These results suggested that either (a) the initial cleavage produces a single labeled $M_r = 32K$ fragment plus another fragment(s) containing no BH label or (b) the $M_r = 32K$ band consists of two labeled fragments which are not resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis or by gel filtration in $NaDodSO_4$.

In order to distinguish between these possibilities, we labeled α biosynthetically with a mixture of five ^3H-labeled amino acids and digested α. Figure 3 shows that papain digestion of ^3H-labeled α resulted in a distribution of label similar to that observed with BH-labeled α. Quantitative analysis of the distribution of counts gave the following results: (a) The degree of digestion was similar, being 87% and 83% complete for the BH-labeled and biosynthetically labeled α, respectively. (b) The counts in the broad $M_r \simeq 32K$ band represent 83% and 75% of the total digested material for BH label and biosynthetic label, respectively. In each case over 90% of the counts applied were recovered.

These results are not consistent with the possibility that the α chain was cleaved such that a significant proportion of the polypeptide is in fragments other than those in the $M_r \simeq 32K$ peak. The α chain has $\simeq$15K daltons of carbohydrate, and studies (below) show that incorporated glucosamine counts are all in the $M_r \simeq 32K$ peak following proteolytic cleavage. If the latter peak represented only a single component, then its size would allow for only half of the polypeptide portion [which itself is $\simeq$35K daltons (Kanellopoulos et al., 1980; Figure 8 and Table II)] to be present there. Thus 50% of the counts incorporated into the polypeptide would have appeared elsewhere; this is clearly not the case either with labeling with ^3H-labeled amino acids or the Bolton–Hunter reagent.

These data therefore strongly support the alternative possibility that α is initially cleaved into two similarly sized fragments of $M_r \simeq 32K$ and that these can be further degraded to smaller products. This analysis therefore demonstrates that no major fragment had escaped detection in previous experiments because it was not labeled by the Bolton–Hunter reagent.

An experiment similar to that of Figure 3 was performed with bromelain. In this case four cleavage products ($M_r \simeq$ 32K, 24K, 16K, and 10K) were seen with each isotope, and again a similar distribution of each isotope among the four fragments was observed (not shown). The similar distribution of BH label and biosynthetic amino acid label among the fragments indicates that the extrinsic Bolton–Hunter label incorporates quite uniformly into the polypeptide portion of α and justifies using BH-labeled α for routine cleavage experiments.

Use of Differential Labeling of α Chains. When α was isolated from cells whose surface proteins were catalytically iodinated with lactoperoxidase and subjected to a 2-h bromelain digestion, the same result was consistently observed: only a single band of $M_r \simeq$ 21–24K was seen following autoradiography. This contrasts with BH-labeled α digested under similar conditions where multiple bands were regularly seen (Figure 1, lower left). This observation prompted the following experiments in which ^{131}I-labeled α isolated from surface radioiodinated cells was mixed with [^{125}I]BH-labeled α prior to enzymatic digestion. The results of papain cleavage are shown in Figure 4. The distribution of Bolton–Hunter label (^{125}I) is consistent with previous results which show only two resolved digestion products: a broad band of $M_r \simeq 32K$ and a band of low molecular weight ($M_r \simeq 12K$). The distribution of ^{131}I counts in the $M_r \simeq 32K$ peak suggests that the latter consists of (at least) two components of approximate $M_r = 34K$ (α_1) and $M_r = 30K$ (α_2), only the smaller of which contains significant surface label. The differential distribution of the two isotopes in this peak was most clearly seen during the early stages of digestion (Figure 4A) and became less noticeable after longer digestion times. This appears to result from subsequent preferential degradation of α_1 by papain as judged by the appearance of low molecular weight material containing primarily ^{125}I label. Only after prolonged papain treatment was a small peak of $M_r \simeq 20K$ containing surface label observed. In another experiment, a similar dual-labeled α mixture was subjected to bromelain digestion (Figure 5). The results again were consistent with initial cleavage to produce two poorly resolved fragments of $M_r \simeq 34K$ and $M_r \simeq 30K$, only the smaller of which contained surface label. In contrast to papain however, bromelain readily, and completely, further degrades α_2 to a $M_r \simeq 24K$ species. After quantitative conversion of α_2 to the $M_r \simeq 24K$ fragment, a BH-labeled peak of $M_r \simeq 34K$ remains in the area of the original broad $M_r = 32K$ band (Figure 5B). This observation demonstrates the existence of a discrete $M_r \simeq 34K$ fragment (α_1) which contains little or no surface label, which is further cleaved by bromelain relatively slowly, and which, together with $M_r \simeq 30K$ fragment (α_2) which does contain surface label, constitutes the broad $M_r = 32K$ band that is the initial cleavage product of the digestion. In addition to the major fragments discussed above, some low molecular weight material con-

