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COMPARISON OF THE VARIOUS IgE RECEPTORS OF RAT BASOPHILIC LEUKEMIA CELLS

by

Patricia A. Roth

A Thesis

Submitted to the Faculty of Graduate Studies in

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ABSTRACT

The present study has provided evidence that the two IgE receptors of rat basophilic leukemia (RBL) cells which have molecular weights of 45kDa and 55kDa and which have been designated R and H, respectively, are structurally distinct molecules. Limited digestion of these receptors with papain generated two sets of peptides which, when analyzed by SDS-PAGE, produced two distinct profiles. In addition, tryptic peptides of the receptors were analyzed by both one- and two-dimensional methods. Surface labelled R and H shared no peptides in common, thus proving that the two receptors are indeed distinct molecular entities.

Attempts to produce IgE-binding fragments of each of the two receptors was made. Using the proteolytic enzymes papain, trypsin and chymotrypsin, only an IgE-binding fragment of H could be produced. The effect of these enzymes on R was variable. The IgE-binding site on all R molecules was destroyed by papain, while trypsin and chymotrypsin destroyed the binding site on some R molecules and left the other molecules intact. The binding fragments of H produced by the three enzymes were similar in molecular weight (36-41kDa). This indicated that H possesses a protease sensitive portion as well as a relatively resistant portion which contains the IgE-binding site. This finding suggested the presence of a domain structure in the H receptor.

The present study confirmed results which had indicated that the reduction of the IgE receptor designated 71K produced a molecule with an electrophoretic mobility similar to that of R. A comparison of the tryptic peptides of the R receptor to those of 71K revealed that these two molecules posses a great degree of homology. The tryptic peptides

which were produced from both reduced and unreduced R and 71K were analyzed by one-dimensional SDS-PAGE. The results clearly showed a great deal of similarity between these two molecules, both in the reduced and unreduced forms. Two dimensional analysis demonstrated that while not all peptides from R and 71K had the same mobilities, many did share a common position. These results suggested that 71K is composed of the R molecule disulfide-bonded, either to another R molecule or to some other polypeptide chain which is not surface labelled.

A two-dimensional SDS-PAGE analysis of receptors from biosynthetically labelled RBL cells was performed to determine the subunit composition of 71K. Reduction of receptors in the second dimension demonstrated that, aside from the molecule with a mobility similar to that of R, no other molecule was produced upon reduction of 71K. This strongly suggested that 71K is composed of two, disulfide-linked R molecules.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CNBr	cyanogen bromide
CPM	counts per minute
CTMC	connective tissue mast cell
DAAD	direct allogenic anaphylactic degranulation
DNP	dinitrophenyl
DTT	dithiothrietol
EDTA	ethylene diamine tetraacetate
FcR	receptor for Fc portion of Ig
FCS	fetal calf serum
HARE	horse anti-rat IgE
kDa	kilodaltons
2-ME	2-mercaptoethanol
MEM	minimum essential medium
MMC	mucosal mast cell
M_r	apparent molecular weight
NP-40	Nonidet P-40
nRGG	IgG fraction from normal rabbit serum
PBS	phosphate buffered saline
PCA	passive cutaneous anaphylaxis
P-K	Prausnitz-Kustner
PMSF	phenyl methyl sulfonyl chloride
RAR	rabbit anti-receptor immunoglobulin
RBL	rat basophilic leukemia
R_f	relative mobility
RMC	rat mast cell

SC secretory component

SDS sodium dodecyl sulfate

SDS-PAGE polyacrylamide gel electrophoresis in sodium dodecyl sulfate

SRS-A slow reacting substance of anaphylaxis

TLC thin layer chromatography

CHAPTER I

INTRODUCTION

[A] HISTORICAL PERSPECTIVE

1. Allergy and skin sensitizing antibodies

The presence of a humoral factor able to transfer specific allergic sensitivities from allergic to non-allergic patients was first demonstrated by Prausnitz and Kustner (1921). In their study, Prausnitz sensitized his own forearm to fish by intracutaneous injection of serum from an allergic individual. Upon challenge with the specific antigen, the skin site gave an erythema-wheal reaction, a symptom indicating the presence of the tissue injury commonly elicited during an allergic response. Subsequently, the passive transfer of immediate hypersensitivity to other allergens was observed and, in 1925, Coca and Grove (1925) applied the term "reagin" to the skin-sensitizing antibodies found in allergic sera. Investigation of the nature of reaginic antibodies then had to await the development of new methods for fractionation of serum proteins.

Improvement of protein separation techniques allowed for the classification of antibodies into specific classes and subclasses based predominantly on distinct antigenic characteristics as well as on net charge and molecular weight. It was established that, regardless of size and charge, antibodies share a 4-polypeptide chain structure consisting of two heavy and two light chains. The structures of the heavy chains differ among classes and subclasses while all share common light chains. By the early 1960's, use of the newly developed method of immunoelectrophoresis aided in the discovery of three classes of human serum immunoglobulins. IgG and IgM were found to correspond to the 7S- and 19S-type immunoglobulin(Ig) antibody, respectively, whereas IgA

represented a new Ig class based on its antigenic properties. After the properties of this new class of Ig were examined, its precipitation and electrophoretic characteristics suggested a resemblance to reaginic antibody. Furthermore, Heremans and Vaerman (1962) demonstrated that the IgA fraction of allergic patients' sera had skin-sensitizing activity, while antiserum against IgA destroyed reaginic activity (Fireman et al., 1963). Finally, Ishizaka et al. (1963) showed that the IgA fraction of normal serum could block the passive sensitization of human skin with reagin but neither IgG nor IgM would do so. However, by the late 1960's, further fractionation of 'pure' IgA from reaginic serum revealed that reaginic activity did not parellel IgA concentration (Ishizaka and Ishizaka, 1966). The negative results obtained from experiments on blocking of passive sensitization by myeloma IgA proteins instead of normal serum IgA fractions strongly suggested that reaginic antibodies do not belong to the IgA subclass but represent a contaminant in many IgA fractions. Upon discovery of yet another antigenically distinct Ig class - IgD - evidence for its relationship to reaginic antibodies was sought, but the data obtained (based on immunochemical and physicochemical properties) did not support such a relationship (Ishizaka et al., 1966a, Perelmutter et al., 1966). In addition, because of its size (8S) and the antigenic differences (Ishizaka et al., 1967a), reaginic antibody was not considered to be of the IgM class. Upon ruling out identity between reaginic antibodies and IgG antibodies (Ishizaka et al., 1967a), the search was begun for a unique immunoglobulin class which had not yet been described.

2. Immunoglobulin E

The first attempt to identify the protein responsible for

reaginic activity was made with antibodies to a reagin-rich fraction from ragweed-sensitive serum. After absorption with normal IgG and IgA and IgD myeloma proteins, the antiserum reacting with the reagin-rich fraction showed a single band which, upon immunoelectrophoresis, had a γ1 mobility. Binding of radioactively labelled ragweed antigen to this band demonstrated that it contained antibody binding activity. This apparently unique immunoglobulin class was tentatively designated γE (Ishizaka et al., 1966b). Further studies revealed a correlation between the disappearance of skin-sensitizing activity in allergic sera and removal of γE by immunoprecipitation with anti-γE in the ragweed system (Ishizaka et al., 1966c) as well as in other allergen systems (Ishizaka and Ishizaka, 1968). Purification of γE (from reaginic sera of ragweed-sensitive patients) using ammonium sulfate precipitation and DEAE-cellulose chromatography and absorption with antisera to other immunoglobulin classes provided more conclusive evidence for the identity of this protein and reaginic antibody. This purified preparation contained only γE antibodies specific to ragweed, as determined by radioimmunodiffusion, and both reaginic activity and γE were completely removed by precipitation with anti-γE (Ishizaka and Ishizaka, 1967). These results clearly show that γE antibody is responsible for reaginic activity.

The extremely low levels of γE normally found in humans would have made investigation of its biochemical and biological properties very difficult. Fortunately, Johansson and Bennich (1967) discovered a human myeloma protein, IgND, which, by antigenic analysis using antisera to both ND and γ-E globulin, seemed to be structurally similar to the protein previously described by the Ishizakas (Bennich et al., 1969).

Biological evidence for the structural relationship between IgND and reaginic antibody was obtained by specifically inhibiting the P-K reaction with the myeloma protein ND (Stanworth et al., 1968). Physicochemical analysis of protein ND revealed a carbohydrate content of about 11%, a sedimentation constant of 7.9S, and an apparent molecular weight of 196kDa. Reduction and alkylation produced 2 components, the molecular weights of which indicated the presence of the 2 heavy and 2 light chains common to all other immunoglobulin classes (Bennich and Johansson, 1971). In conformity with immunoglobulin nomenclature, γE is now designated IgE.

Digestion of human IgE by papain produces Fab and Fc fragments (Bennich and Johansson, 1971), and Stanworth et al. (1968) found that only the Fc fragment was required to inhibit the P-K reaction. Heating of the IgE molecule at 56 C for four hours inactivates its skin-sensitizing ability (Ishizaka et al., 1970a), as does reduction with 0.1M mercaptoethanol followed by alkylation (Ishizaka and Ishizaka, 1969a). A more detailed study performed by Takatsu et al. (1975) on a myeloma IgE protein (PS) indicated that cleavage of one of the two inter-heavy-chain disulfide bonds is required for the complete loss of biological activity. Furthermore, IgE molecules do not fix complement to activate complement by the classical pathway, even in an aggregated form (Ishizaka et al., 1970b), although aggregated IgE does activate complement by the alternate pathway (Ishizaka et al., 1972b).

3. Target organs, tissues and cells of reaginic hypersensitivity reactions

The elicitation of a P-K reaction with reaginic antibodies demonstrated that skin is one of the organs susceptible to the

hypersensitizing effects of IgE. In monkeys, human reaginic serum produced a passive cutaneous anaphylaxis (PCA) (Layton et al., 1962) and this sensitizing activity was found only in a fraction containing IgE (Ishizaka et al., 1967b). In addition, Arbesman et al. (1964) reported that monkey ileum, passively sensitized in vitro with human reaginic sera, contracted upon exposure to allergen. Further examination of this phenomenon showed that an IgE-rich ction of reaginic serum sensitized the ileum and that this activity was removed by anti-IgE (Wicher et al., 1968).

One of the most extensively studied organs in relation to reaginic hypersensitivity is the lung. Goodfriend et al. (1966) demonstrated that in monkey lung sensitized with human reaginic serum, challenge with antigen induced histamine release. Another mediator of hypersensitivity, SRS-A, was released from human asthmatic lung upon challenge with allergen (Brocklehurst, 1960). It was subsequently shown that IgE was the agent responsible for sensitizing monkey lung for the release of both histamine and SRS-A (Ishizaka et al., 1970c). Thus it became apparent that reaginic hypersensitivity reactions involved the effect of IgE on some component(s) of the various tissues studied.

At the cellular level, the mast cell became the prime candidate for the target of reaginic antibody. In 1967, Parish sensitized human lung slices with human, heat-labile antibodies and was able to demonstrate a correlation between histamine and SRS-A release and morphological changes in the mast cells. Tomioka and Ishizaka (1971) found that the major cell type in monkey lung which binds IgE is the mast cell. This study also demonstrated such binding to mast cells in skin, omentum and the lamina propria of the small intestine of monkeys.

While Lichtenstein and Osler (1964) established the methodology for antigen-induced histamine release from peripheral blood leukocytes in 1964, it was not until after IgE was characterized that evidence for its binding to the target cell was obtained. Ishizaka et al. (1969) showed that leukocytes of atopic patients (and some normal individuals) released histamine upon treatment with anti-IgE, which indicated that IgE was indeed bound to some of these cells. The identity of this target cell was determined when radiolabelled IgE was demonstrated on the surface of basophil granulocytes but not on other leukocytes (Ishizaka et al., 1970d). In addition, incubation of radiolabelled anti-IgE or anti-IgG with cell suspensions from passively sensitized monkey lung resulted in the binding of anti-IgE to mast cells, whereas anti-IgG combined only with macrophages and neutrophils (Ishizaka et al., 1972a). Thus, the binding of IgE to basophils and mast cells, which are the major source of histamine in the tissues and blood, suggested that antigen-antibody reactions on these cells result in the release of histamine from these cells.

4. Non-IgE homocytotropic antibodies

Although the IgE class of antibody clearly fits the definition of homocytotropic antibodies, which are immunoglobulin molecules capable of binding to cells of the same or closely related species, it has become apparent that it is not the only class with such a capacity. IgG passively sensitizes human lung and skin tissues, although the results have been inconsistent. These variable results appear to be due to the ease with which the antibody is washed off (Parish, 1978). Sensitization appears to be short term, lasting only 2-6 hours. The subclass responsible for this activity is unknown, although in one study,

Stanworth and Smith (1973) found a myeloma IgG_4 that blocked sensitization of skin by IgE. Providing more direct evidence for binding, Ishizaka et al. (1979) demonstrated that human basophil granulocytes which had been incubated with aggregated IgG could bind radiolabelled anti-IgG and that the receptor that bound IgG was distinct from IgE receptors on the same cells.

In the rat, a non-IgE antibody belonging to the IgG class, capable of inducing a PCA reaction with a 2-6 hour latent period, and generating the release of histamine and SRS-A, has been identified. This antibody is recognized as belonging to the IgG_{2a} subclass (Stechschulte, 1978). In addition to eliciting a PCA reaction, IgG_{2a} is involved in the complement dependent release of SRS-A from neutrophils in the peritoneum (Orange et al., 1968). Studies on the mast cell by Bach et al. (1971) revealed that IgE could inhibit IgG_{2a}-induced PCA reactions and that IgG_{2a} inhibited IgE sensitization of mast cells in vitro, indicating that interaction of these two immunoglobulin classes with the target cell involves a common receptor. More recently, Halper and Metzger (1976) used a direct binding inhibition assay to demonstrate that only IgG_{2a} in immune complexes could inhibit the binding of IgE to rat basophilic leukemia(RBL) cells. Monomeric IgG was not effective. This led the authors to suggest that the affinity of the receptor for IgG was much lower than that for IgE and that only multi-point attachment of immune complexes allows for inhibition. However, further studies have since shown that RBL cells carry two kinds of Fc receptors, one having a higher affinity for IgG than the other (Segal et al., 1981, Kepron et al., 1982).

[B] Basophils and Mast Cells

1. Biology of basophils and mast cells

In the late 19th century, two cell populations were described which contained prominent cytoplasmic granules with an affinity for certain basic dyes. One cell, the basophil, circulated in the blood while the mast cell resided in connective tissue. Both cell types are a major source of the bioactive amines involved in a wide spectrum of inflammatory and immunologic processes. They also express plasma membrane receptors that specifically bind the Fc portion of IgE antibody with high affinity and, upon sensitization with IgE and subsequent challenge with antigen, undergo anaphylactic degranulation which releases chemical mediators into the external medium. More recent evidence has implicated both basophils and mast cells in certain cellular immune processes which occur through non-anaphylactic degranulation (Galli et al., 1984).

Despite these similarities, basophils and mast cells are clearly distinct populations. Basophils, like other granulocytes, differentiate and mature in the bone marrow, and circulate in the blood. Mast cells are distributed throughout connective tissues, often situated around blood and lymphatic vessels and beneath epithelial surfaces. They mature locally in connective tissue from precursor cells which contain few cytoplasmic granules (Combs et al., 1965). In vitro experiments by Ginsburg and Lagunoff (1967) were the first to provide evidence that mast cell precursors are present in mouse thymus and other lymphoid tissue although it has been demonstrated more recently that rat mast cells are derived from bone marrow, not thymus (Crowle and Reed, 1984). Morphologically, basophils generally contain electron dense aggregates of

cytoplasmic glycogen, multi-lobed nuclei, and a nuclear chromatin pattern similar to that of other granulocytes. The plasma membrane contains short, blunt, irregularly distributed processes. Although mast cell populations are heterogeneous in morphology, they generally lack aggregates of cytoplasmic glycogen, have unilobed nuclei and have plasma membranes possesing uniformly distributed, thin, elongated folds and processes (Galli et al., 1984). In addition, while mast cells retain at least a limited proliferative capacity, there is no evidence that mature basophils do so (Galli et al., 1984).

Although cells that undergo anaphylactic degranulation have been categorized as basophils or mast cells, each of these populations contains a good deal of heterogeneity. Mast cells have been subdivided into two distinct subpopulations, based on their location. Other than differences in location, there is much evidence for the distinction between mucosal mast cells (MMC) and connective tissue mast cells (CTMC) (Shanahan et al., 1984, Barret and Metcalfe, 1984). Mucosal mast cells (MMC) are generally small and pleomorphic, have a uni- or bi-lobed nucleus and few granules, while CTM cells are large and ovoid, with an ovoid, unilobed nucleus and many granules (Shanahan et al., 1984, Barret and Metcalfe, 1984). Functionally, the cells respond differently to pharmacological agents such as the secretagogues 48/80 bee venom 401, and ionophore A23187 (Pearce et al., 1982). Although both populations are derived from bone marrow stem cells, MMC require thymic influence for their development, whereas the development of CTMC is thymus-independent (Crowle and Reed, 1984). Basophils have also recently been tentatively divided into two subpopulations based on density (as measured by isopycnic centrifugation), and histamine content, although very little

other data is available at this time (Barret and Metcalfe, 1984).

In recent years, methods have been developed for the long-term culture of mast cells. Nabel et al. (1981) found that mouse mast cell clones cultivated from fetal liver, required for their survival a macromolecular growth factor which was derived from $Ly1^{+}2^{-}$ T cells. Although the identity of this factor has not yet been determined, it shares some characteristics with the lymphokine called interleukin-3 (Galli, et al., 1984). The dependence of these cultured cells on a T-cell derived factor may have some relevance to the dependence of mucosal mast cell proliferation on thymic influence (Ruitenberg and Elgersma, 1976). Human basophils have now been cultured as well. Ishizaka et al. (1985) were able to maintain cord blood basophils in culture for 2-3 weeks and demonstrated that basophils from these cultures were functionally mature (as determined by histamine content). These developments facilitate the study of cells which had previously been difficult to obtain in large numbers.

2. Release of chemical mediators

Immediate hypersensitivity reactions are initiated by the binding of antigen to basophil and mast cell-bound IgE. This event ultimately leads to the secretion of a variety of mediators which are responsible for the pathology of the reaction. These mediators include preformed secretory granular components such as histamine, serotonin, heparin proteoglycan and the chondroitin sulfates, as well as newly generated mediators (from mast cells) such as prostaglandin D_2, platelet-activating factor and leukotrienes (Galli et al., 1984). The cellular process involved in the release of granular components is termed noncytolytic degranulation. A two stage process begins with the fusion of membranes

surrounding the granules to the plasma membrane (and sometimes to each other) so that granules are exposed to the cell exterior and concludes with the release of heparin-bound histamine by a process of cation exchange (Thon and Uvnas, 1967, Rohlich et al., 1971). In basophils, the mucopolysaccharides chondroitin sulfates A and C appear to replace heparin as the histamine-binding cation-exchanger (Sue and Jacques, 1974). Mucosal mast cells also contain a chondroitin sulfate-like glycosaminoglycan instead of heparin (Tas et al., 1977). Both cell types remain viable after degranulation and indeed, undergo regranulation in anticipation of another exocytotic event (Galli et al., 1984, Burwen, 1982).

[C] Cell Surface Phenomena and Transmembrane Signals

1. Plasma membrane structure

The first important hypothesis on the structure of biological membranes, proposed by Davson and Danielli in 1932, and later modified into the unit-membrane hypothesis, features a bilayer of mixed polar lipids, with their hydrophobic hydrocarbon ends oriented inward and their hydrophilic heads pointed outward. In 1972, Singer and Nicolson introduced the fluid-mosaic model. This model postulated that the phospholipids of membranes are arranged in a bilayer to form a fluid, liquid-crystalline core in which lipid molecules can move laterally. Intrinsic proteins are partially or completely embedded in the membrane, depending on their hydrophobic amino acid orientation, while extrinsic proteins are associated with the membrane by ionic or hydrogen binding forces. Many membrane proteins which are anchored in the membrane by hydrophobic interactions contain oligosaccharide side chains facing the

outside of the cell. Some proteins may traverse the bilayer or, alternatively, form part of a complex which traverses it and therefore is in contact with both the external and cytoplasmic sides of the membrane. These molecules can provide the means of communication across the lipid bilayer either through the flow of certain solutes or by transducing "signals" from "messengers" such as hormones, neurotransmitters antibodies or other cells. Thus, membrane proteins (often glycoproteins) play a specific role in the response of cells to environmental stimuli. The response involves three types of events: first, recognition (specific binding) of an extracellular molecule by the receptor protein; then a transduction of a signal across the membrane leading to interactions between the membrane and the cytoplasmic milieu which, ultimately, results in a cellular response that modulates the environment.

2. Ligand-receptor interaction

By definition, a true receptor is a distinct molecular entity whose function is to bind an endogenous ligand and thereby achieve a physiological effect (Goldstein et al., 1979). The receptor should normally be a product of the cell itself and the binding of specific ligand to the receptor site should be a necessary but not necessarily a sufficient step in the sequence leading to changes in the cell. The receptor must further meet the qualifications of (i) saturability, i.e. the cell should possess a finite number of sites which can be filled at high ligand concentrations; (ii) specificity, i.e. the receptor should demonstrate a preference for binding of a known effector compared to negative control; and (iii) it must demonstrate a high affinity interaction (Dorrington, 1976).

3. Transduction of receptor mediated signals

Once biochemical messages in the form of neurotransmitters, hormones, and immunoglobulin ligands are recognized by and bind to specific receptors on the cell surface, chemical and physical changes take place in the membrane which, in turn, allow cells to carry out their specific function. The sequence of changes has been the target of many investigations in recent years and has led to the elucidation of several systems involved in transduction of receptor mediated signals. Using red blood cell ghosts which have surface adrenergic receptors, Hirata et al. (1979) studied the effect of stimulating adrenergic receptors with catecholamine agonists on phospholipid methylation, membrane fluidity and adenylate cyclase. The potent agonist, L-isoproterenol was found to increase incorporation of [^{3}H]methyl group into phospholipids. Furthermore, it was found that this stimulation caused a flip-flop of the methylated phospholipids from the cytoplasmic side to the outer surface of the membrane as well as an increase in membrane fluidity. This fluidity, in turn, appears to facilitate the coupling of the receptor to adenylate cyclase facing the cytoplasmic side of the membrane. The coupling factor in this sequence was shown to be the guanyl nucleotide binding protein (Cassel and Zelinger, 1978). The subsequent generation of cyclic AMP then activates a variety of processes, including glycogenolysis and lipolysis and more specifically control of electrical activity in cells of the nervous system.

Another system studied in detail is that of histamine release from rat mast cells. Through the use of rabbit antibodies against IgE receptors, Ishizaka et al. (1980) demonstrated that bridging of receptors leads to phospholipid methylation followed by increased Ca^{++} uptake and histamine release. They also demonstrated that intracellular cyclic AMP

rises with kinetics parellel to that of phospholipid methylation (Ishizaka et al., 1981). Similar results were found using isolated plasma membranes, indicating a close association between IgE receptors and both methyltransferases and adenylate cyclase. In addition, participation of a guanyl nucleotide-dependent factor in this system is indicated. A theory had been proposed suggesting that several types of receptors floating in the same cell membrane might all interact with a single adenylate cyclase (Cuatrecasas, 1974). Indeed, the similarities between the adrenergic receptor and the IgE receptor systems support this theory.

The cyclic AMP transmembrane signaling system is one of several well studied mechanisms for communication between extra- and intra-cellular compartments. Others include the Ca^{++}-dependent phospholipase A_2 activation leading to histamine release in mast cells (Hirata and Axelrod, 1980) and the Ca^{++} influx-dependent contraction in muscle cells. There may be several systems working concommitantly in any one cell type so that a variety of factors can regulate a particular cellular process.

[D] Receptors for the Fc Portion of Immunoglobulins

The binding of immunoglobulin (Ig) complexes to lymphocytes was described several years ago (Uhr and Phillips, 1966). The phenomenon was further characterized when the interaction was shown to be a function of the Fc portion of Ig (Basten et al., 1972a, Basten et al., 1972b) thereby leading to the concept of Fc receptors (FcR) (Paraskevas et al., 1972). This entity can operationally be defined as a site on the plasma membrane which is capable of binding to the Fc portion of Ig molecules. In recent

years, FcR were found to be quite ubiqitous in its appearance on distinct cell types. Originally recognized on macrophages and monocytes, they have since been demonstrated on neutrophils, B and T lymphocytes, mast cells, basophils and eosinophils, as well as on yolk sac membranes, epithelial cells, liver and kidney cells and virus-infected fibroblasts (Zuckerman and Douglas, 1978). In addition, all Ig classes have been shown to bind to some target cell, with some cells binding more than one class or subclass (Froese and Paraskevas, 1984).

1. Receptors for IgG ($Fc_{\gamma}R$) on Lymphocytes and Macrophages

Monomeric IgG usually binds weakly to cell surfaces; therefore, most investigators have been led to use one of several multivalent forms of IgG for the detection of Fc receptors for IgG ($Fc_{\gamma}R$). Among the several approaches taken, most involve the labelling of receptor-bearing cells with heat denatured or chemically aggregated IgG, antigen-antibody complexes or antibody-coated erythrocytes (Dickler, 1976). These techniques have been effective in detecting $Fc_{\gamma}R$, on a variety of cells, although they provide little information about the physical characteristics of these receptors.

Among the many FcR systems available for study, those from the macrophage and lymphoid cells have been some of the most extensively investigated. Studies on the murine macrophage and macrophage-like cell lines have indicated the presence of more than one class of receptor, based on subclass specificity. One line of evidence was provided by Unkeless and Eisen who observed that murine IgG_{2a} bound to cells with higher affinity than IgG_{2b} (Unkeless and Eisen, 1975). Furthermore, Unkeless (1977) found that the macrophage line, $P388D_1$, has two receptors, one which binds monomeric mouse IgG_{2a} and is sensitive to

trypsin and another which binds rabbit IgG in antibody-antigen complexes and is trypsin-resistant. With the aid of a monoclonal antibody against FcR, Unkeless (1979) found that the latter receptor was actually specific for aggregated mouse IgG_{2b} and IgG_1. It did not bind IgG_{2a}. The use of Scatchard analysis of the binding of IgG_{2a} and IgG_{2b} to $P388D_1$ cells confirms the existence of at least two classes of FcR on murine macrophages (Haeffner-Cavaillon et al., 1979). A recent study has also demonstrated the presence of a specific receptor for IgG_3 on mouse macrophages (Diamond and Yelton, 1981).

Investigation of the molecular nature of these receptors required the development of isolation techniques. One effective procedure has been affinity chromatography, using either the specific ligand (IgG) (Loube and Dorrington, 1980) or monoclonal antibody (Mellman and Unkeless, 1980). Alternatively, some studies have used immune complexes to co-precipitate receptors from detergent-solubilized cells (Bourgois et al., 1977). Thus, Loube and Dorrington (1980) isolated a protein from the $P388D_1$ cell line with a M_r of 57kDa, and Mellman and Unkeless (1980) found two components from the macrophage cell line J774 with M_r of 60kDa and 47KDa. In contrast, Bourgois et al. (1977) described a 120kDa molecule isolated from lymphocytes, macrophages and fibroblasts which, if precautions against proteolysis were not taken, could be reduced to fragments of 75, 45, 20, and 10kDa.

The presence of $Fc_\gamma R$ on lymphocytes has been demonstrated in a variety of species. Receptors on B cells have been detected in mice (Basten, et al., 1972a, Cline et al., 1972) and man (Dickler and Kunkel, 1972), either by direct or indirect binding of labelled antibody, and, while T cell $Fc_\gamma R$ were initially difficult to demonstrate, sensitive

assays did finally detect them in the mouse (Anderson and Grey, 1974, Basten et al., 1975) and in man (Dickler et al., 1974, Moretta et al., 1975). Indeed, additional detection of IgM FcR on a great percentage of human T cells (Moretta et al., 1975) subsequently became the basis for distinction between helper and suppressor T cell subsets (Moretta et al., 1977). Furthermore, participation of a non-B, non-T lymphocyte (K cell) in antibody-dependent cell-mediated cytotoxicity (ADCC), is known to involve $Fc_{\gamma}R$ on that cell (Mingari et al., 1984). Another interesting finding was the detection of a soluble Ig binding factor (IBF) secreted or shed by $Fc_{\gamma}R+$ T cells which can suppress antibody response (Rabourdin-Combe et al., 1984).

Several investigations of the subclass specificity of murine lymphocyte $Fc_{\gamma}R$ produced some disagreement on comparative affinities for IgG subclasses, although most agree that IgG_1, IgG_{2a} and IgG_{2b} are readily bound (Basten et al., 1972b, Cline et al., 1972, Anderson and Grey, 1974) by both B and T cells. In humans, IgG_1 and IgG_3 bind to B cells with much greater affinity that IgG_2 and IgG_4 (Dickler, 1976), a finding that parellels results with human macrophages (Anderson, 1984).

There has been a great deal of diversity of molecular weights found for lymphocyte $Fc_{\gamma}R$. The purification of receptor from a lymphoblastoid cell line produced a multimeric glycoprotein with a subunit M_r of 46kDa (Takacs, 1980), while $Fc_{\gamma}R$ isolated from chronic lymphocytic leukemia cells (B cells) were unglycosylated, phospholipid-associated polypeptides with a M_r of 30kDa (Suzuki et al., 1980a). The latter molecule also possessed phospholipase A_2 activity (Suzuki et al., 1980b). Other studies revealed molecules with molecular weights ranging from 10-130kDa (Bourgois et al., 1977, Cunningham-Rundles

et al., 1980, Cohen et al., 1983, Rask et al., 1975, Frade and Kourlisky, 1977). Indeed, there appears to be some consistency in the differences found, leading at least one group to propose a model which explains much of the data. Kahn-Perles et al. (1980) suggest that $Fc_{\gamma}Rs$ from many cell types, including murine and human macrophages and lymphocytes, are similar in molecular structure. The model they proposed contains 5 globular domains linked by regions accessible to proteolytic enzymes and at least two inter-domain disulfide bridges. Each domain has a M_r of 23kDa. Thus, depending on the isolation conditions (whether or not reducing agents and/or inhibitors of proteolytic enzymes are used), IgG-binding molecules of 115, 90, 70, 45 or 23kDa can be obtained. $Fc_{\gamma}R$ from many different cell types may have a similar overall structure consisting of comparable domains but may differ in number of domains or position of disulfide bonds.

The data on $Fc_{\gamma}R$ suggests there exists a general structure common to many cell types from several different species. Experimental differences may either be due to changes brought about by cellular evolution or perhaps by different local (membrane) environments. A study of the species specificity of IgG binding to the $P388D_1$ cell line suggested that $Fc_{\gamma}R$ on rabbit, guinea pig, mouse and human cells are structurally related and that primary structure in the Fc portion of the IgG molecule has diverged in parellel with Fc receptor strucure (Haeffner-Cavaillon et al., 1979).

2. Receptors for IgM ($Fc_{\mu}R$)

Since phagocytic cells express $Fc_{\gamma}R$, it seemed likely that they would also express $Fc_{\mu}R$. However, for some time, receptors for IgM were difficult to demonstrate. Several studies reported that peripheral blood

monocytes lacked $Fc_{\mu}R$ (Huber et al., 1968, Lawrence et al., 1975, Walker, 1976). Finally, a study using human and rabbit monocytes demonstrated that these molecules exist but are present in low frequency (Haegert, 1979). Furthermore, the number of cells giving $Fc_{\mu}R$ positive rosettes could be greatly increased by treatment with neuraminidase, most likely due to unmasking of cryptic receptors. Aggregated IgM is not necessary for binding, although this does not point to high affinity receptors, since native IgM is pentameric and may thus be involved in multi-point attachment to target cells.

One of the first reports of IgM-specific receptors on human peripheral T cells described the expression of these molecules following a period of in vitro incubation in IgM-free media containing fetal calf serum (Moretta et al., 1975). The receptors could be removed by treatment with pronase, and their expression required protein synthesis. More recent studies indicated that the expression of $Fc_{\mu}R$ by freshly isolated T cells depends upon synthesis of $Fc_{\mu}R$ (Romagnani et al., 1979) and it was suggested that extremely labile receptor is shed during preparation of T cells and it is re-synthesized during a 37°C incubation period. Neuraminidase treatment of Fc R+ lymphocytes decreases $Fc_{\mu}R$ expression (in direct contrast to findings on monocytes) and simultaneously increases $Fc_{\gamma}R$ expression (Schulof et al., 1980), implying that the treatment acts directly on sialic acid-containing $Fc_{\mu}R$ while it removes a blocking moiety to expose $Fc_{\gamma}R$.

Using a rosette assay, a subpopulation of normal B cells was found to have $Fc_{\mu}R$ (Ferrarini et al., 1977) as did neoplastic cells (Ferrarini et al., 1977, Pichler and Knapp, 1977). Neoplastic B cells expressed $Fc_{\mu}R$ immediately after isolation from peripheral blood and

washing. However, normal B cells required overnight incubation at 37°C for the expression of receptors, suggesting that the receptors on these cells were blocked by serum IgM until after this incubation. In addition, individual neoplastic cells often rosetted with indicator cells separately coated with IgG, IgM or IgE, meaning that one cell can carry one, two or three different classes of FcR (Spiegelberg and Dainer, 1979). T cells also have the potential for expressing FcR for both IgM and IgG as demonstrated by the transition from $Fc_{\gamma}R+$ to $Fc_{\mu}R+$ after stimulus with insoluble immune complexes (Pichler et al. 1978) and from $Fc_{\mu}R+$ to $Fc_{\gamma}R+$ cells after concanavalin A treatment (Gupta et al., 1979).

Studies focusing on the various functional activities displayed by peripheral T cells bearing FcR have demonstrated the $Fc_{\gamma}R+$ and $Fc_{\mu}R+$ T cell populations play an antithetical role in pokeweed mitogen-driven B-cell differentiation (Moretta et al., 1977). Thus, $Fc_{\mu}R+$ cells act as helpers while $Fc_{\gamma}R+$ cells act as suppressors. However, since this study was done, it has become clear that identification of functional subsets based only on the presence of FcR is not satisfactory. As described above, FcR are not stable surface structures. In addition, only a small proportion of the cells in a subset identified by FcR may be involved in the functional activity so that assignment of function based on surface markers may only be valid for the subset as a whole and not for single cells (Mingari et al., 1984).

3. Other FcR

A search for receptors for IgA on lymphocytes has produced data which suggests a possible regulatory role in IgA synthesis for these Fc_{α} R+ cells. Thus, a rosette assay of rabbit lymphoid cells detected IgA-specific receptors in the major IgA-producing organs such as

mesenteric lymph nodes, Peyer's patches and appendix, and, to a lesser extent, in systemic lymphoid tissue (Stafford and Fanger, 1980). As with $Fc_{\mu}R$, these receptors could only be detected after an overnight incubation. Furthermore, polyclonal activation of B cells with goat anti-rabbit Fab significantly increased the number of $Fc_{\alpha}R+$ cells in Peyer's patches and spleen, indicating that receptor modulation may play a role in the regulation of IgA immune responses. Another study demonstrated that both T and B cells from human peripheral blood have receptors for IgA (Sjoberg, 1980a).

