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Structural homology of the GABAa/ benzodiazepine receptor as demonstrated by monoclonal antibodies and limited proteolysis

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STRUCTURAL HOMOLOGY OF ALPHA AND BETA SUBUNITS OF THE GAMMA-AMINOBUTYRIC RECEPTOR AS DEMONSTRATED BY MONOCLONAL ANTIBODIES AND LIMITED PROTEOLYSIS

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Structural Homology of Alpha and Beta Subunits of the $GABA_a$/Benzodiazepine Receptor as Demonstrated by Monoclonal Antibodies and Limited Proteolysis

A Thesis

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Requirements for the Degree of

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by

Robert Thomas Malison

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Dedicated to

Eugenia Marie, my lifelong companion

&

Robert and Colleen Malison, my parents

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Abstract

Structural Homology of Alpha and Beta Subunits
of the $GABA_a$/Benzodiazepine Receptor as Demonstrated
by Monoclonal Antibodies and Limited Proteolysis

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Molecular aspects of $GABA_a$/benzodiazepine receptor structure, including putative receptor heterogeneity, were studied. Using affinity chromatographic techniques, a partially purified benzodiazepine receptor preparation was obtained from rat brain. A major protein band of 48.5 kD and several minor bands (68, 58, 54.5, and 50 kD) were isolated. Electrophoretic analysis in one and two dimensions demonstrates co-localization of this 48.5 kD protein with the photo-labeled alpha subunit. Using this benzodiazepine receptor preparation, 6 murine monoclonal cell lines were generated, as tested for by solid phase radioimmunoassay. Western blots to receptor proteins separated electrophoretically demonstrate the presence of two immunoreactive bands of 48.5 and 54.5 kD, corresponding to the alpha and beta subunits. Specificity of one monoclonal antibody, E9, for the alpha subunit (48.5 kD) was confirmed by two dimensional gel electrophoresis. Immunoblot studies using another monoclonal antibody, H10, suggest a common epitope is shared by both subunits. The molecular basis underlying subunit relatedness was explored by limited protelytic degradation of the iodinated

benzodiazepine receptor preparation. Results indicate that degradation of both 48.5 kD and 54.5 kD proteins yielded several shared limiting peptides as determined by molecular weight. Homology of these receptors may, thus, exist at the level of primary protein structure.

Introduction

Gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter, is widely distributed in the mammalian central nervous system. Concentrations in the human brain, though regionally variable, are measured in μmoles/gram, three orders of magnitude greater than concentrations typical of the monoamines (Fahn and Cote, 1968). Estimates suggest that GABA may be a neurotransmitter at 30% of synapses in the brain. The primary action of GABA is inhibitory, resulting in an increased chloride flux across and subsequent hyperpolarization of neuronal cell membranes (Krnjevic,1974; Curtis & Johnston,1974). With recognition of its role as one of the brain's major inhibitory transmitters, substantial efforts have been made to implicate GABA in the pathophysiology of numerous neurologic and psychiatric disorders, including epilepsy (Olsen et al, 1985), Huntington's disease (Van Ness et al, 1982), affective disorders (Emrich et al, 1980), and anxiety (Tallman et al, 1980).

Various lines of investigation have shown that a host of compounds, including the 1,4-benzodiazepines, are capable of modifying post-synaptic responses to GABA. Specifically, electrophysiologic studies demonstrated enhanced GABA-ergic transmission in the presence of benzodiazepines (Schmidt et al, 1967; review by Tallman et al 1980 & Haefley et al, 1981). Investigations into the molecular basis for the benzodiazepine's effect resulted in the demonstration of a high-affinity binding site (receptor) in the CNS (Squires & Braestrup, 1977; Mohler & Okada, 1977). Binding

studies in vitro fulfill criteria for a pharmacologic receptor; namely, binding is rapid, reversible, stereospecific and saturable. In addition, the rank order potency of benzodiazpines' ability to displace diazepam from this central site correlates significantly with both activity in animal behavioral tests predictive of benzodiazepine potency (Braestrup & Squires, 1978; Mohler & Okada, 1978) and therapeutic doses in humans (Tallman et al, 1980).

This high affinity central receptor has been shown to be functionally linked to the chloride channel-linked GABA receptor, known as the $GABA_a$ receptor (a second, G-protein-linked $GABA_b$ receptor has been described by Bowery & Hill, 1980, and is not associated with benzodiazepine binding). Binding of GABA and related agonists to a low affinity (1-5 μM) site on this $GABA_a$ receptor complex facilitates the binding of benzodiazepines (Tallman et al, 1978). This binding is blocked by the GABA antagonist (+)bicuculline. Thus, the central binding sites for both GABA and the benzodiazepines appear to be allosterically linked and most likely reside within the same protein complex.

As research into the molecular nature of the $GABA_a$ /benzodiazepine receptor complex has progressed, the existence of benzodiazepine receptor subtypes in different brain regions has been hypothesized (Seighart & Karobath, 1980). Initial evidence supporting this hypothesis came from binding studies using the triazolopyridazine, CL218,872, a compound shown to interact at the benzodiazepine receptor. Results were consistent with receptor heterogeneity or negative cooperativity in a number of brain regions, most notably the hippocampus (Squires et al, 1979; Klepner et al,

1979). Autoradiographic localization at the light microscopic level supported a heterogeneity of receptors, as well (Young & Kuhar, 1980; Young et al, 1981; Niehoff et al, 1982).

In addition to pharmacologic and autoradiographic lines of evidence, photo-labeling studies with several photo-activated compounds have pointed to a structural heterogeneity of receptor subtypes at the molecular level. Early alkylating compounds, irazepine (Rice et al, 1979) and kenazepine (Williams et al, 1980), were able to distinguished between apparently different receptor subtypes (Skolnick et al, 1982). Introduction of the nitro-containing benzodiazepine, [^{3}H]-flunitrazepam, not only enabled the autoradiographic localization studies mentioned above, but also allowed electrophoretic analysis of the molecular structure of the benzodiazepine receptor under denaturing conditions. Such photo-labeling experiments have demonstrated the existence of a major band, the alpha subunit, in rat brain with a molecular weight of approximately 50 kD (Mohler et al, 1980; Thomas & Tallman, 1981). A second protein band on acrylamide gels, the beta subunit, demonstrates a molecular weight of approximately 55 kD. It appears to exist in hippocampus and striatum (Sieghart & Karobath, 1980; Sieghart et al, 1983), as well as being expressed early in postnatal development (Eichinger & Sieghart, 1986). Additional minor bands of 53 kD and 59 kD have also been described (Sieghart et al, 1983). The relationship of these various proteins to the $GABA_a$ /benzodiazepine receptor complex and to one another remains to be defined.

With improved affinity chromatography and specific ligand elution methods, significant purification of benzodiazepine receptor subtypes has been achieved (Ransom et al, 1986; Sigel et al, 1983; Schoch et al, 1984; Tallman & Gallager, 1979). Using rat cortex, a partially purified benzodiazepine receptor preparation was obtained. In this preparation a major protein band of 48.5 kD and several minor bands (68, 58, 54.5, and 50 kD) were isolated. Analysis of the 48.5 kD protein in two dimensions shows it to co-localize with the photo-labeled alpha subunit. Using this benzodiazepine receptor preparation, 6 murine monoclonal cell lines were generated, as tested for by solid phase radioimmunoassay. Western blots to receptor proteins separated electrophoretically demonstrate the presence of two immunoreactive bands of 48.5 and 54.5 kD. These results are similar to those observed in bovine brain (Haring et al, 1985). In addition, immunoblot studies using one monoclonal antibody, H_{10}, suggest a common epitope is shared by both subunits. In an attempt to further delineate the molecular structure and to explore the possibility that homology between the alpha and beta subunits of the $GABA_a$/benzodiazepine receptor exists, these proteins were analyzed by methods of limited proteolysis. Results indicate that degradation of both 48.5 kD and 54.5 kD proteins yielded several limited peptides as determined by molecular weight. Homology of these receptors may, thus, exist at the level of primary protein structure.

Materials & Methods

Benzodiazepine Receptor Purification

Purification of the benzodiazepine receptor was carried out by affinity chromatographic methods utilizing a benzodiazepine coupled agarose resin. Preparation of the ligand affinity column and isolation of the benzodiazepine receptor have been discussed in detail elsewhere (Ransom et al, 1986; Sigel et al, 1983; Schoch et al, 1984; Tallman & Gallager, 1979). Briefly, the flurazepam derivative, RO7-1986/001, was coupled to an agarose gel support at the carboxy-terminus of a positively charged 15-atom spacer arm (succinyl-methylimino-bis-propylamino carboxylmethyl agarose carboxamide). A volume of 100 ml of the N-hydroxysuccinimide ester of this gel (Affi-Gel 15, Bio-Rad) was washed with absolute ethanol and suspended in a150 ml volume. The agarose was reacted with 100 mg of ligand at 4° C overnight. The coupled gel was washed with 1 liter of distilled water using a sintered glass funnel under light vacuum. The agarose gel was resuspended in 25 mM potassium phosphate buffer at pH 7.3 and filtered just prior to use.