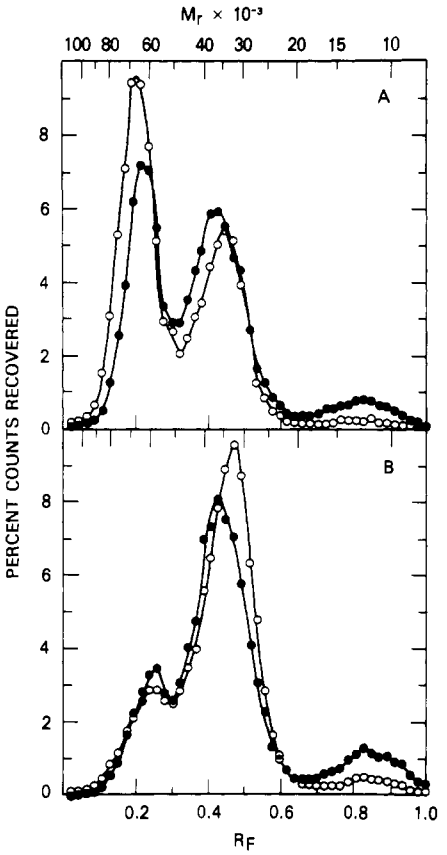

FIGURE 4: Radioactivity profiles from $NaDodSO_4$–polyacrylamide gel electrophoresis on 12.5% acrylamide gels of a mixture of [^{125}I]-BH-labeled α (●) and α isolated from cells labeled on their surface by lactoperoxidase-catalyzed iodination with ^{131}I (○) after papain treatment. Digestion was carried out with 0.5 μg/mL preactivated papain for (A) 2 min and (B) 10 min.

taining more BH than surface label was also produced after extensive digestion. This likely represents further breakdown of α_1 and α_2.

Bromelain digestion in 0.5% $NaDodSO_4$, which was previously shown to stop after the initial cleavage event (Figure 1, lower right), showed the same differential distribution of Bolton–Hunter and surface labels within the $M_r \simeq 32$K peak (not illustrated).

Figure 6 shows the $NaDodSO_4$–polyacrylamide gel electrophoresis patterns generated by aliquots taken at different times from a digestion with bromelain of a mixture of α chains labeled either intrinsically with [^{14}C]glucosamine or extrinsically with [^{125}I]Bolton–Hunter reagent (see Materials and Methods). Recovery of ^{14}C counts at 5, 10, and 30 min was 103%, 97%, and 114% of the "0" time values, respectively. The corresponding recoveries in the gel patterns for the ^{125}I counts were 93%, 75%, and 80%, respectively. In this analysis the molecular weight standards stained too weakly to assess their position accurately so that the molecular weight scale had to be estimated from our experience with other 17% acrylamide gels. Virtually all the ^{14}C counts of the digested α chain are either in a $M_r \simeq 33$K component or a 24K component (85% at 30 min). The latter, which we have shown to be generated from α_2, shows much less labeling than the residual $M_r \simeq 33$K component which chiefly represents undigested α_1. These results are consistent with α_1 being richer in incorporated glucosamine than α_2. Under the conditions used here, a previous study (Kulczycki et al., 1976) showed that RBL cells incorporated [^{14}C]glucosamine into the α chain as *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid almost exclusively.