The demonstration of "up-regulation" of $Fc_{\alpha}R$ on murine T cells by IgA is further evidence for the regulatory role of these receptors (Yodoi et al., 1982). These authors also found that a T cell hybridoma expresses both $Fc_{\alpha}R$ and $Fc_{\gamma}R$ (Yodoi et al., 1983a). Upon incubation with IgA, the hybridoma releases an IgA binding factor which can competitively inhibit binding of IgA to $Fc_{\alpha}R$ and, which suppresses IgA synthesis (Yodoi et al., 1983b). These are findings similar to those for $Fc_{\gamma}R+$ T cells (Rabourdin-Combe et al., 1984) and $Fc_{\varepsilon}R+$ T cells (see section E [2]).

Secretory component (SC) is a glycoprotein constituting an integral part of secretory IgA. Several studies have confirmed that SC functions as an immunoglobulin receptor (Crago et al., 1978, Orlans et al., 1979, Socken et al., 1979). It was identified as the receptor for IgA and IgM on a colonic carcinoma cell line (HT-29) and on epithelial cells of human fetal tissue (Crago et al., 1978). The nature of the receptor was established by inhibition of binding upon treatment with anti-SC antibody. Cultured rat hepatocytes were found to synthesize SC (Socken et al., 1979) and binding of polymeric IgA to these cells could also be inhibited with anti-SC reagents (Orlans et al., 1979, Socken et

al., 1979).

Very few studies have been done on the receptor for the IgD immunoglobulin class. This is not surprising since IgD is primarily considered to be a surface immunoglobulin and, in humans, circulates with comparatively low serum concentrations (30mg/L vs. 10g/L for IgG). By using IgD-coated latex particles, one investigator detected Fc R receptors on a small percentage of human T and non-T lymphocytes (Sjoberg, 1980b). The functional significance of these receptors is unknown.

[E] IgE Fc Receptors ($Fc_{\epsilon}R$)

1. Mast cells and basophils

One of the most extensively characterized FcR to date is that for IgE on mast cells and basophils. This receptor plays a role in immediate hypersensitivity reactions, in which the interaction of a specific antigen with a specific ligand-receptor complex on target cells initiates the release of pharmacological agents into surrounding tissues. The concept of a receptor which plays a role in anaphylaxis was first proposed by Dale (1913) and Weil (1913), before the role of mast cells and basophils was understood, and without knowledge of the nature of the ligand involved. By definition, anaphylaxis is an immunological reaction in which previous sensitization with a specific antigen has elicited homocytotropic antibodies whose target cells are mast cells and basophils. Upon renewed appearance, the antigen binds to cell-bound antibody and causes the release of pharmacologically active agents.

The demonstration of IgE binding to mast cells (Ishizaka et al., 1972a) and basophils (Ishizaka et al., 1970c) suggested the presence of a

specific receptor for IgE on these cells, and prompted a search for the molecule(s) responsible for this binding. Thus, isolation and characterization of these receptors should lead to a better understanding of the triggering mechanisms of allergic reactions at the molecular level.

(a) Evidence for the localization of receptors: visualization on intact cells

Direct evidence for the localization of receptors for IgE on the plasma membrane of human basophils was obtained by Ishizaka et al. (1970c), using autoradiography after allowing the cells to react with either ^{125}I-anti-IgE or ^{125}I-IgE, and by Becker et al. (1973) who used fluorescein-conjugated instead of radiolabeled ligand. The presence of receptors for human IgE on monkey lung mast cells (Tomioka and Ishizaka, 1971) and for rat IgE on rat peritoneal mast cells (Ishizaka et al., 1975) was detected using similar techniques. A more detailed demonstration, providing more direct evidence for the existence of these receptors, was achieved by electron microscopy of human basophils which had been exposed to IgE, rabbit anti-human IgE and a hybrid burro antibody to rabbit IgG and to ferritin (Sullivan et al., 1971). Using this approach, it was observed that the bound IgE molecules were diffusely distributed over the surface of the cells and could be redistributed with anti-IgE to form patches and caps. Such a redistribution is analogous to that seen by surface immunoglobulins on lymphocytes after treatment with anti-immunoglobulin antibodies (Taylor et al., 1971). This finding agrees with the generally accepted concept that cell surface receptors are mobile. Fluorescein-conjugated anti-IgE

was used to show a similar movement of receptors on rat basophilic leukemia (RBL) cells (Carson and Metzger, 1974). A more recent study, in which binding of fluoresceinated IgE tetramers to RBL cells induced large-scale aggregation of receptors (1000-10,000 receptors per cluster), indicated that ligand-induced clustering is accompanied by cell-induced clustering, possibly via cytoskeletal attachment of receptors (Menon et al., 1984). This phenomenon may be relevant to the triggering signal for degranulation.

The finding that brief exposure of human basophils (Ishizaka and Ishizaka, 1974) or RBL cells (Kulczycki and Metzger, 1974) to pH 4 or lower removed the bound IgE while the cells retain their capacity to bind fresh IgE, provided further evidence for surface localization of the receptors. Quantitation of eluted IgE has also been used to measure the number of receptors on the cell (Conroy et al., 1977).

(b) Number of receptors on target cells, kinetics and valency

The cell bound IgE molecules on human basophils were enumerated by Ishizaka et al. using the C1 fixation and transfer technique (Ishizaka et al., 1973). The method consists of addition of excess anti-IgE to basophils, fixation of the C1 component of complement by anti-IgE and subsequent lysis of sensitized erythrocytes by the C1. The assay revealed 10,000-40,000 IgE molecules per basophil and, by saturating the receptors with myeloma IgE molecules, the number of receptor sites was estimated to be 30,000-85,000. Furthermore, they found that there was no correlation between the number of endogenous IgE molecules per cell and the serum IgE concentration. The affinity constant was estimated to be from 10^8 to $10^9 M^{-1}$.

The availability of relatively pure (75-95%) preparations of rat peritoneal mast cells (RMC), of RBL cells and of myeloma rat IgE made studies of this system very attractive. Direct binding of ^{125}I-IgE to RMC (Conrad et al., 1975) and to RBL cells (Kulczycki and Metzger, 1974, Conrad et al., 1975) demonstrated that the number of receptors on these cells ranged between 0.3-1x10^6 per cell. Two protocols were applied to quantitatively study these receptors. Kulczycki and Metzger (1974) showed that receptors on RBL cells can be saturated by adding excess ^{125}I-IgE to the cells. After correcting for non-specific binding, the amount of IgE bound to saturated cells indicated that there were 0.3-1.0x10^6 binding sites per cell. The varialbility was accounted for by changes in the number of receptors during the cell cycle (Isersky et al., 1975). Measuring binding of IgE to RBL cells and to RMC under non-saturating conditions, Conrad et al., (1975) plotted their binding data according to the Scatchard equation (Scatchard, 1949). They found that RBL cells have a substantially higher number of receptor sites than RMC - approximately 6x10^5 versus 3x10^5, respectively. A study of mouse mast cells found 0.6x10^5 sites per cell (Mendoza and Metzger, 1976a). Thus, normal cells from both human and rat appear to have 2-10 times fewer Fc$_\epsilon$R per cell than neoplastic rodent cells.

The study of Kulczycki and Metzger (1974) also showed that the binding between IgE and the receptor is reversible since ^{125}I-IgE could be displaced with a 50-100 fold excess of unlabelled IgE. Thus, direct binding measurements demonstrated that the binding of IgE to RBL-1 cells is governed by a simple reversible bimolecular reaction:

$$\text{IgE + receptor} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{receptor-IgE complex}$$

in which the equilibrium constant, K_a, can be defined as follows:

$$K_a = \frac{k_1}{k_{-1}}$$

where k_1 is the foward rate constant and k_{-1} is the reverse rate constant.

For RBL-1 cells, Kulczycki and Metzger (1974) calculated the foward rate constant, (k_1), to be $9.6 \times 10^{4} M^{-1} sec^{-1}$ and the dissociation constant, k_{-1}, to be a maximum of $1.6 \times 10^{-5} sec^{-1}$, thereby yielding a minimum K_a of $6 \times 10^{9} M^{-1}$. Similar results were obtained for rat mast cells (Mendoza and Metzger, 1976a). Conrad et al. (1975) obtained an equilibrium constant of $1 \times 10^{9} M^{-1}$ for the binding of IgE to both RBL cells and RMC, from the slope of the Scatchard plot. The equilibrium constant using cell-free particles was approximately one order of magnitude greater than that for intact RBL cells, partially due to a smaller dissociation constant (Metzger et al., 1976). This difference may be explained by the fact that cell-free particles are not affected by receptor turnover as are intact cells. Reactions involving solubilized receptors have dramatically greater K_a due to a much slower dissociation rate (Rossi et al., 1977), a finding that is not yet well understood. Overall, the affinity of mast cell and basophil receptors for IgE is much greater than that of the immunoglobulin-receptor interactions of other Ig classes on most cell types (10^{4}-$10^{8} M^{-1}$) (Froese, 1984). This characteristic of the IgE receptor has greatly facilitated attempts to isolate the molecule (see section F[1]).

Many of these quantitative studies assume that the receptor for IgE is monovalent. Mendoza and Metzger (1976b) addressed this question by saturating cells with a mixture of two distinguishable types of rat IgE and determining whether, by redistributing (patching and capping) one of them with specific antibody, the other would co-migrate. Thus, receptors binding rhodamine-IgE did not co-migrate with receptors occupied by fluorescein-IgE when treated with anti-fluorescein, but did so when reacted with anti-IgE, indicating that these IgE receptors are monovalent. In contrast, the results of Ishizaka and Ishizaka (1975) led to a different conclusion. In this study, human basophils, the receptors of which were partially occupied, were either saturated with unlabelled IgE or left untreated, and were then induced to cap with anti-IgE. The cells were subsequently exposed to pH 4 to dissociate IgE-anti-IgE complexes and, upon neutralization, were reincubated with ^{125}I-IgE and examined by radioautography. Cells which had and had not been pre-saturated showed a similar distribution of grains, exhibiting capped or asymmetrical distribution of the label. This suggests that there was co-migration of empty receptors with receptors to which IgE was bound and that the receptors are at least divalent. Differences in the results obtained in these two studies may have been due to differences in species and cell type as well as due to the methods used. However, the recent study of Menon et al., (1984) which describes a cell-induced (as opposed to ligand-induced) aggregation of IgE receptors upon cross-linking of receptors with tetramers of IgE, indicates that while the receptors themselves may be monovalent, there may be an intracellular association, possibly mediated by cytoskeletal elements. The demonstration that soluble receptors interact with IgE with a 1:1 ratio indicates that they

are indeed monovalent in such a state (Newman et al., 1977).

It should be noted that the IgE used in the rodent studies (Kulczycki and Metzger, 1974, Conrad et al., 1975, Mendoza and Metzger, 1976a, Metzger et al., 1976, Rossi et al., 1977, Mendoza and Metzger, 1976b,) is a myeloma protein obtained from rats bearing the IR 162 tumor (Bazin et al., 1974). A comparison of this IgE and IgE obtained from rat reaginic serum demonstrated that both preparations behave similarly with respect to physicochemical and RBL cell-binding characteristics (Kulczycki and Metzger, 1974).

(d) Specificity of receptors

There has been a great deal of interest in both the species and Ig class specificity of the mast cell and basophil IgE receptors. There appears to be considerable cross-reactivity between receptors and IgE from closely related species. Ishizaka et al. (1970c) observed that monkey lung fragments could be passively sensitized with human atopic serum to release histamine upon treatment with anti-IgE. Tomioka and Ishizaka (1971) were also able to demonstrate, using autoradiography with radiolabelled anti-IgE, that human myeloma IgE binds to monkey skin mast cells. In rodents, it is generally accepted that mouse IgE binds to rat mast cells. Since it was shown that mouse reaginic antibody sensitizes rat skin for passive cutaneous anaphylaxis (PCA) (Mota and Wong, 1969), rats are commonly used for assaying IgE levels in mouse serum. It was also shown that absorption with intact RBL cells and solubilized receptors could deplete PCA activity from mouse reaginic serum (Conrad et al., 1976). Furthermore, the binding of rat IgE to mouse mast cells has been demonstrated (Prouvost-Danon et al., 1975) and the binding constant for this reaction is slightly lower than that for the homologous reaction

(Mendoza and Metzger, 1976), indicating that while there is great similarity in structure between rat and mouse receptors, they are not identical. Indeed, Sterk and Ishizaka (1982) have found that while rat mast cells bind rat and mouse IgE equally well, mouse mast cells have one type of receptor which binds rat and mouse IgE with the same affinity and a separate receptor which binds mouse IgE almost exclusively.

The need for a simple assay of human reaginic serum has led to studies of the interaction between human IgE and rat mast cells and to some contradictory results. While Ishizaka et al. (1970b) failed to sensitize the skin of rats with human reaginic serum, an in vitro study demonstrated that RMC could be degranulated by incubation with human reaginic serum and subsequent challenge with allergen (Perelmutter and Khera, 1970). Washing mast cells after sensitization prevented degranulation, indicating that the interaction was rather weak. There was also a great deal of non-specific degranulation of RMC upon addition of human serum. These observations may partly explain some of the divergent results. A more direct attempt to demonstrate binding of human IgE to rat cells was made by Kulczycki et al. (1974), who failed to show significant inhibition of ^{125}I-rat IgE to RBL cells by human myeloma IgE.

The ability of human IgG to passively sensitize human lung and skin tissues (Parish, 1978) has led to speculation on the nature of the receptor for this immunoglobulin class and its relationship to the IgE receptor. Demonstration that a myeloma IgG_4 could inhibit passive sensitization of human skin by IgE suggested that the two immunoglobulin classes cross-reacted with the same receptor (Stanworth and Smith, 1973).

In the rat, IgG_{2a} was found to induce PCA reactions, and the sensitization could be blocked by IgE. Conversely, IgG_{2a} could inhibit

sensitization of rat peritoneal mast cells with IgE in vitro, thus, it was postulated that a common receptor is involved (Bach et al., 1971). No direct binding of ^{125}I-IgG_{2a} to RBL cells was detected by Halper and Metzger (1976), and even a 1000-fold exess of monomeric IgG failed to inhibit IgE binding while partial inhibition was observed if IgG immune complexes were used. These results suggest that IgE and IgG_{2a} share a common FcR but that the IgG-receptor interaction is a rather weak one which requires multi-point attachment for adequate binding. Conrad and Froese (1978a) were similarly unable to demonstrate inhibition of solubilized receptor-binding to IgE-Sepharose with normal rat IgG. All of these results would be reconcilable if it is assumed that the IgG_{2a} used by Bach et al. (1971) contained some aggregated immunoglobulin.

Daeron et al. (1975) described a phenomenon in the mouse known as direct allogeneic anaphylactic degranulation (DAAD), in which IgG_1 antibodies specific for mouse mast cell histocompatability antigens can degranulate these cells. They postulated that the bridging caused by the interaction of the IgG Fc with FcR and the Fab with histocompatability antigens is a sufficient signal for degranulation. Further investigation revealed that myeloma IgG_1 produced partial inhibition of DAAD, while pre-saturating cells with rat myeloma IgE failed to do so (Daeron et al., 1980). Since the rat IgE-mouse mast cell interaction has a relatively high affinity constant (Mendoza and Metzger, 1976), it seemed unlikely that mouse IgG could replace the IgE on the same receptors, thus it was concluded that the two immunoglobulin classes were interacting with separate receptors. Results seemingly contradictory to these were obtained by Mossman et al., (1979) who used a similar system in the rat and who demonstrated that rat IgG_{2a} alloantibodies

(anti-histocompatability antigen) could inhibit IgE-mediated histamine release from RMC and rat IgE could inhibit IgG_{2a}-mediated DAAD.

This confusing picture was somewhat clarified by the work of Segal et al., (1981). Using chemically cross-linked rat IgG oligomers of different sizes, they demonstrated an increasing affinity of RBL cells for oligomers of increasing size. Thus, affinity constants were calculated to be 6.6×10^6, 1.6×10^7 and $8 \times 10^7 M^{-1}$ for dimers, trimers and higher oligomers, respectively. As previously demonstrated (Halper and Metzger, 1976), binding of monomeric IgG was too weak to be detected by direct measurement. Furthermore, binding of IgE monomer could not be inhibited by rabbit IgG aggregates which effectively block uptake of rat IgG oligomers or by rat oligomers themselves. Monomeric IgE could inhibit IgG oligomer binding more efficiently than monomeric IgG could. Association constants for IgE monomer and IgG monomer binding to IgG binding receptors were estimated to be 1.6×10^6 and $4.2 \times 10^5 M^{-1}$, respectively. These results indicate that RBL cells carry two kinds of receptors. One of these is specific for IgE and cannot be inhibited by monomeric IgG or aggregated IgG; the other binds oligomers of IgG and this binding can be inhibited by monomeric IgG and IgE. However, monomeric IgE has a greater affinity for the "$Fc_\gamma R$" than than does monomeric IgG. These observations were extended by Kepron et al. (1982), who made very similar observations with solubilized receptors (see section [F2]).

2. Lymphocytes

One of the first studies demonstrating Fc R on lymphocytes was performed by Lawrence et al. who found a small but significant uptake of radiolabelled, aggregated IgE (Lawrence et al., 1975). Rosette assays

were used to enumerate and characterize IgE-binding lymphocytes from human peripheral blood lymphocytes (PBLs) (Gonzalez-Molina and Spiegelberg, 1977, Hellstrom and Spiegelberg, 1979, Yodoi and Ishizaka, 1979a) and the results indicate that 1-4% of PBLs are $Fc_{\epsilon}R+$. When lymphocytes were separated into B and T cells, Yodoi and Ishizaka (1979a) found that $Fc_{\epsilon}R+$ lymphocytes were enriched in the B cell fraction, while the T cell fraction contained an insignificant number of rosette forming cells. Only cells from some atopic patients contained a significant number of $Fc_{\epsilon}R+$ T cells. Indeed, Spiegelberg et al. (1979) also found that severely atopic patients had elevated numbers of $Fc_{\epsilon}R+$ lymphocytes. In addition, no $Fc_{\gamma}R$ were co-expressed with $Fc_{\epsilon}R$ on these cells. The presence of $Fc_{\epsilon}R$ on the cells of several human lymphoblastoid cell lines was investigated as well and it was found that the majority of these lines are $Fc_{\epsilon}R+$ (Gonzalez-Molina and Spiegelberg, 1976). Many chronic lymphatic leukemia patients had high proportions of $Fc_{\epsilon}R+$ lymphocytes (11-82%) and many of these lymphocytes expressed $Fc_{\gamma}R$, $Fc_{\mu}R$ and $Fc_{\epsilon}R$ simultaneously (Spiegelberg and Dainer, 1979).

In vitro investigations of $Fc_{\epsilon}R+$ rat lymphocytes carried out by Yodoi et al. (1979) showed that the proportion of these cells markedly increased after incubation with isologous IgE. Other immunoglobulins, such as human IgE, rat IgG or rabbit IgG were not effective. Yodoi and Ishizaka (1979b) found this increase to be the result of a transition of $Fc_{\gamma}R+$ to $Fc_{\epsilon}R+$ cells. The results from this study suggest that IgE can bind to either $Fc_{\epsilon}R$ or $Fc_{\gamma}R$ and provide a signal for the formation of new $Fc_{\epsilon}R$. In addition, culture of mesenteric lymph node cells from rats infected with the parasite, N. brasiliensis (which enhances IgE synthesis) results in the release of a soluble IgE-binding factor that

competes with Fc R on rat lymphocytes in a rosette assay (Yodoi and Ishizaka, 1980). The release correlates with the increase in $Fc_{\epsilon}R+$ cells, suggesting that the factor may be derived from these cells. The major source of the factor was shown to be the T cell population. Further investigation of this factor demonstrated that it has an enhancing or suppressive effect on the IgE antibody response, depending on its state of glycosylation. More recently, it has been shown that B cell lines also release an IgE-binding factor capable of regulating the IgE response (Sarfati et al., 1984). These investigations led to the conclusion that $Fc_{\epsilon}R+$ lymphocytes and $Fc_{\epsilon}R$-like molecules from these cells play an important role in the regulation of the IgE immune response, much like the IgG- and IgA-binding factors already discussed (sections D[1] and D[3], respectively).

The molecular nature of the lymphocyte $Fc_{\epsilon}R$ was determined from studies on detergent solubilized receptors from a variety of cells. Meinke et al. (1978) found that the molecules precipitated from a lymphoblastoid cell line by an anti-receptor antiserum had M_r of 86, 47 and 23kDa as determined by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), the smallest being a proteolytic degradation product. The affinity of the receptor for monomeric IgE was relatively low (10^6-$10^7 M^{-1}$) (Spiegelberg and Melewicz, 1980). Conrad and Peterson (1984) found that the molecule isolated from normal murine B lymphocytes with IgE-Sepharose had a molecular weight of 49kDa and comparison to the 45kDa receptor from human B cells by two-dimensional gel analysis showed great similarities between these two molecules. The high affinity $Fc_{\epsilon}R$ from RBL cells produced a very different 2-D pattern. Furthermore, the murine B cell receptor was found to be multivalent, as demonstrated by

the fact that radiolabelled IgE, when bound to the B cell $Fc_{\epsilon}R$, will co-isolate with the Fc R on an IgE-Sepharose column (Lee and Conrad, 1984). Along with the fact that antiserum to lymphocyte $Fc_{\epsilon}R$ will not release histamine from basophils (Meinke et al., 1978), these results suggest that $Fc_{\epsilon}R$ from a variety of lymphocytes are structurally different from mast cell and basophil high affinity $Fc_{\epsilon}R$.

3. Macrophages and monocytes

Approximately 20% of peripheral blood monocytes (latex-ingesting cells) from healthy human donors were found to form rosettes with IgE-coated indicator cells (Melewicz and Spiegelberg, 1980). This would suggest that a subpopulation of monocytes is involved in the phagocytosis and lysis of IgE-coated target cells. Severely allergic patients had significantly greater proportions of $Fc_{\epsilon}R+$ monocytes (Melewicz et al., 1981), indicating that these cells also play a role in the regulation or expression of allergic disease.

In the rat, Dessaint et al. (1979b) found weak binding of monomeric IgE to macrophages, and were able to show increased binding when using aggregated IgE. It was also discovered that the majority of peritoneal and alveolar macrophages bear $Fc_{\epsilon}R$ (Boltz-Nitulescu and Spiegelberg, 1981), and the proportion increases with increased serum IgE levels (Boltz-Nitulescu et al., 1984). Capron et al. (1975) discovered that normal rat peritoneal macrophages incubated with serum from rats immune to the parasite S. mansoni, become highly cytotoxic for schistosome larvae, a phenomenon shown to be mediated by IgE antibody specific for the parasite. These macrophages were further shown to release lysosomal enzymes in response to incubation with IgE and anti-IgE (Dessaint et al., 1979a). These results provide evidence that IgE

interaction with the macrophage and its subsequent cross-linking leads to the activation of macrophage killing ability, important in the defense against IgE-coated parasites.

4. Eosinophils

The involvement of eosinophil leukocytes and rat IgG_{2a} in the antibody-mediated cytotoxicity against S. mansoni (Capron et al., 1978), and several accounts of IgE binding to eosinophils from allergic patients (Hubscher and Eisen, 1971, Hubscher, 1975), led to the investigation of the role of eosinophils as effector cells in the killing of parasites. Thus, Capron et al. (1981b) found that rat eosinophils and human eosinophils from hypereosinophilic patients bear $Fc_{\epsilon}R$, and that the proportion of cells bearing receptor increased upon incubation with eosinophil chemotactic factor of anaphylaxis (ECF-A), a mast cell-derived mediator. They also demonstrated that rat eosinophils are directly involved in the killing of schistosomula, and the presence of mast cells (ECF-A in particular) enhanced cytotoxicity (Capron et al., 1981a). This effect was mediated by IgE antibodies. Similar findings for the IgG_{2a}-mediated eosinophil cytotoxicity (Capron et al., 1978) point to a relationship between anaphylactic antibodies and phagocytic cells in the cytotoxic mechanisms against certain parasites, with mast cells playing a central role as well.

An interesting finding by Capron et al. (1984) was that an antiserum directed against the $Fc_{\epsilon}R$ of a human lymphoblastoid cell line (Meinke et al., 1978) could inhibit IgE-rosette formation by human eosinophils suggests that the receptors on these cells are antigenically related.

F. Physicochemical characterization of solubilized Fc R from RBL cells and mast cells.

1. Isolation of receptors

One factor which greatly facilitates the analysis of a receptor is a convenient source of cells bearing the receptor. Initially, rat peritoneal mast cells were routinely obtained in relatively pure preparations. The induction of a rat basophilic leukemia by transformation with β-chlorethylamine (Eccleston et al., 1973) provided an IgE binding cell which could be maintained in vitro. A large part of the characterization of the rat mast cell/basophil $Fc_\epsilon R$ has been done with this cell line.

A thorough analysis of the receptors for IgE requires that they be solubilized without loss of IgE-binding capacity. This ability has been demonstrated both for rat mast cells (RMC) and RBL cells by Conrad et al. (1976) who used the non-ionic detergent, Nonidet P-40 (NP-40), to solubilize the plasma membrane. They found that radiolabelled IgE bound to RMC and RBL cell prior to NP-40 solubilization eluted ahead of free IgE alone upon gel chromatography. The increased molecular weight of IgE was due to complex formation with receptors and indicated that receptors in this extract retain their IgE-binding capacity in NP-40. An assay for solubilized receptor was developed by Rossi et al. (1977), based on the fact that the solubility of free ^{125}I-IgE is much greater in a 42% ammonium sulfate solution than that of ^{125}I-IgE-receptor complexes.

To isolate and further characterize solubilized receptors, Conrad and Froese (1976) surface iodinated RMC and RBL cells by the lactoperoxidase-catalyzed reaction. Radiolabelled, solubilized receptors were then isolated by a variety of methods. A sandwich precipitation

technique using rat myeloma IgE (IR162), ϵ-specific rabbit anti-rat IgE, and goat anti-rabbit Ig was used in this study to isolate receptor. Alternatively, precipitates were obtained with IgE and rabbit anti-IgE at equivalence, or the complex was bound by anti-IgE-Sepharose. Protein A-Sepharose has also been used to insolubilize the complex through binding of anti-IgE (Conrad and Froese, 1978a). Isolation of receptors free of IgE was achieved using IgE-Sepharose as the affinity gel and KSCN (Conrad and Froese, 1978a), 0.5M acetic acid or 6M guanidine HCl (Kulczycki and Parker, 1979) as eluting reagents. In the latter study, repetitive binding of receptor material to IgE-Sepharose was shown to yield highly purified receptor as determined by binding of receptor from biosynthetically labelled RBL cells.

An alternate form of affinity chromatography was developed in two laboratories to decrease the elution of cellular material nonspecifically bound to affinity gels. Thus, either dinitrophenol residues (Conrad and Froese, 1978b) or arsanilic acid molecules (Kanellopoulos et al., 1979) were coupled to IgE which subsequently formed IgE-receptor complexes. These complexes were adsorbed onto conjugates of Sepharose and the appropriate anti-hapten antibody and then eluted by addition of the corresponding hapten.

Several different anti-receptor antisera have been produced (see Section F[4]) and, these reagents have been used to isolate receptors. Isersky et al. (1978) used polyethylene glycol to faciltate precipitation of receptor-anti-receptor complexes, while others used protein A-Sepharose to isolate immune complexes (Conrad et al., 1978, Conrad et al., 1979).

2. <u>The number of different receptor molecules and their molecular weight</u>

Initial attempts to determine the molecular weights of the solubilized receptors involved gel filtration or density gradient centrifugation in the presence of non-ionic detergents. Gel filtration on a Bio-Gel A-15 agarose column of IgE-receptor complexes, prepared by addition of ^{125}I-IgE to whole RMC or RBL cell extracts, yielded an approximate apparent molecular weight of 3.5-5.5x10^5 daltons and thus a M_r of approximately 2-4x10^5 daltons for the receptor (Conrad et al., 1976). Receptor eluted from IgE-Sepharose in the presence of NP-40 produced a very broad peak upon gel filtration that led to an estimate of 2x10^5 daltons for the molecular weight (Conrad and Froese, 1978a). Rossi et al. (1977) ran IgE-receptor complexes and free receptor on a Sepharose 6B column and obtained estimates of 410kDa for the complexes and 250kDa for the free receptor.

Using density gradient centrifugation, Newman et al. (1977) arrived at an apparent molecular weight of 130kDa for the receptor in the presence of NP-40, and, by considering the partial specific volume, estimated that the receptor would be about 77kDa in a detergent-free solution. Using the same technique, Conrad and Froese (1978a) arrived at an estimate of 150kDa for the receptor in the presence of NP-40.

This heterogeneity of molecular weights has been attributed, in part, to the variable binding of micellar non-ionic detergent to the receptor, which would also lead to a high molecular weight. Thus, when molecular weights of the receptors were determined by SDS-PAGE, generally lower values were found. Using 5% gels and a receptor preparation isolated with IgE and anti-IgE, Conrad and Froese (1976) isolated a single cell surface component from both RMC and RBL cells with an apparent molecular weight of 62kDa. These results were confirmed by

Kulczycki et al., (1976) who showed that on 10 and 12% gels, the molecular weight was 45 to 50kDa. A slightly larger molecule (58kDa) was reported by Isersky et al., (1977) who also used 10% gels. The dependence of the apparent molecular weight on the porosity of the gel suggested that the receptor was a glycoprotein (Weber and Osborn, 1975). More recently, Holowka and Baird (1984) detected a 33kDa IgE-binding molecule on vesicles prepared from RBL cells, which could only be iodinated in the presence of low-ionic strength buffer and which did not seem to be associated with the other, higher moleculer weight receptors.

Isolation of surface-iodinated receptors from RBL cells with IgE-Sepharose and analysis by SDS-PAGE on 10% gels yielded major 2 peaks with apparent molecular weights corresponding to 45kDa and 55kDa (designated R and H receptors, respectively), as well as a minor peak corresponding to a M_r of 71kDa (later designated 71K by Helm and Froese, 1981a) (Conrad and Froese, 1978a). In contrast, receptor isolated by precipitation with IgE and anti-IgE (Conrad and Froese, 1978a) or with IgE-DNP and anti-DNP (Conrad and Froese, 1978b) yielded R receptors and the minor component, 71K, but not H receptor. In addition, a study by Froese (1980) demonstrated the presence of both R and H on RMC when IgE-Sepharose was used for isolation, although the relative amount of H detected was less than that from RBL cells.

Other laboratories performing studies with a cell line from the same source (Eccleston et al., 1973) found conflicting results. Only one major peak was detected on SDS-PAGE profiles, regardless of whether the receptor was isolated by immune precipitation (Kanellopoulos et al., 1976, Kulczycki et al., 1976, Isersky et al., 1977) or by affinity chromatography (Kulczycki and Parker, 1979, Kulczycki et al., 1976).

However, the molecular weight of this peak was not the same in all of these studies. Cells maintained at the NIH in Bethesda had a receptor with an apparent molecular weight of 58kDa (Isersky et al., 1977) and 53kDa (Kanellopoulos et al., 1979), while cells from St. Louis expressed a receptor with an apparent molecular weight of 45-52kDa (Kulczycki et al., 1976) and 50kDa (Kulczycki and Parker, 1979).

To reconcile these differences, a collaborative study was performed, in which cell lines from different laboratories were analyzed in one laboratory (Froese et al., 1982a). Thus, receptors from different cell lines were analyzed on the same SDS-PAGE gels using a dual-labelling procedure. By employing a sequential absorption using first IgE and anti-IgE-Sepharose, then IgE-Sepharose, it was confirmed that R- and H-like molecules could be isolated from all cell lines tested. However, R- and H-like receptors from some RBL cell lines were shown to have apparent molecular weights which are too close to be resolved. Further evidence for the presence of these two molecules on RBL cell lines and RMC was obtained by Pecoud and Conrad (1981), who demonstrated with tryptic peptide maps that R- and H-like molecules are distinct entities and that each shares the same general pattern from line to line.

To date, only the R-like receptor has been implicated in the histamine release process (see section [F4]). The function of the H receptor has recently been further investigated. Kepron et al. (1982) found that solubilized R and H bound to both IgE- and IgG-Sepharose. Free IgE could inhibit binding of both receptors to either affinity gel. However, free IgG could not inhibit the binding of R to IgE-Sepharose and its inhibition of H binding to IgG-Sepharose was more effective than that to IgE-Sepharose. In addition, absorption of cell extract with

IgG-Sepharose removed more H than R. These results indicate that H has a higher affinity for IgG than does R although both are bound more effectively by IgE-Separose than IgG-Sepharose. These authors suggest that the R and H receptors investigated in this study are most likely the same receptors studied by Segal et al. (1981). The latter investigators studied receptors on intact RBL-2H3 cells and concluded that there is an IgE receptor which binds monomeric IgE with high affinity and an IgG receptor which binds both monomeric IgG and IgE but with a lower affinity than the IgE receptor for its ligand. In addition the IgG receptor binds to IgE with a greater affinity than to IgG. Thus, in both studies, the "Fc$_\gamma$R" appears to have a greater affinity for IgE than IgG. However, in vivo, where the concentration of IgG is by far higher than that of IgE, it may indeed act primarily as a receptor for IgG. Both of these studies used whole rat IgG so that the subclass specificity of the IgG (H) receptor was unknown. In a more recent study, the binding of R and H to rat IgG subclasses was evaluated (Kepron et al., 1984). By inhibiting binding of receptors to whole rat IgG-Sepharose, it was demonstrated that R had a greater affinity for IgG_{2a} and H had a greater affinity for both IgG_1 and IgG_{2b}. These results are interesting in light of the fact that Mossman et al. (1979) have shown that IgE-mediated histamine release from rat mast cells could be inhibited with rat IgG_{2a} but not with IgG_1. Halper and Metzger (1976) also found that immune complexes containing IgG_{2a} could inhibit IgE binding to RBL cells. However, it is not certain which of the two receptors (R or H) was involved in either study.

3. Biochemical nature of the receptor.

It has already been suggested that Fc$_\epsilon$R from RBL cells are glycoproteins because of the dependence of their apparent molecular

weight on the porosity of the gel used in SDS-PAGE. Further evidence for the presence of carbohydrate on these molecules comes from their ability to biosynthetically incorporate radiolabelled sugars (Kulczycki and Parker, 1979, Kulczycki et al., 1976, Helm and Froese, 1981a). While the studies of Kulczycki and his colleagues (Kulczycki and Parker, 1979, Kulczycki et al., 1976) did not distinguish between R and H receptors, that of Helm and Froese (1981a) did establish that both of these molecules incorporate sugars and that 71K is also a glycoprotein. It has also been suggested that the carbohydrate composition of R and H are different as demonstrated by the difference in affinity of these two molecules for different lectins (Helm and Froese, 1981b, Helm et al., 1979). Recent compositional analysis by Kanellopoulos et al. (1980) has demonstrated that the R-like molecule (which has now been designated subunit in some laboratories) has a carbohydrate content of 32%, consisting of fucose, mannose, galactose and N-acetyl-glucosamine. No sialic acid could be detected by direct analysis in this study. However, Pecoud et al. (1981) obtained some indirect evidence for the presense of this molecule by showing that neuraminidase treatment of the cells enhances surface labelling with ^{3}H-$NaBH_4$. The effect of tunicamycin, a glycosylation-inhibiting antibiotic, on the RBL cell $Fc_{\epsilon}R$ was investigated by Hempstead et al. (1981b). They found that the diffuse SDS-PAGE gel receptor band routinely seen in several studies (Conrad and Froese, 1978a, Kanellopoulos et al., 1979, Kulczycki et al., 1976), spanning molecular weights of 45-60kDa, was replaced with a sharper band after treatment with the antibiotic, indicating that most of the microheterogeneity previously observed can be accounted for by carbohydrate heterogeneity.