Whole rat brain was homogenized in10 volumes of 25 mM potassium phosphate buffer containing 0.1 mM phenylmethylsulfonylfluoride (PMSF), 2.5 µg/ml trypsin inhibitor, 5 mM ethylene-diamine-tetraacetic acid (EDTA) and 50 µM benzamidine at pH=7.3 (PIB) using a Polytron tissue homogenizer (Brinkman Instruments). The homogenate was then centrifuged at 40,000 x g for 20 minutes and the pelleted membranes homogenized

and centrifuged twice more in the same manner. The washed pellets were then frozen overnight at -80° C.

The following morning, pellets were again homogenized and centrifuged in the same buffer twice. The membranes were suspended in 10 volumes of 1.0% Lubrol PX (Sigma Chemical Company) in PIB and stirred at 4° C for 1 hr. The solubilized membrane suspension was ultracentrifuged at 105,000 x g for 30 minutes. Supernatants were pooled and stirred with the RO7-1986/001 coupled agarose (approx. 3:1, V/V) for 1 hr. at 4 ° C. The Affigel was washed and filtered 2 times with 1.0% Lubrol PX and 4 times with 5 mM 3-(chloramidopropyldimethyl-amino)-1-propansulfonate (CHAPS) in comparable volumes. The benzodiazepine receptor was eluted from the Affigel overnight with1.0 mM Flurazepam in PIB with CHAPS at 4 ° C.

The next morning the Affigel was filtered and the filtrate dialyzed against 1 liter of PIB with CHAPS using an Amicon filtration apparatus (membrane size PM30). Following the dialysis, the receptor mixture was then concentrated to a final volume of 2-3 mls and stored at - 80 ° C for future use. The benzodiazepine coupled Affigel was washed with a generous volume of distilled water and stored for reuse in 70% ethanol at 4 ° C.

The partially purified benzodiazepine receptor preparation was further purified by gel exclusion chromatography. Sephadex G-200 resin (Pharmacia) was preswollen for 48 hours in distilled water, a 200 ml bed volume poured, and the column eluted with 2 column volumes of 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). A 2.0 ml sample of the purified benzodiazepine

receptor preparation (as described above) containing 1 mg/ml Dextran-2000 Blue was applied to the column and eluted with 5 mM HEPES at 4 ° C. Fractions of 1.0 ml were collected and 10 µl samples of each fraction analyzed by gel electrophoresis. Samples enriched in the benzodiazepine receptor (48.5 kD band) were pooled and lyophilized.

Anti-Benzodiazepine Receptor Antibody Screening by Solid Phase Radioimmunoassay

Initial screening of polyclonal and monoclonal antibody titers was carried out by a previously described solid phase radioimmunoassay (Kun et al, 1978). Briefly, 50 µl of receptor preparation at a concentration of approximately 1 µg/ml was passively absorbed to polyvinyl chloride microtiter wells (approximately 0.05 µg receptor protein). Unreacted sites were then blocked with a 5% solution of bovine serum albumin (BSA). Polyclonal serum or monoclonal supernatant was then incubated in wells for 24 hours at 4 ° C. After extensive washing, wells were then reacted at room temperature for 2 hours with ^{125}I-goat anti-mouse IgG having a specific activity of 500,000 cpm/ml. Following extensive washing, wells were then counted in a Beckman Model 5000 gamma counter.

Production of Monoclonal Antibodies

Monoclonal antibodies to the benzodiazepine receptor were produced using the methods originally described by Kohler & Milstein (1975) with modifications (Kohler, 1980). Female BALB/c

mice, ages 18-20 weeks, were injected intraperitoneally with purified benzodiazepine receptor preparation according to the following schedule: Week 1, 75 µg receptor protein in 0.5 mls Freund's complete adjuvant; Week 2, 50 µg receptor protein in 0.5 mls Freund's incomplete adjuvant; Week 3, 50 µg receptor protein in 250 µl 25 mM potassium phosphate buffer, pH 7.3. Mice were bled retroorbitally and the serum polyclonal antibody titer determined by solid phase radioimmunoassay as described above.

Two days after final injection, mice with the highest polyclonal titers were sacrificed and spleens dissected under sterile technique. Splenic lymphocytes from a single mouse were suspended in 10 mls Hank's Balanced Salt solution (Gibco) and pelletted. One confluent 75 cm^2 tissue culture flask of the non-secreting myeloma cell line, AG6-53 (obtained from Dr. E. Hawrot, Yale), was pelleted, washed, and recentrifuged in an identical fashion. Cells were then co-suspended in 20 mls of Hank's ,pelleted, and the supernatant discarded. Cells were resuspended in a minimal volume of Hank's by gentle agitation and 1.0 ml of 50% polyethylene glycol (Sigma Chemical Company, 3000-3700) added dropwise over 1 minute with gentle swirling. In a similar fashion, a total volume of 20 mls of high glucose formula Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal calf serum, 50 µM hypoxanthine, 0.2 µM aminopterin, and 8 µM thymidine (Sigma Chemical Company) was added. Cell suspensions were then aliquoted dropwise in 96 well microtiter plates from which hybridoma lines were selected. Clones testing positive to receptor preparation by solid phase radioimmunoassay were expanded in 75 cm^2 tissue culture flasks using high glucose

DMEM supplemented with 10% fetal calf serum and 50 μM hypoxanthine and 8 μM thymidine.

High titer ascitic fluid was produced by passing positive hybridoma cell lines into syngeneic mice by intraperitoneal injection. Mice were initially primed with intraperitoneal injection of 0.5 ml of pristane five days before injection of approximately 4 x 10^6 cells in 1.0 ml serum free medium. Ascitic fluid obtained by paracentesis was stored at -80 ° C.

Gel Electrophoresis

Separation of receptor proteins for molecular weight determination, analysis by immunoblot, 2-dimensional electrophoretic analysis, and limited proteolytic digestion was attained utilizing a discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel system as described by Laemmli (1970). Routinely, 7.5% acrylamide separating gels were used for analysis of purified benzodiazepine receptor preparations and western blotting, whereas 15% gels were required for resolution of peptide fragments derived from limited proteolysis. Prior to application on gels, samples were boiled for 5 minutes in 0.2 M Tris, pH 6.8, containing 2-mercaptoethanol, 10% glycerol, 2.3% SDS and 0.001 % bromphenol blue. Low molecular weight standards (Dalton VII-L, Sigma) were used to determine approximate molecular weights of receptor proteins and proteolytic fragments. Gels were stained in a solution of 0.25% Coomassie brilliant blue, 50% methanol, 10% acetic acid on an Eberbach clinical rotator. Subsequent destaining of gels was carried out with rotary shaking in a solution of 50% methanol, 10% acetic

acid. As needed for preservation or autoradiography, gels were dryed on chromatography paper (Whatman 3mmChr) at 80 ° C for 2 hours under vacuum using a slab gel dryer (Bio-Rad, Model 224). For autoradiography, dried gels were placed against Kodak X-omat film in an intensifying cassette for 72 hours at -80 ° C.

Screening of Monoclonal Antibodies by Western Blotting

Either washed membrane or purified receptor preparations were run on 7.5% polyacrylamide slab gels as described above. Separated proteins were then transferred to nitrocellulose paper by high-intensity transverse electrophoresis for 2 hours at 1.0 A in a buffer containing 25 mM Tris, 192 mM glycine and 20% (V/V) methanol (Towbin et al, 1979). Unreacted sites on the nitrocellulose paper were blocked by three 10-minute incubations in 10 mM phosphate buffered saline, 140 mM NaCl, pH 7.4 with 0.05% Tween (PBS/Tween). Primary incubation of ascitic fluid at a dilution of 1:1000 in PBS/Tween was performed overnight at 4 ° C. The nitrocellulose was then washed at room temperature with PBS/Tween to eliminate unreacted primary antibody. For the secondary incubation ^{125}I-sheep anti-mouse IgG was used at a final specific activity of 750,000 cpm/ml for 2 hours. This was succeeded by 3 more 10-minute incubations in PBS/Tween. The nitrocellulose was then allowed to air dry, placed against Kodak X-omat film and loaded in an intensifying cassette for up to 18 hours at -80 ° C.

Iodination of the Benzodiazepine Receptor

Iodination of the purified benzodiazepine receptor preparation was carried out by the method described by Bolton and Hunter (1973) with the following modifications: 250 μC ^{125}I-Bolton/Hunter reagent (^{125}I-BH, New England Nuclear) was gently concentrated to dryness using a dessicant-filtered N_2 stream (15 min.). Approximately 10 μg of lyophilized receptor protein in 25 μl of 100 mM Na_2HPO_4 , pH 8.0 was then added to the ^{125}I-BH vial and the reaction carried out for 30 min. at 0 ° C. Unreacted ^{125}I-BH was removed by adding 400 μl of 0.2 M glycine in 100 mM Na_2HPO_4 , pH 8.0 and allowing the reaction to proceed another 5 minutes. The reaction mixture was then chromatographed on a Sephadex G-50 (fine) column using a solution of 50 mM Na_2HPO_4 , pH 7.4 containing 0.25 % gelatin, 0.02 % NaN_3 , and 0.1 % SDS as eluant. Fractions of 200 μl were collected. Aliquots (5 μl) of each fraction were counted on a Beckman Model 5000 gamma counter. Radioactive fractions were then subjected to gel electrophoresis. and autoradiography, as described above. Fractions containing ^{125}I-labeled benzodiazepine receptor were then aliquoted into 100 μl volumes and stored at - 80 ° C.