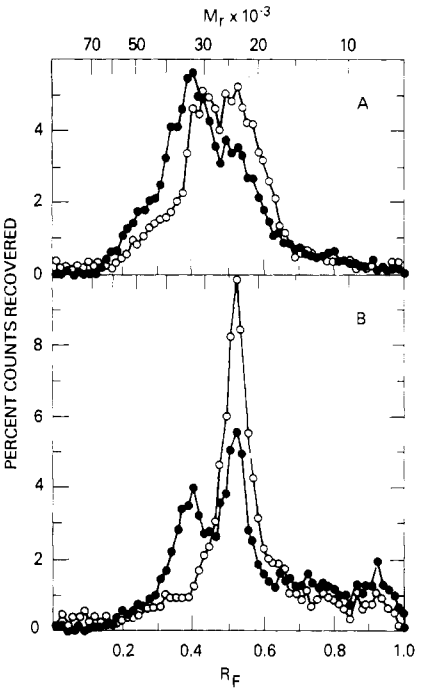

FIGURE 5: Radioactivity profiles from $NaDodSO_4$–polyacrylamide gel electrophoresis of a mixture of [^{125}I]BH-labeled α (●) and α isolated from cells labeled on their surface by lactoperoxidase-catalyzed iodination with ^{131}I (○) after bromelain treatment. (A) Digestion was carried out in $NaDodSO_4$-digest buffer (Materials and Methods) with 1 μg/mL bromelain in the absence of carrier protein for 2 h. Results were taken from a 17% acrylamide gel. (B) Digestion was carried out as in (A) but with 10 μg/mL bromelain in the presence of 1 mg/mL ovalbumin carrier for 2 h. Results were taken from a 17% acrylamide gel.

Table I: Apparent Molecular Weights of Size-Fractionated α Chains and α-Chain Products of Bromelain Digestion[a]

species	$M_r \times 10^{-3}$ undigested	$M_r \times 10^{-3}$ digested[b]
unfractionated	57	33
fraction 1	60	34
2	54	30
3	50	29
4	44	26
fraction 1 minus 4	16	8

[a] The analysis was performed on the gel shown in Figure 7.
[b] The molecular weight of the principal digestion product only is shown.

Attempts To Separate Proteolytic Fragments. Since the differential labeling techniques indicated the existence of two poorly resolved primary digestion products (α_1 and α_2), we tried to resolve these components directly. One approach consisted of fractionating the broad α band into several narrower bands of differing molecular weight and subjecting each of these to protease treatment. As shown below the broadness of the α band on gels appears to arise from carbohydrate heterogeneity. We reasoned that if either α_1 or α_2 was relatively homogeneous in this respect, size fractionation of intact α chains might lead to better resolution of α_1 from α_2 after protease treatment. Figure 7 shows that bromelain treatment of the unfractionated as well as the size-fractionated BH-labeled α bands led to the appearance of the usual bands on $NaDodSO_4$–polyacrylamide gel electrophoresis. The largest of these bands, which consists of still unresolved α_1 and α_2, reflects the molecular weight of the parent fractionated α band (Table I). Significantly, the molecular weight range of the four ($\alpha_1 + \alpha_2$) digest bands (8000 daltons) is precisely half

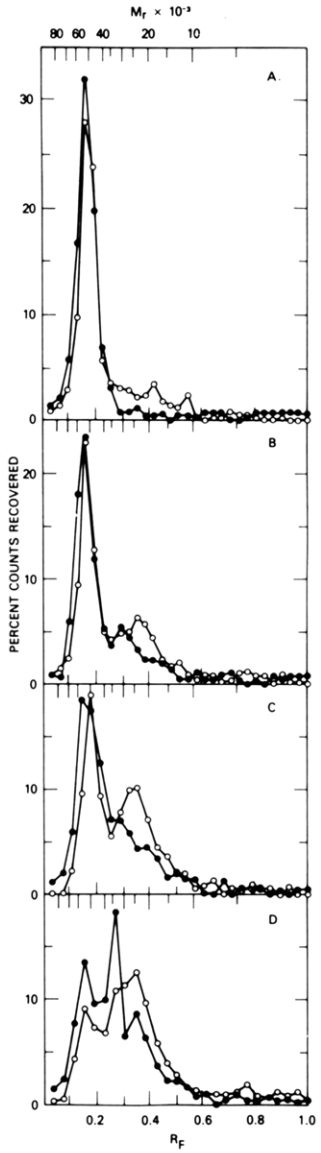

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of bromelain-treated α on 17% acrylamide gel after reduction: (●) [^{14}C]glucosamine-labeled α; (○) BH-labeled α. The samples were mixed prior to digestion. Digestion times of (A) 0, (B) 5, (C) 10, and (D) 30 min are shown.