Evidence for the protein nature of these receptors was first provided by Kulczycki et al., (1976) who were able to eliminate receptor bands from SDS-PAGE gels by treatment with pepsin and pronase. This study and that of Kulczycki and Parker (1979) also demonstrates the biosynthetic incorporation of tritiated amino acids into a single receptor band. In addition, Helm and Froese (1981a) showed that all three receptors, R, H, and 71K could be labelled with ^{3}H-leucine and ^{3}H-tyrosine. Amino acid analysis of the α subunit (see section F[5]) has been performed and demonstrated a relatively high proportion of acidic amino acids (20%) and low numbers of hydrophobic ones (Kanellopoulos et al., 1980). A total of seven cysteic acid residues per molecule was found. Indeed, the protein nature of these receptors has allowed for their further characterization in several studies (See Section F [5] and results of the present study).

In addition to being a glycoprotein, there is some evidence that the α subunit from rat mast cells is a phosphoprotein. Incorporation of ^{32}P-orthophosphate into the receptor band of an SDS-PAGE gel was demonstrated and was markedly enhanced in cells exposed to the divalent cation ionophore A23187 (Hempstead et al., 1981a). The radiolabel was found in a phosphoserine moiety, indicating that a protein kinase was involved in the phosphorylation. A similar enhancement of phosphorylation was seen after antigenic stimulation of sensitized rat mast cells (Hempstead et al., 1983). Because increased phosphorylation is noted within seconds of incubation with antigen, it was suggested that it may be a part of the secretory process. Phosphorylation of receptor associated proteins will be discussed in Section F [5].

4. Antisera to receptors

Several attempts have been made to produce antibodies to either intact RMC or RBL cells (Yiu and Froese, 1976, Basciano et al., 1981), IgE-receptor complexes (Ishizaka et al., 1977) or partially purified receptor (Isersky et al., 1978, Conrad et al., 1979). Each of these preparations was shown to inhibit the binding of IgE to the cell which served as the source of the receptor. The anti-RMC antiserum produced by Yiu and Froese (1976) precipitated several surface components, one of which had a mobility similar to the receptor for IgE on SDS-PAGE analysis. The preparation was cytotoxic for mast cells, although its histamine-releasing capabilities was not tested.

Bridging of receptor-bound IgE molecules by multivalent antigen or divalent anti-IgE has been shown to induce the release of chemical mediators from target cells (Ishizaka and Ishizaka, 1969b, Siriganian et al., 1975). It was then suggested that interaction of adjacent receptor molecules may induce activation of enzymes which lead to mediator release (Ishizaka et al., 1978). To test this hypothesis, antibodies specific for the portion of receptors that is exposed to the cell exterior were necessary. Antibodies produced in rabbits by immunization with receptor-IgE-anti-IgE precipitates were purified by adsorption to and elution from RBL cells (Ishizaka et al., 1977) or, alternatively, an antiserum was produced after immunization with partially purified receptor and purified by absorption with IgE columns and lymphoid cells (Isersky et al., 1978).

Both antibody preparations were then tested for their capacity to induce mediator release. Both whole antibody molecules and $F(ab')_2$ dimers were capable of inducing mediator release from RMC and RBL cells (Isersky et al., 1978, Ishizaka et al., 1977, Ishizaka et al., 1978),

while Fab monomers were not effective. The antibodies were shown to precipitate molecules from extracts of surface labelled RBL cells which had a relative mobility identical to that of the receptor for IgE (Isersky et al., 1978, Conrad et al., 1978). In one study, the anti-receptor antibodies were shown to precipitate IgE-receptor complexes (Isersky et al., 1978), whereas the other antiserum had little activity towards such complexes (Ishizaka et al., 1977), indicating that the two antisera were specific for different antigenic determinants. These results demonstrate that aggregation of receptors rather than cross-linking of cell-bound IgE molecules is the necessary event for antigen-induced mediator release. In light of the more recent findings by Froese et al. (1982a) which demonstrated the presence of two distinct IgE receptors (R and H or their analogs) on all the RBL cell lines tested, the antisera produced by Ishizaka et al. (1977) and Isersky et al. (1978) (designated anti-Rec_{Balt-1} and anti-Rec_{NIH}, respectively) were further evaluated by Froese et al. (1982b) to determine which of these receptors was involved. By precipitating receptors from Winnipeg RBL cells with various anti-receptor antisera, it was determined that anti-Rec_{Balt-1} (Ishizaka et al., 1977) was specific for the R receptor, as had already been demonstrated by Conrad et al. (1978), while anti-Rec_{NIH} (Isersky et al., 1977) isolated predominantly H receptor. The results with the latter antiserum are somewhat surprising since this reagent was produced by immunization with receptors isolated with IgE-anti-IgE, a method which has been shown to isolate only R (Conrad and Froese, 1978). One possible explanation is based on the fact that H has been shown to have a higher affinity for IgG than R (Kepron et al., 1982, see section [F2]). The antibody in anti-Rec_{NIH} may react with H through

the Fc portion of the IgG as well as with R through its antigen specific Fab portion. The fact that this antiserum is capable of inducing mediator release may mean that the relatively small numbers of R receptor crosslinked by antibody provided a sufficient release signal, but the possibility of crosslinking of H through aggregated IgG cannot be ruled out.

Conrad et al. (1979) produced an antiserum by injecting a KSCN eluate from IgE-Sepharose (which contained both R and H) into rabbits. The resulting antibody preparation (RAR) precipitated predominantly H receptor, though R was bound to a lesser extent. RAR was not able to induce histamine release, and the fact that the antiserum was only able to bind to H on intact cells may explain this. To date only R has been shown to be involved in the histamine release process. Since a mixture of R and H were used for immunization, it is possible that H was more immunogenic than R and thus elicited the greater proportion of the response.

A monoclonal antibody to the $Fc_{\epsilon}R$ of a clone of RBL cells was induced in mice by immunizing them with intact RBL-2H3 cells (Basciano et al., 1981). This preparation was found to inhibit binding of IgE to RBL cells but was unable to induce histamine release. More recently, four monoclonal antibody preparations were produced, all of which inhibited IgE-binding (Basciano et al., 1984). Only three induced histamine release, and these could also precipitate a broad band with a M_r of 50-55kDa. The fourth could not induce histamine release but was able to modulate it, indicating that this preparation binds to a molecule which is closely associated with the IgE receptor and which is involved in the regulation of histamine release.

5. Subunit composition of the receptors

When it had been demonstrated that RMC and RBL cells have several surface glycoproteins which specifically bind monomeric IgE, attention turned to non-surface, receptor associated proteins. Thus, crosslinking studies revealed a polypeptide with a molecular weight of 30-35kDa which could not be surface labelled, and which was found to be associated with the R-like receptor (designated α) (Holowka et al., 1980). This polypeptide, which was called β, could be radiolabelled with the hydrophobic probe, 5-iodonaphthyl-1-azide (^{125}I-INA) (Holowka et al., 1981). These results indicate that β is embedded in the membrane. A molecule with a similar molecular weight was intermittently found by Helm and Froese (1981a) when RBL cells were biosynthetically labelled with ^{3}H-amino acids. Kulczycki and Parker (1979) also noted that a polypeptide which could be biosynthetically labelled with amino acids but not sugars, co-purified with the IgE-binding receptor and was absent after extensive purification. These results, and the fact that rigorous washing of IgE-receptor complexes with non-ionic detergent results in dissociation of β from the α-IgE complex (Holowka and Metzger, 1982), strongly suggest that the association between α and β is a non-covalent one which is weak in the presence of detergents. The interaction could be stabilized by the presence of phospholipids in an appropriate lipid:detergent ratio (Rivnay et al., 1982).

Under conditions in which the α-β interaction is stabilized, another protein band was discovered (Perez-Montford et al. 1983b). A 20kDa molecule, which, when reduced became a 10kDa molecule, co-purified with the IgE receptor and was termed γ chain. The molecule is not surface iodinated but can be labelled by the hydrophobic reagent INA and

can incorporate ^{3}H-leucine. A functional interaction between these polypeptides was demonstrated by monitoring changes in extent of phosphorylation of the molecules upon immunological stimulation (Perez-Montford et al., 1983a). It was shown that the amount of ^{32}P associated with γ decreases as that associated with β increases after binding of a multivalent hapten to cell-bound IgE. The physiological significance of these results remains, as yet, undetermined, although the involvement of protein kinase in cell triggering has been demonstrated (Teshima et al., 1984).

An analysis of the domain structure of the α and β subunits of the IgE receptor from RBL cells and their spatial relationship to one another was attempted with the use of proteolytic enzymes. Goetze et al. (1981) cleaved isolated α chain with a variety of enzymes, including papain, pronase and trypsin, and found that all gave similar results. Two fragments were repeatedly produced: a 30kDa fragment which was associated with the surface label and the majority of the carbohydrate, and a 34kDa fragment. Extensive digestion produced many low molecular weight molecules. In addition, the β subunit was found to be cleaved when it was isolated in the absence of protease inhibitors, producing a 23kDa fragment which bound the INA label and which appeared to make contact with α subunit, as well as a smaller fragment (Holowka and Metzger, 1982). An integration of these results led to the proposal of a possible model for the disposition of the IgE receptor in the membrane. Thus, the α subunit consists of 2 'domains' joined by a loosely coiled segment which is susceptible to proteolytic cleavage. Both domains are exposed on the surface, since they both contain carbohydrate, and the receptor may therefore exhibit a bilateral symmetry with respect to IgE

binding. The fragment of β which is bound to α is completely intramembranous, while the remaining fragment is exposed on the cytoplasmic side. Evidence for this last point comes from the finding that lactoperoxidase iodination of the cytoplasmic side of RBL cell plasma membrane vesicles labels both the β and γ subunits (Holowka and Baird, 1984).

SCOPE OF THE PRESENT INVESTIGATION

The aims of this investigation were to establish that R and H receptors of rat basophilic leukemia (RBL) cells having molecular weights of 45kDa and 55kDa, respectively, are distinct entities and to produce fragments of these molecules which retain IgE-binding capacity. The study went on to determine the relationship between R or H receptors and the 71K component and to determine the subunit composition of the 71K molecule.

In Chapter II, the isolation and characterization of surface-iodinated receptors is described. Techniques which allowed for the isolation of separate R and H receptors had already been described by others and were performed as a requisite to the comparative analysis of these molecules. Limited proteolysis of the receptors is then described as a method of comparing the composition of these two receptors.

Chapter III contains the description of digestions of the receptor with a variety of reagents in an attempt to produce IgE binding fragments. Some of these fragments were further characterized with two dimensional maps to establish their receptor origin.

In Chapter IV, the preparative isolation of 71K, R and H receptors by sequential affinity chromatography is described. Comparisons of the isolated receptors were performed by one- and two-dimensional mapping of tryptic digests. Purified receptors were also isolated by repetitive affinity chromatography. This method allowed for the analysis of the subunit composition of the 71K receptor by two dimensional SDS-PAGE. This chapter establishes the relationship among the three receptors.

Finally, the results obtained in this study are discussed in

detail in a section entitled "General Discussion".

CHAPTER II

Comparison of Peptides Generated From R and H IgE Receptors of RBL Cells

INTRODUCTION

Using surface-iodinated cell preparations, the IgE receptors from rat mast cells (RMC) and rat basophilic leukemia (RBL) cells have been isolated by a variety of techniques. In our laboratory, different methods of isolation have yielded different receptor preparations. Thus, immunoprecipitation of IgE-receptor complexes with anti-IgE (Conrad and Froese, 1976) or DNP-IgE-receptor complexes with anti-DNP (Conrad and Froese, 1978b) isolated predominantly the 45kDa R receptor, with traces of the 71kDa receptor also appearing when the latter method of isolation was used. Alternatively, the use of the anti-receptor antiserum (RAR), in conjunction with either anti-rabbit Ig or protein A-Sepharose, yielded predominantly the 55kDa H receptor (Conrad et al., 1979), and all three receptors were found to bind to and elute from IgE-Sepharose (Conrad and Froese, 1978a, Helm and Froese 1981a).

Studies from other laboratories had provided conflicting results, in as much as all methods of receptor isolation appeared to yield only a single molecular entity (Kanellopoulos et al., 1979, Kulczycki et al., 1976, Kulczycki and Parker, 1979), although the apparent molecular weight (M_r) of the isolated receptor was variably reported to fall into the 40-50kDa range or 50-60kDa range. A later collaborative study had established that RBL cell lines maintained in other laboratories are also associated with both R- and H-like receptor molecules, and that the M_r of the two molecules are very similar, making it difficult to distinguish between them by SDS-PAGE (Froese et al., 1982a).

Thus, one of the major discriminating features between R and H receptors of all RBL cell lines was their reactivity with IgE-Sepharose on one hand, and IgE and anti-IgE on the other. Differences between R and H receptors were also established on the basis of their binding to lectins. In fact, it was shown that the two receptors had dissimilar affinities for lentil (Helm and Froese, 1981a), pea and gorse lectins (Helm and Froese, 1981b).

In spite of these differences, the possibility still remained that the two receptor molecules were related in terms of their amino acid sequences or that the smaller R molecule was an enzymatic degradation product of the larger H. Thus, to further compare the two receptor molecules, it was decided to treat the isolated molecules in the presence of SDS with proteolytic enzymes and to compare the peptides produced by performing SDS-PAGE. The method was developed by Cleveland et al. (1977), who demonstrated that one-dimensional SDS-PAGE profiles of peptides generated from given proteins are unique to these proteins.

Before the receptors are compared on this basis, the procedures for isolation of receptors are described. These procedures are the basis for all subsequent experiments and it was thus necessary to confirm previous results, which showed that different receptors are isolated using different isolation procedures.

MATERIALS AND METHODS

Buffers:

The following buffers were used where indicated:

(a) Phosphate buffered saline (PBS) (0.14M NaCl, 0.01M PO_4, pH 7.4) with 0.05% bovine serum albumin (PBS/BSA).

(b) PBS containing Nonidet P-40 (Particle Data Laboratories Ltd., Elmhurst, IL.) was used routinely for cell disruption and experimental procedures (0.1 or 0.5% NP-40/PBS).

Rat Basophilic Leukemia (RBL) Cells

The rat basophilic leukemia cells were propagated *in vivo* in Wistar rats as described by Eccleston et al. (1973) and Kulczycki et al. (1974). A total of $1x10^6$ RBL cells in 0.1 ml PBS were injected subcutaneously into the nuchal region of newborn (1-48 hour-old) ICI Wistar rats. Tumours, which routinely developed within 10-15 days in about 70-80% of the rats, were aseptically removed and minced in cold MEM with Spinner's salts. Single cell suspensions were obtained by trypsinization (0.05% trypsin [Difco Laboratories, Detroit, MI] in MEM) at 0 C and filtration through wire screens. Cells were adapted to tissue culture as described by Conrad et al. (1976), using Eagle's minimal essential medium (MEM) with Earle's salts (Difco Laboratories, Detroit, MI). The medium was supplemented with non-essential amino acids, vitamins, 100 units/ml of penicillin, 100µg/ml of Streptomycin (all purchased from Grand Island Biological Co., Burlington Ont.), and 15% heat-inactivated fetal calf serum (purchased from either GIBCO or Flow Laboratories). Filtered tumour cells were aliquoted at $5-10x10^5$ cells/ml in either 30 or 50 ml Falcon flasks. Cultures were then incubated in a 5% CO_2 atmosphere and maintained at 37°C in a humidified incubator with

constant air flow. When cells reached a density of 2-3x10^6 cells/ml, they were utilized for experiments or passaged to new flasks by mechanical stripping of adherent cells or by treatment of cells with EDTA. During the initial phases of this study, cells maintained in vitro were passaged through animals at regular intervals (3-4 months), as described above.

RBL Cell Iodination and Disruption

RBL cells used in the course of this study were obtained from tissue culture. Cells were washed 5 times in PBS and labelled by the lactoperoxidase procedure described by Conrad and Froese (1976). Briefly, 2-3x10^7 cells were suspended in 0.5ml of PBS, and 0.5mCi of carrier-free $Na^{125}I$ (Amersham Corp. Oakville, Ont.) was added. Three aliquots of lactoperoxidase (Calbiochem, San Diego, CA) (10 μl of 166 IU/ml) and hydrogen peroxide (Fisher Scientific Co., Canada) (10 μl of 0.03% H_2O_2) spaced one minute apart were then added with intermittent agitation. The reaction was quenched by adding the cells to 10 ml of PBS/BSA at 0°C and cells were collected by centrifugation. In order to reduce the presence of ^{125}I bound to low molecular weight lipids, intact labelled cells were incubated in PBS/BSA for 1 hour at 37°C with intermittent agitation. The iodinated RBL cells were then centifuged through 1-2ml of FCS, and washed twice with PBS/BSA. Cells were solubilized by mixing with 0.5% NP-40/PBS at a concentration of 1x10^8 cells/ml for 30 min. at 4°C. Particulate material was removed by centrifugation at 12,000 g in an Eppendorf microcentrifuge (Model 3200) and the supernatant material was used for further experiments.

Preparation of Rat Monoclonal IgE

Rat ascitic fluid was obtained from Lou/M/Wsl rats bearing the

IgE-secreting IR-162 immunocytoma (Bazin et al., 1974). Both rats and immunocytoma were originally a gift from Dr. H. Bazin. Subsequently, ascitic fluid was harvested from intraperitoneally grafted members of a LOU/M/Wsl rat colony maintained in this department. IgE was routinely purified in this laboratory by Ms. K. McNeill using the following procedure. The ascitic fluid was adjusted to 50% saturation of $(NH_4)_2SO_4$, stirred for 2 hours at room temperature and the precipitate was removed by centrifugation, washed with 50% saturated $(NH_4)_2SO_4$, dissolved and dialyzed against 0.2M Tris-HCl, 0.15M NaCl, 0.5% NaN_3, pH 8.0, overnight at 4°C. Gel filtration chromatography of 500mg of IR-162 was then carried out on either Biogel P-300 (Bio-Rad Laboratories, Richmond, CA) or, more recently, on Ultra-gel ACA 34 (LKB Produktor, Broma, Sweden). Proteins comprising the second peak were collected and adjusted to 40% of $(NH_4)_2SO_4$ and the supernatants from this precipitation step were dialyzed against 0.01M glycine HCl, pH 7.6, overnight at 4°C. Dialyzed samples were then subjected to isoelectric focusing using 1% carrier ampholytes, pH 4.0 - 6.5 (LKB) in a sucrose gradient of 0 - 40%. Samples were applied at the center of the preformed ampholine gradient (to prevent precipitation of IgE) and run at 600V for 48 hours. Material focusing at pH 5.9 was collected and dialyzed against PBS to remove ampholyte and the sucrose. The IgE concentration was determined using an extinction coefficient of 13.6 ($E^{1\%}_{280nm}$ (Carson and Metzger, 1974), and the preparations were stored at -70°C until used. IgE to be coupled to Sepharose CL-4B for use in affinity chromatography was the only preparation not subjected to isoelectic focusing.

Preparation of Antisera

Monospecific horse anti-rat IgE (HARE) was kindly supplied by Dr.

K. Kelly of the Department of Immunology, University of Manitoba and was prepared as described by Kelly et al. (1979).

The preparation of anti-receptor antiserum (RAR) has been previously described (Conrad et al., 1979). Briefly, the NP-40/PBS extract from 1-3x10^9 RBL cells was incubated for 1 hour with IgE-Sepharose. The gel was then washed with NP-40/PBS and receptor material was eluted with 3M KSCN that contained 0.1% NP-40. Eluates were dialyzed against PBS, then used as antigen to immunize rabbits. A crude immunoglobulin preparation was made by precipitation of the immune serum with 40% $(NH_4)_2SO_4$. After dissolving the precipitate, the Ig preparation was absorbed twice with IgE-Sepharose and was thereafter used at a concentration of 9mg/ml. This preparation was routinely available in the laboratory and had been prepared by Dr. D. H. Conrad.

Immunosorbents

IgE-Sepharose CL-4B was routinely prepared using the cyanogen bromide procedure of Cuatrecasas and Anfinsen (1971). Sepharose CL-4B (Pharmacia, Uppsala, Sweden) was activated by mixing 150-200mg of cyanogen bromide per ml of washed Sepharose beads. The reaction was carried out in distilled H_2O, by maintaining the pH between 9 and 11, either for 20 min. or until it failed to drop below 9. The activated Sepharose was then washed with 0.1M $NaHCO_3$, pH 8.0. Usually, 10-20mg of IgE were reacted overnight at 4°C with 1ml of packed, activated Sepharose. More recently, the IgE concentration was reduced to 5mg/ml of gel. The gel was then washed to remove uncoupled protein and quenched with 5ml of 0.5M ethanolamine, pH 8.0, for 3-4 hours. Coupling efficiency, which ranged between 80-95%, was determined by spectrophotometric measurement of the supernatant. A control

immunosorbent was prepared by activating Sepharose CL-4B with CNBr and quenching it with ethanolamine. All gels were washed with PBS containing 0.1% sodium azide and stored at 4°C.

Protein A-Sepharose was purchased from Pharmacia in the form of dehydrated beads which were rehydrated with PBS containing 0.1% sodium azide to yield a 1:1 gel slurry.

Affinity Chromatography with IgE-Sepharose

The affinity gel was first equilibrated with 0.1% NP-40/PBS by washing the gel two times with this solution. A 0.1ml aliquot of a 0.5%NP-40/PBS extract of surface labelled RBL cells, containing $1x10^7$ cell equivalents, was added to 0.1ml of the IgE-Sepharose immunosorbent in plastic tubes at 4°C and rotated on a Fisher Roto Rack (Fisher Scientific Co. Ltd., Canada) for 60-90 min. The immunosorbent beads were then washed four times in 3-4ml of 0.1% NP-40/PBS and once in 0.0625M Tris, pH 6.8. Receptor material was eluted by boiling in 0.0625M Tris, pH 6.8, containing 2% SDS, in preparation for polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE).

Affinity Chromatography with Protein A-Sepharose

A 0.1ml aliquot of a NP-40 extract containing $1x10^7$ cell equivalents of surface labelled RBL cells was mixed with a) 900 µg of RAR in 100 1, the concentration demonstrated to be optimal by Conrad et al. (1979) or b) 10 µl of IgE (1mg/ml) and 100 µl of anti-IgE (HARE) (9mg/ml) for 60 min. at 37°C. Each sample was then added to 0.1 ml of washed protein A-Sepharose and rotated for 60 min. at 4°C. Immunosorbent gels were washed and receptor material was eluted as described above.

Polyacrylamide Gel Electrophoresis in SDS (SDS-PAGE)

The Tris-buffered system described by Laemmli (1970) was used

throughout this study. For separation of radiolabelled components, specifically bound to affinity gels, elution was carried out by boiling in 0.0625M Tris, pH 6.8, containing 2% SDS. After cooling, 10% glycerol and 5 1 of bromphenol blue tracking dye were added. Gels cast in tubes (6mm i.d.) consisted of a 140mm separating gel of either 10 or 15% acrylamide and a 30mm stacking gel. To separate immunosorbent-purified material, samples were applied to 10% gels and electrophoresis was carried out at 1mA/gel for stacking and 3-4mA/gel for separation. Gels were then sliced into 2mm fractions with a Gilson Model B-100 or B-200 fractionator (Gilson Medical Electronics Inc., Middleton, WI). In experiments where fractions containing receptor material were subsequently collected, gels were sliced using 0.125M Tris, pH 6.8 as eluant. For analysis of receptor digests, 15% gels were used and the sample buffer was 0.125M Tris, pH 6.8, containing 2% SDS. Following slicing, radioactivity in each fraction was counted in a Beckman Model 8000 gamma counter.

The system was calibrated using proteins of known molecular weights as standards: ϵ-chain of IR-162 IgE, 75kDa; catalase, 58kDa (Sigma Chemical Co., St. Louis, MO); γ-chain of MOPC21a IgG, 53kDa; pepsin, 35kDa (Worthington Biochemical Corp. Freehold, NJ); light chain of MOPC21a, 22.5KDa; chymotrypsin, 21.6kDa (Sigma Chemical Co., St. Louis, MO); myoglobin, 17.4kDa (Miles Laboratories, Kankakee, IL); cytochrome c, 12.3kDa (Sigma Chemical Co., St. Louis, MO). Protein standards were radiolabelled by the chloramine T procedure (McConahey and Dixon, 1966). Following electrophoresis, gels were sliced into 2mm fractions and counted. Standard curves were constructed by plotting the relative migration distance (R_f) of each standard versus the logarithm of

its molecular weight. More recently, standardization of slab gels was carried out using a BioRad Molecular Weight Standard Kit (BioRad Laboratories, Richmond, CA) containing the following markers: phosphoryslase B, 92.5kDa; bovine serum albumin (BSA), 66.2kDa; ovalbumin, 45kDa; carbonic anhydrase, 31kDa; soybean trypsin inhibitor, 21.5kDa; lysozyme, 14.4kDa. These gels were stained with a modified procedure described by Fairbanks et al. (1971). Briefly, the gels were fixed and stained simultaneously in 0.05% Coomassie blue, 25% isopropyl alcohol and 10% acetic acid for 18-24 hours. This solution was then replaced with 0.025% Coomassie blue, 10% isopropyl alcohol and 10% acetic acid for an additional 18-24 hours. The gels were then destained to clearness with 10% acetic acid containing 5% glycerol, leaving the protein bands stained blue. R_f values were used to construct the standard curves which were routinely used to calculate molecular weights of unknown samples.

Limited Proteolysis in the Presence of SDS

Partial purification and separation of R and H receptors was achieved using differential affinity chromatography. The NP-40 extract of surface iodinated cells was treated with either IgE and HARE or RAR as described above. Complexes were insolubilized by mixing with protein A-Sepharose. After washing with NP-40/PBS, then 0.0625M Tris, pH 6.8 (sample buffer), receptor material was eluted by boiling in sample buffer containing 2% SDS. The samples were applied to 10% polyacrylamide gels and electrophoresis was performed. Partially purified receptors were then digested using a modification of a procedure described by Cleveland et al. (1977). After electrophoresis, fractions sliced on the Gilson fractionator, using 0.125M Tris, pH 6.8 as eluant, were counted in a

Beckman Model 8000 gamma counter. Following an overnight incubation at room temperature, buffer from sliced and minced fractions constituting the major peaks from each gel were collected, pooled, concentrated with Sephadex G-200 and dialyzed against 0.125M Tris, pH 6.8 for 18 hours. Samples were then boiled to destroy any endogenous proteolytic activity. They were treated with either 116 μg/ml mercuripapain (Worthington Biochemical Corp., Freehold, NJ) and 1.2×10^{-2}M 2-mercaptoethanol (2-ME) or with 2-ME alone for 30 min. at 37°C. Samples were boiled to destroy proteolytic activity and, after adjusting the SDS concentration to 2%, were analyzed by SDS-PAGE using 15% gels.

RESULTS

Isolation of Receptors from NP-40-Solubilized Surface-Labelled RBL Cells

The flow diagram (Fig. 1) represents some of the experimental conditions used in this investigation to isolate receptors for IgE on RBL cells. All other procedures used throughout this study were variations of these basic methods. Protocol A outlines the isolation of receptors using IgE-Sepharose as the immunosorbent as described in Materials and Methods. Routinely, the NP-40 extract of surface-labelled RBL cells was mixed with a quantity of IgE-Sepharose which was previously found to bind receptors quantitatively. Thus, a 0.1ml aliquot, containing $1x10^7$ cell equivalents, was mixed with 0.1ml of packed IgE-Sepharose for one hour at 4°C. After washing four times with 0.1% NP-40/PBS and once with 0.0625M Tris sample buffer, receptors were eluted with Tris SDS. IgE-Sepharose generally bound between 1 to 3% of the total radiolabel in the extract. Of those counts, 55-65% could be eluted by boiling in SDS-sample buffer.

Protocols B and C represent the procedures used to isolate receptors with IgE and anti-IgE or with anti-receptor antiserum, respectively. A 0.1ml aliquot of the NP-40 extract of surface-labelled RBL cells was mixed with 10 μg of IgE, then 900 μg of HARE. Alternatively, such an aliquot was mixed with 900 μg of RAR. The samples were incubated for 30 min. at 37°C, then added to 0.1ml of washed protein A-Sepharose and rotated/incubated for 60 min. at 4°C. After washing with NP-40/PBS and Tris sample buffer, bound material was eluted by boiling for 90 sec. in sample buffer containing 2% SDS. Protein A-Sepharose bound 0.7-1.3% of the total radiolabel in the extract when IgE-anti-IgE was used, while 0.5-1.0% was bound when RAR was used. These figures are

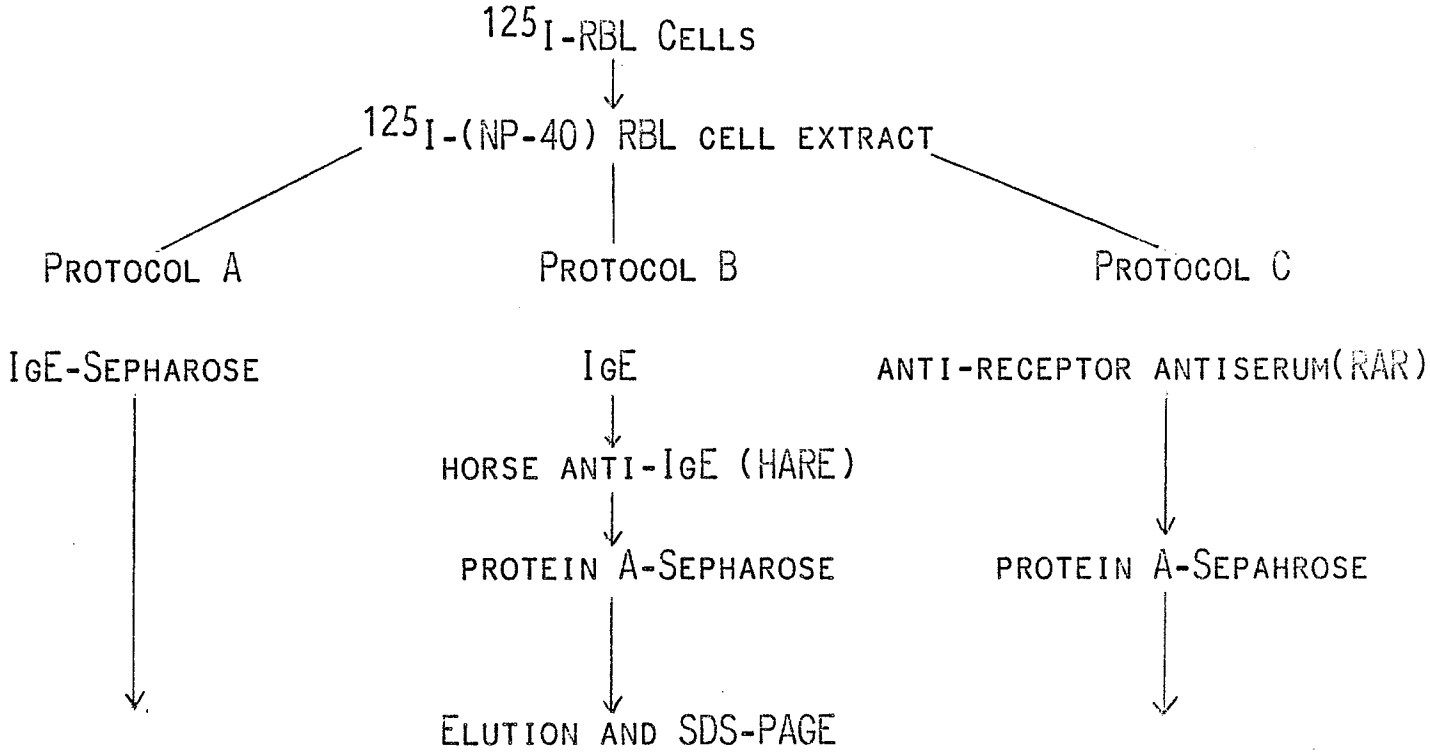


Figure 1: Isolation of the IgE receptors from radiolabelled rat basophilic leukemia (RBL) cells.

in agreement with those for IgE-Sepharose binding, if one assumes that IgE-anti-IgE and RAR bind different components, both of which bind to IgE-Sepharose (see below). Eluting with sample buffer-SDS yielded 60-70% of bound counts when IgE-anti-IgE was used and 65-75% of bound counts when RAR was used. Since batchwise elution was used, most of the remaining counts most likely represent material which remained trapped in the matrix of the gel.

SDS-PAGE of Affinity Chromatography-Purified Surface Components from Solubilized RBL Cells

Calibration of the 10% gels used in the Laemmli SDS-PAGE system is shown in Fig. 2. Radiolabelled proteins were run on tube gels and the molecular weight was plotted versus the relative mobility on a semilogarithmic scale.

The distribution of radiolabelled material eluted from IgE-Sepharose, and run on 10% gels is shown in Fig. 3. It can be seen that two major peaks were found having an M_r of approximately 45kDa and 55kDa. These two molecules will henceforth be called R and H, respectively. In addition, a considerably smaller peak was found, the M_r of which was estimated to be 71kDa and which will be called 71K.

Figure 4 represents the material eluted from protein A-Sepharose after treatment of radiolabelled cell extract with either IgE-HARE or RAR. One major peak was observed when IgE and HARE were used to isolate receptor (Fig. 4a). Its M_r was estimated to be 44K daltons. A smaller peak near fraction 18, was found to have a mobility corresponding to an M_r of 72kDa. Both the 72 and 44kDa peaks have a broadness which is generally characteristic of R and 71K receptors (Conrad and Froese, 1978a, Conrad and Froese, 1976). In addition, a third, sharper peak with

Figure 2: Calibration curve for the SDS-PAGE system according to Laemmli (1970) using 10% polyacrylamide tube gels. Proteins were labelled with ^{125}I by the chloramine T method (McConahey and Dixon, 1966). Proteins (5 µg) were dialyzed against 0.0625M Tris buffer, adjusted to 2% SDS and analyzed by SDS-PAGE. The following proteins were used to calibrate the gels: ε-chain of rat monoclonal IgE (IR-162), 75kDa; catalase, 58kDa; γ-chain of mouse monoclonal IgG (MOPC 21a), 53kDa; pepsin, 35kDa; light chain of MOPC 21a, 22.5 kDa; chymotrypsin, 21.6 kDa.