Peptide Mapping by Limited Proteolysis

Peptide mapping of the benzodiazepine receptor was carried out by limited proteolysis as described by Cleveland, et al (1977) with the following modifications. This ^{125}I-BZR benzodiazepine receptor preparation was first subjected to gel electrophoresis on a 7.5% polyacrylamide gel as described above. Visualization by

coomassie blue staining was enabled by the addition of "cold" carrier receptor, as necessary. Protein bands were then cut from the slab gel and the gel slices washed with agitation for 30 minutes in 125 mM Tris-HCl, pH 6.8 containing 0.1% SDS (Tris buffer). Gel pieces were then applied to the wells of a long SDS-polyacrylamide gel (4.5 cm, 3% stacking gel; 11.5 cm, 15% separating gel). Gel slices were then overlayed with approximately 50 µl of Tris buffer containing 20% glycerol (V/V) and a trace of bromphenol blue. Staphylococcus aureus V8 protease (Sigma) was diluted in Tris buffer containing 10% glycerol (V/V) and a trace of pyronin Y. The desired amount of protease solution (0.001 - 0.1 µg) was layered on top and the protein subjected again to electrophoresis overnight at 60 V. Gels were dried and placed against Kodak X-omat film in an intensifying cassette for 72 hours at -80 ° C.

Two Dimensional Gel Electrophoresis

This method makes use of the substitution of a nonionic detergent for SDS, thus allowing isoelectric focusing in the first dimension before size separation in the second. Samples (20 µl) of ^{125}I-labeled benzodiazepine receptor were brought to a final volumn of 80 µl with a solution containing 9.0 M urea, 4% NP-40 (nonidet P-40, Sigma), 2% ampholines pH 3-10 (Biorad), and 5% ß-mercaptoethanol. The samples were then subjected to 2D electrophoresis according to the procedure described by O'Farrell (1975), and modified by Ames & Nikiado (1976); 7.5% acrylamide gels were used for the second dimension as described above.

Results

Receptor Purification & SDS-PAGE Analysis

Benzodiazepine receptor was initially purified from rat cortical membranes by affinity chromatography. Results of SDS electrophoretic analysis of this partially purified benzodiazepine receptor preparation are seen in Figure 1. Several protein bands ranging in molecular weight from 45 to 68 kD are visualized by coomassie blue staining. Of these, 48.5 kD and 68 kD bands represent the major protein constituents. In addition to these bands, proteins of molecular weights 50 kD, 54.5 kD, and 58 kD copurify by affinity chromatography. A fifth minor protein band weighing 45 kD represents the major phosphoprotein in this preparation and has been implicated to be the enzyme pyruvate dehydrogenase (Gallager, Sweetnam, and Tallman, in preparation). In addition, there exist other minor bands of higher (70-90 kd) and lower (<35 kD) molecular weights.

Gel filtration chromatography (G-200) of the partially purified benzodiazepine receptor preparation resulted in fractions of varying protein composition as evidenced by SDS gel electrophoresis (Figure 2). Fractions enhanced in the major protein bands (48.5 kD, 54.5 kD, 58 kD, and 67 kD) eluted early with the Blue Dextran front and were pooled for iodination and subsequent proteolytic analysis. Interestingly, the 50 kD protein eluted later and was pooled separately. Figure 3 illustrates the relative purification of the

receptor preparation with elimination of the 50 kD and lower molecular weight proteins.

Monoclonal Antibodies & Immunoblot Analysis

Balb-C mice were successfully immunized using the benzodiazepine receptor preparation as indicated by solid phase RIA (Figure 4). Three animals with the highest polyclonal titers were sacrificed for splenic lymphocytes and cell lines established as described in Materials & Methods. Following 7-10 days in culture, solid phase RIA demonstrated antibody positive cell lines with counts of 800-1500, whereas supernatants from lines not producing anti-benzodiazepine receptor yielded background values of 400-500. Positive lines were retested and then expanded in tissue culture flasks. In total, 6 cell lines secreting anti-benzodiazepine receptor antibodies were passed into mice and high titer ascitic fluid prepared. Figure 5 shows the solid phase RIA data of diluted ascites against a limited receptor concentration (upper limit of assay approximately 1500 cpm). Ascitic fluid tested positive in all cases and at dilutions of 1:10,000 in the case of some cell lines (e.g. E_9, D_5, and B_2).

Following initial screening by solid phase RIA, specificity of antibodies was determined by Western blot analysis. Immunoblots of ascitic fluid demonstrated two distinct binding patterns, as characterized by the cell lines E_9 and H_{10}. As seen in Figure 6, E_9 primarily labels a protein of molecular weight 48 kD - 50 kD. Ascites from cell line H_{10}, in addition to labeling the protein band at 48 kD - 50 kD, demonstrated recognition of a band of 55 kD. Both antibodies

appeared to demonstrate minor labeling of a protein of molecular weight 45 kD. Monoclonal antibodies from cell lines B_2 and D_5 shared binding patterns similar to E_9, whereas A_2 duplicated immunoblots seen with H_{10}. Western blot analysis in the ensuing data is comprised of the two representative cell lines E_9 and H_{10}.

Iodination of the BZR

Purified benzodiazepine receptor obtained by affinity and gel exclusion chromatography methods served as substrate for iodination. Following conjugation of ^{125}I-Bolton/Hunter (^{125}I-BH) reagent to 10 μg of the reconstituted receptor lyophilizate, the iodination mixture was subjected to Sephadex G-50 chromatography with the elution of 2 major radioactive peaks (figure 7). Analysis of the fractions by SDS acrylamide electrophoresis and autoradiography (Figure 8) demonstrated the first of these to represent the ^{125}I-BH-labeled receptor preparation (^{125}I-BZR). Electrophoresis and autoradiography of fractions comprising the second, larger radioactive peak failed to demonstrate any protein bands. It is most likely that this peak represents ^{125}I-BH-conjugated glycine. Radioactivity incorporated into the benzodiazepine receptor fraction represented less than 5% of the starting 250 μCi total.

Limited Proteolytic Map of the 125I-BZR

Iodination of the receptor preparation yielded 4 major radioactive bands in the region of the suggested receptor subunits. These corresponded to the 48.5kD, 54.5 kD, 58 kD and the 68 kD bands. These bands were subjected to limited proteolytic

degradation using increasing concentrations of Staph. aureus V8 protease (SAP). A sufficient concentration range of protease was employed to digest major bands completely into (SAP) limiting peptide fragments. Conditions employed, nevertheless, utilized small amounts of SAP, as all bands seemed sensitive to the protease (optimum range of 0.001 - 0.03 μg). Proteolytic fingerprints generated for each of the major bands are illustrated in Figures 9 and 10.

Once the conditions for limited proteolysis were optimized, a second digest experiment was conducted in an attempt to compare proteolytic fingerprints. In this experiment, each of the individual protein bands were run in adjacent lanes and subjected to enzymatic degradation with an identical amount SAP (0.001 and 0.01 μg). Comparison of neighboring proteolytic fingerprints was thus facilitated (Figure 11).

2-Dimensional Gel Analysis

The iodinated benzodiazepine receptor preparation was subjected to two dimensional electrophoresis as demonstrated in Figure 12. The experiments were performed in quadruplicate, in the presence and absence of molecular weight/isoelectric standards. In addition, ^{125}I-labeled benzodiazepine receptor preparation was run separately in the second dimension for reference. Results indicated that the four major protein bands (48.5 kD, 54.5 kD, 58 kD, and 67 kD) focused within the experimental pH range of 7.0 - 4.5. Isoelectric focussing revealed the 54.5 kD and 58 kD bands to be comprised of more than one protein constituent The two 58 kD

constituents demonstrated nearly identical pI's to those of two 54.5 kD proteins (approximately 6.0). In addition, isoelectric focussing of the 54.5 kD band reveal it to be comprised of a larger, slightly more basic protein having an apparent pI of 6.5 - 7.0. 67 kD and 48.5 kD bands were slightly more acidic, with pI's of 4.7 and 4.8, respectively. Apparent molecular weights and isoelectric points of all bands were unchanged by the presence of the protein standards.

Electrophoretic analysis in two dimensions enabled comparison of the iodinated benzodiazepine receptor preparation to receptor preparation subjected to coomassie blue staining and E_9 immunoblotting, as well as to rat cortical membranes subjected to photolabeling with ^{3}H-Flunitrazepam (Figures 13 & 14). The apparent molecular weight and isoelectric point of the 48.5 kD band were similar to those of the immunoreactive and photolabeled protein. These were in agreement as well with the coomassie blue stained major protein.