the molecular weight range of the fractionated undigested α bands (16000 daltons). These data provide strong independent support for a model in which $\alpha \rightarrow \alpha_1 + \alpha_2$ is the primary cleavage event. It also indicates that α_1 and α_2 make quantitatively similar contributions to the heterogeneity of α. The two smaller digestion products ($M_r \simeq$ 22K and 15K) show a smaller systematic shift in molecular weight, indicating that they contribute little to the heterogeneity of α presumably due to their low carbohydrate content.

A second potential approach for better resolving α_1 from α_2 involved the use of RBL cells maintained in another laboratory. Those cells (RBL_{wpg}) have been reported to have an α component of $M_r \simeq$ 45000 on 10% acrylamide gels (Conrad & Froese, 1978), some 10000 daltons smaller than the size we consistently observe for α isolated from RBL cells maintained in our laboratory. It was hoped that the basis of the lower molecular weight of α_{wpg} might be asymmetrically distributed between α_1 and α_2, thereby accentuating any difference in molecular weight between them. Unfortunately, α isolated from RBL_{wpg} cells which had been cultured in our laboratory for several weeks did not differ significantly in

FIGURE 7: Autoradiograph of BH-labeled, size-fractionated α after bromelain treatment and analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis on 4–30% acrylamide gradient gel. BH-labeled α was run on a 12.5% acrylamide slab gel and the broad band was sliced into four segments of differing average molecular weight. After elution from the gel and concentration, these four fractions as well as unfractionated α were separately treated with bromelain. Shown from left to right are the following: unfractionated α before and after bromelain treatment; size-fractionated α bands in order of decreasing molecular weight (each shown before and after bromelain treatment).

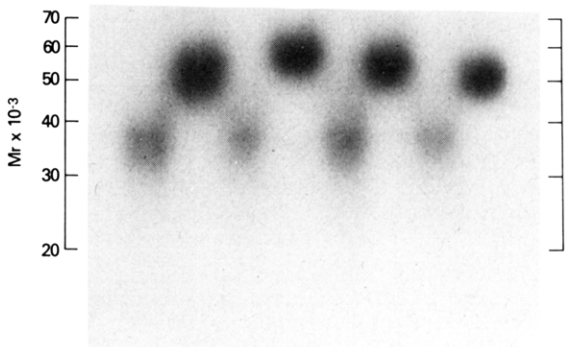

FIGURE 8: 4–30% acrylamide gradient gel analysis of size-fractionated α after treatment with α-D-*N*-acetylgalactosaminyloligosaccharidase. Digestion was carried out as described under Materials and Methods. Shown from left to right are unfractionated α after digestion, unfractionated α prior to digestion, and size-fractionated α bands in order of decreasing molecular weight (each shown after and prior to digestion, respectively).

molecular weight, or in the nature of its digestion products, from α isolated from our cell line.

A third approach utilized glycosidase treatment of α. Such material might have a lesser heterogeneity and might have given better resolution for this reason or because the carbohydrate content of α_1 and α_2 was substantially different. Treatment with neuraminidase, endo-β-*N*-acetylglucosaminidase D (endoglycosidase D), endo-β-*N*-acetylglucosaminidase H (endoglycosidase H), or a mixture of exoglycosidases caused small but reproducible decreases in the molecular weight of α. Prior incubation of α with neuraminidase enhanced the decrease in molecular weight observed with endoglycosidase D and with mixed exoglycosidases.[2] Treatment of α with α-D-*N*-acetylgalactosaminyloligosaccharidase resulted in a decrease in apparent molecular weight of α to 36K and was unaffected by prior neuraminidase treatment. When size-fractionated α bands were treated with this enzyme, a common product of $M_r \simeq$ 35–36K was obtained both on gradient gels (Figure 8) and on 12.5% acrylamide slab gels. Furthermore, results on the latter (Table II) showed that the half-height widths of the size-fractionated undigested material were clearly smaller than that of unfractionated α.