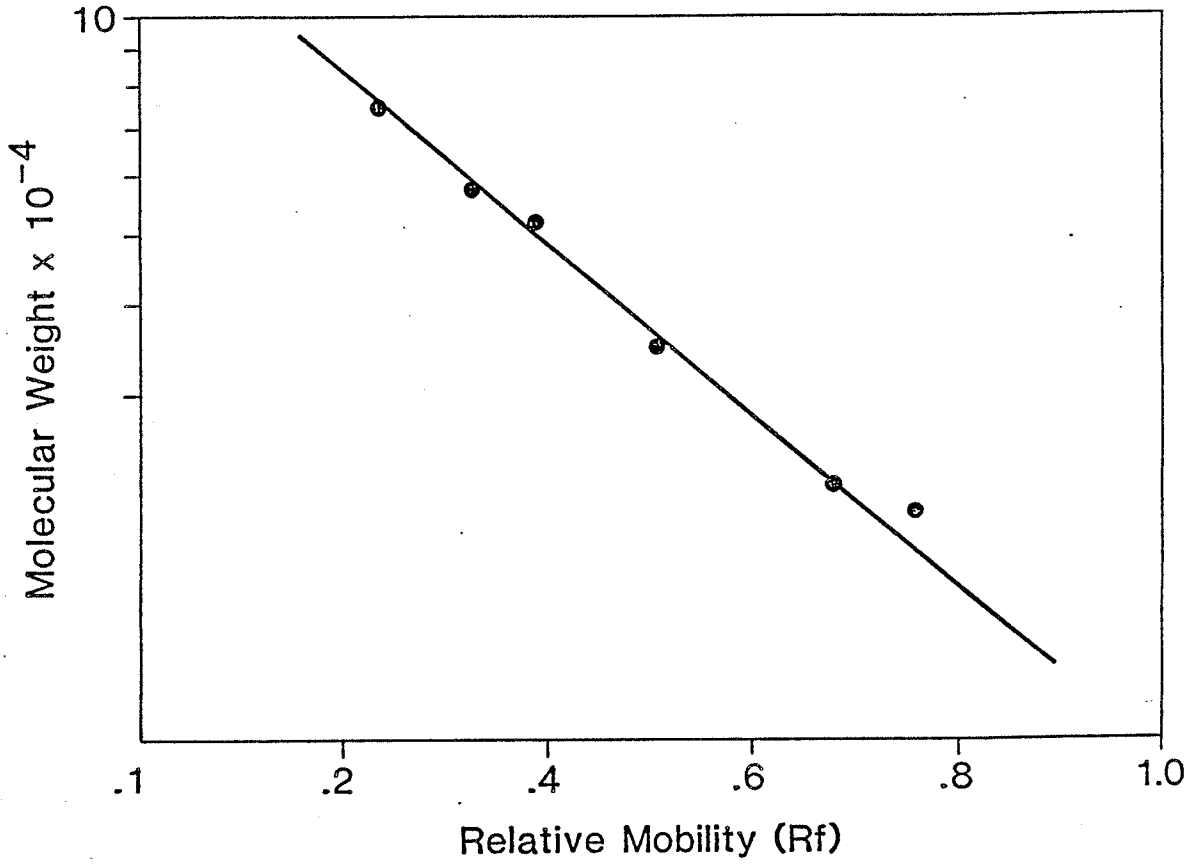
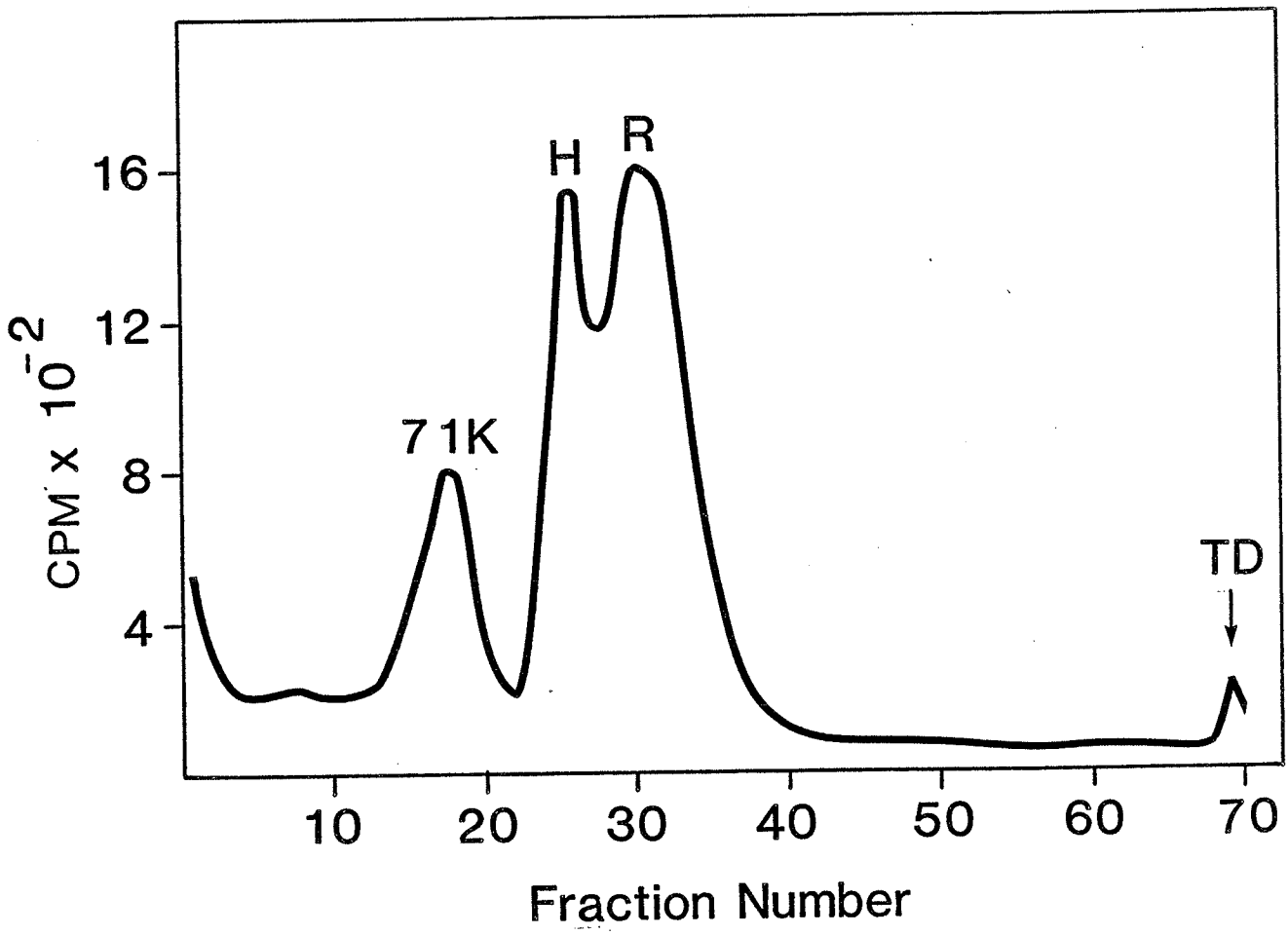

10
Molecular Weight x 10^{-4}
.1
.2
.4
.6
.8
1.0
Relative Mobility (Rf)

Figure 3: SDS-PAGE analysis on 10% gels of surface labelled RBL cell components bound by IgE-Sepahrose. RBL cell equivalents (1 x 10^7) in the NP-40 extract were added to 0.1 ml of IgE-Sepharose and were allowed to mix for 60 min. Gels were washed four times with 3-4 ml of 0.1% NP-40/PBS and once with 0.0625M Tris. Receptor material was eluted with Tris sample buffer containing 2% SDS. The samples were heated in a boiling water bath and eluates were analyzed by SDS-PAGE. TD marks the position of the tracking dye.

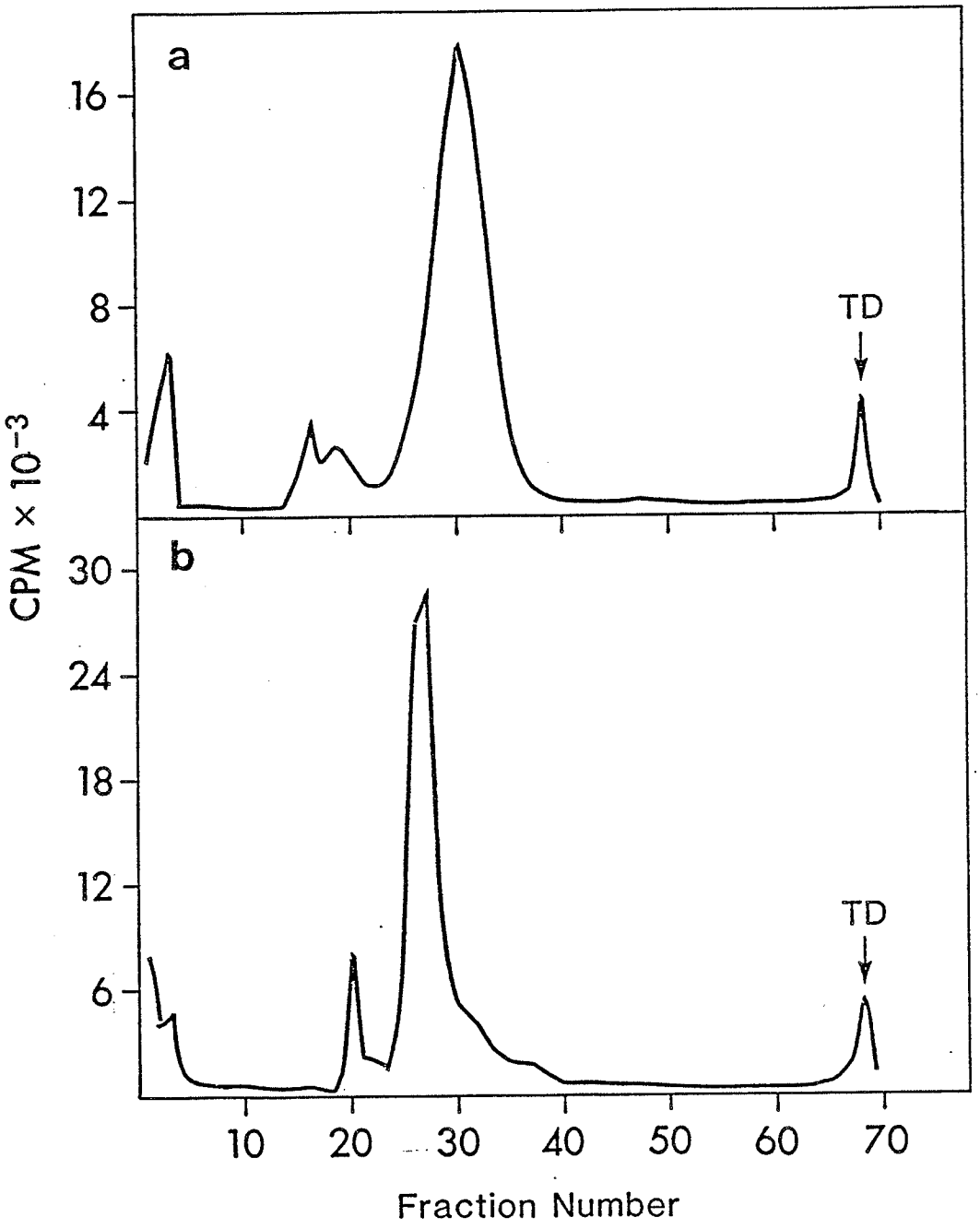

CPM x 10^{-2}
16
12
8
4
H
R
7 1K
TD
10
20
30
40
50
60
70
Fraction Number

a mobility corresponding to an M_r of 77kDa was observed. Molecules isolated by means of RAR are also represented by one major peak (Fig. 4b), with an M_r of 53kDa. It is less broad than the band isolated with IgE and HARE. The minor peak, isolated in fraction 20, has an M_r of 67kDa and is sharper than the band usually attributed to 71K. In addition, a peak which travels with the tracking dye was observed in both profiles and most likely represents unsaturated lipids which had been labelled by ^{125}I (Zimmerman and Chapman, 1977). These results are in agreement with those from previous studies using IgE-Sepharose (Conrad and Froese 1978a), IgE and anti-IgE (Conrad and Froese, 1978a, Conrad and Froese, 1976) and RAR (Conrad et al., 1979). They indicate that R and H receptors are the entities represented by the major peaks shown in Figs. 4a and 4b, respectively, and that these two molecules can be isolated free of each other.

Comparison of Papain-Generated Peptides From R and H Receptors

The radioactive material in the fractions constituting the major peaks in Figs. 4a and 4b and containing R and H receptors, respectively, was collected from gel slices by elution into 0.125M Tris over an 18 hour period. The eluant was found to contain approximately 75% of the total radioactivity in the gel slices. After concentration and dialysis against 0.125M Tris, samples were subjected to proteolysis by 2ME-activated papain (116 μg/ml) for 30 min. at 37°C. Control aliquots were treated with 2-ME alone. After adjusting the SDS concentration to 2% and boiling for 2 min., the samples were analyzed by SDS-PAGE on 15% polyacrylamide gels. Calibration of 15% gels is shown in Fig. 5 as a plot of the relative mobility of radiolabelled standards versus molecular weight. Analysis of peptides generated by proteolysis with papain and

Figure 4: SDS-PAGE analysis on 10% gels of surface labelled RBL cell molecules isolated by IgE and HARE and RAR. a) 1 x 10^7 cell equivalents in an NP-40 extract were mixed with 10 μg of IgE and 900 μg of horse anti-rat IgE (HARE) for 60 min. at 37°C. b) 1 x 10^7 cell equivalents were mixed with 900 μg of rabbit anti-receptor (RAR) antiserum for 60 min. at 37°C. Samples from (a) and (b) were then added to 0.1 ml of protein A-Sepharose and allowed to mix for 60 min. at 4°C. Gels were washed and receptor material was eluted by boiling in Tris sample buffer containing 2% SDS. Eluates were analyzed by SDS-PAGE.


a
b
TD
TD
16
12
8
4
30
24
18
12
6
CPM × 10⁻³
10
20
30
40
50
60
70
Fraction Number

that of 2-ME-treated controls is depicted in Fig 6. The single peaks seen in the gel profiles of control solutions indicate that 2-mercaptoethanol alone did not reduce or degrade either R or H to any extent. The mobilities of these peaks correspond to molecular weights of 45kDa and 55kDa, respectively. The gel profile of digested R receptor is shown in Fig. 6a. It indicates that a group of peptides, the majority of which have molecular weights ranging from 23K to 15K daltons, were generated by the proteolysis of R. No undigested material remained, as seen by the lack of any radioactivity in the region where undigested receptor would normally be found. The digestion of H receptor (Fig. 6b) apparently generated fewer fragments than that of R. In addition, all of these peptides had apparent molecular weights of less than 15K daltons. Indeed, most of the radioactivity resides in one sharp peak the mobility of which corresponds to slighly less than 12K daltons. In this case, a small amount of radioactive material appeared to remain undigested.

Figure 5: Calibration curve for the SDS-PAGE system using 15% tube gels. Proteins were labelled with ^{125}I by the chloramine T method. Proteins were dialyzed against 0.0625M Tris, adjusted to 2% SDS and analyzed by SDS-PAGE. The following proteins were used to calibrate the gel: catalase, 58kDa; pepsin, 35kDa; chymotrypsin, 21.6kDa; myoglobin, 17.4kDa; cytochrome c, 12.3kDa.

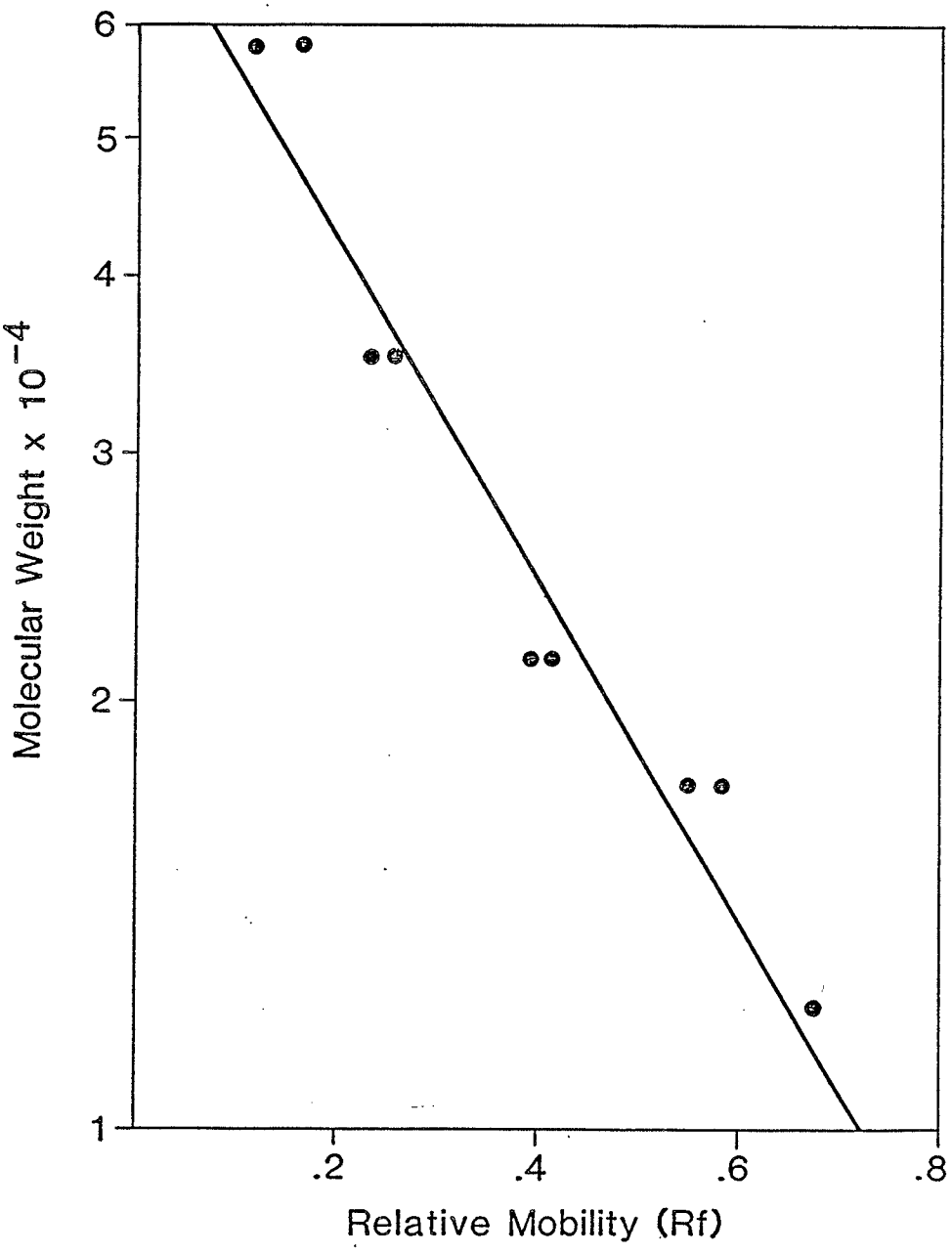
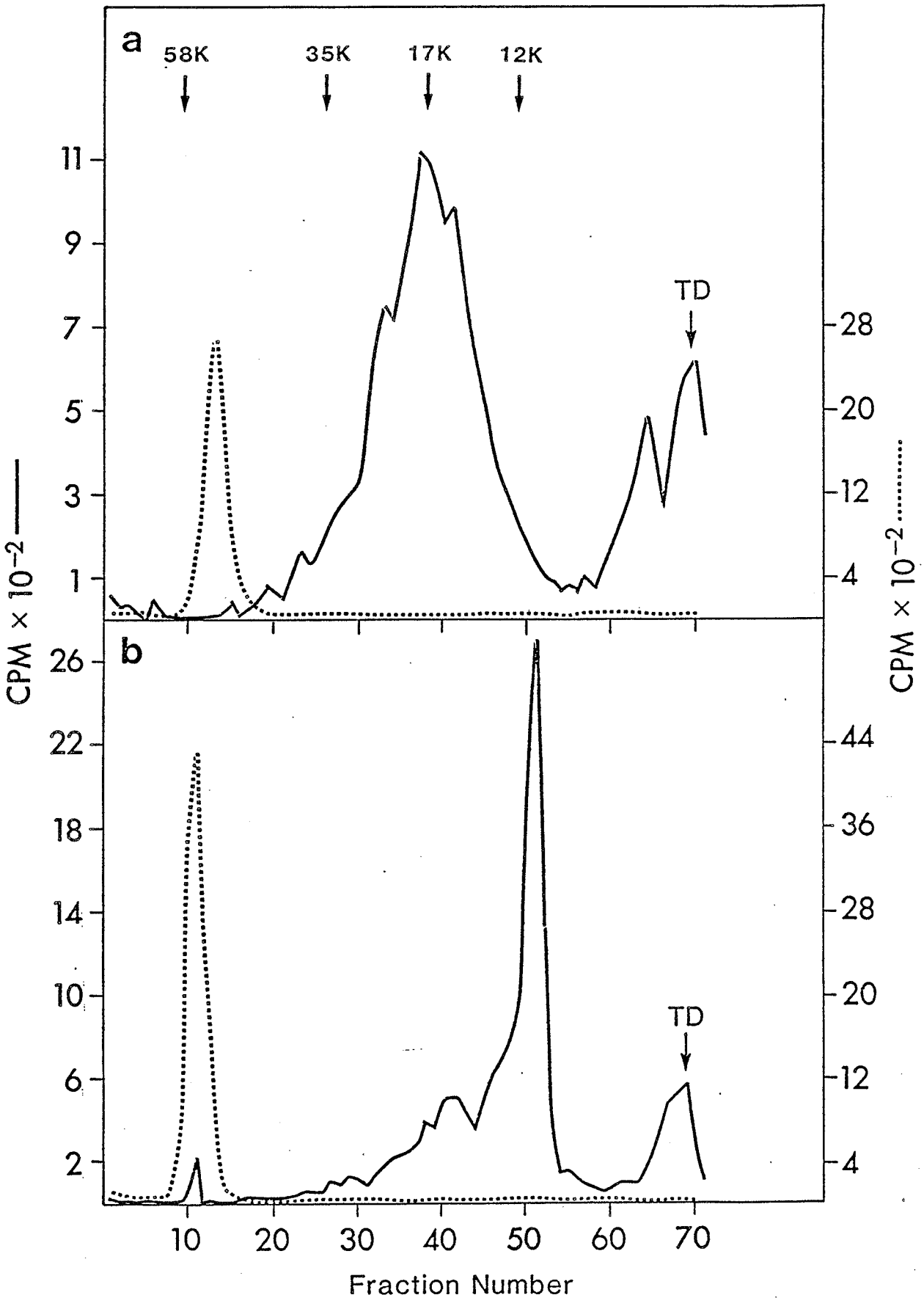

6
5
4
3
2
1
Molecular Weight x 10^{-4}
.2
.4
.6
.8
Relative Mobility (Rf)

Figure 6: SDS-PAGE analysis on 15% gels of papain generated peptides of R and H receptors. Material in fractions constituting the major peaks in Figs. 4a and 4b was collected by eluting from gel slices. Samples were treated with 2-ME-activated papain (final concentration: 116 μg/ml) (——) or 2-ME alone (----) for 30 min. at 37°C. The SDS concentration was adjusted to 2%, samples were boiled and analyzed by SDS-PAGE. Digests of material isolated from gel represented in (a) Fig. 4a, containing R receptor and (b) Fig. 4b, containing H receptor. Molecular weight markers in descending order from left to right are: catalase; pepsin; myoglobin; and cytochrome c.


a
58K
35K
17K
12K
TD
b
TD
CPM × 10⁻² ——
CPM × 10⁻² .....
Fraction Number

DISCUSSION

The purpose of the studies undertaken in this Chapter was two-fold. It was first necessary to confirm results from previous studies (Conrad and Froese, 1978a, Conrad and Froese 1976, Conrad et al., 1979) in which the receptors for IgE on RBL cells are isolated by different means. Secondly, the different types of receptors, designated R and H, were compared through their papain-generated fragments to determine whether these two molecules are structurally similar.

In agreement with the finding by Conrad and Froese (1978a), isolation of receptors with the aid of IgE-Sepharose yielded two molecules having apparent M_r of 45kDa and 55kDa, designated R and H. In addition, a third surface molecule was routinely present and it had apparent M_r of approximately 72kDa. This molecule was also occasionally isolated by Conrad and Froese (1978a) though to a lesser extent. More recently, Helm and Froese (1981a) regularly isolated small amounts of this molecule and named it 71K. The significance of this molecule and its relationship to other receptors is the subject of subsequent chapters.

These experiments also confirmed the finding of Conrad and Froese (1976) by isolating R receptor, but not H, when using IgE and anti-IgE. Again, 71K co-purified with R. The reason for the differences in types of receptors isolated by different procedures is not known. Kepron et al. (1982) showed that the affinity of IgE for R is higher than that for H. They suggested that the amount of IgE used in the IgE-anti-IgE procedure is not in high enough excess to react with significant amounts of H. Another possibility is that the H-IgE complexes formed are

dissociated upon interaction with anti-IgE and this may also be due to the lower affinity of IgE for H (Conrad and Froese, 1978a). The identitiy of the peak near fraction 16 (Fig. 4a) is not known. Its M_r is too high (77kDa) to represent any of the known receptors for IgE. It was not regularly observed.

The use of anti-receptor (RAR) prepared by Conrad et al., (1979), as shown previously, yielded predominantly H receptor in the present study. The identity of the sharp peak near fraction 20 is not known. A sharp band of similar mobility is seen regularly when receptors are isolated by means of other RAR preparations and occasionally when IgE- or IgG-Sepharose is used (M. Kepron, personal communication). Neither free IgE nor IgG could inhibit binding of this molecule, although a molecule of similar mobility does bind to human transferrin-Sepharose (M. Kepron, personal communication). Thus, this band may represent transferrin which was absorbed by cells during tissue culture, or it may represent one of the chains of the dimeric transferrin receptor itself. The ability to isolate R and H receptors separately greatly facilitated the comparison of these molecules, by making it possible to digest relatively pure receptor preparations. The minor bands in Fig. 4 were not expected to interfere in the comparison of peptides derived from R and H, since only material comprising the major peaks was collected and used for subsequent experiments.

The effects of proteolytic and other enzymes on cell surface receptors have been used by others to establish differences between these receptors. Unkeless (1977) found that the two $Fc_{\gamma}R$ on the mouse macrophage line $P388D_1$ could be differentiated by their sensitivity to trypsin. In the present study, the different sensitivities of the two

solubilized receptors for IgE on RBL cells to the proteolytic enzyme, papain, was used to distinguish between them. Thus, the finding that digestion of surface-labelled R and H generated two distinct sets of fragments suggest that these two molecules differ in amino acid composition or sequence, and are therefore two distinct entities. It also indicates that R is not a fragment of H or that both are not fragments of the same larger molecule as was found for the isolated $Fc_{\gamma}R$ on a variety of mouse cells (Bourgois et al., 1977, Kahn-Perles et al., 1980). These results were later supported by those of Pecoud and Conrad (1981), who demonstrated significant differences between the two-dimensional tryptic peptide maps of R and H receptors.

In considering the data shown in Fig. 6, it should be kept in mind that only surface iodinated molecules were used and that, as a consequence, only peptides containing iodinated amino acids, i.e. primarily tyrosine and originating from the exposed portions of the molecule were detected. The finding by Holowka et al. (1981) that an R-like molecule on their RBL cell line cannot be labelled by the hydrophobic probe 5-iodonaphthyl-1-azide (INA) suggests that very little of the molecule is buried in the membrane, therefore a significant portion of R should have been available for iodination. Similar information on H is unavailable. Another possibility to be considered is based on the fact that there are demonstrable differences in the carbohydrate content between R and H (Helm et al., 1979). These differences may extend to the fragments produced by digestion and may be partly responsible for the differences in mobilities of fragments of R and H. It has also been demonstrated that the broadness of the intact R peak is mainly due to carbohydrate heterogeneity (Hempstead et al.,

1981b). This heterogeneity may affect the susceptibility of certain sites in some receptor populations, leading to the demonstrable heterogeneity in R fragments. The differences found between these two molecules does suggest that they are distinct entities and this will be further explored in subsequent chapters.

CHAPTER III

Production of IgE-Binding Fragments of Receptors from RBL Cells

INTRODUCTION

The study of cell surface molecules has played an important part in the investigation of ligand-receptor interactions. These studies have often required the removal of molecules from the lipid bilayer without destroying their ligand-binding activities. The use of non-ionic detergents such as NP-40 and Triton X-100 has greatly facilitated this isolation by solubilizing integral membrane proteins. They also keep normally insoluble amphiphilic proteins soluble in aqueous solutions while preserving conformational structure to retain biological activity. Early studies suggested that the receptor for IgE on RBL cells bound a significant amount of NP-40 (Newman et al., 1977, Conrad and Froese, 1978) and demonstrated that this molecule came out of solution if a maximum of the solubilizing NP-40 detergent was removed (unpublished observation). This indicated that the receptor was hydrophobic in nature. Thus, in the present study, as an alternative to studying the IgE receptor in the presence of detergents, it was decided to produce fragments of the receptor(s) which retain binding capacity but can be manipulated in aqueous solutions. More recently, however, compositional analysis of the R-like, or α receptor has demostrated that it is a rather hydrophilic protein (Kanellopoulos et al., 1980). The fact that this molecule could not be labelled by the hydrophobic probe, 5-iodonaphthyl-1-nitrene supports these results (Holowka et al., 1981). However, the lack of solubility in the absence of detergents may have been due, at least in part, to the β and α chains, which are hydrophobic and are known

to be associated with R (α). Indeed, Kumar and Metzger (1982) were able to analyze the receptor in a 6M guanidine HCl solution in the absence of any detergent.

The availability of binding fragments of the IgE receptor(s) nonetheless provides the opportunity to study an active receptor molecule with a lower structural complexity than that of intact receptor. This would facilitate the elucidation of the structure (both primary and tertiary) of the receptor binding site. In another system, this concept prompted Bartfeld and Fuchs (1979) to produce an active portion of the acetylcholine receptor by digestion with trypsin. They demonstrated that the 27kDa fragment of intact receptor (40kDa) could bind specific ligand as well as induce an autoimmune myasthenia gravis in rabbits when it was injected with complete Freund's adjuvant. This indicates that the fragment contained both the binding site and the mediator of a physiological response.

This chapter describes the search for binding fragments of the IgE receptor(s) on RBL cells. In order to isolate an IgE-binding molecule, the digestion had to take place before the receptor was exposed to any denaturing reagents. Thus, an approach different from the one taken in Chapter II was needed. Therefore, in this study, receptors were digested before isolation with affinity gels. In addition, several different proteases were used in an attempt to generate a variety of different fragments. These attempts have provided further information on the structure of the IgE receptors.

MATERIALS AND METHODS

The buffers, preparation of IgE and IgE-Sepharose, RBL cell iodination and disruption have already been described in Chapter II, Materials and Methods.

Digestion of Receptors in the Presence of NP-40 or SDS

Extracts of RBL cells which were surface labelled with either ^{125}I or ^{131}I were mixed with IgE-Sepharose for one hour at 4°C. The gel to which the ^{125}I-extract had been added was then transferred to a 1.0 ml Pasteur pipette column and was washed with 15-20 ml of 0.1% NP-40/PBS. Receptor material was eluted with 3.0 M KSCN containing 0.1% NP-40 and the eluate dialyzed against 0.125M Tris, pH 6.8, containing 0.1% NP-40. The gel to which ^{131}I-receptor material had been added was washed 4 times with 0.1% NP-40/PBS and once with 0.125M Tris. Bound receptors was eluted by boiling in sample buffer containing 2% SDS. Both eluates were treated with 116 µg/ml mercuripapain and in the presence of $1.2x10^{-1}$M 2-ME for 30 min. at 37°C. Control aliquots were adjusted to $1.2x10^{-1}$M 2-ME. SDS was added to KSCN eluates to a final concentration of 2%. All samples were boiled for 2 min. and then applied to 15% polyacrylamide gels for SDS-PAGE analysis. Both digests were applied to one gel, as were both controls.

Digestion of RBL Cell Extract to Produce an IgE-Binding Fragment of IgE Receptors

RBL cells, surface-labelled with ^{125}I, were solubilized with 0.1% NP-40/PBS. An aliquot of the extract containing $6x10^7$ cell equivalents was digested with 1.16mg/ml papain in the presence of $1.2x10^{-1}$M 2 ME while only 2 ME was added to a control aliquot containing $1x10^7$ cell equivalents. Samples were incubated for 90 min. at 37°C and then treated

with $1.5x10^{-1}$M iodoacetamide (Serva, Heidelberg, W. Germany) for 45 min at 4°C. Receptors from the control aliquot were isolated with 0.2 ml IgE-Sepharose, while their fragments were isolated with a variety of reagents (as described in the Methods and Materials section of Chapter II), including 0.2 ml IgE-Sepharose; IgE (10 μg), HARE (900 μg) and 0.2 ml protein A-Sepharose; and RAR (900 μg) and 0.2 ml protein A-Sepharose. The controls of these various methods of isolation were: 0.2 ml activated and quenched Sepharose; HARE and protein A-Sepharose; and normal rabbit gamma globulin (nRGG) (900μg) and protein A-Sepharose, respectively. Material eluted from these gels was analyzed on 10% polyacrylamide gels.

To determine the origin of papain-derived fragments, fractions of the PAGE gel constituting the peaks were cut out and the receptor material contained in these fractions were extensively digested with trypsin. The resulting peptides were analyzed by a two dimensional procedure. The details of these methods will be described in Chapter IV. A tryptic map of intact H receptor was prepared in a similar fashion, using RAR and protein A-Sepharose to isolate the receptor from a surface-labelled RBL extract.

In a separate experiment, aliquots of surface-labelled RBL cells containing $3.6x10^{7}$ cell equivalents were treated with either trypsin (Schwartz/Mann, Orangeburg, NY) or chymotrypsin (Sigma Scientific Co. St. Louis, MO) at a final concentration of 5mg/ml of the enzyme in PBS or with PBS alone. After a 2 hour incubation at 37°C, phenyl methyl sulfonyl fluoride (PMSF) (Sigma Scientific Co. St. Louis, MO) was added to all samples to a final concentration of $8x10^{-3}$M to stop digestion. Receptors and their fragments were once again isolated with IgE-Sepharose, IgE, HARE and protein A-Sepharose or RAR and protein

A-Sepharose. SDS-sample buffer eluates from affinity gels were then analyzed by SDS-PAGE on 10% gels.

RESULTS

Digestion of R and H Receptors in the Presence NP-40 and SDS

The experiments described in Chapter II had demonstrated that R and H receptors are susceptible to digestion by papain in the presence of the anionic detergent, SDS. However, if an attempt to prepare an IgE-binding fragment of these molecules was to be made, the digestion would have to take place before receptors came into contact with a denaturing reagent. Thus, it seemed appropriate to attempt the digestion while receptors were in the NP-40 extract, before their isolation by affinity chromatography. The following experiment was performed to determine whether digestion of receptor molecules was possible in the presence of NP-40, and to compare the extent of this digestion to that in the presence of SDS.