Discussion

In recent years, extensive investigation into the $GABA_a$ receptor has lead to a fuller understanding of its molecular structure and function. It has become increasingly clear that GABA-ergic transmission occurs when GABA binds to a post-synaptic receptor, resulting in increased chloride flux through an associated protein channel. The 1,4- benzodiazepines bind directly to this complex, increase the likelyhood of channel opening in response to GABA binding (Choi et al, 1981), and thus, serve to enhance GABA-ergic activity. Binding has been shown to be rapid, reversible, and saturable, as well as to be influenced by the presence of GABA. The complex has thus been referred to as the $GABA_a$/benzodiazepine receptor. A variety of molecular approaches, including affinity purification, electrophoretic analysis, and photo-labeling experiments have been used in an attempt to elucidate the proteins associated with this complex. Such approaches have revealed two different benzodiazepine binding proteins associated with the $GABA_a$ receptor having molecular weights of 50 and 55 kD, respectively. Further studies using antibodies specific for these proteins have lead to their respective description as the alpha and beta subunits of the $GABA_a$/benzodiazepine receptor complex. The relationship between these proteins remains to be clarified.

In this paper, aspects of the molecular structure, including the putative heterogeneity of the benzodiazepine receptor were studied. Various methods of investigation in addition to those above were employed, including proteolytic mapping, and molecular studies

using monoclonal antibodies. Experimental results using these combined modalities suggest, rather, that some structural similarity among receptor subunits may exist.

Initial detergent extraction with Lubrol PX has been previously shown to differentially solubilize the "central" or clonazapam specific/GABA-enhanced benzodiazepine binding site from a "peripheral" receptor (defined by high affinity binding of the benzodiazepine analog RO5-4864) (Yosufi et al, 1979). This partial purification results in an 800-1000 fold increase in benzodiazepine binding activity over crude homogenates. This activity is characterized by high-affinity (<10 nM) binding sites for both benzodiazepine agonists (^{3}H-Flunitrazepam) and antagonists (RO15-1788), as well as both high and low affinity sites for GABA. In addition to co-purification of the benzodiazepine and GABA sites, enhancement of benzodiazepine binding by GABA in such preparations has been demonstrated as well, indicating preservation of allosteric properties of the complex.

Analysis of this partially purified benzodiazepine receptor preparation by SDS gel electrophoresis and coomassie blue staining reveals the presence of multiple protein bands (48.5 kD, 50 kD, 54.5 kD, 58 kD, and 68 kD). The major band in this preparation, 48.5 kD, has been shown to co-migrate on polyacrylamide gels with the major photo-labeled species (data not shown), and is thought to represent the alpha subunit of the $GABA_a$ receptor. This was confirmed by analysis of the receptor preparation using 2-dimensional electrophoresis. This technique enables resolution of proteins based not only on molecular weight, but also on the basis of the protein's

isoelectric point (pI). Autoradiography of the ^{125}I-labeled benzodiazepine receptor preparation demonstrated a spot with an apparent molecular weight of 48.5 kD and a pI of approximately 4.8. This coincided with findings demonstrating co-localization in 2-dimensions of both the coomassie blue stained protein and the photo-labeled receptor (M_r=48.5 kD, pI approximately 4.8). Barnard (1984) has previously reported the pI of the GABA/benzodiazepine receptor complex as 5.6, though isoeletric focusing of individual subunits has yet to be reported. In the literature, there remains a variety of apparent molecular weights described as representing the major photo-labeled moiety ranging from 48.5 - 53 kD (Sigel et al, 1983; Kuriyama et al, 1984; Sigel & Barnard, 1984; Mohler et al, 1984; Fischer & Olsen, 1986). Part of this variability may be explained by the different species studied (e.g. rat, bovine), or may represent differences in electrophoretic techniques, including the composition of gels (percentage of acrylamide) and electrophoresis time. Two other bands of apparent molecular weight 55 kD and 47 kD incorporate relatively minor amounts (<5%) of ^{3}H-flunitrazepam under optimal conditions for photo-activation (Sweetnam, Nestler, & Tallman, in preparation). These were detectable only by gel slice techniques and were not visualized by fluorography. Sieghart & Karobath (1980) have reported receptor heterogeneity for the benzodiazepines by covalent labeling of bands with apparent molecular weights of 50 kD, 53 kD, 55 kD, and 59 kD using the photo-activated nitrogen containing benzodiazepine, ^{3}H-flunitrazepam. No attempt to inhibit proteolysis was made in these experiments, and endogenous proteases shown to be present in membrane fractions

(Klotz et al, 1984) might account for this observed heterogeneity. Several protease inhibitors (benzamidine, soybean trypsin inhibitor, and PMSF) are employed in our purification protocol. For this reason it is unlikely that endogenous proteolytic activity contributes to our photo-labeling results. We routinely label only one major protein band in our preparation corresponding to the alpha subunit.

Monoclonal antibodies to the rat benzodiazepine receptor were raised in mice using the partially purified receptor preparation. Techniques using secondary antibody recognition suggest these antibodies to be of the IgM subclass (Sweetnam, personal communication). This would be consistent with the rapid immunization and harvesting schedule utilized in our protocol. As demonstrated by immunoblotting techniques to both purified receptor and whole brain homogenates, our monoclonal antibodies demonstrate specificity for the major receptor band of 48.5 kD (50 k band or alpha subunit). 2-Dimensional electrophoretic analysis has confirmed the specificity of monoclonal line E_9 for the alpha subunit. Western blots to 2-D gels using E_9 show recognition of a protein with apparent molecular weight of 48.5 kD and an isoelectric point of around 4.8 (as described above).

Several of the monoclonals, as typified by H_{10}, recognize a second higher molecular weight species of approximately 55 kD on 1-dimensional gels. Subsequent subcloning of these cell lines has confirmed that a single antibody is responsible for the recognition of both bands. The molecular basis for this dual recognition remains at the present uncertain. Gallombardo (in press) has shown H10 (and E9) capable of increasing the affinity of the membrane bound

receptor for ^{3}H-Flunitrazepam, ^{3}H-RO15-1788 and ^{3}H- muscimol. In light of these binding studies to membrane bound receptor, immunoblot data suggests a common cell surface determinant on both 48.5 kd and 55 k bands. However, the molecular basis for this shared epitope cannot be inferred from immunoblot data alone.

With the recent generation of monoclonal antibodies to the bovine benzodiazepine receptor, Mohler and colleagues have utilized similar techniques to explore the molecular structure of the benzodiazepine receptor (Haring et al, 1985). Results in the rat are identical to those obtained using the bovine benzodiazepine receptor. Immunoblot analysis revealed the existence of immunoreactive proteins of 50 and 55 kD in all brain regions examined.

As stated above, the existence of multiple, heterogeneous "central-type" benzodiazepine receptors has been postulated. This hypothesis was initially based on the pharmacology of the triazolopyridazine, CL218,872, in different brain regions (Squires et al, 1979; Klepner et al, 1979). Autoradiographic studies at the light microscopic level seemed to corroborate this finding (Young & Kuhar, 1980; Young et al, 1981). This agreement was not absolute, however, as CL218,872 binding in cerebellum suggested a single receptor subtype, whereas autoradiography demonstrated receptor heterogeneity (Niehoff et al, 1982). Several laboratories have reported the photolabeling of benzodiazepine receptor subtypes in the hippocampus and striatum (BZ_1 and BZ_2), whereas photo-labeling in cerebellum revealed only a single receptor subtype (BZ_1). Although such regional heterogeneity might offer a tenable explanation for the two major receptor subtypes seen, recent

evidence suggests that these subtypes may in fact be subunits of a single receptor species. Photo-labeling studies using the GABA agonist [^{3}H]muscimol demonstrate labeling of the beta subunit in bovine brain (Casalotti et al, 1986). Monoclonal antibodies to the bovine benzodiazepine receptor demonstrate the 55 kD protein in all brain regions studied, including the cerebellum (Haring et al, 1985). Furthermore, by immunoprecipitation with either of two monoclonal antibodies recognizing the different "receptor proteins", this group was able to demonstrate co-precipitation of the entire population of binding sites (benzodiazepine, as well as low and high affinity GABA) found in their purified preparations. These results were interpreted to suggest the existence of a homogenous population of $GABA_a$/benzodiazepine receptors comprised of two different receptor protein subunits (alpha and beta). Thus, multiple subunits of a large macromolecular complex, rather than receptor heterogeneity may account for the 50 and 55 kD bands.

As noted above, allosteric properties of the receptor complex remain intact in our partially purified preparation, implicating conformational integrity of the GABA/benzodiazepine receptor complex. With this in mind, attempts to further purify the receptor complex on the basis of its macromolecular size were pursued. Gel exclusion chromatography on Sephadex G-200 enabled enhancement of the major bands (48.5 kD, 54.5 kD). Many bands of higher and lower molecular weights, as well as the 50 kD band, eluted later from the column. The early elution of the major bands with the blue dextran marker (M_r= 2000 kD) is consistent with the possibility of their association as a large macromolecular complex. Utilizing a

variety of approaches, investigators have implicated the GABA/benzodiazepine receptor complex to have an apparent molecular weight of 200 - 900 kD (as review by Fischer & Olsen, 1986). In particular, by methods of gel filtration chromatography, the native receptor has been estimated to have a molecular weight of 220 kD (Yosufi et al, 1979; Chang & Barnard, 1982; Martini et al, 1982; Fischer & Olsen, 1986). Thus, the exclusion size of the Sephadex G-200 would be expected to result in elution of the receptor complex with the void volume as seen with the 48.5 and 54.5 kD bands.