[2] In a previous report we failed to detect sialic acid in α by direct analysis (Kanellopoulos et al., 1980). This discrepancy with the present findings might have resulted from a loss of sialic acid residues during dialysis of α against dilute acetic acid in the earlier study.

Table II: Oligosaccharidase Digestion of Size-Fractionated α Chains [a]

	control		digest	
sample	$M_r \times 10^{-3}$	half-height width [b]	$M_r \times 10^{-3}$	half-height width
unfractionated	54.3	0.159	35	0.279
fraction 1	58.5	0.114	35.5	0.261
2	54.2	0.092	35	0.247
3	51.2	0.103	35.5	0.259
4	46.5		35.7	

[a] Analysis based on densitometric tracings of a radioautograph of a 12.5% acrylamide slab gel. The density of fraction 4 was too low to permit accurate quantitation of the half-height width. The molecular weight data are in excellent agreement with those obtained in Figure 8. [b] In R_f units.

Thus both the molecular weight data and the half-height width analyses indicate that the broadness of the α band on NaDodSO$_4$–polyacrylamide gel electrophoresis arises predominantly, if not exclusively, from carbohydrate heterogeneity. Direct analysis indicates a carbohydrate content of 32% for α (Kanellopoulos et al., 1980), a value in excellent agreement with the observed molecular weight decrease upon α-D-*N*-acetylgalactosaminyloligosaccharidase treatment. The specificity of this enzyme implies that the vast majority of this carbohydrate is O-linked to serine and/or threonine residue(s).

It is of obvious importance to rule out contaminating protease activity as being responsible for the observed digestion. The following data are evidence against this possibility: (i) The enzyme as assayed by the supplier released no counts from ^{3}H-labeled bovine serum albumin under conditions containing 100-fold more enzyme than in the standard glycosidase assay. (ii) In some of our own experiments bovine serum albumin was included as a carrier protein and was not visibly degraded as shown by Coomassie blue staining of the gels. (iii) When the oligosaccharidase was assayed for its ability to digest azocasein under conditions identical with those used for α, at most 5–15% digestion of azocasein was observed. This contrasts with 100% digestion of α. (iv) The product obtained after oligosaccharidase digestion of α was reproducibly slightly larger than the $M_r \simeq$ 32K band obtained with every protease we have tested and failed to show a shift when size-fractionated α was examined (cf. Figures 7 and 8). These findings strongly suggest that the observed digestion is due to glycosidic activity. However, we cannot categorically rule out the possibility that contaminating protease activity contributed to the results. For example, we have no direct information which permits us to interpret the broadness of the α-chain band after digestion with the glycosidase (Table II). Because of this broadness we were unable to affect with glycosidase better resolution of α_1 from α_2 generated by subsequent (or prior) digestion of α with proteases.

An alternative approach to separating α_1 from α_2 was to see if they could be selectively absorbed by (or eluted from) an absorbent. Since α binds well to hydroxylapatite in NaDodSO$_4$ (Kanellopoulos et al., 1980), this material was tested. No selective absorption or elution of α_1 vs. α_2 was observed. The ability of concanavalin A to discriminate between α_1 and α_2 was also tested. The materials tested had been exposed to NaDodSO$_4$ and then extensively dialyzed against nonionic detergent. Concanavalin A–Sepharose quantitatively bound α as well as the $\alpha_1 + \alpha_2$ mixture isolated by gel filtration of α digested with bromelain. In another experiment, the different pools of a size-fractionated $\alpha_1 + \alpha_2$ mixture were all found to bind quantitatively to the lectin. These results are consistent with both α_1 and α_2 containing carbohydrate moieties with an affinity for concanavalin A.