Two aliquots of $3x10^7$ cells were surface iodinated, one with ^{125}I the other with ^{131}I. After NP-40 solubilization, each cell extract was added to 0.3ml of IgE-Sepharose and incubated for 1 hour at 4°C. Of the ^{125}I labelled receptor material 1.3% was bound by the affinity gel and 75% of the bound material could be eluted with 3M KSCN/PBS containing 0.1% NP-40. The ^{131}I-labelled receptor material bound to an extent of 1% and 55% of the bound material could be eluted with Tris/SDS. Following dialysis of the KSCN eluate against 0.125M Tris/0.1% NP-40, both eluates were subjected to digestion by papain under conditions identical to those described in Chapter II. Again, control aliquots were treated with 2-ME only. Samples in NP-40 were adjusted to 2% SDS. Digests of both eluates were applied to one 15% polyacrylamide gel and controls were applied to another. SDS-PAGE analysis was then performed. Fig. 7 shows undigested

eluates, and demonstrates that both R and H receptors are eluted with KSCN/NP-40 as well as with SDS. There also appears to be some contaminating labelled non-receptor material in the KSCN eluate. This is most likely due to trapping of non-specific material which was not washed away in the pipette column. It is not present in the SDS eluate. The patterns of digested material appear in Fig. 8 and clearly show that papain retains its ability to digest both R and H receptors in the presence of NP-40 as well as in SDS. In either case, no undigested material remained, as demonstrated by the lack of significant radioactivity in fractions corresponding to molecular weights of 45kDa and 55kDa. When comparing the digests of the two preparations, it is apparent that somewhat larger fragments were generated by digestion in NP-40 than by that in SDS. This is possibly due to the fact that NP-40 is a non-ionic detergent and may not unfold the proteins to as great an extent as the ionic detergent SDS, thus making fewer sites available to proteolysis and leading to production of fewer fragments.

Isolation of IgE-Binding Fragments of Receptors

Once it was established that papain retains its proteolytic activity in the presence of NP-40, the following experiments were performed in an attempt to isolate binding peptides of R and H receptors. Surface iodinated RBL cells ($7x10^7$) were solubilized in 1 ml of 0.1% NP-40/PBS. An aliquot containing $6x10^7$ cell equivalents was incubated with 1.16 mg/ml papain and 2-ME, while the control aliquot, containing $1x10^7$ cell equivalents, was treated with 2-ME only. Both digest and control were then incubated with iodoacetamide to stop the reaction. The control aliquot was incubated with IgE-Sepharose, as was an aliquot of the digested extract. The remainder of the digest was incubated with one

Figure 7: SDS-PAGE analysis (on 15% gels) of surface labelled RBL cell components bound to IgE-Sepharose and eluted in the presence of SDS or KSCN. 3×10^7 equivalents of RBL cells labelled with either ^{125}I or ^{131}I were added to two aliquots of 0.3 ml IgE-Sepahrose and incubated for 60 min. at 4°C, and then washed. The ^{125}I labelled receptor material was eluted with 3M KSCN/PBS containing 0.1% NP-40 (----) and the ^{131}I labelled receptor material was eluted by boiling in 0.125M Tris containing 2% SDS (——). Following dialysis of the KSCN eluate against 0.125M Tris/0.1%NP-40, both samples were treated with 2-ME for 30 min. at 37°C. SDS was added to the KSCN eluate to a final concentration of 2%. Samples were boiled and analyzed by SDS-PAGE on the same 15% tube gel.

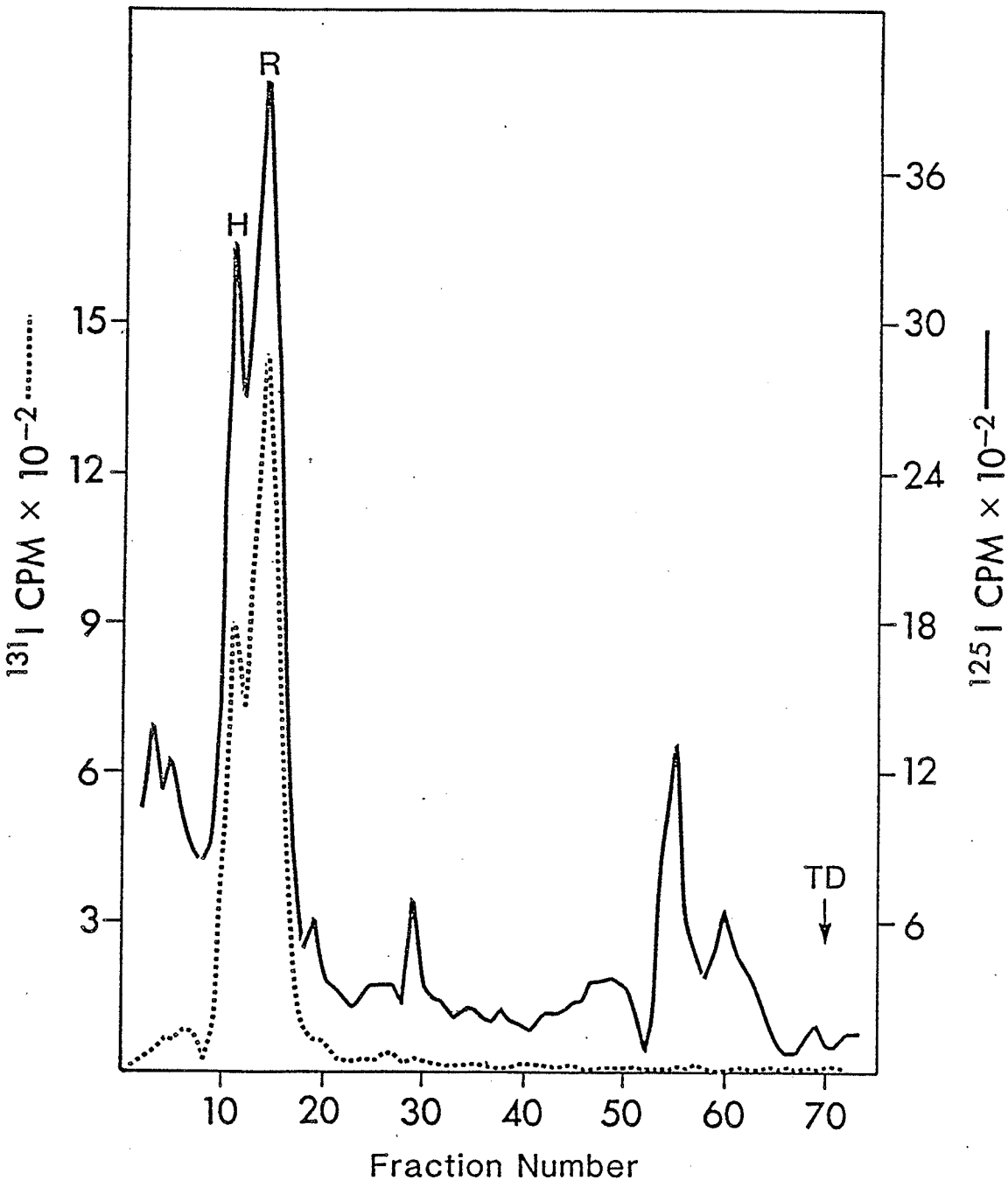
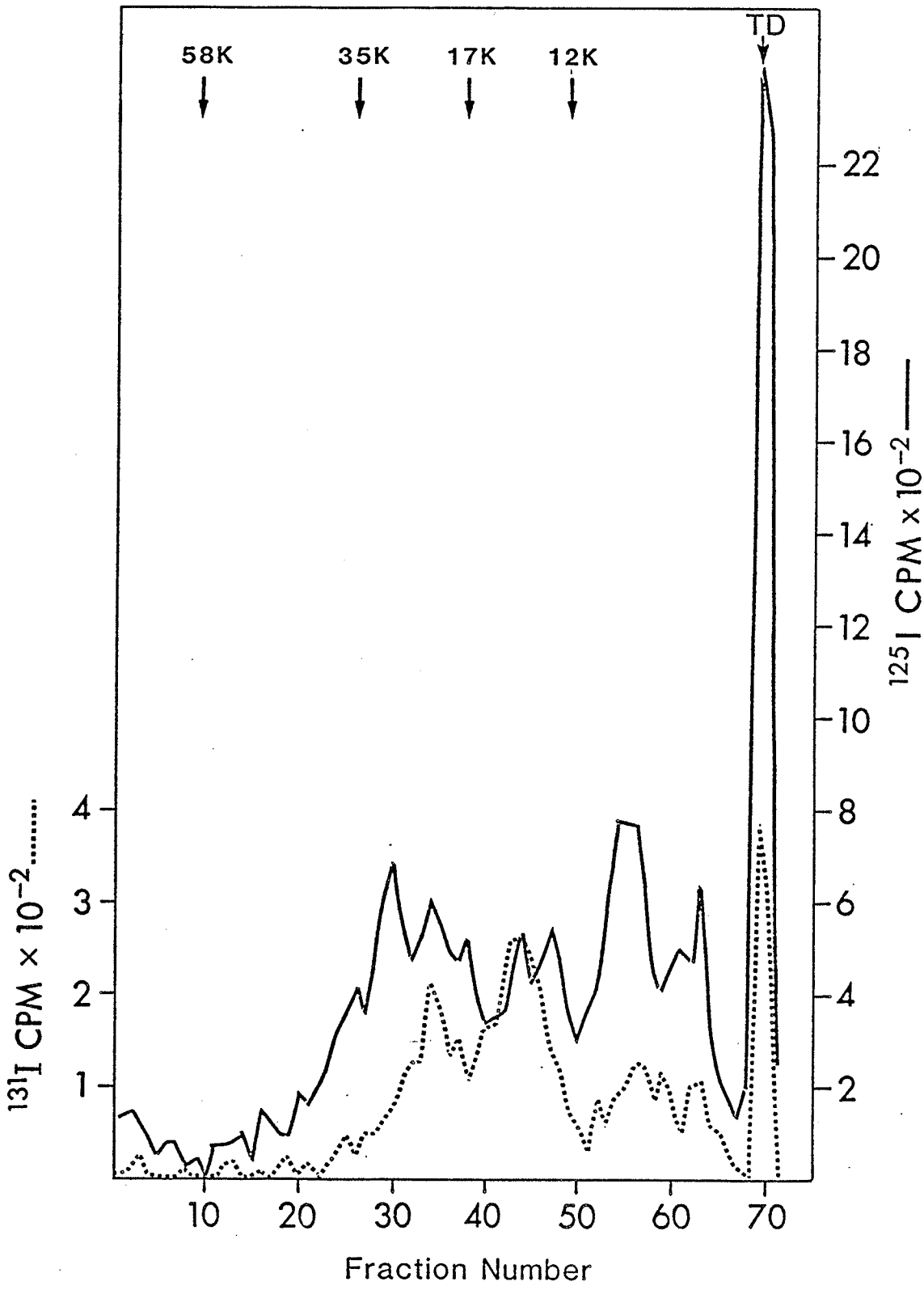

R
H
TD
15
12
9
6
3
36
30
24
18
12
6
10
20
30
40
50
60
70
Fraction Number
^{131}I CPM × 10^{-2}
^{125}I CPM × 10^{-2}

Figure 8: SDS-PAGE analysis of papain digests of receptors digested in the presence of either SDS or NP-40. Samples were processed as in Fig. 7, except that 2-ME-activated papain (final concentration: 116 μg/ml) was added instead of 2-ME. Digests were analyzed by SDS-PAGE on the same 15% gel. Receptors digested in the presence of SDS (----) or NP-40 (———). Molecular weight markers in descending order from left to right are: catalase; pepsin; myoglobin; and cytochrome c.


58K
35K
17K
12K
TD
22
20
18
16
14
12
10
8
6
4
2
4
3
2
1
10
20
30
40
50
60
70
Fraction Number
^{131}I CPM x 10^{-2}
^{125}I CPM x 10^{-2}

of the following sets of reagents: IgE, HARE and protein A-Sepharose; RAR and protein A-Sepharose; activated and quenched Sepharose; HARE and protein A-Sepharose; nRGG and protein A-Sepharose. The last three sets of reagents were used as binding controls. SDS-PAGE analysis of the eluates from these affinity gels were performed on 10% polyacrylamide gels.

Gel patterns of the receptor material isolated from papain-digested extracts are shown in Figs. 9 and 10. The control extract (Fig. 9a) which was not treated with enzyme demonstrates the presence of intact R and H receptors. When IgE-Sepharose was used to isolate receptor material from digested extract, a single peak was generated (Fig. 9b), the mobility of which corresponds to an M_r of 36kDa. The lack of binding of radiolabelled material to activated Sepharose indicates that digestion did not generate any peptides which nonspecifically bound to the gel. IgE, HARE and protein A-Sepharose did not isolate any IgE-binding fragments (Fig. 10a) as was the case for RAR and protein A-Sepharose (Fig. 10b). Interestingly enough, nRGG (Fig. 10b), which was used as a binding control for RAR, did bind a fragment, the mobility of which was similar to that isolated by IgE-Sepharose (Fig. 9b). However, nRGG and protein A-Sepharose bound significantly less material than did IgE-Sepharose.

To establish the origin of the peptide isolated by IgE-Sepharose (and presumably by nRGG and protein A-Sepharose) the material contained in the fractions constituting the peak of the fragment isolated by IgE-Sepharose was digested by trypsin. The tryptic peptides thus generated were analyzed by a two dimensional procedure, the details of which will be described in Chapter IV. The map thus produced was

Figure 9: SDS-PAGE analysis (on 10% gels) of papain generated IgE -binding fragments bound by IgE-Sepharose. a) 1 x 10^7 cell equivalents of surface labelled RBL cells were treated with 2-ME for 90 min. at 37°C, followed by iodoacetamide for 45 min. at 4°C before mixing with 0.2 ml of IgE-Sepharose. b) as in (a), except that 2 x 10^7 cell equivalents were treated with 2-ME-activated papain (final concentration: 1.16 mg/ml) and one half of the sample was incubated with activated Sepharose (----), the other with IgE-Sepharose (——). Gels were washed and eluted and the material was analyzed by SDS-PAGE.

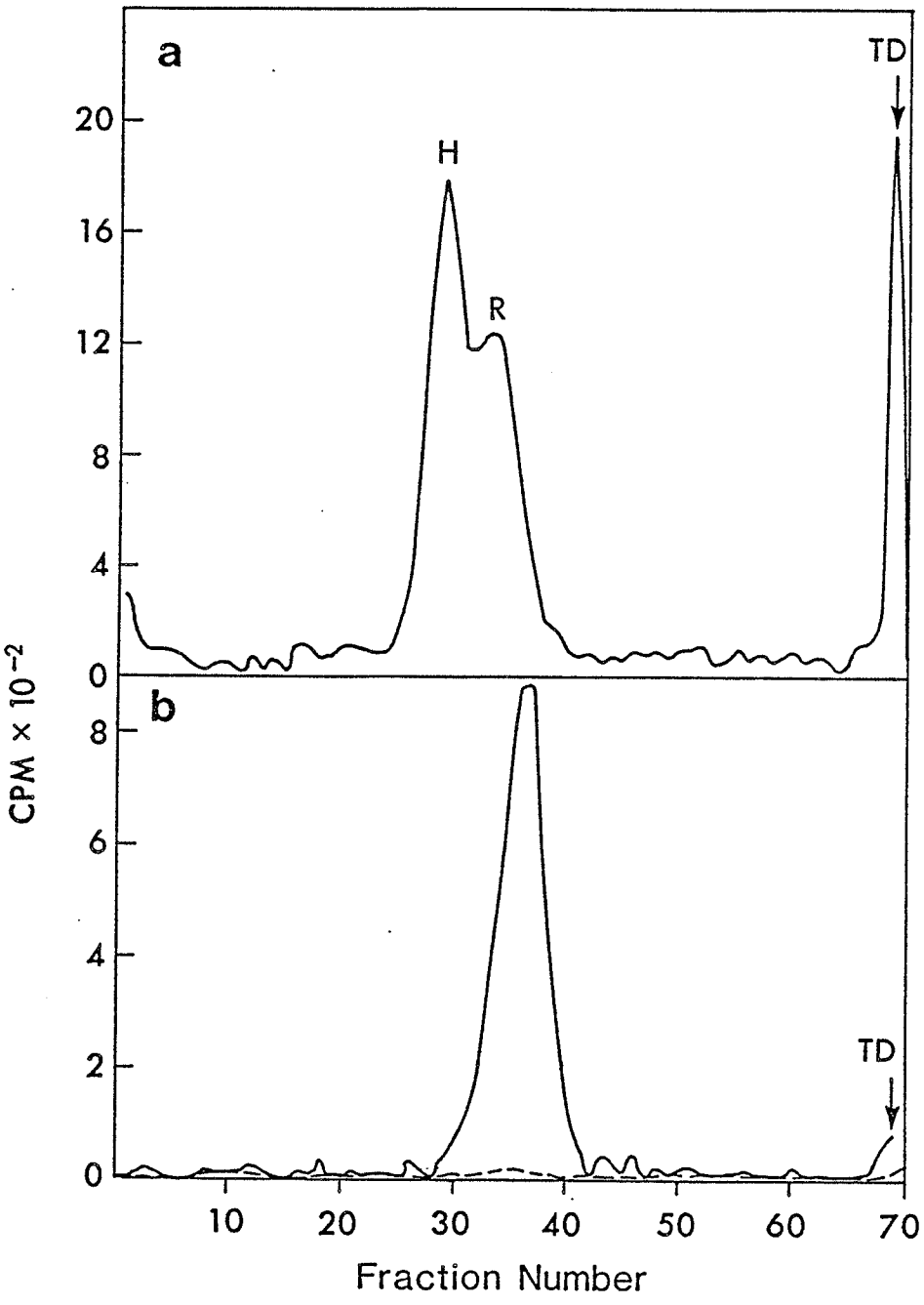
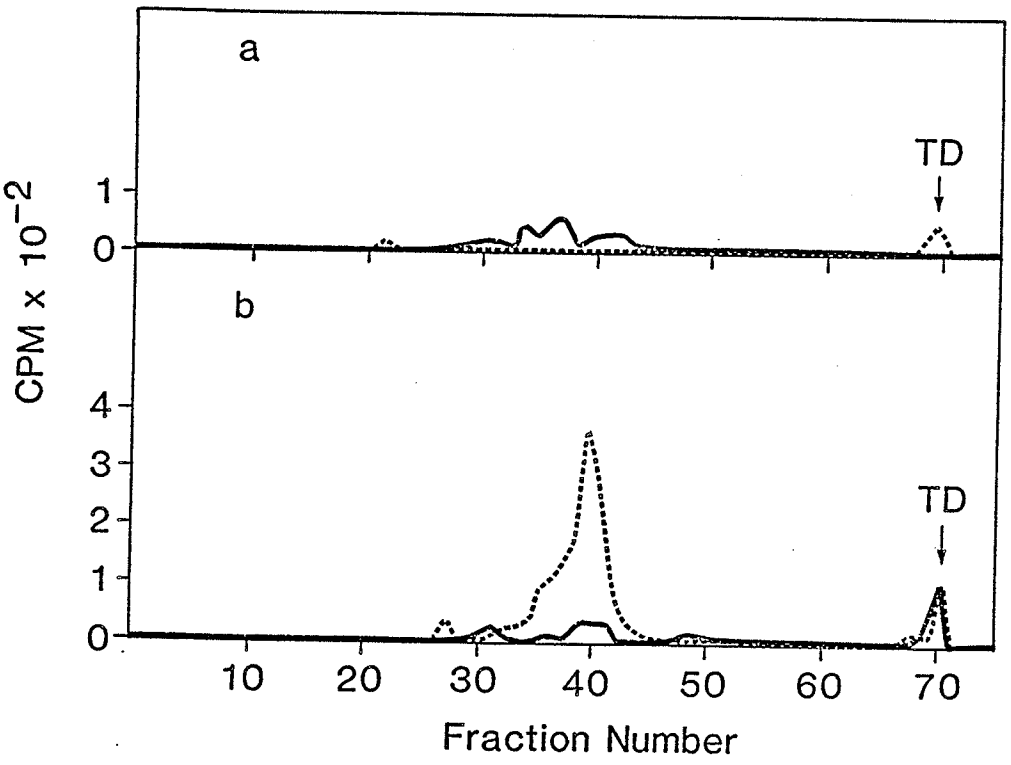

a
b
TD
H
R
TD
20
16
12
8
4
0
8
6
4
2
0
CPM × 10⁻²
10
20
30
40
50
60
70
Fraction Number

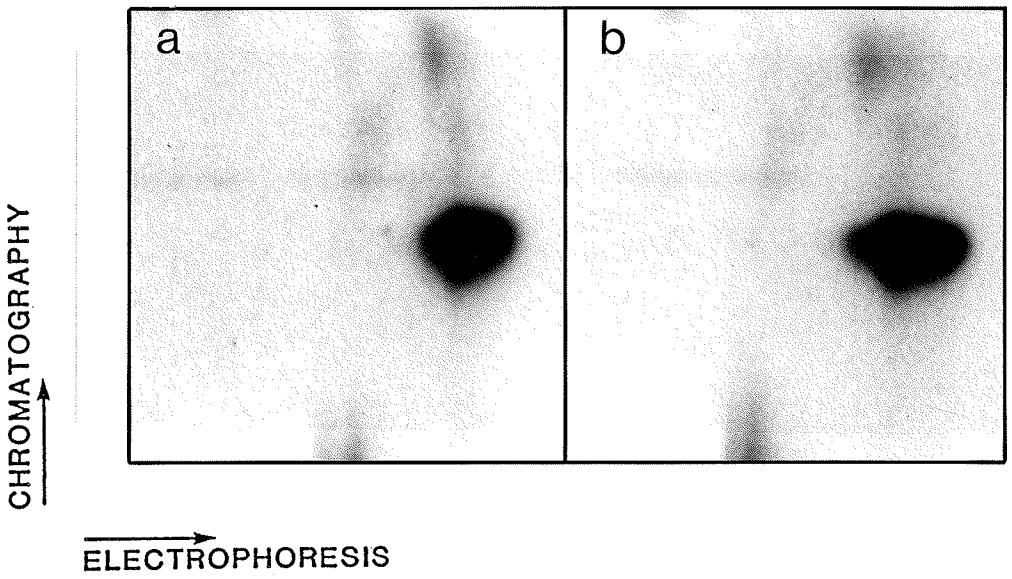
Figure 10: SDS-PAGE analysis of papain generated IgE-binding fragments bound by IgE and HARE and RAR. 4 x 10^7 cell equivalents of surface labelled RBL cells were treated with 2-ME-activated papain (final concentration: 1.16 mg/ml) for 90 min. at 37°C followed by iodoacetamide for 45 min. at 4 C. a) Aliquots of the digest were incubated with IgE and HARE (——) or with HARE alone (----) and then mixed with protein A-Sepharose. b) Aliquots of the digest were incubated with RAR (——) or nRGG (----) and then mixed with protein A-Sepharose. Gels were washed and eluates were analyzed by SDS-PAGE.


a
b
TD
TD
CPM x 10^{-2}
0
1
2
3
4
10
20
30
40
50
60
70
Fraction Number

compared to a map of peptides generated from H receptor as isolated by RAR and protein A-Sepharose, as described in Chapter II. As can be seen from Fig. 11, the peptides from the papain-generated fragment isolated by IgE-Sepharose and those from intact H produce similar if not identical maps. This provides evidence that the single IgE-binding fragment produced by papain digestion is a fragment of H receptor rather than R, the peptide map of which is quite different (as will be shown in Chapter IV).

Trypsin and chymotrypsin were also used in an attempt to produce binding fragments. The NP-40 extract of $1x10^8$ surface iodinated RBL cells was incubated with either 5mg/ml trypsin, 5mg/ml chymotrypsin or PBS at 37°C for 2 hours. All aliquots were then incubated with PMSF at a concentration of $8x10^{-3}$M for 45 min. at 4°C. to terminate the reactions. Each aliquot was then treated with IgE-Sepharose, IgE-HARE-protein A-Sepharose or RAR-protein A-Sepharose. Fig. 12 shows the results of limited tryptic digestion of the receptors. Isolation of receptors from the control aliquot with IgE-Sepharose yielded both R and H in intact forms (Fig. 12a) while isolation with RAR and IgE-HARE isolated H and R separately (Figs. 12b and 12c, respectively). Treatment of the trypsin-digested extract using IgE-Sepharose yielded what appears to be one fragment, the mobility of which corresponded to an M_r of 41kDa (Fig. 12a). Treatment with RAR isolated a fragment with similar mobility, although the peak contained considerably less radioactivity than that obtained from an undigested control extract (Fig. 12b). Treatment of the digested cell extract with IgE and HARE yielded a molecule which, in terms of mobility, did not differ very much from intact R isolated from a control extract though again the amount of radiolabel isolated from the

Figure 11: Two dimensional tryptic peptide map of papain-generated IgE-binding fragment compared to peptide map of H. The IgE binding fragment was fixed in fractions constituting the major peak as it is represented in Fig. 9b. The H receptor was fixed in fractions constituting the major peak as it is represented in Fig. 4b. Fractions containing receptor material were dried and rehydrated with 0.05M NH_4HCO_3 containing 50 μg/ml TPCK-trypsin. After a 20 hour incubation at 37°C, supernatants were removed and gels were incubated with fresh 0.05M NH_4HCO_3. Supernatants from both incubations were pooled and lyophilized. Samples were reconstituted in 88% formic acid and applied in different spots to one cellulose acetate TLC plate. Electrophoresis was performed at 1000V for 45 min. in a formic acid:acetic acid:H_2O buffer. Gels were air dried and subjected to thin layer chromatography in the second dimension using a butanol:pyridine:acetic acid:H_2O solvent. Gels were exposed to X-ray film which was then subjected to autoradiography. Tryptic maps of a)intact H and b) papain generated IgE-binding fragment.


a
b
CHROMATOGRAPHY
ELECTROPHORESIS

digest was less than that from the control (Fig. 12c). The slight shift in mobility of the peak isolated from the digest may indicate that, of the heterogeneous R molecules represented by the broad peak, those with the higher molecular weights were degraded into non-binding fragments. This would also explain why less radiolabel is isolated from the digest.

Analysis of the chymotryptic fragments in an identical manner produced results similar to those obtained with trypsin (Fig. 13). IgE-Sepharose and RAR isolated a fragment with an M_r of 41kDa (Figs. 13a and 13b, respectively). IgE-HARE again, isolated a relatively smaller amount of what appears to be intact R receptor (Fig. 13c).

Figure 12: SDS-PAGE analysis of IgE-binding receptor fragments produced by tryptic digestion. 3.6×10^7 cell equivalents of a surface labelled RBL extract were treated with trypsin (final concentration: 5 mg/ml) (——) or PBS (-----) for 2 hours at 37°C and then PMSF was added to all samples. Samples were incubated with (a) IgE-Sepharose, (b) RAR and protein A-Sepharose and (c) IgE and HARE, and protein A-Sepharose. Gels were washed and eluates were analyzed by SDS-PAGE on 10% gels.

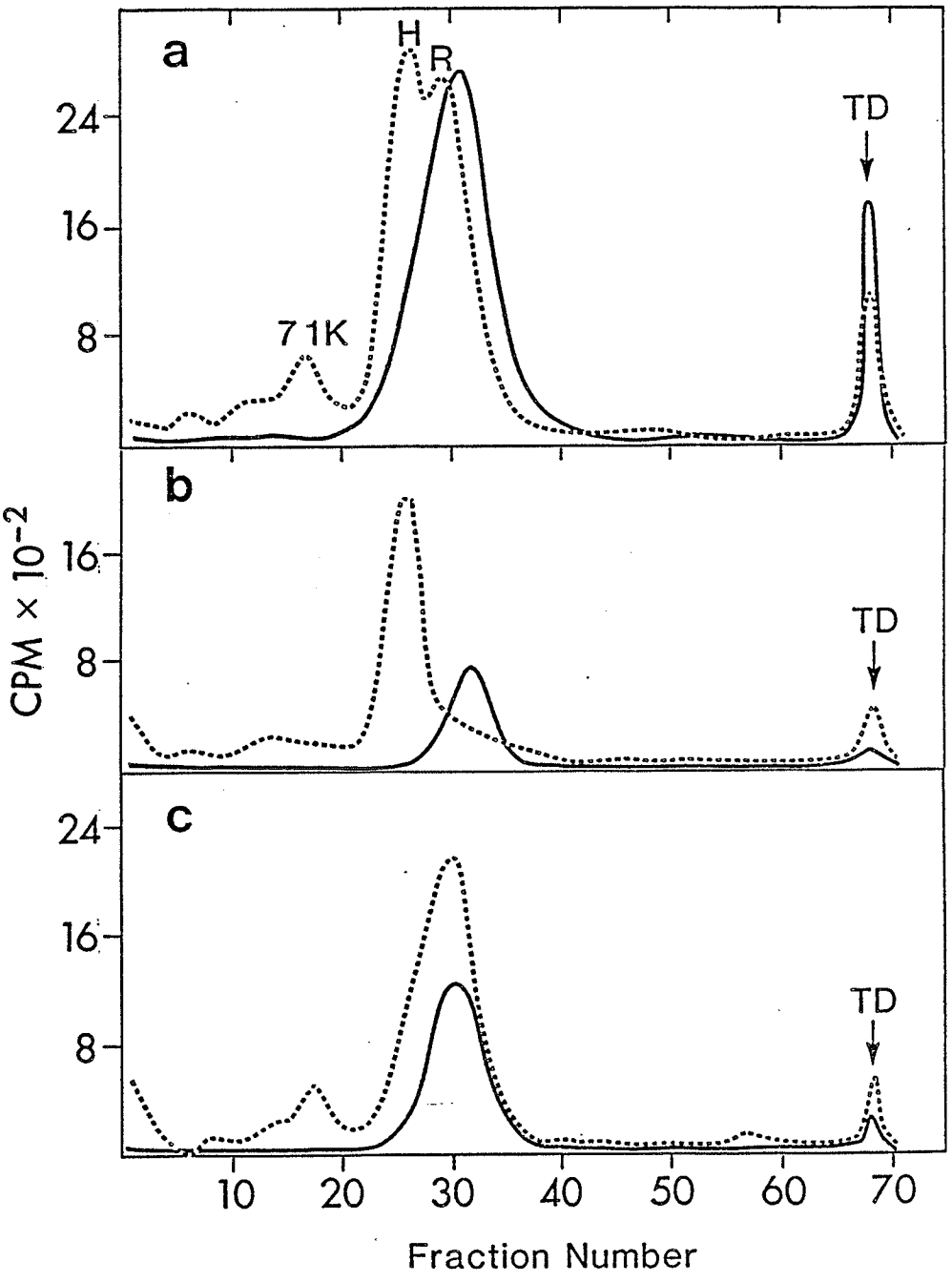
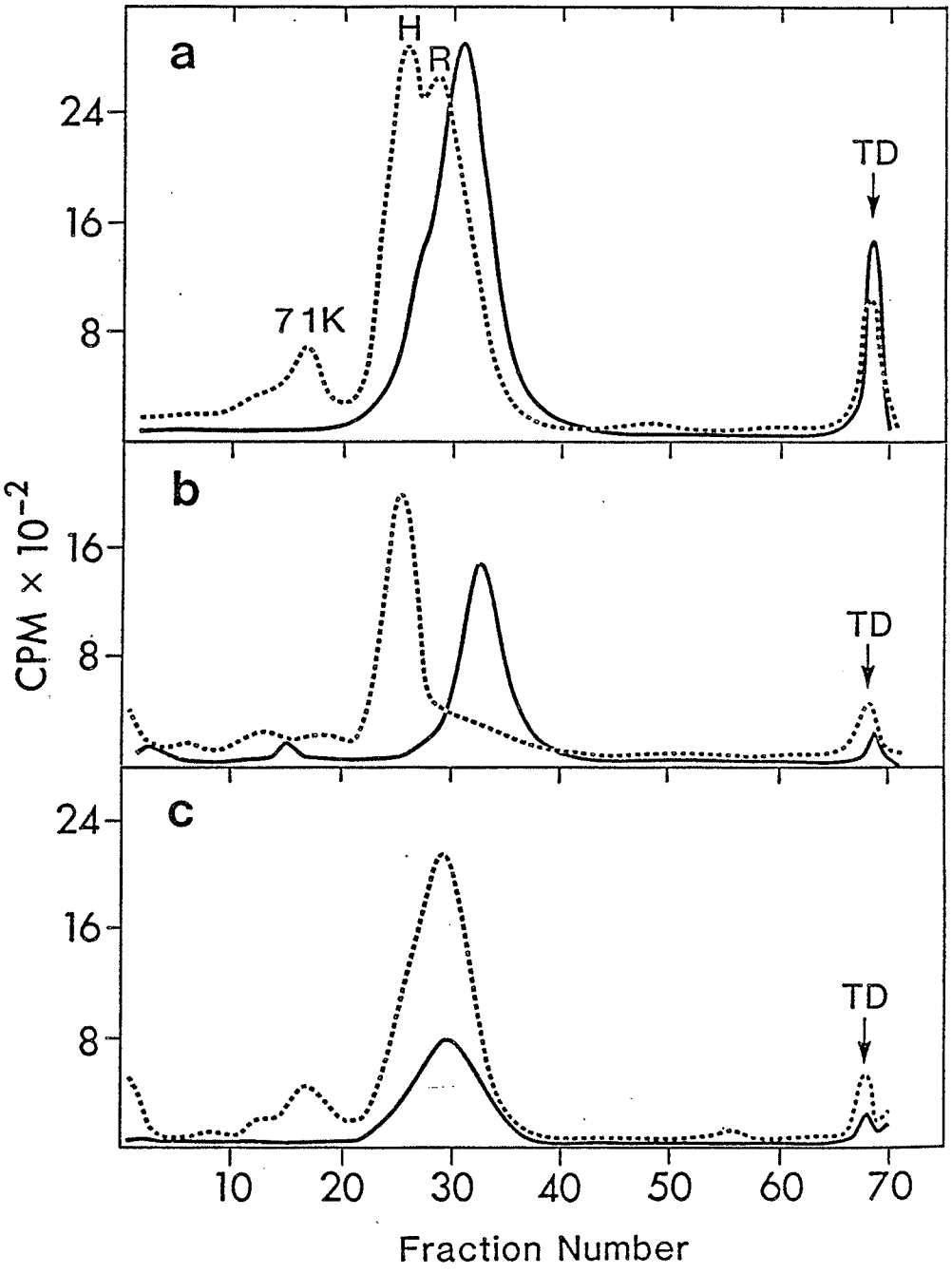

a
H
R
TD
71K
24
16
8
b
16
8
TD
c
24
16
8
TD
CPM × 10⁻²
10
20
30
40
50
60
70
Fraction Number

Figure 13: SDS-PAGE analysis of IgE-binding receptor fragments produced by chymotryptic digestion. As in Fig. 12, except that chymotrypsin (final concentration: 5 mg/ml) was used instead of trypsin. Receptors isolated with (a) IgE-Sepharose, (b) RAR and protein A-Sepharose and (c) IgE and HARE, and protein A-Sepharose.


a
b
c
H
R
71K
TD
TD
TD
24
16
8
16
8
24
16
8
CPM × 10⁻²
10
20
30
40
50
60
70
Fraction Number

DISCUSSION

Attempts to obtain ligand-binding fragments of receptors have been successfully made in the acetylcholine receptor system as well as in the present investigation. In the former study, the affinity chromatography purified receptor was digested with trypsin, resulting in the formation of a fragment which retained capacity to bind its ligands (Barfeld and Fuchs, 1979). Such a receptor derivative may provide investigators with a less complex molecule with which to study ligand-receptor interactions. In addition, it may contribute information on the actual structure of the molecule, particularly its ligand-binding portion. In the present study, receptors were digested with three different proteases in an attempt to produce IgE-binding fragments of both R and H receptors. Since, unlike the acetylcholine receptor, the recovery of biologically active IgE receptors after affinity chromatography is quite poor, these digestions were performed on receptors in the NP-40 extract of surface-iodinated RBL cells prior to the isolation of IgE-binding material. It was established that digestion was possible in the presence of NP-40 (Fig. 8).