With the knowledge that the 48.5 kD and 54.5 kD proteins (alpha and beta subunits) shared a common immunoreactive epitope, it remained interesting to speculate about potential homology between these two proteins. Though much work in the field has focused on the apparent heterogeneity or multiplicity of benzodiazepine receptor proteins, few studies have sought to explore at a molecular level their relatedness. The question of homology was approached using methods of limited proteolytic degradation. In order to adequately visualize peptide fragments, the receptor was first iodinated and results determined by autoradiography. By subjecting the major protein bands in our highly purified receptor preparation to SAP digestion, it was possible to generate a proteolytic map or "fingerprint" of the receptor.

Results show that all of the major bands were susceptible to degradation by the V8 protease within the same concentration range (0.001 - 0.03 μg). The peptide fragments visualized by autoradiography may not represent the "complete" peptide map of

these proteins due to variable incorporation of the ^{125}I-BH based on amino acid composition. Nevertheless, limiting peptides characteristic of individual bands were generated. Most striking among these were those of the 48.5 and 54.5 kD bands. Although variations in their limited peptide maps exist, both proteins demonstrate 2 major limiting peptides of apparent molecular weights 21 and 23 kD. This reproducible peptide pattern may represent some degree of homology at the level of primary protein structure.

Interpretation of this data was supplemented by electrophoresis of the iodinated preparation in two dimensions. Isoelectric focusing of the 48.5 band demonstrated its homogeneity with a pI in the acidic range (approximately 4.8). The 54.5 band, however, appeared to be comprised of several distinct protein foci. The peptide map of this band, therefore, may represent the combined pattern of several, potentially unrelated proteins. Interestingly, both the 54.5 and 58 kD bands seem to share the protein spots with pI's of approximately 6.0. Whether these protein foci represent distinctly different proteins with differing primary structure is uncertain. Alternatively, their similar pI's might be explained by slight differences in carbohydrate content of an otherwise identical protein molecule. Cleavage of a small, neutral peptide, be it physiological or artifactual, might account for the molecular weight differences. In the peptide maps of the 54.5 and 58 kD bands, faint bands of identical molecular weight can be seen. Based on similar pI values and apparently shared proteolytic fragments, it remains interesting to speculate that these various foci represent the same primary protein. In addition to these proteins,

the 54.5 kD band is comprised of a larger, more basic (pI=6.5-7.0) protein. The more diffuse focusing of this protein may represent an artifact of the gradient at the more basic spectrum, or might again be explained by variable glycosylation of the same protein. Both 58 and 68 kD proteins demonstrated unique peptide maps, distinct from either of the two lower molecular weight bands. The shared limiting peptides of the 48.5 and 54.5 kD bands may thus be the result of homology between the more basic 54.5 kD protein (pI=6.5-7.0) and the 48.5 kD band. Immunoblot studies to two dimensional gels with the monoclonal antibody H_{10} may serve to clarify this relationship.

In tryptic digest experiments done by Eichinger & Sieghart (1985), they demonstrated differential degradation of ^{3}H-Flunitrazepam-labeled 51 and 55 kD proteins. They cite this differential degradation pattern as supportive evidence of different receptor subtypes. Analysis of their data shows that although tryptic degradation results in different major limiting peptides, tryptic digestion of the 51 kD protein generates minor bands of 42 and 45 kD. These appear identical to the major [^{3}H]Flunitrazepam-labeled limiting peptide fragments (42 and 45 kD) characteristic of the 55 kD band. Thus, though interpreted to implicate receptor heterogeneity, an argument for structural homology between the same two proteins can be made. Because the fragments generated in their experiments represent photo-labeled peptides, such results would implicate homology at the benzodiazepine binding portion of the receptor. At present, it is not possible from our data to ascribe homology to a given receptor domain. Proteolytic experiments utilizing photo-labeled receptor remain to be done. In addition, immunoblot

experiments using our monoclonal antibodies against these proteolytic maps may enable identification of peptide epitopes, either that specific for the 48.5 or that shared by both the 48.5 and 55 kD proteins. Until the various receptor bands are cloned and their sequences defined, such lines of investigation may help to elucidate the relatedness of these various proteins and their relationship to the $GABA_a$ /benzodiazepine receptor.

In addition to the 48.5 and 54.5 kD proteins discussed above, several other proteins are observed in our partially purified receptor preparation (50 kD, 58 kD and 68 kD). The identity of these bands is uncertain, although they remain unrecognized by our monoclonal antibodies, and the two larger proteins demonstrate unique peptide maps different from either the 48.5 or the 54.5 kD bands. Although these protein bands may be regarded as contaminants, other possible explanations for their co-purification exist. Several proteins may in fact co-purify by our affinity chromatography methods based on their ability to bind benzodiazepines. Even proteins with low affinities for the benzodiazepines, if present in large amounts in tissue, might be expected to represent a significant constituent of the partially purified preparation. Albumin, for example, is known to bind numerous drugs, including the benzodiazepines (Fehske et al, 1979). In addition, other "peripheral" receptors for the benzodiazepines have described, including a low affinity (μM) Ca^{2+}-Calmodulin kinase associated receptor (Bowling & DeLorenzo, 1982).

A second, and as yet unexplored possibility, is that various bands in our preparation represent different stages in the post-translational modification of the receptor. It has been demonstrated

that the receptor is a glycoprotein (Gavish & Snyder, 1981; Stephenson & Olsen, 1983; Sweetnam & Tallman, 1986). Glycosylated versus non-glycosylated, as well as "pre-" and "pre/pro-" forms of the receptor might account for observed "heterogeneity". In addition, multiple allelic variants of the benzodiazepine receptor within the genome may exist. Such genetic variation in enzymes ("isozymes") is a well documented phenomenon in both physiologic and pathophysiologic states in the human. Though no example of such an "iso-receptor" has been demonstrated to date, allelic variants of the benzodiazepine receptor would have significant implications for many neurologic and psychiatric disorders.

Legends to Figures

Figure 1. Partially purified benzodiazepine receptor preparation (Bzr) was run under standard 7.5% SDS-PAGE conditions and visualized by coomassie blue staining as described in "Materials & Methods." A major protein band of 48.5 kD is seen, as well as other minor bands (50, 54.5, 58, and 68 kD). A reference lane of low molecular weight standards (Std) is shown, as well.

Figure 2. The partially purified benzodiazepine receptor preparation was subjected to gel exclusion chromatography as described in "Materials & Methods." Fractions were collected beginning with elution of the Dextran Blue front and were then subjected to standard 7.5% SDS-PAGE analysis. As seen here, fractions 1-8 elute with the Dextran Blue and are enhanced in the 48.5, 54.5, 58, and 68 kD bands. These fractions were pooled as Sample 1. The 50 kD band elutes later (fractions 9-20) and was pooled separately as Sample 2. Reference lanes containing low molecular weight standards (Std) and partially purified benzodiazepine receptor preparation (Bzr) are also shown.

Figure 3. Benzodiazepine receptor fractions collected following gel exclusion chromatography of the partially purified preparation were pooled as shown in Figure 2. The pooled fractions, Samples 1 and 2, were then subjected to standard 7.5% SDS-PAGE and coomassie blue staining as described in "Materials & Methods." Sample 1 shows relative enhancement of the 48.5, 54.5, 58, and 68 kD bands and was

saved for iodination and subsequent limited proteolysis. Sample 2 is contains the 50 kD, as well as other higher and lower molecular weight bands separated by gel exclusion purification. Reference lanes containing low molecular weight standards (Std) and partially purified benzodiazepine receptor preparation (Bzr) are also shown.

Figure 4. Balb-C mice were immunized using the partially purified benzodiazepine receptor preparation. Sera obtained from each mouse before, following primary, and following secondary immunizations was subjected to solid phase radioimmunoassay as outlined in "Materials & Methods." As shown here, immunization resulted in increasing titers of polyclonal sera following each step. Mice with the highest polyclonal titers were then identified for generation of hybridoma cell lines.

Figure 5. High titer ascites from 6 murine monoclonal cell lines was tested by solid phase radioimmunoassay as described in "Materials & Methods." Results here show significant activity at dilutions of 1:1000 to 1:10,000. Monoclonals E_9 and H_{10} demonstrate considerable activity at higher dilutions (1:10,000) and were used for subsequent immunoblot experiments.

Figure 6. Partially purified benzodiazepine receptor preparation was subjected to SDS-PAGE and subsequent transverse electrophoresis onto nitrocellulose as described in "Material & Methods." Immunoblots to the nitrocellulose fixed receptor using E_9, H_{10}, and control ascites at dilutions of 1:1000 are shown. Low

molecular weight standards (Std) and receptor preparation (Bzr) subjected to SDS-PAGE and coomassie blue staining are included for reference. Two immunoreactive proteins with apparent molecular weights of 48.5 and 54.5 kD can be seen. Antibody produced by the monoclonal cell line E_9 is specific for the 48.5 kD or alpha subunit. $H_{10,}$ however, demonstrates cross-reactivity between both the 48.5 (alpha) and 54.5 kD (beta subunit) bands. Subsequent subcloning has shown a single antibody to be responsible for this dual recognition.