Table III: Properties of Major Proteolytic Fragments of α Subunit

fragment	α_1	α_2
M_r (app)	34K	30K
surface labeling	±	++
carbohydrate	++	+
proteolytic cleavage products	15K (papain)	24K (bromelain)

Inhibition of binding of α or its fragments to the lectin by methyl α-D-mannoside was always ≥50% but, in contrast to IgE, was never complete. This can be rationalized by postulating that the high carbohydrate content of α promotes multiple interactions with the immobilized lectin which results in a very high avidity. Some bound glycoproteins are notoriously difficult to dissociate from immobilized concanavalin A. Surprising, nevertheless, is the finding not only that oligosaccharidase-treated α binds to the concanavalin A–Sepharose but also that its binding is similarly difficult to inhibit with methyl α-D-mannoside. While binding could be explained by residual N-linked carbohydrate, a large amount by residual carbohydrate is clearly inconsistent with the carbohydrate composition of α. Therefore the inability to inhibit binding quantitatively is hard to explain. We cannot at present exclude the possibility that α, or at least α that had been exposed to NaDodSO$_4$, interacts with concanavalin A in some other nonspecific manner, possibly through interaction with a hydrophobic site (Davey et al., 1975, 1976) on the immobilized lectin.

Conclusion

The purpose of this study was to see if enzymatic cleavage combined with differential labeling of the α chain of the receptor for IgE would permit us to identify discrete areas of this glycopeptide. Such identification could then provide the basis for studies which will investigate how the α chain is disposed in the plasma membrane.

Our results demonstrate that α is readily cleaved in NaDodSO$_4$ by several proteases into two similar-sized fragments (α_1 and α_2). Table III gives a summary of the properties of α_1 and α_2 so far determined. Each of these fragments can be further fragmented under appropriate conditions. Thus techniques now exist for reproducibly dissecting α into several fragments, each of which can be isolated and studied in terms of location and possible function. For example, α_1 and α_2 are readily, and nearly quantitatively, produced by digesting with papain for short periods of time or with bromelain in 0.5% NaDodSO$_4$. Complete conversion of α_2 to a fragment of $M_r \simeq$ 24K is readily achieved by more extensive bromelain digestion. Cleavage of α_1 is achieved by longer papain digestion which, however, produces low molecular weight fragments which may be heterogeneous. Under these latter conditions α_2 remains largely intact.

Since α contains ≃30% carbohydrate (Kanellopoulos et al., 1980), one of the two major fragments may contain proportionately even more carbohydrate. A high carbohydrate content has been demonstrated to produce abnormal migration on NaDodSO$_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1975; Ferguson, 1964) so that the imperfect agreement between the apparent mass of α (58 000 daltons) and the sum of α_1 and α_2 (64 000 daltons) on 12.5% acrylamide gels is not unexpected. On the basis of the amount of [^{14}C]glucosamine incorporated, α_1 may contain the majority of the carbohydrate present in α and therefore could contain >50% carbohydrate by weight. Its mobility in NaDodSO$_4$ gels