Papain digestion of a cell extract containing R and H yielded a fragment which was bound by IgE-Sepharose (Fig. 9b) but not by IgE and HARE (Fig. 10a). From these results, it was deduced that the isolated fragment, a molecule of 36kDa, was part of the H receptor: if it had been of R origin it presumably would have been bound by IgE and HARE, as well as by IgE-Sepharose, since only R is isolated by both of these approaches. The fact that RAR could not bind this fragment (Fig. 10b) is probably due to the degradation of the antigenic determinant for which

this antiserum is specific. This would not necessarily affect the IgE binding site as it was demonstrated that this antibody preparation interacts with a part of the receptor which does not bind to IgE (Conrad et al., 1979). In subsequent experiments, the papain-derived fragment did at times react with RAR, and this may indicate that the antigenic determinant(s) may be close to a portion of the molecule which is susceptible to proteolysis. It is interesting to note that nRGG, the control for RAR, does bind what appears to be the same fragment as that bound by IgE-Sepharose. This result may be explained by the recent finding that H receptor has affinity for rat IgG as well as IgE (Kepron et al., 1982), and also cross-reacts with rabbit IgG (Kepron et al., 1984). One would expect that this fragment would also bind to the Fc portion of the IgG in RAR, although why this is not the case remains unclear. The IgG fraction from some but not all normal rabbit sera was shown to precipitate a molecule(s) the SDS-PAGE mobility of which is similar to that of H although it has not been demonstrated that this molecule was the IgE receptor (unpublished observation). Such activity could, perhaps, have been due to the presence of natural antibodies in some rabbit sera. The RAR preparation used in these experiments may have been produced in rabbits whose pre-immunization sera did not bind to an H-like molecule. To establish conclusively that the fragment produced by papain originated from H, two dimensional maps of tryptic peptides from the fragment and from intact H were compared (Fig. 11). The striking similarity between these two maps clearly demonstrated that the isolated fragment was a part of H. This similarity also suggests that most if not all amino acid residues labelled by iodine are in the IgE-binding portion of the molecule which, of course, would be expected to be an exposed

portion of the molecule.

Digestion of RBL cell extracts with trypsin or chymotrypsin also yielded IgE-binding fragments, although the effects of these enzymes were somewhat different from those of papain. Both enzymes generated a fragment which bound to RAR as well as to IgE-Sepharose (Figs. 12 and 13, a and b). This would, again suggest that the fragment, which was found to have an M_r of 41kDa, was of H origin. For both enzymes, the amount of radiolabel recovered by RAR precipitation was less than that in undigested preparations (Figs. 12 and 13, b). A possible explanation for this is that again, the site of enzymatic activity on H is close to the antigenic determinant for which RAR is specific. Digestion may thus have produced some fragments which did not react with RAR. If one considers that H receptors are likely to consist of a heterogeneous population of molecules, differences that contribute to this heterogeneity, e.g. carbohydrate content, may also be responsible for the observed differences in enzyme effect.

The effect of trypsin and chymotrypsin on R, seems to have been of an all-or-none nature. The molecule recovered by IgE-HARE precipitation was, most likely, intact R (Figs. 12 and 13, c). The relative decrease of recovered radiolabel suggests that the binding site of a fraction of this receptor was degraded while that of another fraction remained intact. This is supported by the fact that, for both enzymes, the higher molecular weight fractions of R have been degraded. It has been reported that the heterogeneity in R-like molecules is, in part, due to carbohydrate content (Hempstead et al., 1981b). It is possible that this heterogeneity or perhaps heterogeneity in detergent binding was responsible for the differences in enzyme effect, as was

postulated for H. Although a variety of digestion conditions have been attempted, to date, a modified R receptor has not been produced.

These results have led to several conclusions. There seems to be a difference in proteolytic susceptibility, at least in certain parts of the molecule, between R and H. The binding site of R is generally more easily destroyed, using both semi-specific (papain) and highly residue-specific (trypsin and chymotrypsin) proteases than is the binding site of H. This is further supported by the fact that when partially purified receptors are digested extensively, H is degraded into one major radiolabelled fragment, while R is broken down into several radiolabelled fragments. If it is assumed that the portion of the molecule which is surface labelled is at least, in part, the portion of the molecule which binds IgE, as experimental evidence by Conrad and Froese (1976) would suggest, then the degradation of the radiolabelled portions of R into several fragments supports the fact that an IgE-binding fragment of this molecule could not be produced.

The results obtained by limited proteolysis of H also reveal some interesting features of this molecule. The repeated finding of IgE-binding molecules of similar size, after digestion with several different enzymes, points to the existence of a protease sensitive portion of the H molecule, as well as a relatively resistant portion which contains the IgE binding site. This type of arrangement would not be unprecedented in biologically active molecules. The immunoglobulin chains are known to possess short sections which are highly susceptible to enzymatic cleavage and which bridge distinct functional domains (Edelman et al., 1969). It would not be surprising to find a similar domain structure in cell membrane molecules. Indeed, Goetze et al.

(1981) found that when they subjected an R-like molecule from an RBL cell line to limited proteolysis using a variety of enzymes, they repeatedly isolated two fragments of almost equal size (32-34K daltons). Although the digestion was done in the presence of SDS and fragments could not be tested for IgE binding activity, they and later, Holowka et al. (1981), suggested that receptors for IgE may possess one domain involved in IgE binding and another one responsible for anchoring the receptor in the membrane, either directly, or through another membrane bound molecule.

CHAPTER IV

Determination of the Relationship of 71K to the R and H Receptors

INTRODUCTION

The results from Chapters II and III of this study have shown that the R and H receptors of RBL cells differ in several respects: they are isolated with different reagents; they generate different sets of peptides when digested with papain; and, they differ in susceptibility to digestion with several proteases. In this chapter, the relationship of the 71K receptor to R and H is investigated. The first evidence of an IgE-binding molecule with a molecular weight of approximately 70kDa was seen by Conrad and Froese (1978a), who detected this molecule in receptor preparations isolated by means of IgE-Sepharose and IgE and anti-IgE. Because of its sporadic appearance at that time, it was considered to be a contaminant (A. Froese, personal communication). More recently, small amounts of a 71kDa molecule were consistently isolated from surface-labelled cells by IgE-Sepharose (Helm and Froese, 1981a). Moreover, the binding of this molecule to IgE-Sepharose as well as that of R and H could be inhibited by free IgE, indicating that this was indeed an IgE-specific receptor. The molecule was designated 71K. Biosynthetic labelling studies determined that it is a glycoprotein by demonstrating that it can be biosynthetically labelled with tritiated amino acids and sugars (Helm and Froese, 1981a). The same authors, using DNP-IgE and anti-DNP, isolated a molecule from ^{3}H-amino acid labelled RBL cells, the M_r of which was approximately 26K daltons. A molecule of similar size was described by Holowka et al. (1980) as being IgE receptor-associated, although it could not be surface labelled. Because 71K was isolated by the same reagents which isolated R (IgE and anti-IgE,

Froese et al., 1982a, DNP-IgE and anti-DNP, Helm and Froese, 1981a) and, in light of the discovery of a molecule (later named β chain) associated with the R-like receptor (Holowka et al., 1980), it was suggested that 71K may be a more stable adduct of R and 26K (or α and β, to use different nomenclature) (Helm and Froese, 1981a). While early attemps to reduce 71K to yield polypeptide chains of lower M_r had failed (Helm and Froese, 1981a), a more recent attempt, by M. Rao of this laboratory, using higher concentrations of reducing agents, produced a surface labelled molecule with an M_r identical to that of R (M. Rao, personal communication). This led to speculation that 71K might consist of R disulfide linked to some other molecule. This chapter describes the experiments designed to determine whether there is a structural relationship between R and 71K.

Tryptic peptides of surface-labelled 71K and R and H are compared by one- and two-dimensional mapping. Comparison of R and H confirmed the results obtained in Chapter II, which indicate that these two receptors are different molecular entities. Comparison of 71K to these receptors established a relationship between 71K and R and thus led to subsequent experiments using biosynthetically labelled receptors, to determine the 'subunit' composition of 71K.

MATERIALS AND METHODS

The iodination and disruption of RBL cells was performed as described in Chapter II.

Preparation of Anti-IgE-Sepharose (HARE-Sepharose) Immunosorbent: Horse anti-rat IgE (HARE) (kindly supplied by Dr. K. Kelly) was coupled to Sepharose CL-4B at a concentration of 2.5 mg/ml using the procedure described in the Methods and Materials section of Chapter II. The coupling efficiency was 90-95%.

Sequential Affinity Chromatography Using HARE-Sepharose and IgE-Sepharose: Receptors were isolated according to a method described by Froese et al. (1982a), modified to become a preparative procedure. To isolate R and 71K, the NP-40 extract of $3\text{-}4\times10^8$ surface-iodinated RBL cells (5×10^7 cells/ml) was mixed with 300-400 μg of IgE, incubated for 30 min. at 37°C, then added to 6-8 ml of HARE-Sepharose and rotated for 90 min. at 4°C. To remove any residual R and 71K, the supernatant was removed, then added to 3-4 ml of fresh HARE-Sepharose and incubated for 60 min. at 4°C. To isolate H, the supernatant was again removed, added to 4-6 ml of IgE-Sepharose and rotated overnight at 4°C. Affinity gels were washed 4 times with 0.1% NP-40/PBS and once with 0.0625M Tris, pH 6.8 (sample buffer). Elution was performed by boiling with equal volumes of sample buffer containing 2% SDS. Eluates were concentrated and dialyzed overnight against sample buffer.

SDS-PAGE on Slab Gels: 10% polyacrylamide slab gels were prepared according to Laemmli (1970) with a 12 cm separating gel and a 1.5 cm stacking gel. Bromphenol blue and glycerol were added to samples which were then applied to slab gels. Electrophoresis was performed using a Protean cell (BioRad, Richmond, CA) at 30mA/gel. Upon completion of the

run, a 0.5 cm wide vertical section of the gel was removed and manually cut into 2 mm fractions. The fractions were counted on a Beckman 8000 gamma counter. Sections of the slab gel containing receptor peaks were then removed and either immersed in double distilled H_2O or fixed with 10% acetic acid/10% isopropyl alcohol for 1 hour. Fixed gels were then incubated with 25% isopropyl alcohol for 12 hours and 10% methanol for 12 hours. Gels in H_2O were incubated for 48-72 hours at 37°C. Their supernatants were lyophilized, then reconstituted with sample buffer containing 2% SDS and 10% 2-ME and boiled for 2 min. These samples were applied to 10% tube gels for SDS-PAGE. After electrophoresis, fractions containing receptor peaks were fixed as described above.

Tryptic Digestion of Receptors in Polyacrylamide Gels: This procedure was performed according to Elder et al. (1977) and Pecoud and Conrad (1981). After incubation in 10% methanol, gels were dried under a heat lamp and subsequently rehydrated with 0.05M NH_4HCO_3 pH 7.8, containing 50 µg/ml TPCK trypsin (Worthington, Freehold, NJ). After a 20 hour incubation at 37°C, supernatants were removed and gels were incubated with fresh 0.05M NH_4HCO_3 for 24 hours at 37°C. Supernatants from both incubations were pooled and lyophylized.

One-Dimensional Analysis of Tryptic Digests: Receptor digests were reconstituted with sample buffer containing 2% SDS, glycerol and bromphenol blue, then applied to a 15% tube gel for SDS-PAGE. Gels were sliced into 2 mm fractions and counted in a Beckman 8000 gamma counter.

Two-Dimensional Analysis of Tryptic Digests: Alternatively, receptor digests were reconstituted with 88% formic acid (Fisher Scientific Co.) and applied to 20 x 20 cm Eastman Chromagram cellulose-coated thin layer chromatography plates (Eastman Kodak, Rochester, NY). Electrophoresis

was performed at 0°C on a high-voltage electrophoresis apparatus (Desaga, Heidelberg, W. Germany) with an electrode buffer of acetic acid : formic acid : H_2O (15:5:8) for 90 to 120 min. at 1000 V. migration was monitored by the progression of a dye (0.25% acid fuchsin [Matheson Co. Cincinnati, OH], 0.125% Orange G [Sigma Scientific Co. St. Louis, MO] prepared in electrode buffer) spotted on the other side of the plate and which migrated in a direction opposite to that of the samples. Following electrophoresis, plates were dried and tryptic peptides were then subjected to thin-layer chromatography in the 2nd dimension using a butanol : pyridine : acetic acid : H_2O (32.5:25:5:20) buffer.

Biosynthetic labelling of RBL cells: A procedure described by Kulczycki and Parker (1979) was used. RBL cells entering the stationary growth phase were harvested, washed and resuspended at a concentration of 1.5 x 10^6 cells/ml in leucine-free Eagle's MEM (Gibco) supplemented with 15% fetal calf serum. L-[4,5-^{3}H] leucine (Amersham, Oakville, Ont.) was added under sterile conditions at a concentration of 50 μg/ml. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 20-24 hours. Cells were then harvested using EDTA, washed 3 times with PBS/BSA and their viabilities determined by trypan blue exclusion. Incorporation of ^{3}H was determined by measuring the radioactivity in 10 μl of the washed cells and of the culture supernatant. The 10 μl aliquots were each mixed with 10 ml of Econofluor (New England Nuclear, Lachine, Que.) and counted in a Beckman liquid scintillation counter, Model LS-335 (Beckman Instruments, Fullerton CA). This procedure was used to monitor radioactivity throughout the subsequent purification.

Repetitive Affinity Chromatography: Rat IgG, which was precipitated with ammonium sulfate from normal rat serum and filtered through an ACA 34

column, was prepared by Mr. M. Kepron of this laboratory as described in Kepron et al. (1982) and 4x crystallized BSA was purchased from ICN Pharmaceuticals (Cleveland, OH). IgG-Sepharose and BSA-Sepharose were prepared as described in Chapter II at a concentration of 5mg/ml. Receptors were isolated according to Kulczycki and Parker (1979). The NP-40 extract of 2-3 x 10^8 biosynthetically labelled RBL cells was mixed with 4-6 ml of IgG-Sepharose overnight at 4°C. The supernatant was then removed and mixed with 2-3 ml of IgE-Sepharose for 2 hours at 4°C. The IgE-Sepharose was transferred to Pasteur pipette columns in 10 aliquots and the gel was washed with a total of approximately 120 ml of PBS/0.1% NP-40 at 4°C. Bound material was eluted by quickly passing 0.5 ml of 0.5N acetic acid containing 1% NP-40 through each gel, and immediately neutralizing the eluate with 0.185 ml of 2.0M Tris, pH 8.6. The eluate was added to 1-2 ml of BSA-Sepharose and incubated at 4°C for 30 min. The supernatant was then removed and mixed overnight with 1-2 ml of IgE-Sepharose at 4°C. After washing 4 times with PBS/0.1% NP-40 and once with 0.0625M Tris, pH 6.8 (sample buffer), receptor material was eluted by boiling in sample buffer containing 2% SDS.

Analysis of Purified Receptors: Eluates from the second IgE-Sepharose incubation were applied to 10% polyacrylamide slab gels for SDS-PAGE analysis. Electrophoresis was performed at 30mA/gel. Gels were then stained with Coomassie blue, as described in Chapter II, destained with 10% acetic acid, then treated with En^{3}Hance (New England Nuclear, Lachine, Que.) for one hour. Gels were dried in a BioRad Gel Dryer (Richmond, CA), autoradiography was performed and films were scanned on a Quick Scan densitometer (Helena Laboratories, Beaumont, TX). Alternatively, electrophoresis was performed on purified receptor

material using 10% tube gels on eluates separated into two aliquots. Gels were frozen overnight in 0.0625M Tris. Thawed gels were placed in troughs above 10% slab gels. The tube gels were secured on top of the slab gels with 1.5% agarose prepared in electrode buffer. The upper electrode buffer contained 0.04% bromphenol blue (0.001%) as the tracking dye. During the electrophoresis, performed under reducing conditions, the agarose contained 7.5% 2-ME and the upper electrode buffer contained 1% 2-ME. The same 2-dimensional procedure was applied to surface-iodinated receptors isolated by a single incubation with IgE-Sepharose. Slab gels containing biosynthetically labelled material were treated with En^{3}Hance. Gels were then dried and autoradiography was performed.

Autoradiography: Plates were exposed to Kodak X-Omat AR x-ray film (Eastman Kodak, Rochester, NY) and Cronex Lightening Plus intensifying screens (Dupont, Wilmington, DE) at -70^{o}C for 2-4 weeks. Films were developed using Kodak Liquid X-Ray Developer and Replenisher and Kodak X-Ray Rapid Fixer (Eastman Kodak, Rochester, NY). Films which were exposed to tritiated material were hypersensitized before exposure according to the method of Laskey and Mills (1977).

RESULTS

Investigation of the relationship between the R and 71K IgE receptors began in this laboratory with the finding that reduction of 71K produced a molecule whose SDS-PAGE electophoretic mobility was practically identical to that of R. The results of these experiments, performed by Dr. Mangala Rao, are shown in Figures 14 and 15. When receptors for IgE, isolated by means of IgE-Sepharose, were reduced by 5% 2-ME and subsequently analyzed by SDS-PAGE (Fig. 14), the peak corresponding to 71K disappeared, and that corresponding to R increased. To show more clearly that 71K was converted to a molecule with R-like mobility, receptors were isolated from an extract of surface iodinated RBL cells using IgE-Sepahrose and separated by SDS-PAGE on 10% slab gels. Radiolabelled material from fractions constituting R and 71K receptor peaks were collected and aliquots of the 71K receptor were reduced or run unreduced. Figure 15c depicts the SDS-PAGE profile of unreduced 71K. Fig. 15, a and b show the SDS-PAGE profiles of isolated 71K, reduced with 25mM dithiotrietol and 5% 2-ME, respectively. The mobilities of these peaks are similar to that of R (Fig. 15d). This result suggested that 71K might be composed of R receptor, disulfide linked either to itself or to some non-iodinated molecule. The following experiments were performed to investigate this possibility.

Isolation of R, H and 71K receptors using sequential affinity chromatography

Isolation of the receptor material, necessary for tryptic mapping, required a preparative procedure capable of resolving R, H and 71K receptors. While the methods of receptor isolation described in Chapters II and III provided a means of separating R and 71K on the one

Figure 14: SDS-PAGE analysis of reduction of IgE receptors. The NP-40 extract from surface labelled RBL cells was mixed with IgE-Sepharose. The washed gel was divided into two aliquots and receptor material was eluted by boiling in (a) 0.0625M Tris containing 2% SDS or (b) 0.0625M Tris containing 2% SDS and 5% 2-ME. Eluates were analyzed by SDS-PAGE on 10% gels. (Figure kindly provided by Dr. M. Rao).

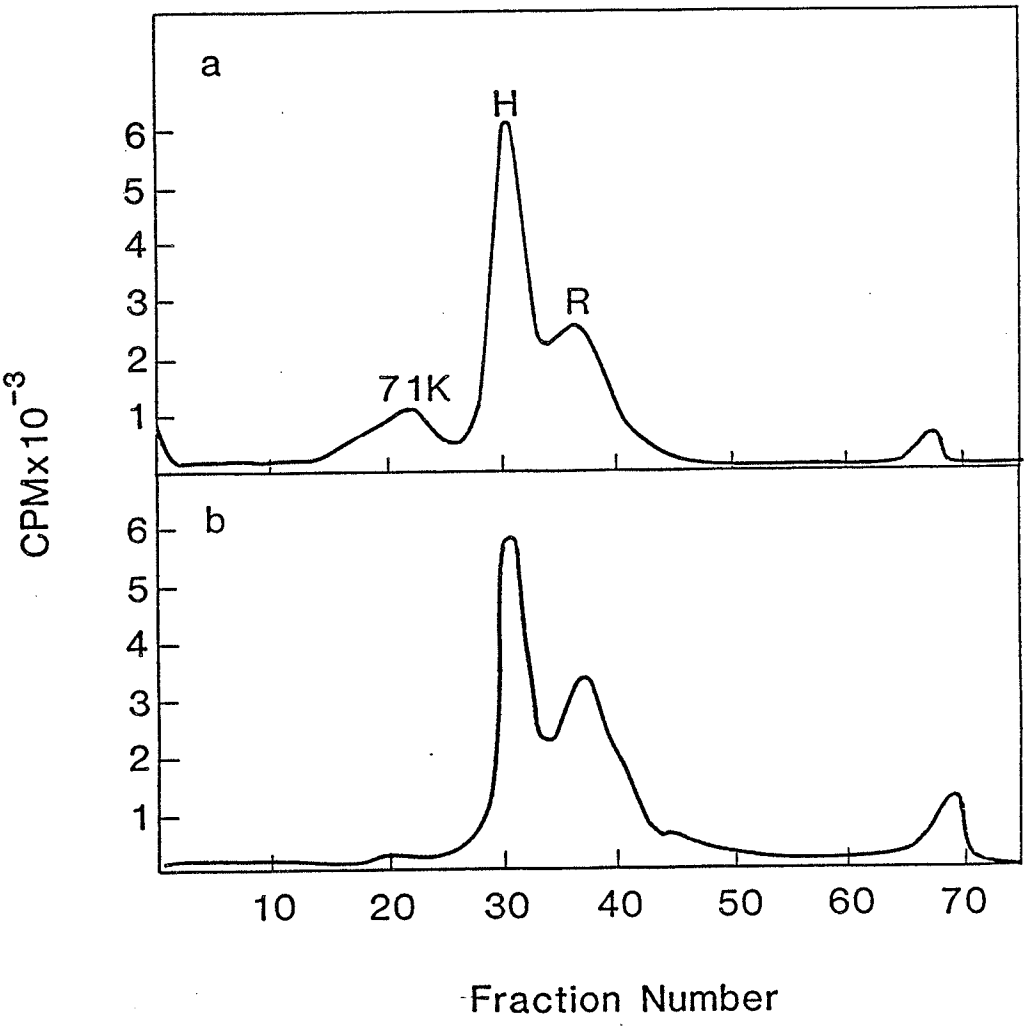
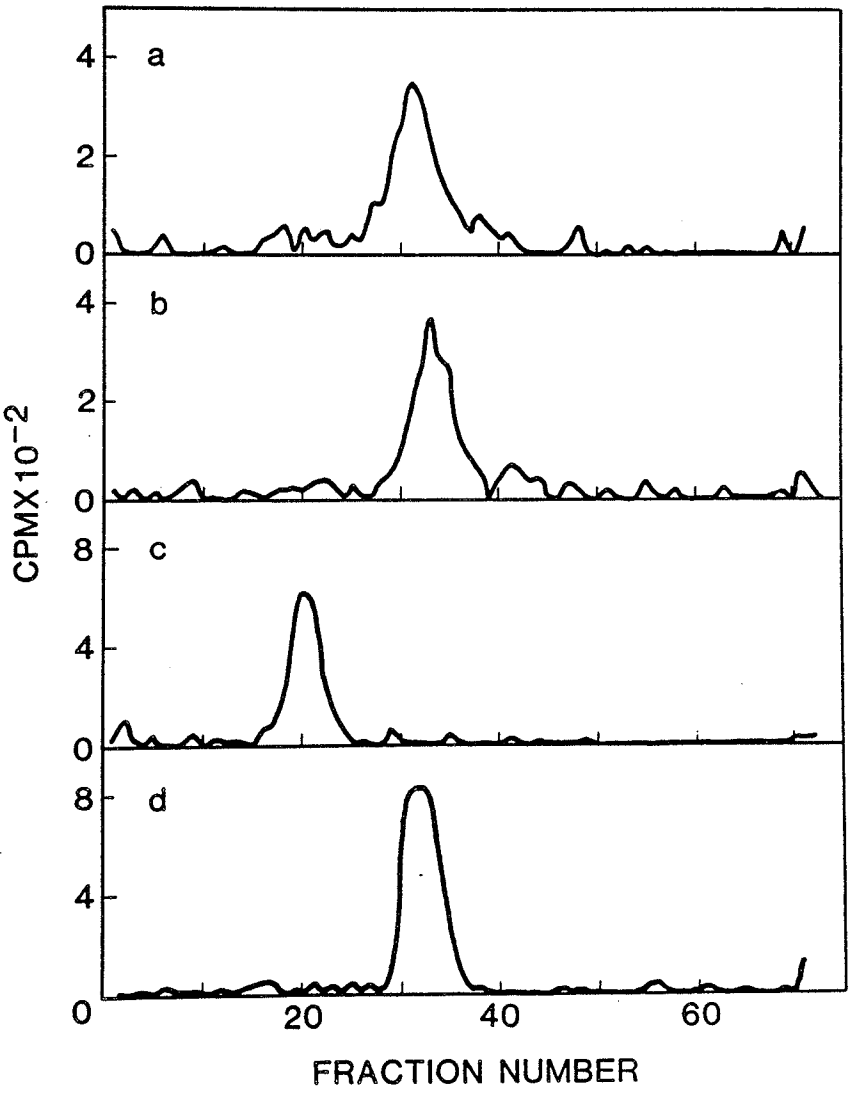

a
H
R
71K
b
CPMx10^{-3}
1
2
3
4
5
6
10
20
30
40
50
60
70
Fraction Number

Figure 15: SDS-PAGE analysis of reduced 71K receptor. The extract of surface labelled RBL cells was mixed with IgE and then HARE-Sepharose. The eluate was subjected to SDS-PAGE and R and 71K receptors were eluted from fractions constituting the major peaks. Samples were dialyzed against 0.0625M Tris, adjusted to 2% SDS (c and d), 2% SDS and 25mM dithiothrietol (DTT) (a) or 2% SDS and 5% 2-ME (b) and analyzed by SDS-PAGE. (Figure kindly provided by Dr. M Rao).


a
b
c
d
4
2
0
8
4
0
20
40
60
CPMX10^{-2}
FRACTION NUMBER

hand (IgE and anti-IgE) and H on the other (RAR), these methods resulted in a good deal of wasted receptor material, since H receptor, in aliquots treated with IgE and anti-IgE, and R and 71K receptors in aliquots treated with RAR would not be isolated. Thus, a procedure which was used analytically by Froese et al. (1982), was modified to become preparative (as outlined in Materials and Methods) for this study. The first incubation of a NP-40 extract of ^{125}I-labelled RBL cell extract with IgE and HARE-Sepharose resulted in the binding of 2-3% of the total radioactivity in the extract, while the second incubation bound only an additional 0.05% of the total radioactvity. This indicates that the first incubation removed virtually all of the receptor material that such a quantity of this reagent is capable of removing. The subsequent incubation of extract with IgE-Sepharose led to the binding of an additional 0.5-1% of the total radioactivity. Receptor material was eluted by boiling in equal volumes of sample buffer containing 2% SDS.

Eluates from HARE-Sepharose and IgE-Sepharose were applied to separate 10% polyacrylamide slab gels for SDS-PAGE according to Laemmli (1970). Upon completion of the run, a 0.5cm wide vertical section of each gel was removed and manually cut into 2mm fractions. These fractions were counted and the resulting profile is shown in Fig. 16. The eluate from HARE-Sepharose contained two major radiolabelled peaks the mobilities of which corresponded to 43kDa and 67kDa (Fig. 16a). This indicates that R and 71K receptors are isolated using this reagent, which is in agreement with the results obtained in Chapter II. The double peak representing R is most likely due to the fact that these gels were manually sliced, leading to slight differences in the fractions. The IgE-Sepharose eluate (Fig. 16b) contained one major peak with a mobility

corresponding to a molecular weight of approximately 52kDa. The small, higher molecular weight peak has already been described in Chapter II. The small peak on the descending side of the major peak most likely represents residual R receptors which were not isolated with IgE-HARE-Sepharose. With this possibility in mind, and to insure maximal purity of the receptor preparations, only fractions corresponding to the shaded areas were used for further experiments. In a typical experiment, an 8mm horizontal section of the gel corresponding to the 4 fractions containing 71K and a 16mm section of the gel corresponding to the 8 fractions containing R were removed from a 120mm gel for further analysis. In a similar fashion, an 8mm section of the gel separating the IgE-Sepharose eluate, which contained H receptor, was also removed.

Analysis of Tryptic Peptides of Receptors

The relationship between R, 71K and H receptors was investigated using one- and two dimensional mapping of receptor tryptic peptides which were prepared according to Elder et al. (1977) and Pecoud and Conrad (1981). Before digestion, sections of the slab gels containing receptor material which was to undergo reduction, were incubated for 48-72 hours in distilled water. After incubation, the supernatants were found to contain approximately 50% of the radioactivty in the original 71K band and 65% of the radioactivity in the R band. The supernatants were dialyzed against distilled water and lyophilyzed. The reconstituting sample buffer (0.0625M Tris) contained 2% SDS and 10% 2-ME. Samples were then subjected to SDS-PAGE in tube gels which were subsequently manually sliced into 5mm fractions. Figure 17 depicts the distribution of radioactivity after electrophoresis. As expected, and, in agreement with Fig. 15, the reduction of both R and 71K yielded

Figure 16: SDS-PAGE analysis of receptors isolated using preparative sequential affinity chromatography. The NP-40 extract of 4 x 10^8 surface labelled RBL cells was incubated with 400µg of IgE for 30 min. at 37°C, and with 8 ml of HARE-Sepharose for 90 min. at 4°C. The supernatant was incubated with fresh HARE-Sepharose for 60 min. at 4 C. The supernatant was again removed and incubated with 6 ml of IgE-Sepharose overnight at 4°C. Gels were washed and receptor material was eluted with equal volumes of sample buffer containing 2% SDS. Eluates from the (a) first batch of HARE-Sepharose and from (b) IgE-Sepharose were concentrated and applied to 10% slab gels for SDS-PAGE. Following electrophoresis, a 0.5cm wide vertical section from each gel was manually cut into 2mm fractions, which were counted in a gamma counter. Shaded areas denote fractions representing sections of slab gels which were removed and used for subsequent experiments.

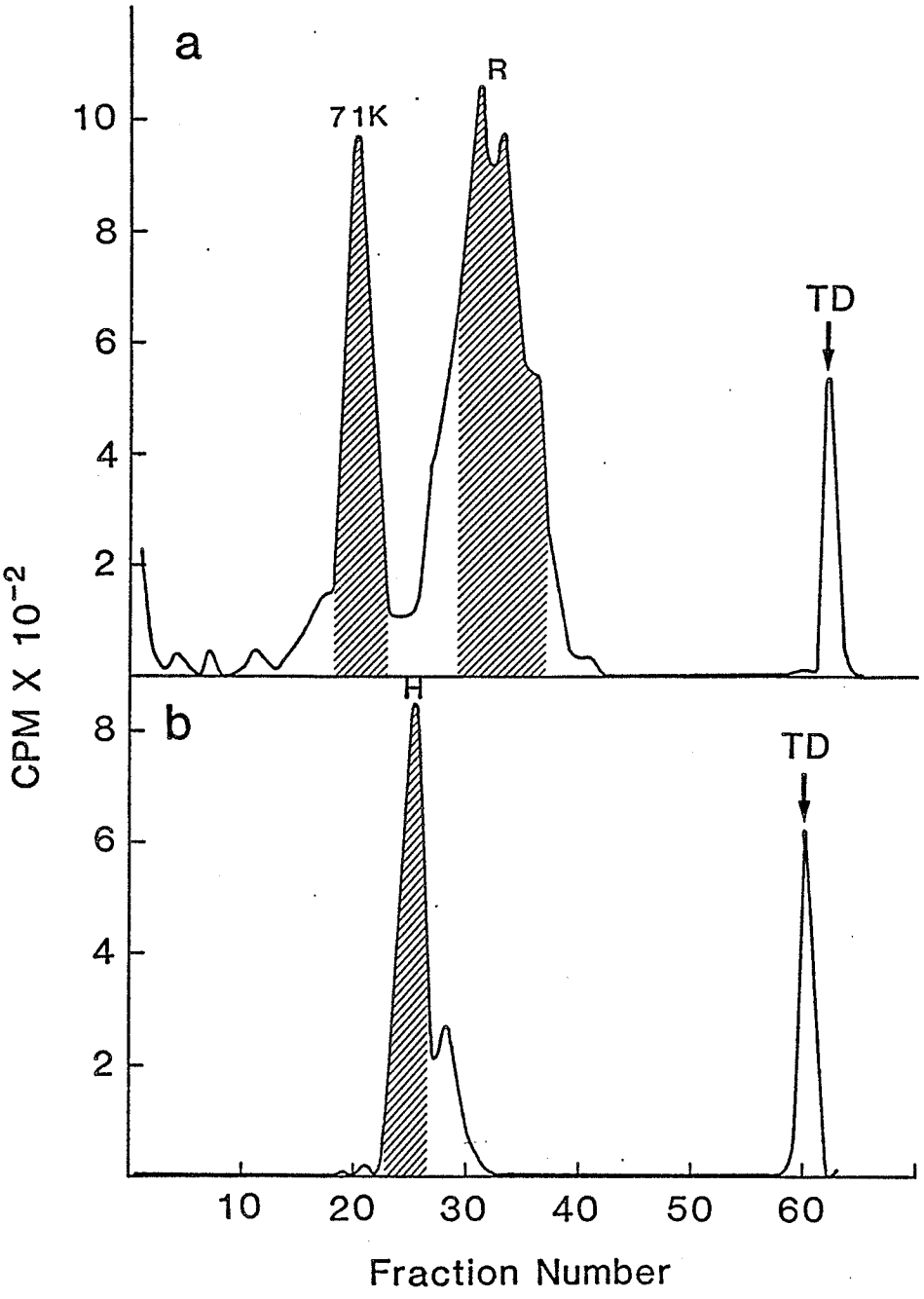
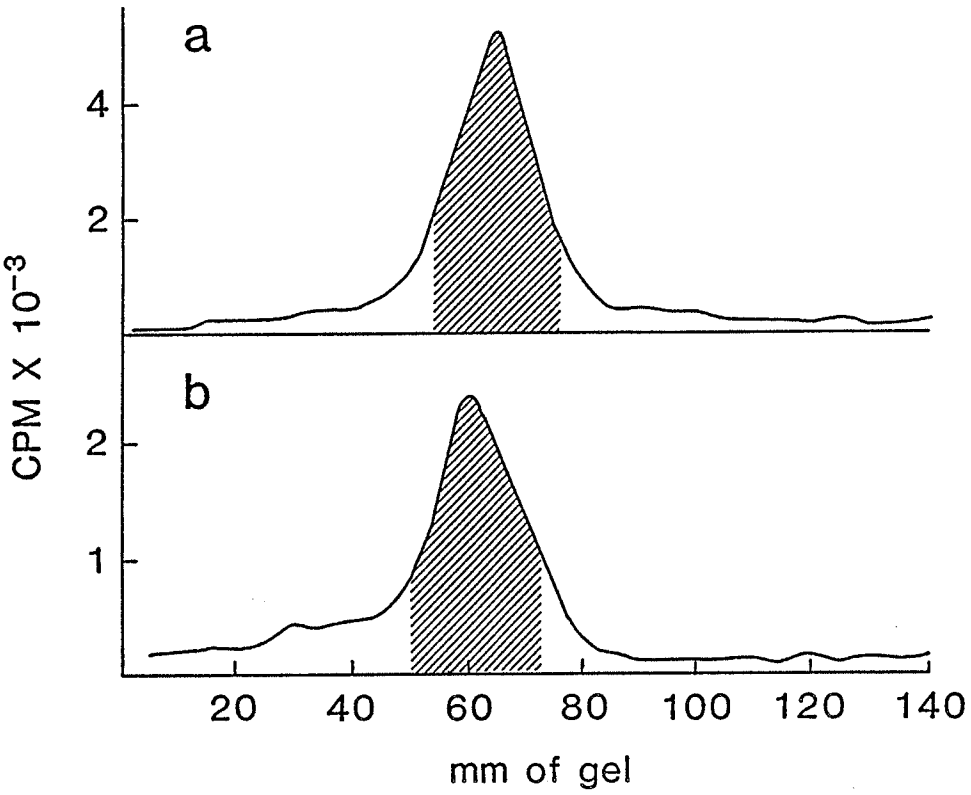

a
71K
R
TD
10
8
6
4
2
b
H
TD
8
6
4
2
CPM X 10⁻²
10
20
30
40
50
60
Fraction Number

Figure 17: SDS-PAGE analysis of reduced receptors. R and 71K receptor material was isolated using IgE and HARE-Sepharose as described for Fig. 16a. Sections of gel containing receptor material were incubated in distilled H_2O for 48-72 hours. Supernatants were dialyzed against dH_2O and lyophilized. Samples were reconstituted with 0.0625M Tris containing 2% SDS and 10% 2-ME and were subjected to SDS-PAGE in tube gels. Gels were manually sliced into 5mm fractions. (a) Reduced R and (b) reduced 71K. Shaded areas denote fractions representing sections of tube gels used for further experiments.