Figure 7. Purified benzodiazepine receptor obtained by affinity and gel exclusion chromatography methods was subjected to iodination as described in "Materials & Methods." The profile of radioactivity eluted from the Sephadex G-50 column is shown here. Two radioactive peaks can be seen to elute from the column. The minor peak appearing with the void volume represents the iodinated receptor (^{125}I-BZR) as demonstrated in Figure 8. The second, major peak most likely represents Bolton/Hunter reagent coupled to glycine.

Figure 8. Following elution of the iodinated benzodiazepine receptor from the Sephadex G-50 column, individual fractions were sampled (5µl) and analyzed by 7.5% SDS-PAGE and autoradiography as described in "Materials & Methods." The autoradiogram demonstrates the iodinated receptor (^{125}I-BZR) in fractions 2-5, the first peak seen in Figure 2.

Figure 9. The iodinated benzodiazepine receptor was run under 7.5% SDS-PAGE conditions, 58 kD and 54.5 kD bands cut from the gel, and the gel slices subjected to limited proteolysis on 15% SDS-PAG's as described in "Materials & Methods." The autoradiogram pictures the individual bands in the absence of staph. aureus V8 protease (SAP) in lanes 1. Lanes 2-5 demonstrate increasing degradation of the original protein bands into limiting peptide fragments in the presence of 0.001, .003, 0.01, and 0.03 μg of SAP, respectively. The iodinated receptor is included for reference. In addition, arrows indicate appropriate positions of the low molecular weight standards.

Figure 10. The iodinated benzodiazepine receptor was run under 7.5% SDS-PAGE conditions, 68 kD and 48.5 kD bands cut from the gel, and the gel slices subjected to limited proteolysis on 15% SDS-PAG's as described in "Materials & Methods." The autoradiogram pictures the individual bands in the absence of staph. aureus V8 protease (SAP) in lanes 1. Lanes 2-5 demonstrate increasing degradation of the original protein bands into limiting peptide fragments in the presence of 0.001, .003, 0.01, and 0.03 μg of SAP, respectively. The iodinated receptor is included for reference. In addition, arrows indicate appropriate positions of the low molecular weight standards.

Figure 11. Limited proteolysis of the 68, 58, 54.5, and 48.5 kD bands was conducted in parallel using identical SAP conditions as described in "Materials & Methods." As seen from left to right, the 68, 58, 54.5, and 48.5 kD bands were run in the absence (1), with 0.001 μg (2), and with 0.01 μg (3) of SAP. Comparison of the

neighboring peptide maps demonstrate the presence of two shared limiting peptides (21 and 23 kD) by the 48.5 and 54.5 kD bands. A sharply focussed U-shaped band positioned between the 21 and 23 kD peptide fragments of the 54.5 kD band represents artifact (see figure 9 for comparison).

Figure 12. Utilizing 2D gel analysis as described in "Materials & Methods" the iodinated benzodiazepine receptor preparation was examined. The major 48.5 kD protein appears homogeneous in composition and has a pI value of approximately 4.8. The 68 kD band appears as a single spot at around pH 4.7. Both the 54.5 and 58 kD bands can be seen to resolve into multiple spots with different pI's. Several of these with pI's of approximately 6.0 appear to be duplicated in both 54.5 and 58 kD bands. In addition, the 54.5 kD band is composed of a more basic protein spot having a pI of 6.5-7.0.

Figures 13 & 14. In parallel experiments, whole photo-labeled membranes were subjected to 2D electrophoresis on three sister gels as described in "Materials & Methods." The first was stained for total protein by coomassie blue (A): this revealed proteins to be generally acidic (pI's 4.5-7.0). The second gel was subjected to autoradiography (B) and the third was immunoblotted with E_9 ascites. These gels reveal a single major protein spot with pI's identical to the 48.5 kD protein of the iodinated receptor preparation (Figure 12) and the partially purified receptor preparation as visualized by coomassie blue staining (D).

Figure 1

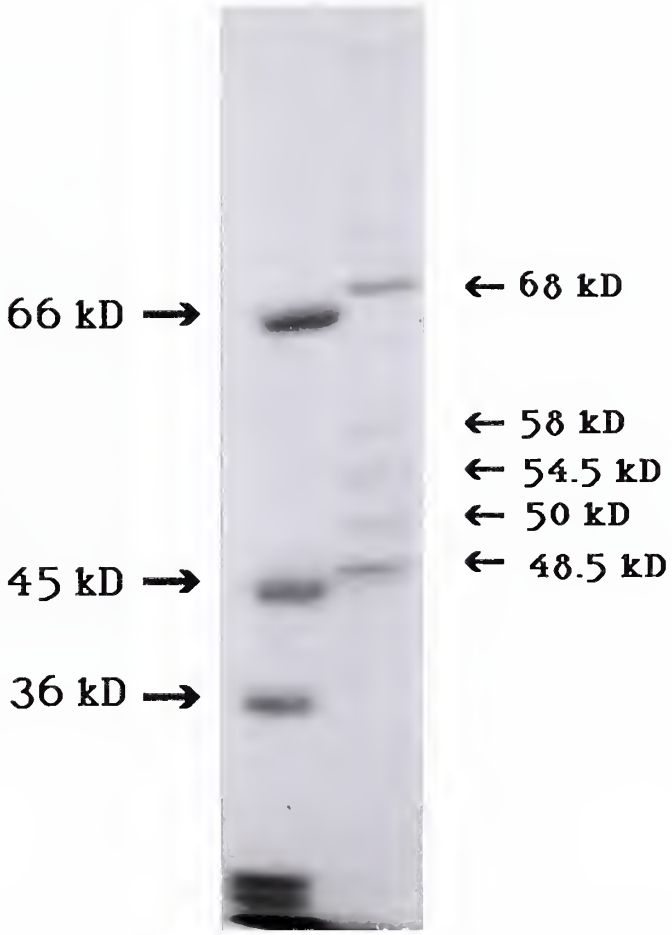


Figure 2


66 kD →
45 kD →
36 kD →
← 68 kD
← 58 kD
← 55 kD
← 50 kD
← 48.5 kD
Std
Bzr
1 2 3 4 5 6 7 8 9 10 11
1
2 →