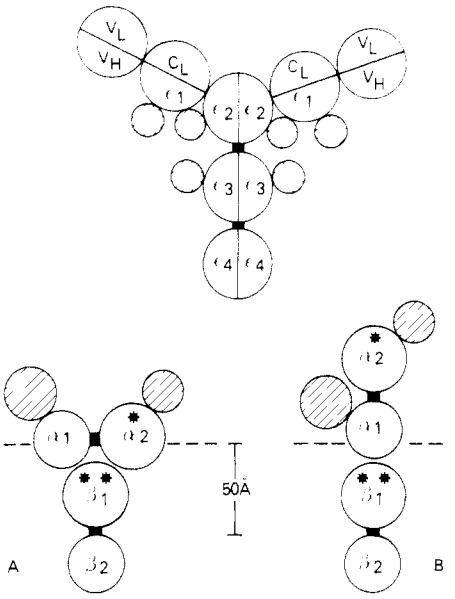

FIGURE 9: Schematic representation of plausible models for IgE and the receptor to which it binds. The polypeptide portions and carbohydrate moieties (striped) are depicted as globular units whose volumes are proportional to the apparent masses. The regions depicted in black are those most susceptible to cleavage by proteases. Top: The model for IgE is based largely on data for human IgE (Bennich & Von Bahr-Lindstrom, 1974); however, the sites of susceptibility to proteases are based on data for rodent IgE (Ellerson et al., 1978; J. M. Kanellopoulos and R. Montefort-Perez, unpublished observations). Bottom: Models for the α chain are based on data largely from this paper; that for the β chain from data in Holowka et al. (1981) and Holowka & Metzger (1981). The single asterick shows the general location of the principal residue(s) modified by surface labeling; the double asterick shows where labeling with an intramembranous probe occurs (Holowka et al., 1981; Holowka & Metzger, 1981). The dashed line represents the extracellular aspect of the plasma membrane. The scale marker is for 50 Å—the approximate thickness of a phospholipid bilayer. (A) A U-shaped model with both α_1 and α_2 penetrating the lipid bilayer to an indeterminant extent and both potentially interacting with the β chain embedded in the bilayer. (B) A linear model in which only one of the α-chain domains interacts with the bilayer and the β chain. This is presumed to be the α_1 component solely on the basis of its minimal participation during surface labeling.

might therefore be more aberrant than that of α_2. If so, the polypeptide portion of α_1 and α_2 would be rather similar in size and this would be consistent with the results obtained with α labeled biosynthetically or extrinsically with the Bolton–Hunter reagent. Obviously preparative isolation of α_1 and α_2 followed by compositional analysis will be necessary to determine the precise distribution of polypeptide in the two "domains" of the α chain.

Of the two major fragments, α_2 contains the large majority of the label from surface radioiodinated cells. Since iodination of α in situ is completely inhibited in the presence of IgE (Conrad & Froese, 1976), α_2 by this criterion contains the site which binds IgE. This, along with the presence of carbohydrate on α_1 and α_2, implies that both fragments are exposed on the cell surface. The finding that the majority of the surface label present in α_2 is recovered in a $M_r \simeq 24$K fragment (Figure 5B) shows that the IgE binding site can be further localized to a subfragment of α_2.

Although we have no data as yet concerning the degree of exposure of α on the cytoplasmic face of the plasma membrane, two differing models for the topological orientation of α must now be considered. In the first model, α would be a schematically "U-shaped" molecule (Figure 9A) penetrating the membrane at least twice (but possibly more times). Both α_1 and α_2 would contain intramembranous sequence(s). This model has the attractive feature that it could result in a binding site with 2-fold pseudosymmetry which could interact with the Fc portion of IgE which itself contains a 2-fold symmetry axis (see, however, below). An alternative model (Figure 9B) would require only one of the two major fragments to be anchored in the membrane. This model appears to be inconsistent with the observation that, in situ, α is resistant to protease treatment (Metzger et al., 1976). At this time, we therefore favor the first model since it contains a potential proteolytic cleavage site accessible to enzymes in solution but not in situ.

This latter argument is not as persuasive as it might be because we are here comparing the susceptibility to digestion of native α chains in situ with α chains in $NaDodSO_4$. Data on the susceptibility of native α chains in solution are scanty (Kulczycki et al., 1976; Rossi et al., 1977); however, what data there are suggest that under such conditions the α chain is also highly sensitive to proteases. $NaDodSO_4$ may alter the folding of proteins, in some cases actually increasing the helicity of the polypeptide chain compared to its native conformation [for a recent discussion see Wu et al. (1981)]. We have some experimental data which show that α chains transferred from $NaDodSO_4$ solutions to solutions of nonionic detergents yield digestion patterns similar to those described in the present work. Nevertheless we recognize that our interpretations drawn from the results of proteolysis in $NaDodSO_4$ can be applied to the native structure of α only provisionally. Some independent results from our group using target analysis of the α chain do support the proposal that the native α chain contains discrete domains. Thus, using radiation inactivation, Fewtrell et al. (1981) have measured a target size of ~28 000 daltons, in solution, for that portion of the receptor which binds IgE. It is tempting to speculate that this target represents the postulated α_2 domain of the α chain. A target size which represents only a portion of an intact polypeptide chain implies, in the present case, a high degree of functional isolation of the IgE binding domain (α_2) from the remainder of the polypeptide. These results therefore suggest that α_1 may not contribute importantly to the combining site for IgE.