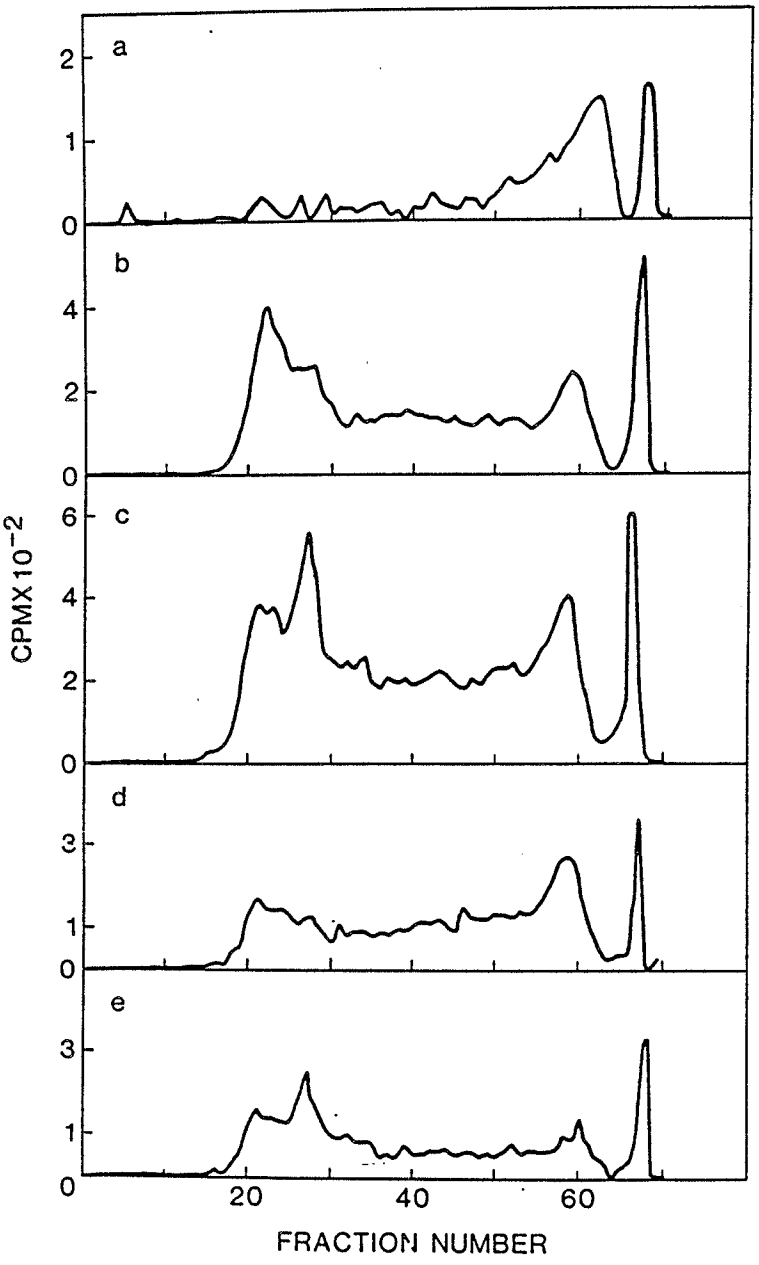

a
b
4
2
2
1
CPM X 10^{-3}
20
40
60
80
100
120
140
mm of gel

molecules with similar mobilities. Fractions constituting the radioactive peaks (5 fractions each) were collected. These, as well as the slab gel sections containing unreduced receptor material were fixed with 10% acetic acid/10% isopropyl alcohol. They were washed in 25% isopropyl alcohol, then 10% methanol, to remove the SDS. Gels were then dried under a heat lamp and rehydrated with 0.05M ammonium bicarbonate containing TPCK-trypsin. After two 20-hour incubations at 37 C, the supernatants were found to contain 70-80% of the counts originally in the receptor bands. Samples were lyophilized.

a) <u>One dimensional analysis</u>

Aliquots of all samples were reconstituted with SDS-sample buffer, then applied to 15% polyacrylamide gels for SDS-PAGE. Profiles of the digests are shown in Fig. 18. Digestion of H produced few labelled peptides, none of which had apparent molecular weights greater than 10kDa (Fig. 18a). In contrast, digestion of R and 71K, in both reduced and non-reduced forms, produced several peptides. Their exact number was difficult to determine, due to the broadness of the peaks (Fig. 18, b-d). Also in all cases, the larger peptides had molecular weights between 20-35kDa. It is clear that the patterns generated from reduced and unreduced R and 71K were very similar. However, some changes in the distribution of peptides were apparent after reduction of both R and 71K. In comparing Fig. 18b to 18c and Fig. 18d to 18e, one can see that the relative amounts of higher molecular weight peptides change upon reduction of R and 71K. This change appeared to be similar for both receptors. Thus, while digestion of unreduced R and 71K produced similar patterns, digestion of the reduced receptors made the similarity even more apparent.

Figure 18: SDS-PAGE analysis of tryptic peptides of receptors on 15% gels. R, H, and 71K receptors were isolated as described in Figs. 16 and 17. Gel sections containing receptor material were dried and rehydrated with 0.05M NH_4HCO_3 containing 50 µg/ml of trypsin. After a 20 hour incubation at 37°C, supernatants were removed and gels were incubated with fresh 0.05M NH_4HCO_3. Supernatants from both incubations were pooled and lyophilized. Digests were reconstituted with sample buffer containing 2% SDS and applied to 15% tube gels for SDS-PAGE analysis. Tryptic digest of (a) H, (b) R, (c) reduced R, (d) 71K and (e) reduced 71K.


a
b
c
d
e
2
1
0
4
2
0
6
4
2
0
3
1
0
3
1
0
CPMX10^{-2}
20
40
60
FRACTION NUMBER

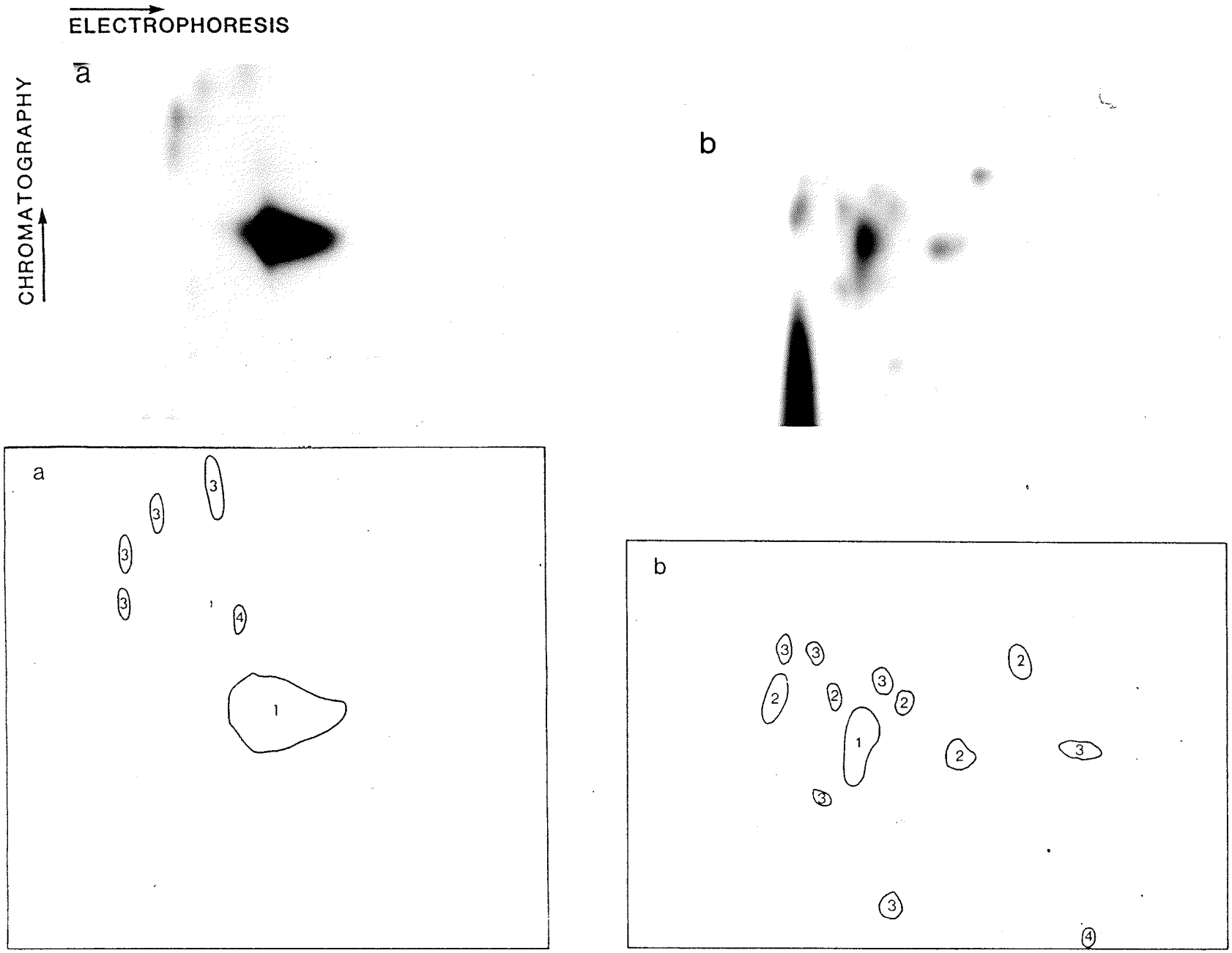
b) Two dimensional analysis

The one-dimensional analyses presented in this Chapter and in Chapter II demonstrate beyond a doubt that R and H receptors have little if any similarity in primary structure. By contrast, results of one-dimensional tryptic mapping, presented in this Chapter, indicate a good deal of similarity between R and 71K. However, it was felt that greater resolving power was required to establish more definitively the extent of the similarities. Thus, a two dimensional separation of tryptic peptides was performed in an attempt to establish more clearly the differences and similarities between these two molecules.

Tryptic peptides dissolved in formic acid were applied onto a thin-layer chromatography plate coated with cellulose acetate gel. High-voltage electophoresis was performed with an acetic acid : formic acid : H_2O electrode buffer. The plates were then dried and the samples were subjected to thin layer chromatography for separation in the second dimension, using a butanol : pyridine : acetic acid : H_2O solvent. Plates were exposed to X-ray film for 2-4 weeks and then developed.

Tryptic peptide maps of H and R receptor digests are shown in Fig. 19 a and b, respectively. A schematic representation of each map is depicted below the corresponding map. The digestion of surface labelled R generated 12-15 labelled peptides (Fig. 19b) while that of H produced 5-6 peptides (Fig. 19a). Approximately equal amounts of radiolabel were applied. One peptide derived from R appears to have a greater intensity than the other peptides, which share the remaining radiolabel fairly evenly. In contrast, almost all the radiolabel in H peptides was associated with a single spot, with a small proportion of the radiolabel being found in the remaining 4-5 peptides. In addition, as can be seen,

Figure 19: Two dimensional analysis of tryptic peptides of H and R. Tryptic peptides were prepared as described in Fig. 18. Digests were reconstituted in 88% formic acid and applied to cellulose acetate gel thin layer chromatography plates. High voltage electrophoresis using a formic acid:acetic acid:H_2O electrode buffer was performed. Following electrophoresis, dried plates were subjected to chromatography in the second dimension in a butanol:pyridine:acetic acid;H_2O solvent. Plates were then exposed to X-ray film and autoradiography was performed. Digests of (a) H and (b) R. The drawing below each pattern is a schematic representation of the pattern. The number inside each spot corresponds to the intensity of the spot on the original film. 1 = most intense, 4 = least intense.


ELECTROPHORESIS
CHROMATOGRAPHY
a
b
a
b

there is no overlap of peptides from R with those from H. Thus, digestion of R and H produced labelled peptides which differed both in number and in position.

The peptide map of R is compared to that of 71K in Fig. 20. In this case (as well as in Fig. 21), schematic representations of the maps allow visualization of peptides which appeared on the film but were too faint to appear in the photographs. It should be noted that 17,000 cpm of R and 8,000 cpm of 71K were applied. In comparing Figs. 20, a and b, peptides from 71K are fewer (11) and less intense than those of R. This would be expected, since fewer counts of 71K digest were applied. Of the ones which are visible, 5 peptides derived from 71K have positions in common with peptides from the R digest. These are represented by shaded spots in the schematic drawing. The remainder of the peptides are in different positions. This indicates a certain degree of homology between labelled peptides of R and 71K.

The peptide maps of R and 71K which were reduced before tryptic digestion are shown in Fig. 21. In this case, 71K peptides comprising 4,000 cpm and R peptides amounting to 7,000 cpm were applied. Of the 11 71K peptides (Fig. 21b) and the 12 R peptides (Fig. 21a), 5 share a common position (shaded in the schematic drawings). Of these 5 peptides, 3 also share positions with peptides from unreduced R and 71K (Fig. 20 a and b). The remaining peptides have no positions in common. Thus, a similar degree of homology does appear to exists between reduced 71K and R as does between unreduced 71K and R. If maps of unreduced and reduced R are compared (Figs. 20a and 21a, respectively), 4 peptides are found to share common positions. Three peptides from unreduced and reduced 71K also share positions (Figs. 20b and 21b). This indicates that reduction

Figure 20: Two dimensional analysis of tryptic peptides of R and 71K. Maps were prepared as in Fig. 19. Digests of (a) R and (b) 71K. In this figure, the schematic drawings contain shaded spots which represent peptides sharing common positions in both (a) and (b).

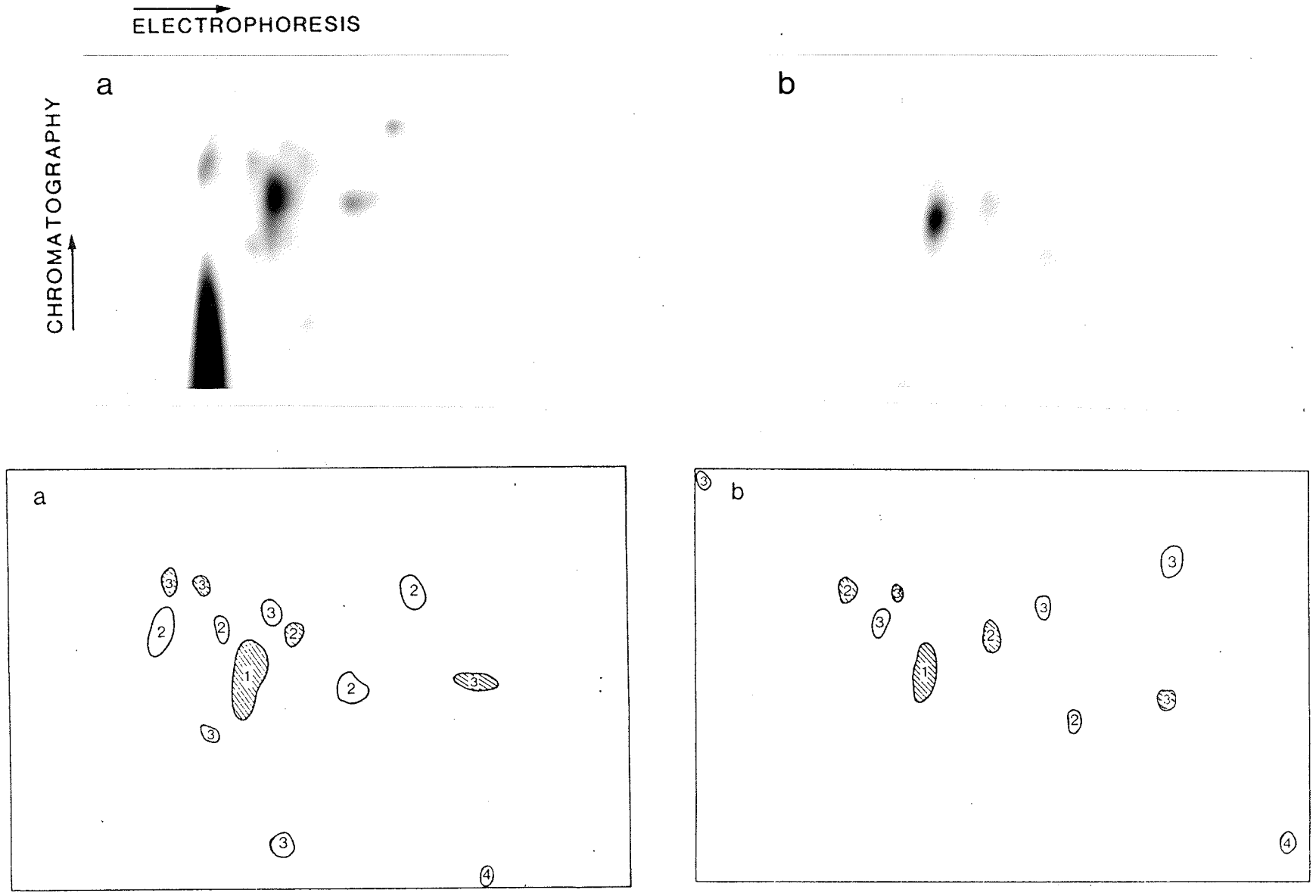
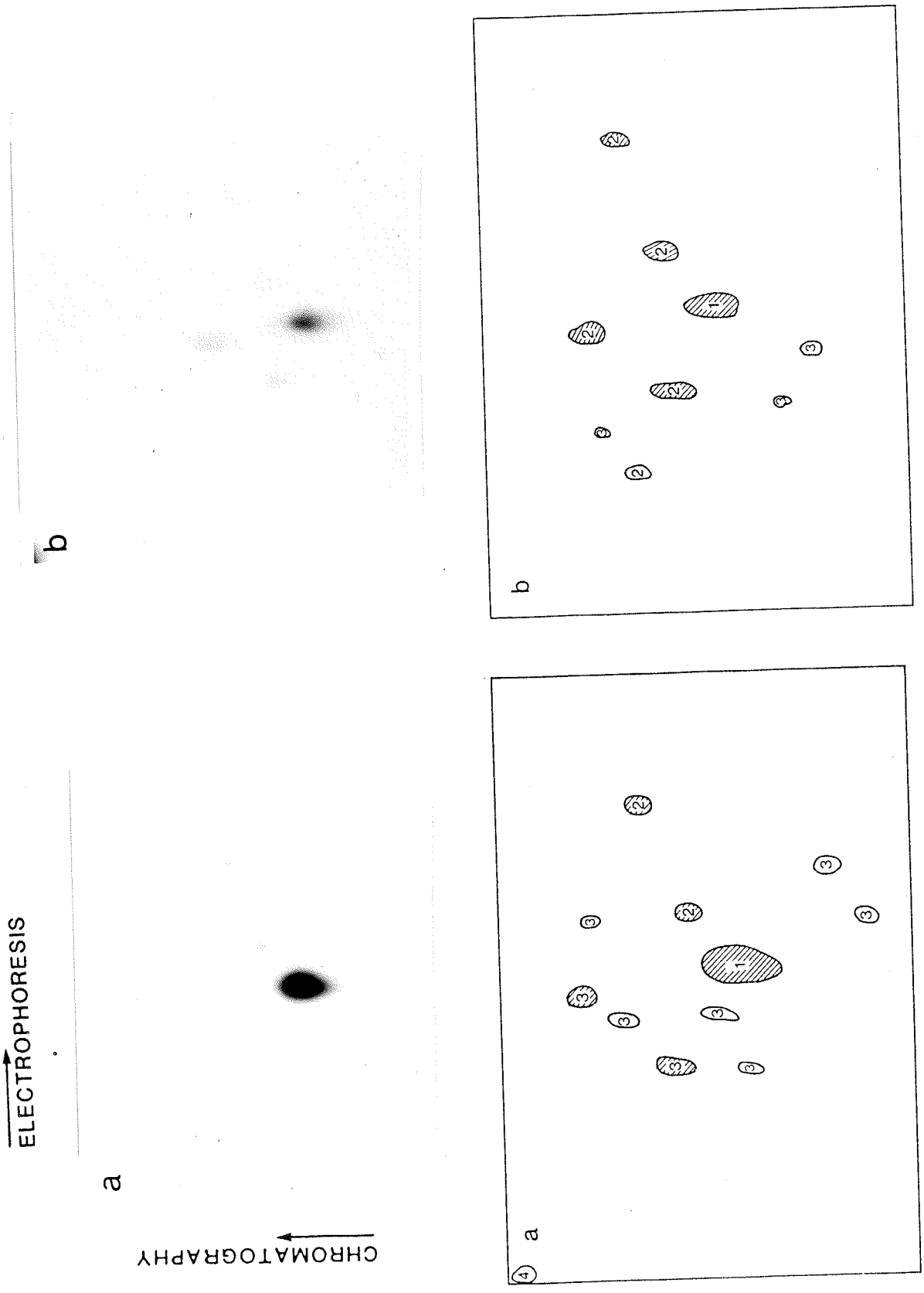

ELECTROPHORESIS
CHROMATOGRAPHY
a
b
a
b

Figure 21: Two dimensional analysis of tryptic peptides of reduced R and 71K. Maps were prepared as in Fig. 19. Digests of (a) reduced R and (b) reduced 71K. The shaded spots in the schematic drawings represent peptides sharing common positions in both (a) and (b).


ELECTROPHORESIS
CHROMATOGRAPHY
a
b
a
b

leads to some change in both 71K and R molecules which results in the generation of several different peptides. This change appears to be more pronounced for 71K than R.

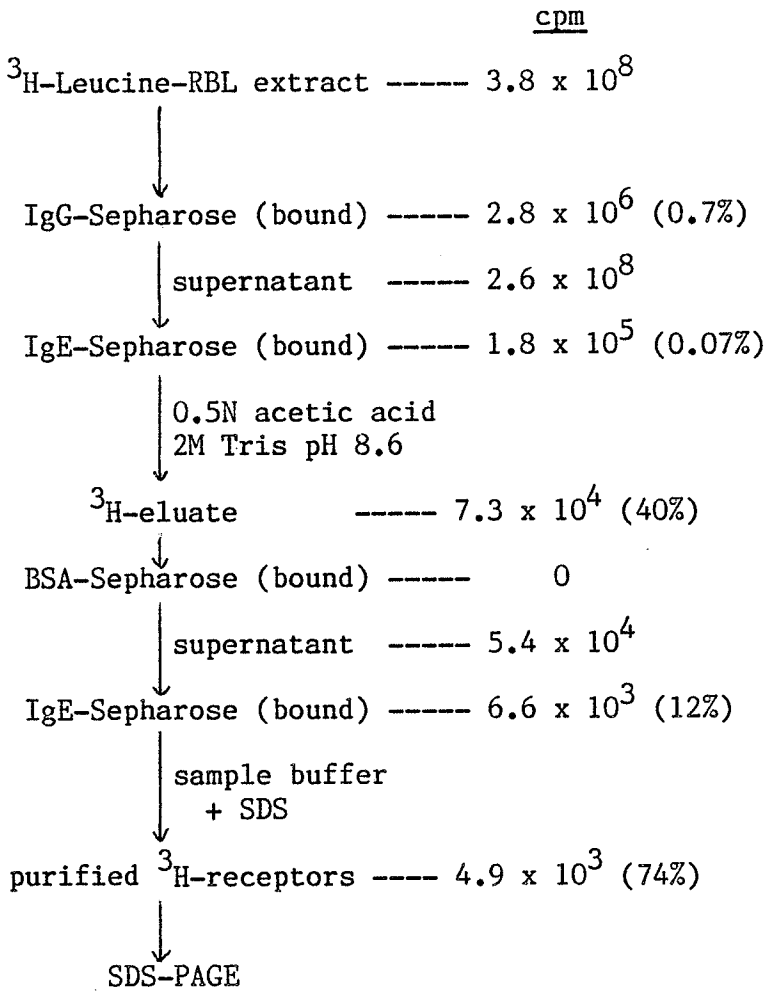
Isolation of Purified Receptors By Repetitive Affinity Chromatography

The results described in the preceeding section strongly suggested that 71K is a receptor consisting of R or an R-like molecule disulfide-bonded to another polypeptide chain. Since only one peak is apparent after SDS-PAGE of reduced, surface labelled 71K (Figs. 15 and 17), the other polypeptide chain would either have to be another R or R-like molecule or a molecule which is not surface labelled. To test the latter possibility, RBL cells biosynthetically labelled with ^{3}H-leucine were used as the source of receptors. As demonstrated by Helm and Froese (1981a), isolation of tritiated receptors from RBL cells using IgE-Sepharose results in a complex SDS-PAGE pattern. The presence of free IgE was unable to inhibit the binding of several peaks, indicating that there was a good deal of non-specific binding. The presence of molecules non-specifically bound to IgE-Sepharose would make a pattern, run under reducing conditions, difficult to interpret. In the present study, receptors from RBL cells which were biosynthetically labelled with ^{3}H-leucine (cells incorporated approximately 75% of radiolabel) were purified according to a procedure described by Kulczycki and Parker (1979). The flow chart representing this procedure is depicted in Fig. 22. The first column, rat IgG-Sepharose, bound 0.7% of the total radioactivity in the extract after it was washed. However, the supernatant, which was transferred to IgE-Sepharose, contained only 75% of the original extract activity, indicating that approximately 25% of the extract material was physically trapped in the gel or was very

loosely bound to IgG. IgE-Sepharose bound 0.07% of the IgG-Sepharose supernatant. This percentage is less than that bound in the case of surface-iodinated extract i.e. 1-3% (see Results, Chapter II), and is probably due to the fact that in biosynthetically labelled cells, both surface and non-surface proteins were labelled and therefore the original cell ^{3}H-extract could be expected to have contained many more labelled molecules which did not bind to IgE-Sepharose. Binding to IgE-Sepharose was also less than that to IgG-Sepharose. This suggests that the IgG column mainly bound what would otherwise have been bound non-specifically by the IgE column and that the use of IgG-Sepharose for the removal of non-specifically bound molecules was therefore appropriate. Acetic acid treatment of IgE-Sepharose resulted in the elution of 40% of bound counts. This percentage varied depending on the amount of time which the gel was in contact with the acetic acid. This also determined the amount of IgE-binding activity remaining in the eluate, thus, exposure of the receptors to low pH for more than 5-10 sec. destroyed almost all activity. The IgE-Sepharose eluate was then mixed with BSA-Sepharose, which did not bind significant counts. Rebinding of receptors in the BSA-Sepharose supernatant to IgE-Sepharose was approximately 12%.

Analysis of purified receptors was carried out by SDS-PAGE on 10% polyacrylamide slab gels. After staining, gels were treated with En^{3}Hance, dried and exposed to X-ray film. The resulting track was examined on a Helena densitometer and the pattern is depicted in Fig. 23. Three major peaks are apparent in this profile. Their mobilities correspond to 72, 48 and 27kDa. The 48kDa peak has a prominent shoulder on its ascending side. This is most likely due to the presence of H receptor. In addition, a peak migrating with the tracking dye can also

Figure 22: Flow chart of purification of ^{3}H-receptors by repetitive affinity chromatography. Numbers in parenthesis represent the percentage of available material which was bound or eluted.

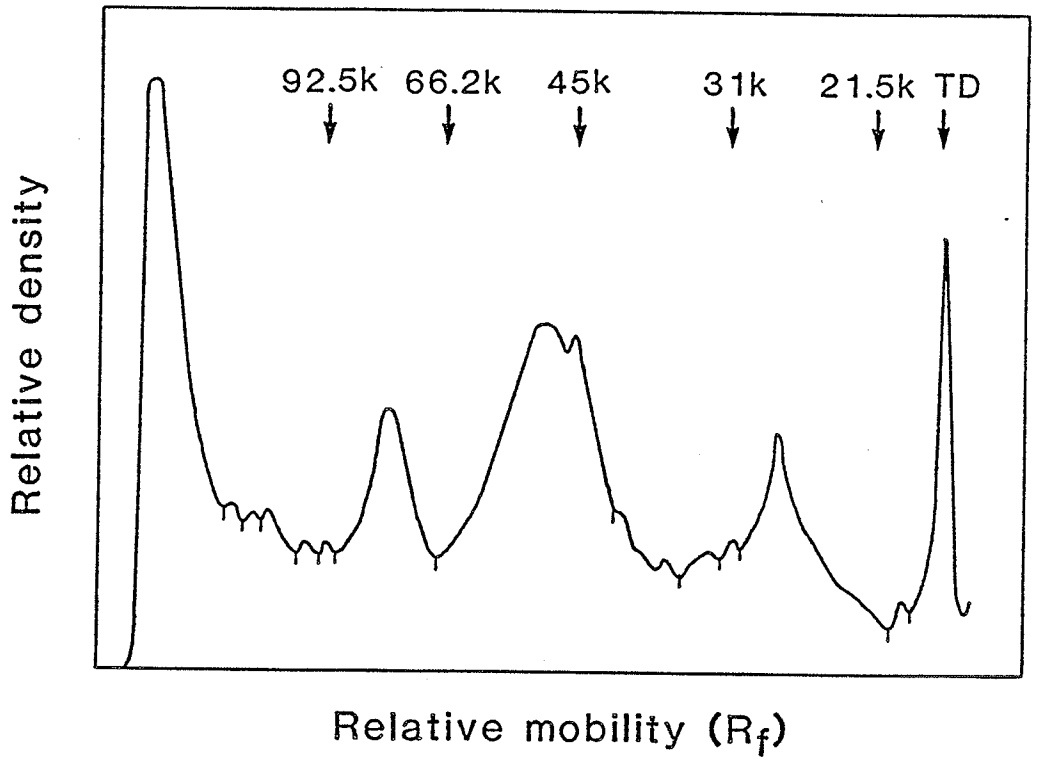

cpm
^{3}H-Leucine-RBL extract ----- 3.8 x 10^8
IgG-Sepharose (bound) ----- 2.8 x 10^6 (0.7%)
supernatant ----- 2.6 x 10^8
IgE-Sepharose (bound) ----- 1.8 x 10^5 (0.07%)
0.5N acetic acid
2M Tris pH 8.6
^{3}H-eluate ----- 7.3 x 10^4 (40%)
BSA-Sepharose (bound) ----- 0
supernatant ----- 5.4 x 10^4
IgE-Sepharose (bound) ----- 6.6 x 10^3 (12%)
sample buffer
+ SDS
purified ^{3}H-receptors ---- 4.9 x 10^3 (74%)
SDS-PAGE

be seen. From previous results (see Chapter II), two distinct peaks, representing H and R were expected. However, this procedure included a pre-incubation with rat IgG-Sepharose, extensive washing of gels as well as rebinding of eluted receptors to IgE-Sepharose. According to Kepron et al. (1982), there is evidence for the preferential binding to IgG-Sepharose of H over R, thus, in this procedure, more H than R may have been removed by the pre-incubation with IgG-Sepharose. In addition, the study by Kepron et al. also indicated that H has a lower affinity for IgE-Sepharose than does R. This may explain why, in the present study, rigorous washing and rebinding of eluted receptors may have resulted in a greater loss of H. Since this loss did not interfere with the investigation of 71K and R, it was not considered detrimental.

Two-Dimensional SDS-PAGE Analysis of Purified Receptors

To determine whether 71K could be reduced into more than one biosynthetically labelled polypeptide chain, receptors were analyzed by a two-dimensional procedure, the first dimension being SDS-PAGE under non-reducing conditions and the second, SDS-PAGE under reducing conditions. A preliminary experiment was performed using surface iodinated receptors which had been purified by a single incubation with IgE-Sepharose. SDS-PAGE was performed under non-reducing conditions in three 10% polyacrylamide tube gels. Two of these gels were then placed over 10% polyacrylamide slab gels and SDS-PAGE was performed under either non-reducing or reducing conditions. The other tube gel was sliced into 2 mm fractions which were counted in a gamma counter. The slab gels were fixed, dried and subjected to autoradioagraphy. Figure 24a shows the two-dimensional SDS-PAGE analysis of surface-iodinated receptors which were not reduced in the second dimension. As expected, all visible bands

Figure 23: One-dimensional SDS-PAGE analysis of receptors purified by repetitive affinity chromatography. ^{3}H-labelled receptors purified using the protocol depicted in Fig. 22 were analyzed on a 10% slab gel. The gel was fixed and stained with Coomassie Blue, then treated with En^{3}Hance. The dried gel was exposed to X-ray film at -70°C and autoradiography was performed. The gel was scanned with a desitometer. The arrows represent the positions of protein standards in order of their decreasing molecular weigh from left to right: Phosphorylase B, 92.5kDa; bovine serum albumin, 66.2kDa; ovalbumin, 45kDa; carbonic anhydrase, 31kDa; soybean trypsin inhibitor, 21kDa.


92.5k
66.2k
45k
31k
21.5k
TD
Relative density
Relative mobility (R_f)

fell onto a diagonal line, indicating that all molecules had the same mobility in both dimensions.

When receptors were run under reducing condition, (Fig. 24b) the spot corresponding to 71K in the first dimension has a greater mobility in the second dimension, i.e. similar to the mobility of the spot corresponding to R. The spot corresponding to 71K thus falls off the diagonal line. The profile depicted over each pattern represents the receptor mobility in the first dimension gel, as determined by slicing and counting the third tube gel. It should be noted that the spots representing receptor material are very diffuse in both the reduced and unreduced patterns. This is not unexpected in light of the fact that one-dimensional SDS-PAGE analysis produces diffuse receptor bands. When these already heterogeneous bands are subjected to second-dimensional SDS-PAGE, this heterogeneity would be expected to become even more apparent.