Figure 3

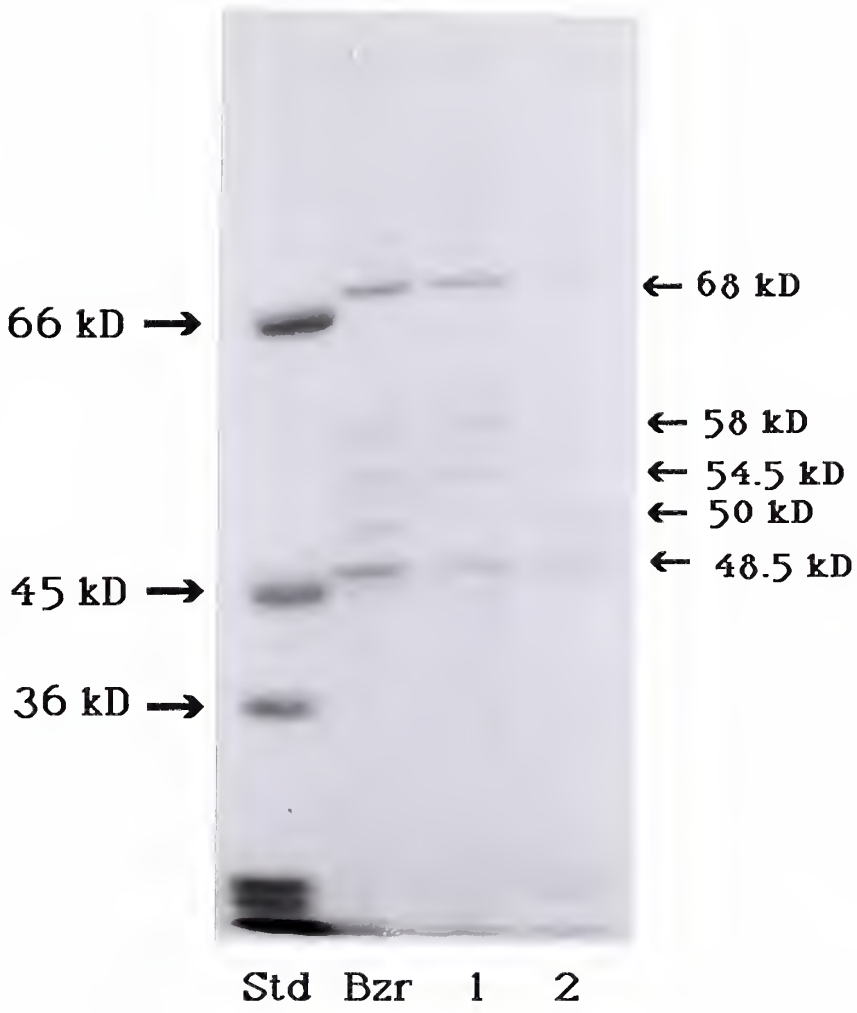


Figure 4

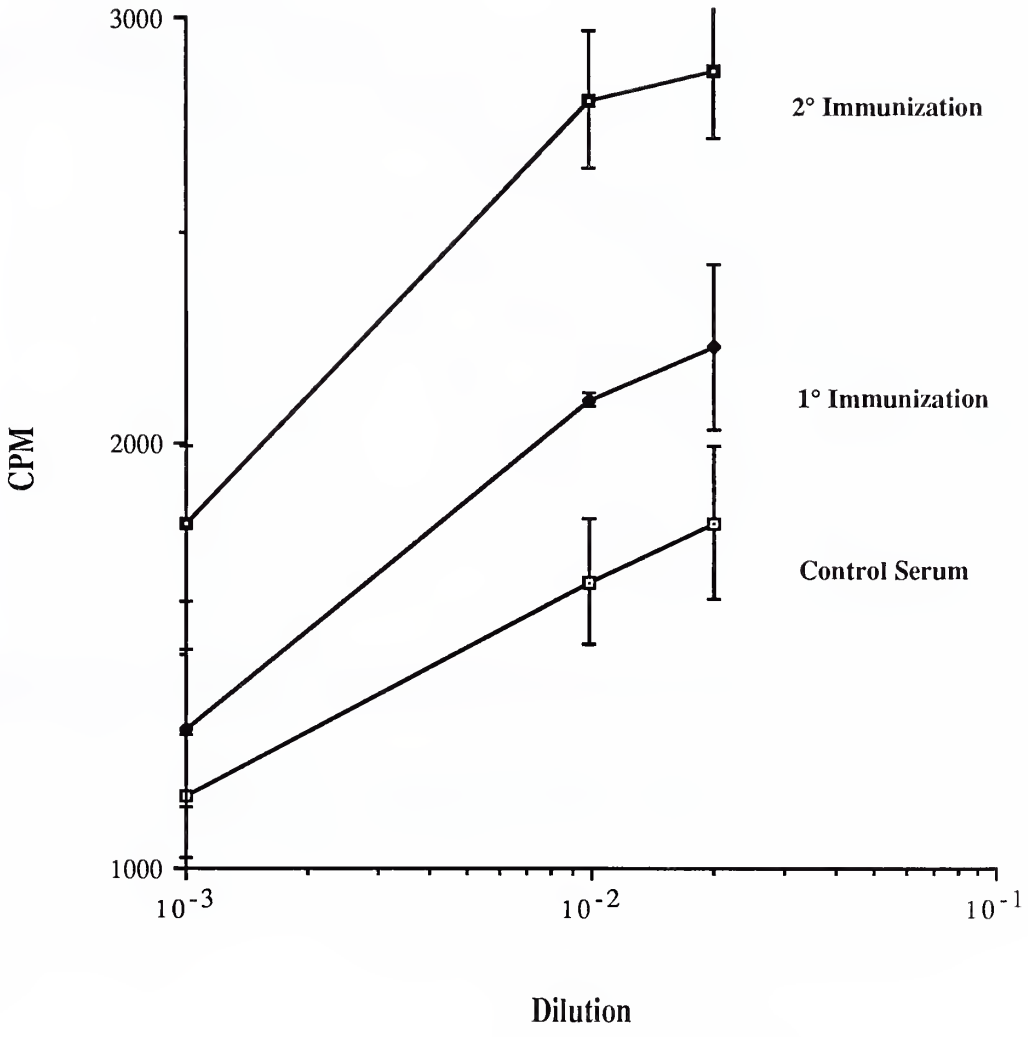

3000
2000
1000
CPM
2° Immunization
1° Immunization
Control Serum
10^{-3}
10^{-2}
10^{-1}
Dilution

Figure 5

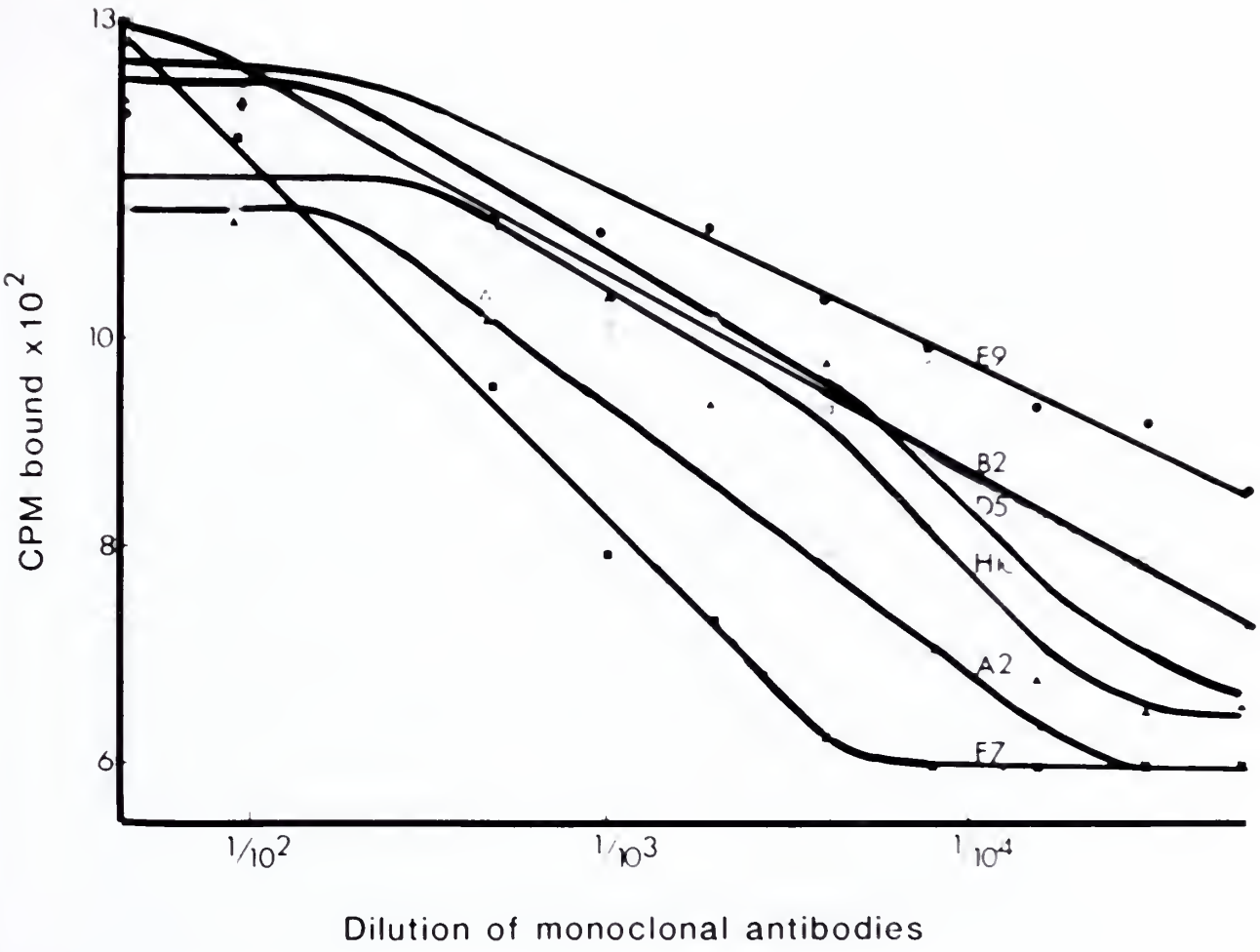

13
10
8
6
CPM bound x 10^2
E9
B2
D5
A2
E7
1/10^2
1/10^3
Dilution of monoclonal antibodies