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Inhibition of the Classical Complement Pathway by Synthetic Peptides from the Second Constant Domain of the Heavy Chain of Human Immunoglobulin G†

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ABSTRACT: The $C_\gamma2$ domain of immunoglobulin G (IgG) is reported to have a tryptophan residue and cationic residues at or near its C1q-binding site. The present study has used synthetic IgG peptides to explore the involvement of the 275–290 region of the $C_\gamma2$ domain of the heavy chain of human IgG1 in binding to C1. This region (Phe-Asn-Trp-Tyr-Val-Asp-Gly-Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys) contains Trp-277 and the cationic residues His-285, Lys-288, and Lys-290. The following peptides were synthesized by the solid-phase method and purified to homogeneity by using reverse-phase high-pressure liquid chromatography: the hexadecapeptide 275–290 and its N^t-formyl derivative 275–290F containing both Trp-277 and the cationic residues; the N^α-acetylpentapeptide 275–279A comprising the hydrophobic region around Trp-277; and the cationic decapeptide 281–290. When examined in the Augener assay for inhibition of C1-mediated immune hemolysis, peptides 275–290F and 281–290 were about half as active as monomeric 7S human IgG on a molar basis and essentially as active on a site basis. Since both peptides containing residues 281–290 inhibited hemolysis in a manner similar to the $C_\gamma2$ domain, the cationic 281–290 region containing His-285, Lys-288, and Lys-290 may be a part of the C1q-binding site of $C_\gamma2$. These results are consistent with the tertiary structure of the Fc fragment of IgG, in which the 275–279 region is part of the hydrophobic core of the $C_\gamma2$ domain and the 281–290 region is exposed on the surface.

When immunoglobulin G (IgG)[1] binds to a bacterium or virus, the resulting immune complex binds to C1q, which leads to the activation of the classical complement pathway and the destruction and removal of the invading organism (Müller-Eberhard, 1975). During the past two decades, many studies have explored the location and chemical nature of the C1q-binding site of IgG. Several studies of IgG fragments have suggested that this site resides in the $C_\gamma2$ domain. Progressive proteolysis of the Fc region of IgG, which is responsible for complement activation (Ishizaka et al., 1962), abolished complement binding when the amino-terminal portion was degraded (Utsumi, 1969). Kehoe & Fougereau (1969) found that a 62-residue fragment (residues 253–314) of the $C_\gamma2$ domain of murine IgG2a inhibited complement fixation when bound to polystyrene latex beads. This observation has been supported by the observations that a $C_\gamma2$-containing fragment from intact IgG (Colomb & Porter, 1975), the isolated $C_\gamma2$ domain (Yasmeen et al., 1976), and a 54-residue fragment (residues 253–306) of human IgG1 (Lee & Painter, 1980) each inhibit C1-mediated hemolysis. But the direct binding of the $C_\gamma2$ domain or an IgG fragment to C1q has not been demonstrated.

Two chemical features of the C1q-binding site of IgG have been reported. IgG aggregates bearing modified tryptophan residues fail to activate complement, which suggests that a tryptophan residue is at or near the C1q-binding site (Allan & Isliker, 1974a,b). In addition, a peptide corresponding to residues 277–281 of IgG and containing Trp-277 was reported

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[1] Abbreviations used: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; C1q, C1r, C1s, C2, C3, and C9, protein components of the serum complement cascade; $C_\gamma2$, second constant domain of the γ (heavy) chain of IgG; For, formyl; HPLC, high-pressure liquid chromatography; IgG, immunoglobulin G; I_{50}, 50% inhibition of C1-mediated immune hemolysis; TLC, thin-layer chromatography; TLE, thin-layer electrophoresis; DMF, dimethylformamide.

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