The identical experiment was performed using extensively purified receptors from RBL cells biosynthetically labelled with ^{3}H-leucine. Figure 25a illustrates the two-dimensional analysis of tritiated receptors which were not reduced in the second dimension. Clearly, all visible spots fall within the diagonal line. Due to cracking of the gel during the drying process, the spot with high mobility corresponding to the low molecular weight band (27kDa), seen in Figure 23, is difficult to distinguish. Its presence is clear, however, in Fig. 25b, which represents analysis of purified receptors reduced in the second dimension of SDS-PAGE. In this case all spots, except, one fall on a diagonal line, the exception being the spot with the lowest mobility which, in agreement with the pattern in Fig. 24b, represents 71K. The spot which

represents the 27kDa molecule clearly remained on the diagonal line and was therefore not drastically affected by reduction. So did a spot which in the first dimension, must have migrated with the tracking dye. Of great interest in this pattern is the fact that while the spot representing the 71K receptor falls off the diagonal upon reduction, no other spots are apparent, either at the level of the spot representing the 27kDa molecule or anywhere else on the vertical under 71K, where a reduction product of 71K would be expected to appear.

Figure 24: Two-dimensional SDS-PAGE analysis of ^{125}I-Labelled receptors. ^{125}I-labelled receptors were isolated from the NP-40 extract of surface labelled RBL cells by a single incubation with IgE-Sepharose. SDS-PAGE was performed in the first dimension in three 10% tube gels. Two of these gels were then placed over 10% slab gels and fixed in position with 1.5% agarose. For the gel run under reducing conditions, the agarose contained 7.5% 2-ME and the upper electrode buffer contained 1% 2-ME. Following electrophoresis, the gels were dried, exposed to X-ray film and autoradiography was performed. Second dimension run under (a) non-reducing and (b) reducing conditions. The profiles over each pattern represent the receptor mobility in the first dimension gel, as determined by slicing and counting the third tube gel.

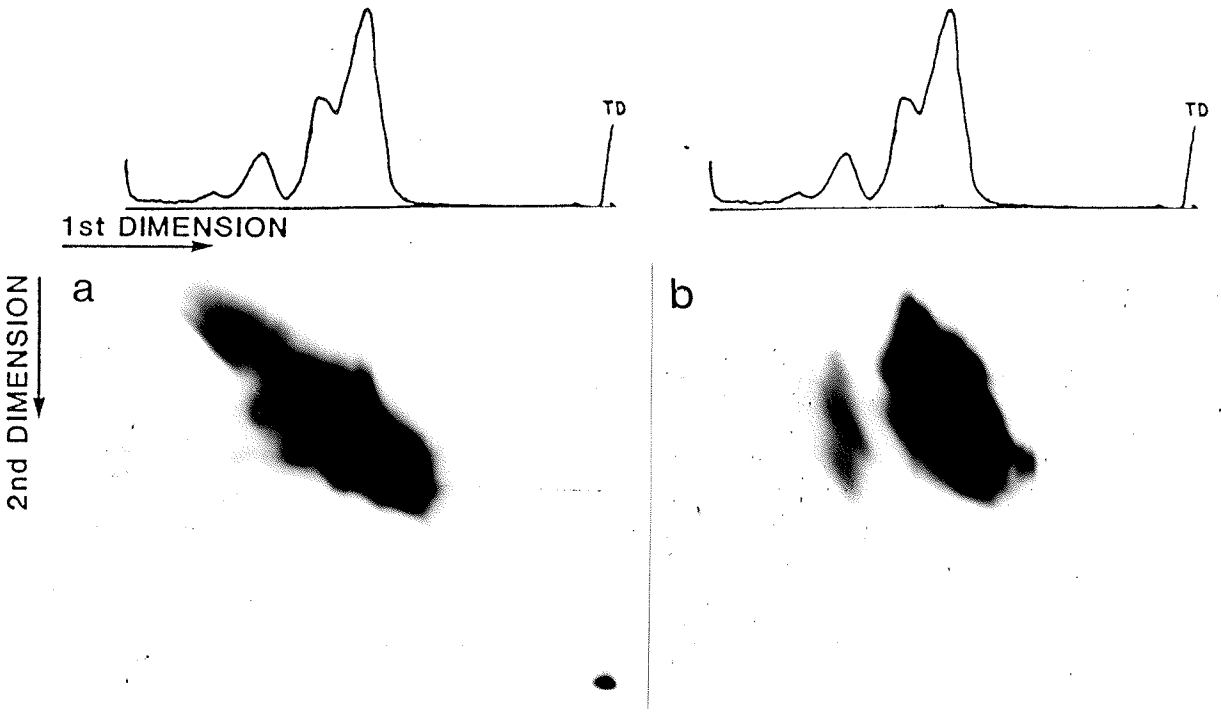
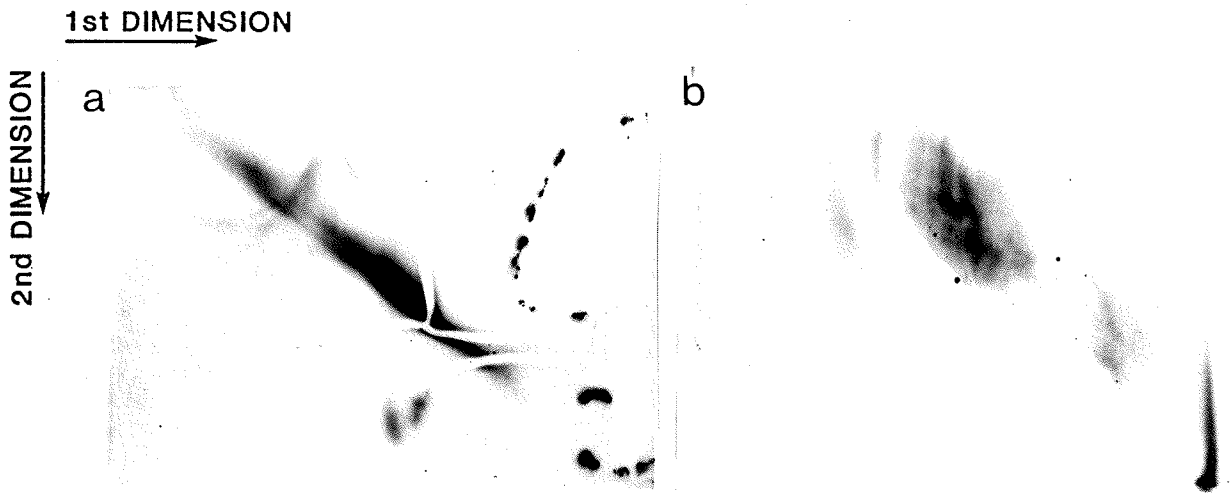

TD
TD
1st DIMENSION
2nd DIMENSION
a
b

Figure 25: Two-dimensional SDS-PAGE analysis of ^{3}H-labelled receptors. ^{3}H-labelled receptors were purified using the protocol depicted in Fig. 22. Two dimensional analysis was performed as in Fig. 24 for ^{125}I-labelled receptors. Slab gels were treated with En^{3}Hance, dried and exposed to X-ray film. Autoradiography was performed. Second dimension run under (a) non-reducing conditions and (b) reducing conditions.


1st DIMENSION
2nd DIMENSION
a
b

DISCUSSION

The results in Chapters II and III of this thesis as well as several other studies (Kepron et al., 1982, Conrad and Froese, 1978a, Helm et al., 1979) indicate that the R and H receptors have many different properties. The R receptor has a higher affinity for IgE-Sepharose than H, although the reverse appears to be true for IgG-Sepahrose (Kepron et al., 1982). While both R and H can be isolated with IgE-Sepharose, only R can be isolated by using IgE and anti-IgE (Conrad and Froese, 1978a). Furthermore, differences in binding to a variety of lectins were demonstrated (Helm et al., 1979), indicating differences in the carbohydrate make-up of these two molecules. In the present study, it has been shown that papain digestion of R and H produce different peptide profiles (Chapter II) and that the two molecules differ in their susceptibility to digestion with several enzymes (Chapters II and III). Results in this chapter provide further evidence that R and H are different molecules.

Exhaustive tryptic digestion of the receptors produced peptides which were analyzed by one- and two-dimensional procedures. It is apparent from Fig. 18, a and b that tryptic digestion of surface-labelled R and H produces different patterns as detected by one-dimensional SDS-PAGE. The most obvious difference is in the number of peptides generated, R being degraded into more peptides than H. Since tryptic digestion is specific for lysine and arginine residues, this suggests that the surface labelled portions of these molecules differ in amino acid sequence. This result is in agreement with data in Chapter II in which papain digestion produced fewer peptides from H than from R. Two-dimensional analysis was performed on these two sets of peptides to

compare them in more detail, and, involving properties other than molecular weight. When separated with respect to charge in the first dimension and partition coefficient in the second dimension, a clear-cut difference between the peptides from R and H can be seen (Fig. 19). The difference in the number of peptides is still apparent. More significantly, there is no similarity in the positions of peptides from R and H. These data confirm the results of Pecoud and Conrad (1981), who compared R and H using the same procedure. They too found that digestion of H generated fewer peptides than that of R (3 vs. 18) and that none of the peptides coincided in position. Although there are some differences between the patterns obtained in that study and the present one, the conclusions to be drawn are the same: there is little, if any homology between R and H.

In contrast to the comparison of R and H, that of R and 71K consistently suggested a close relationship between these two molecules. Every method that isolates R also isolates 71K (Froese, 1984). In addition, they bind to the same lectins (Helm et al., 1979). More recently, experiments performed in this laboratory by Dr. M. Rao and illustrated in Fig. 15, demonstrated that reduction of isolated 71K yields a molecule with an SDS-PAGE mobility and thus, molecular weight which is very similar to that of R. An earlier attempt to reduce 71K failed to show any effect upon treatment with 2-ME (Helm and Froese, 1981a). However, in that experiment, the concentration of 2-ME used was only 2%, compared to 5% in the more recent experiment (Fig. 15), and 5-10% in the present study. Furthermore, in the earlier experiment, 2-ME was used in the presence of IgE-Sepharose containing about twice as much IgE per ml of Sepharose as the affinity gel in later experiments. Since

IgE is associated with many disulfide bonds, this change may have contributed to the difference in ability to reduce 71K.

The striking similarities between R and 71K have led to the experiments described in the present study. One-dimensional SDS-PAGE analysis of tryptic peptides provided more evidence for the similarity between these two molecules (Fig. 18). Contrary to the comparison between H and R (Fig. 18, a and b), the patterns representing peptides of unreduced R and 71K are almost identical, differing only in relative counts (Fig. 18, b and d). Although the lack of resolution in some areas of these patterns makes it difficult to compare peaks, it is clear that whatever peptides were generated from R and 71K, they are similar in size and in the relative amount of radioactivity contained in the different peptides. These patterns also point to the possibility that the heterogeneity attributed to R in past studies (Conrad and Froese, 1978a Kanellopoulos et al., 1979, Kulczycki et al., 1976) contributes, in part, to the large number of peptides generated from R. The molecular weights of the larger peptides in Fig. 18b suggest that different populations of R are degraded into different peptides, since not all could have been derived from the same molecule. The microheterogeneity in R was, in part, attributed by carbohydrate heterogeneity (Hempstead et al., 1981b), indicating that either carbohydrate influences the accessibility of some cleavage sites or, that differences in carbohydrate content affect the SDS-PAGE mobility of certain peptides or, both. This may also account, in part, for the generation of apparently fewer peptides of H, since intact H does not appear to be as heterogeneous as R. A pattern of 71K peptides similar to that of R (Fig. 18d) indicates that this receptor's heterogeneity also influenced its digestion and/or mobility of its

fragments.

If the radiolabelled portion of 71K is actually R, then digestion of the reduced receptors would be expected to demonstrate similar if not identical patterns upon SDS-PAGE analysis. Inspection of Fig. 18, c and e suggests a great similarity between the two patterns although again, the broadness of the peaks does not allow a close comparison of individual peaks. Comparison of corresponding reduced and unreduced receptors yields another piece of information. The change in the relative peak heights of the higher molecular weight peptides upon reduction of R (and, less obviously of 71K) suggests that R contains intrachain disulfide bonds in the vicinity of tryptic cleavage sites. Reduction of these bonds results in a shift of peptides to fractions corresponding to lower molecular weight (Fig. 18, b and c). This would also pertain to 71K, although of the inter-chain disulfide bond(s) which has to exist in 71K, would also be expected to affect the SDS-PAGE patterns.

A comparison of two-dimensional maps of unreduced R and 71K on the one hand (Fig. 20) and, the reduced molecules on the other (Fig. 21) indicates that in both instances, R and 71K share 5 peptides, suggesting that there is a great deal of homology between the two molecules. Actually, it is possible that 71K and R may have even more peptides in common since it is conceivable that the two molecules were not iodinated identically, either because they are on a different microenvironment on the plasma membrane or because the tyrosine residues of 71K, due to its different quaternary structure, were not as accessible as those of R. This would hold if 71K were a disulfide-bonded dimer of R or if it consisted of R linked to another polypeptide chain. Reduction results

suggest that 71K consists of only one polypeptide chain (see also following). Thus some peptides of R or 71K may not be labelled or, their specific activity may have been too low to be detected.

By the same token, it can be argued that even if 71K consists of either two disulfide-linked R molecules or one R linked to a non-iodinated polypeptide chain, completely identical maps of unreduced R and 71K could not be expected because the differences in quaternary structure could alter the accessibility of certain peptide bonds of 71K to the enzyme. In any case, the digest of 71K would contain disulfide bonded peptides not present in that of R, although reduction of receptors prior to digestion would be expected to produce even more common peptides, and this did not appear to be the case.

The fact that the peptide maps (one- and two-dimensional) of reduced R as well as those of 71K were altered compared to the unreduced molecules may indicate that both molecules are associated with intra-chain disulfide loops. The potential for the presence of intra-chain disulfide bonds in R exists in view of the fact that this molecule was shown to have 7 cysteine residues per mole (Kanellopoulos et al., 1980).

In summary, the one and two dimensional peptide maps have strongly suggested that 71K consists of at least an R or R-like molecule disulfide bonded either to itself or to another polypeptide chain which cannot be surface iodinated. To test the latter possibility, two-dimensional SDS-PAGE using biosynthetically labelled receptors was carried out. The two-dimensional procedure consisting of electrophoresis under non-reducing conditions in the first dimension and reducing conditions in the second, was performed on receptors from

biosynthetically labelled RBL cells. Thus, reduction in the second dimension demonstrated that a spot with a mobility corresponding to that of 71K shifted off of the diagonal line into a spot corresponding to the mobility of R (Fig. 25b). This result was expected and is in agreement with Figs. 14 and 17, which show that reduction of isolated 71K causes it to shift to a position corresponding to that of R. However, the results do not support the possibility that 71K consists of R disulfide-bonded to a molecule previously seen in this laboratory called 26K (Helm and Froese, 1981a) or to a comparable, 30kDa molecule found on another RBL cell line (Holowka et al., 1980) and which was shown to be associated with R or α. If this had been the case, one would expect to see an additional spot directly under the spot corresponding to 71K, which had shifted upon reduction. Indeed, a band corresponding to a molecular weight of 27kDa is seen in both the one-dimensional SDS-PAGE pattern (Fig. 23) and the two-dimensional pattern (Fig. 25b). In either case, the identity of this band is not entirely certain, since recently, a molecule with a M_r of 33kDa was described which cannot be surface iodinated under normal conditions and which binds IgE directly, in the absence of R or α (Holowka and Baird, 1984). Although the M_r of this molecule (33kDa) is somewhat higher than that found in the present study (27kDa), the possibility still exists that it is this newly described molecule which is seen in Figs. 23 and 25b. In any case, the spot remains on the diagonal after reduction (Fig. 25b), indicating that it was not associated with any other polypeptide during the first-dimension electrophoresis. Inspection of Fig. 25b reveals that no other spot is visible directly under the spot corresponding to reduced 71K. Another possibility became apparent after the completion of these experiments,

when a second receptor associated molecule, named was described by Perez-Montford et al. (1983b). This molecule consists of a disulfide-linked dimer of 10kDa polypeptides which cannot be surface labelled. The sharp, low molecular weight band seen in Fig. 23 may well represent the dimer form of this molecule. A spot corresponding to a low M_r molecule is also apparent in the reduced, two-dimensional pattern (Fig. 25b) although its streaking nature makes it difficult to determine whether it falls on or off the diagonal line. However, no spot corresponding to this molecular weight is visible under the reduced 71K spot, indicating that this is not the molecule which is disulfide-bonded to R. A possible explanation for the fact that no other spot is visible in this line is that the polypeptide which bonds to the R receptor is not biosynthetically labelled during the 24 hour culture period or in the 48 hour period used in the study by Helm and Froese (1981a) because of a low turnover rate. However, this seems unlikely in cells the doubling time of which is 12-16 hours (unpublished observation). Another, more plausible explanation is that 71K is a disulfide bonded dimer of the R receptor, therefore both polypeptide chains are contained in the one spot which is visible in Fig. 25b.

Very recently, and subsequent to obtaining the results described above, it was discovered that the RBL cell line used in the present study was contaminated with a mycoplasma, which was later typed as M. hyorhinis. Moreover, this contamination was found to induce 71K on the surface of RBL cells (Chan et al., 1985). This induction was shown to be reversible, as elimination of mycoplasma resulted in the disappearance of 71K, and re-infection led to its reappearance. This study also showed that ^{125}I-71K was detectable after infection of surface iodinated cells,

indicating that mycoplasma exerted its effect on a molecule present on the cell surface at the time of infection.

It is clear from the present study, which used chronically infected cells, that not all of the R receptors are affected, since at no time could a profile with little or no R be found. Thus, it appears that mycoplasma somehow causes certain R receptors to become disulfide bonded to other polypeptides, perhaps to other R molecules. The heterogeneity of the R receptor has already been described as being mostly the result of carbohydrate heterogeneity (Hempstead et al., 1981b). This may be the basis for selection of R molecules to be effected by mycoplasma. It has been demonstrated that binding of certain mycoplasma species to host cells involves sialic acid on the host cell membrane (Kahane, 1983, Gabridge, 1982). There is some indirect evidence from Pecoud et al. (1981) that IgE receptors on RBL cells contain sialic acid residues. They found that neuraminidase treatment (which removes sialic acid) enhances labelling of receptors with $^{3}H\text{-}NaBH_4$, which labels terminal galactose and galactosamine residues. In this laboratory, it was found that neuraminidase treatment increased the pI of some but not all R molecules (unpublished observation). This indicates a heterogeneity in the sialic acid (or other sugar) content of R receptors which may be the criteria for their alteration by mycoplasma. Such a selection may result in differences in the carbohydrate composition of 71K and R and may thus be partly responsible for some of the differences seen in peptide maps of these molecules. The phenomenon of mycoplasma binding to host cell surface glycoproteins has been demonstrated in T lymphocytes (Stanbridge et al., 1981). M. hyorhinis was found to co-cap along with the Thy-1.1 antigen, indicating that this organism is capable of influencing the

lateral mobility of surface molecules. In a study by Wise, et al. (1982), the Thy-1.1 antigen was found to be localized on the surface of the M. hyorhinis organisms which had colonized the host cells. In addition, they found that a lipid probe inserted into the lymphocyte membrane was translocated into the mycoplasma membrane. These facts suggest that there was some exchange of surface glycoproteins as well as external modulation of the lipid composition of the host cell membrane by the mycoplasma cell. Such phenomena may play a role in the mycoplasmal infection of RBL cells and in the resulting alteration of IgE receptors on these host cells. Indeed, these observations raise the possibility that 71K may consist of R and a mycoplasma derived molecule, although such a molecule could be expected to be labelled under the conditions used in this study.

In light of the fact that cross-linking of R-like receptors was shown to be sufficient for the induction of mediator release (Isersky et al., 1977, Ishizaka et al., 1977, Ishizaka et al., 1978), the possibility that mycoplasma infection may result in the covalent crosslinking of two R receptors and thus in the modulation of mediator release. Even if it should turn out that, in order to produce 71K, R is linked to another molecule, mediator release could be effected since it was shown that bridging of Fc receptors and histocompatibility antigens can also lead to mast cell degranulation (Daeron et al., 1977).

GENERAL DISCUSSION

Studies of the IgE receptors on RBL cells as well as on RMC have led to an increasingly complex picture of these molecules. Some of the earliest investigations of the RBL cell Fc_ϵ receptors revealed a single cell surface molecule capable of binding IgE. Conrad and Froese (1976) used a sandwich precipitation method with rat myeloma IgE, ϵ-specific rabbit anti-rat IgE and goat anti-rabbit Ig, and isolated a single surface iodinated molecule, the SDS-PAGE mobility of which on 5% gels corresponded to an apparent molecular weight of 62kDa. This result was confirmed by Kulczycki, et al. (1976), who found a molecule with an apparent molecular weight of 45-50kDa using 10 and 12% gels. At the same time, this study provided some of the first concrete evidence for the protein nature of the receptor by demonstrating its sensitivity to a variety of proteases. The dependence of the apparent molecular weight on the porosity of the polyacrylamide gel also suggested that the receptor was a glycoprotein (Weber and Osborn, 1975), a characteristic which has since been repeatedly demonstrated (Kulczycki and Parker, 1979 Kulczycki et al., 1976, Helm and Froese, 1981a, Helm and Froese, 1981b). Another study, by Isersky, et al., (1977) reported the presence of a slightly larger molecule (58kDa) on 10% gels, and first suggested the existence of molecular weight heterogeneity between RBL cell lines.

Isolation of receptors using IgE-Sepharose revealed a further complexity in their nature. Conrad and Froese (1978a) found that a molecule with an apparent molecular weight of 55kDa could be recovered along with the 45kDa component, and named the two receptors H and R, respectively. Kulczycki and Parker (1979) found only one receptor with an M_r of 50kDa by this method. A collaborative study involving a number

of laboratories which utilized the different cell lines demonstrated that these seemingly discordant results could be reconciled (Froese et al., 1982). All the cell lines were shown to express both the R and H receptors, though the molecular weights of the receptors, particularly R, were shown to vary from cell line to line. In addition, the relative amounts of each receptor also differed among the lines. Both types of receptors were also found on normal peritoneal rat mast cells (Froese, 1980). To date, only R-like molecules have been implicated in mediator release from RBL cells (Isersky et al., 1978), and from RMC (Ishizaka et al., 1977). No function has been attributed to the H molecule. However, a study from this laboratory has shown that while both R and H bind to IgG-Sepharose, H appears to have the higher affinity for this immunosorbent and a lower affinity for IgE-Sepharose (Kepron et al., 1982).

The present study has compared R and H receptors to determine whether or not there is structural homology between these two molecules. It was prompted by the possibility that R is a degradation product of H. This would be similar to the results obtained with the $Fc_{\gamma}R$ from a variety of cells, in which 70kDa and 90kDa molecules were found to be spontaneous degradation products of a 115kDa molecule (Kahn-Perles et al., 1980). Alternatively, H might represent a precursor of R. The results presented in this study failed to demonstrate any similarity between the two molecules and actually showed that they are different. Digestion of isolated receptors by papain produced strikingly different patterns when peptides were analyzed by SDS-PAGE (Fig. 6). These patterns demonstrate that H (or its surface labelled portion) is more susceptible to cleavage under these conditions than R and thus, smaller

peptides are produced from the former receptor. The differences between R and H were further emphasized when an attempt was made to obtain IgE-binding fragments of the receptors by proteolytic degradation. When the residue-non-specific papain was employed, only a binding fragment of H was produced (Figs. 9 and 10), indicating that, although the surface-labelled portion of R is less susceptible to papain digestion than that of H, its binding site is destroyed by this enzyme. The loss of the IgE-binding activity of R could either be due to a direct effect on the binding site, or to an alteration of secondary or tertiary structure. Digestion by trypsin and chymotrypsin also produced an IgE-binding fragment of H (Figs. 12a, b and 13a, b respectively). In these experiments, however, a population of R receptors remained intact (Figs. 12c and 13c). Since carbohydrate heterogeneity has repeatedly been shown to be responsible for the heterogeneity seen in R (Hempstead et al., 1981b, Goetze et al., 1981), this result suggests that carbohydrates may influence the susceptibility of the R binding site to destruction by these enzymes. When subjecting the equivalent of R on their RBL cell line to controlled digestion with a variety of proteolytic enzymes, Goetze, et al. (1981) consistently produced two fragments of similar size, both of which contained some carbohydrate. They suggested that the α chain, or R, exists in the form of two domains, both of which are exposed on the surface of the cell. They carried out their experiments under denaturing conditions, which did not allow subsequent studies on the IgE-binding capabilities of the isolated fragments or domains. Finally, analysis of peptides of R and H produced by exhaustive tryptic cleavage demonstrate that there is little if any structural homology between the surface labelled portion of these two receptors

(Figs. 17a and b, 18a and b). These results confirm those obtained by Pecoud and Conrad (1981), which were published while this study was in progress. Although only surface-labelled peptides were being compared in the present study as well as in that of Pecoud and Conrad, the differences found between R and H are nonetheless significant. It is not unreasonable to assume that it is the surface-labelled portions of the intact molecules which contain the IgE-binding activity. Therefore, this study provides evidence that the functional portions of these two molecules are structurally different. Thus, it is not surprising that these molecules show differences in their interaction with IgE and IgG (Kepron et al., 1982). Further investigations would be required to determine whether or not the remainder of the molecules, which are not accessible to surface iodination, display any homology.

Rat basophilic leukemia cells are not unique in possessing distinct Fc receptors for different Ig classes or subclasses. Conrad and Peterson (1984) found that the Fc R and the Fc $_{2b}$R on murine B lymphocytes are structurally distinct based on the lack of crossreactivity in Ig-binding. However, both receptors bound to rabbit IgG, indicating that some crossreactivity does exist. Murine macrophages were also found to have at least two distinct receptors for IgG, and, although both bound the two classes of IgG (IgG_{2a} and IgG_{2b}), one had a higher affinity for IgG_{2a}, the other for IgG_{2b} (Haeffner-Cavaillon et al., 1979). Using the same cell line, Unkeless (1977) provided evidence for an IgG_{2a} receptor which was trypsin sensitive as well as a trypsin-resistant aggregated-rabbit IgG receptor. Whether these two receptors were the same two receptors described in the study by Haeffner-Cavaillon, et al. (1979) was not determined. Rat macrophages

were found to have at least three distinct Fc receptors: one for rat IgE; one for IgG_{2a}; one for IgG_1, IgG_{2b} and heterologous IgG (Boltz-Nitulescu et al., 1981). Rat IgE was capable of inhibiting binding to the last receptor. Indeed, there are many cell types which bear distinct, 'specific' receptors for different Ig classes and sublasses. In several cases, the structurally distinct receptors were shown to display affinity for the same Ig classes. A similar situation may exist in RBL cells, which, according to the studies by Kepron, et al. (1982) and Segal, et al. (1981), bear two distinct receptors for IgE and IgG. Both Ig classes bind to either receptor with differing affinities.

The presence of a third receptor for IgE on RBL cells was first detected by Conrad and Froese (1978a). At that time, it was not regularly observed, and was therefore considered a contaminant (A. Froese, personal communication). The molecule, the molecular weight of which was estimated to be 71kDa and which was called 71K, was described as an IgE receptor by Helm, et al. (1981a) who showed that its binding to IgE-Sepharose could be inhibited by presaturating receptors with free IgE. The same study demonstrated the biosynthetic incorporation of ^{3}H-leucine into the three receptors, R, H and 71K, as well as into a 26kDa molecule. A molecule analogous to the 26kDa molecule had earlier been detected on RBL cells by Holowka, et al. (1980), who used a crosslinking reagent to show that a 30-35kDa polypeptide (called β) was associated with the R-like receptor (called α). The polypeptide could not be surface labelled in either study. In addition, in the study by Helm, et al. (1981a), the affinity of 26K for R (if such an association did exist) was weak since the former molecule could not be consistently isolated by IgE-Sepharose or DNP-IgE and anti-DNP. In another

laboratory, using a different RBL cell line, only treatment with crosslinking reagents (Holowka et al., 1980, Holowka et al., 1981) or isolation of receptors in the presence of the appropriate phospholipid:detergent ratio produced a consistent yield of the molecule (Rivnay et al., 1982). These results indicate that the association between α and β (and the putative one between R and 26K) is a weak and non-covalent one.

Although reduction of receptors did not appear to change their mobility in the study by Helm, et al. (1981a), in a more recent experiment, reduction did result in a change in the mobility of the 71K molecule (Fig. 15) (M. Rao, unpublished results). This discrepancy may have been due to the difference in the concentration of reducing reagent used or to the difference in the amount of IgE coupled to Sepharose, or to both. The appearance, upon reduction of isolated 71K, of a molecule the molecular weight of which was similar to that of R led to speculation that 71K was composed of R, disulfide linked to some other polypeptide. The previously demonstrated similarities between the binding characteristics of R and 71K lent further support to this hypothesis. Thus, both receptors could be isolated with IgE-anti-IgE, (Froese et al., 1982, Helm and Froese, 1981a, Figs. 4 and 16, present study), with DNP-IgE-anti-DNP (Helm and Froese, 1981a), as well as with IgE-Sepharose (Froese et al., 1982, Helm and Froese 1981a, Figs. 3 and 23, present study). On the other hand, neither R nor 71K bind significantly when anti-receptor antiserum (RAR) is used to isolate H receptors (Fig. 4). In addition, the lectin binding characteristics of R and 71K are very similar but are somewhat different from that of H (Helm and Froese, 1981b). These facts suggested some structural relationship between R and

71K.

A comparison of tryptic peptides in the present study provides some further information on this relationship. Both one- and two dimensional tryptic peptide maps of R and 71K point to a great deal of similarity between these two receptors. However, differences were also observed and must be accounted for. In Chapter IV, it was suggested that even if 71K consists of either a disulfide-linked dimer of R or an adduct of R and another, as yet undetected polypeptide chain, some differences in peptide maps could perhaps have been expected due to differences in iodination of the two molecules. An alternate or additional explanation could be found in the fact that R exhibits a great deal of microheterogeneity, most likely due to differences in carbohydrate content (Hempstead et al., 1981b, Goetze et al., 1981). Such a heterogeneity could give rise to differences in the properties of peptides generated from different R molecules and thus lead to differences in two-dimensional peptide maps of individual molecules. Differences could be due to variability in charge, i.e. variability in sialic acid content and/or to partition coefficient differences, and would be detected in the first and second dimension, respectively. Therefore, even if R molecules are present in 71K, the microheterogeneity in 71K may differ from that of conventional R receptors.

In the report by Helm and Froese (1981a), it was suggested that 71K might be a more stable conformation of R and 26K, since, along with all the other evidence, the arithmetic also fits this hypothesis, i.e. the sum of 45kDa and 26kDa yields 71kDa. In the present study, this possibility was investigated by separating biosynthetically labelled receptors in two dimensions by SDS-PAGE. The results indicate that

although a biosynthetically labelled molecule similar in mobility to 26K was isolated, it was not disulfide-bonded to R (Fig. 25b). No such molecule was found when 71K was reduced. Indeed, no biosynthetically labelled molecule other than R was seen upon reduction of 71K in the second dimension. It therefore seems unlikely that 71K is a form of R, covalently linked to 26K. Alternatively, 71K may be a disulfide-bonded dimer of R. Simplistically, one could argue that a dimer of R should have a molecular weight of 90kDa. However, differences in microheterogeneity and the fact that 71K, when studied by SDS-PAGE under non-reducing conditions, retains its quaternary structure (which could influence its mobility) may make such simple arithmetic meaningless. Although the results presented in this thesis do not prove conclusively that 71K is a dimer of R, they rule out several other alternatives and provide no evidence to contradict this possibility.

Subsequent to performing the experiments for this thesis, the discovery that infection with mycoplasma (M. hyorhinis) was responsible for the expression of the 71K receptor (Chan et al., 1985) led to a new perspective in the investigation of this molecule. It was demonstrated that uninfected cells begin to express 71K on their surface 24 hours after infection and that the effect of the organism was on receptors pre-existing on the cell suface at the time of infection (Chan et al., 1985). The pre-existing receptors were most likely R since RBL cell lines which have a larger R-like molecule than the one used in the present study appear to express a larger 71K-like molecule upon infection (Froese et al., 1982, B.M.C. Chan, personal communication). It has also been demonstrated that RBL cells infected in the presence of IgE do not express 71K (B.M.C. Chan personal communication), which suggests that the

mycoplasma organism must come into contact with the receptor in order to exert its effect.

The effect of a variety of mycoplasma species on the immune system became a focal point of investigation when it became evident that the pathogenesis of some mycoplasma is due both to the direct effect on tissue cells and to a non-specific stimulation of a host immune response (Brunner, 1981). Both mycoplasmal pneumonia (Brunner, 1981) and arthritis (Jordan, 1981) have been shown to fall into this category. The interactions of mycoplasma with cells of the immune system have since been studied in vitro and have been shown to be responsible for a variety of phenomena. Certain species of mycoplasma are inducers of interferon in spleen cells and thus increase the susceptibility of infected tumor cells to natural killer cell-mediated lysis (Birke et al., 1981). Phagocytic cells are activated upon infection with some species of this organism (Koppel, 1984, Dietz and Cole, 1982). Mycoplasma may either inhibit or stimulate blast transformation of splenic lymphocytes (Stanbridge et al., 1981). The organisms are mitogenic for both B and T lymphocytes but the mechanisms are likely to be different (Stanbridge et al., 1981). In fact, different mycoplasma species exert their effects through different mechanisms. The mitogenic activity that M. arthritidis has for T cells is due to a soluble factor present in the mycoplasma supernatant (Cole et al., 1982). In infections with M. hyorhinis, T cells must bear Thy 1 antigens in order for the organisms to attach (Stanbridge, 1983). Once the organisms are bound, they and the Thy 1 antigen cluster and then co-cap. This capping correlates with the blast transformation of the T cells. Thus, it appears that the mycoplasma mimic multivalent antigens during this interaction. The mycoplasma may

also interact with other glycoproteins on these cells, such as gp70 and some H-2 antigens, since it was also shown to co-cap with these surface molecules.

The demonstration of an interaction between mycoplasma organisms and the glycoproteins on the T cell surface may have some relevance to the present study of IgE receptors on RBL cells. Infection of RBL cells with M. hyorhinis induces the expression of the 71K receptor on the membrane surface. The results of this study suggest that 71K may be a dimer of R, the receptor the crosslinking of which on RBL cells and RMC initiates the pathway leading to mediator release (Isersky et al., 1977, Ishizaka et al., 1978). It is not known how the mycoplasma organism would covalently crosslink two receptors. It has been demonstrated with a lipid probe that there is some transfer of lipid molecules from lymphocyte membrane to the attached M. hyorhinis mycoplasma membrane (Wise et al., 1982). If the M. hyorhinis which has chronically infected the cell line used in the present study is capable of effecting a similar modulation in the RBL cell membrane it might be part of a mechanism which leads to the disulfide bond formation which has been demonstrated.

The possibility of receptor crosslinking by mycoplasmal organisms may be relevant to the pathogenesis of these organisms. Non-IgE-dependent mediator release and the subsequent sequelae could be a possible consequence of in vivo mycoplasma infection. Indeed, it has been demonstrated in this laboratory that mycoplasma infection of RBL cell lines exerts an influence on cellular histamine content, i. e., the total cellular histamine content is increased (Chan et al., 1985). Although the significance of this finding is not known, it does indicate that mycoplasma infection affects not only surface phenomena, but

intracellular events as well. Experiments are now in progress to further elucidate the effects of mycoplasma on RBL cells.

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