Figure 6

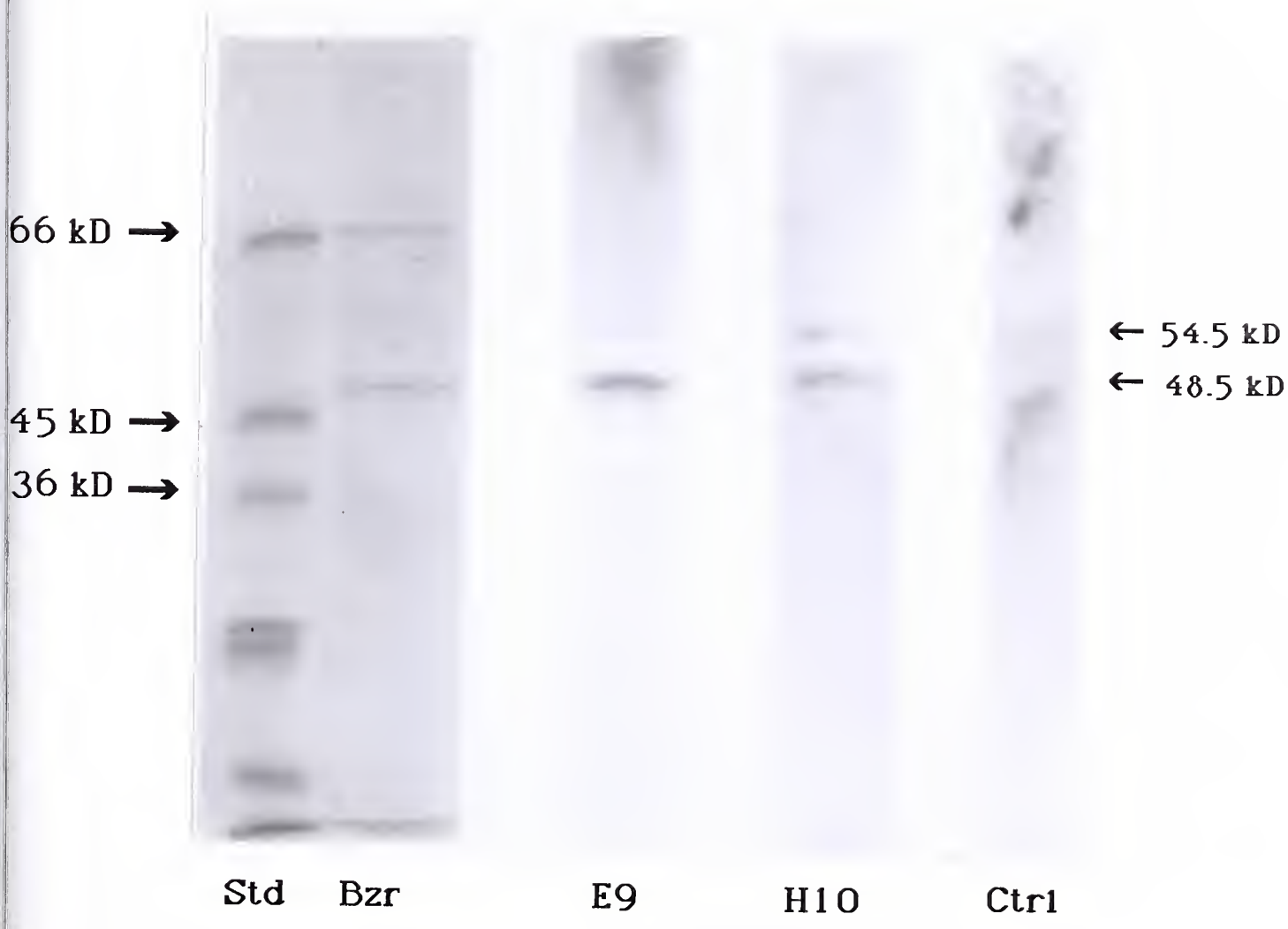


Figure 7

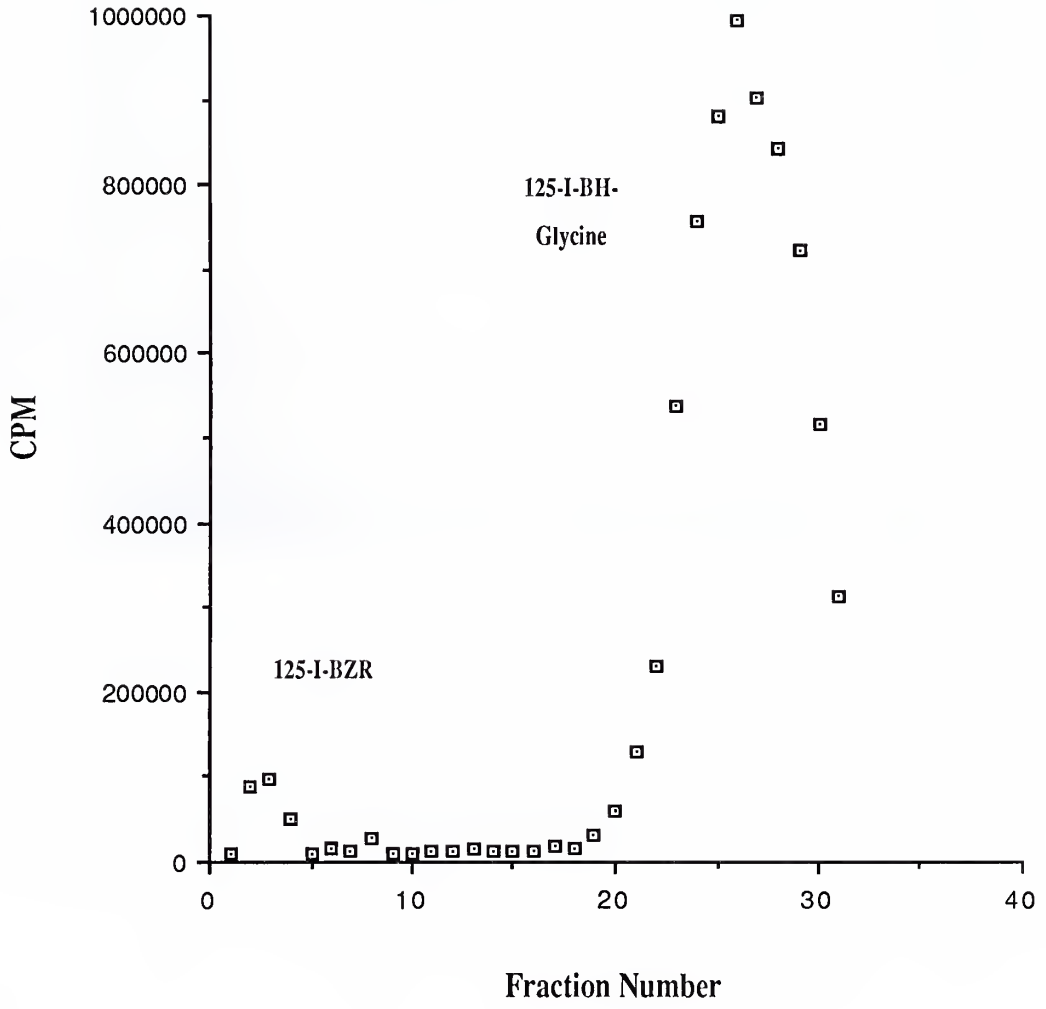

1000000
800000
600000
400000
200000
0
CPM
125-I-BH-
Glycine
125-I-BZR
0
10
20
30
40
Fraction Number

Figure 8

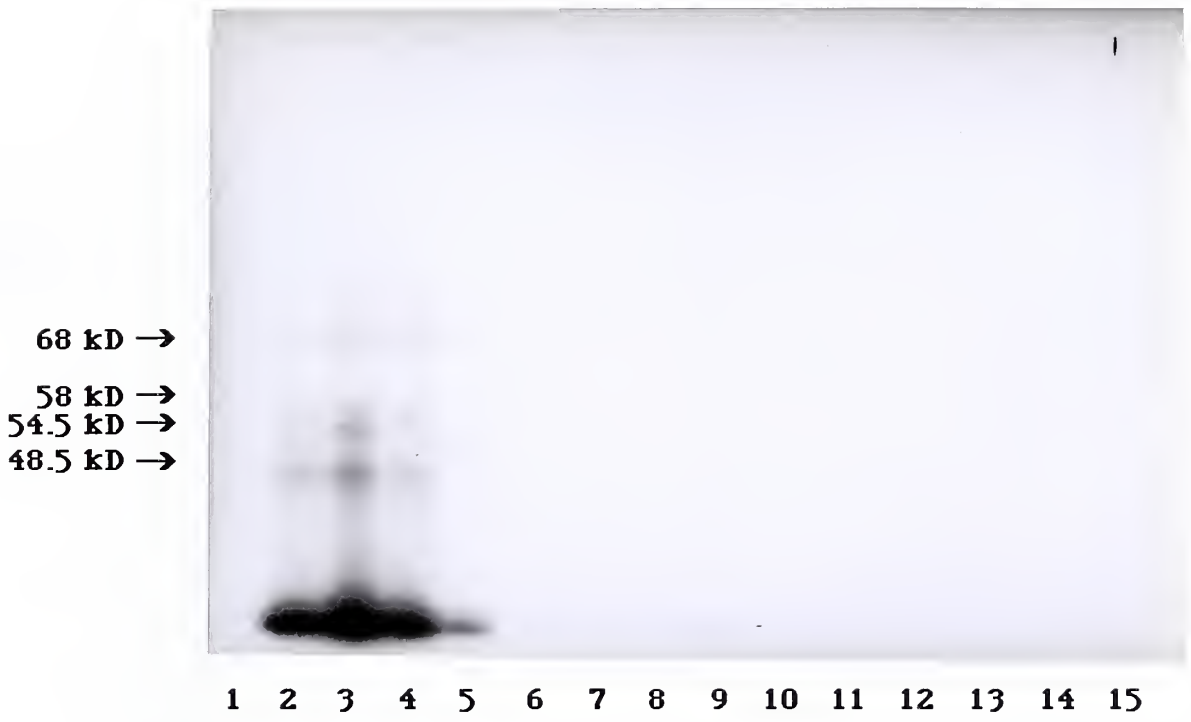


Figure 9

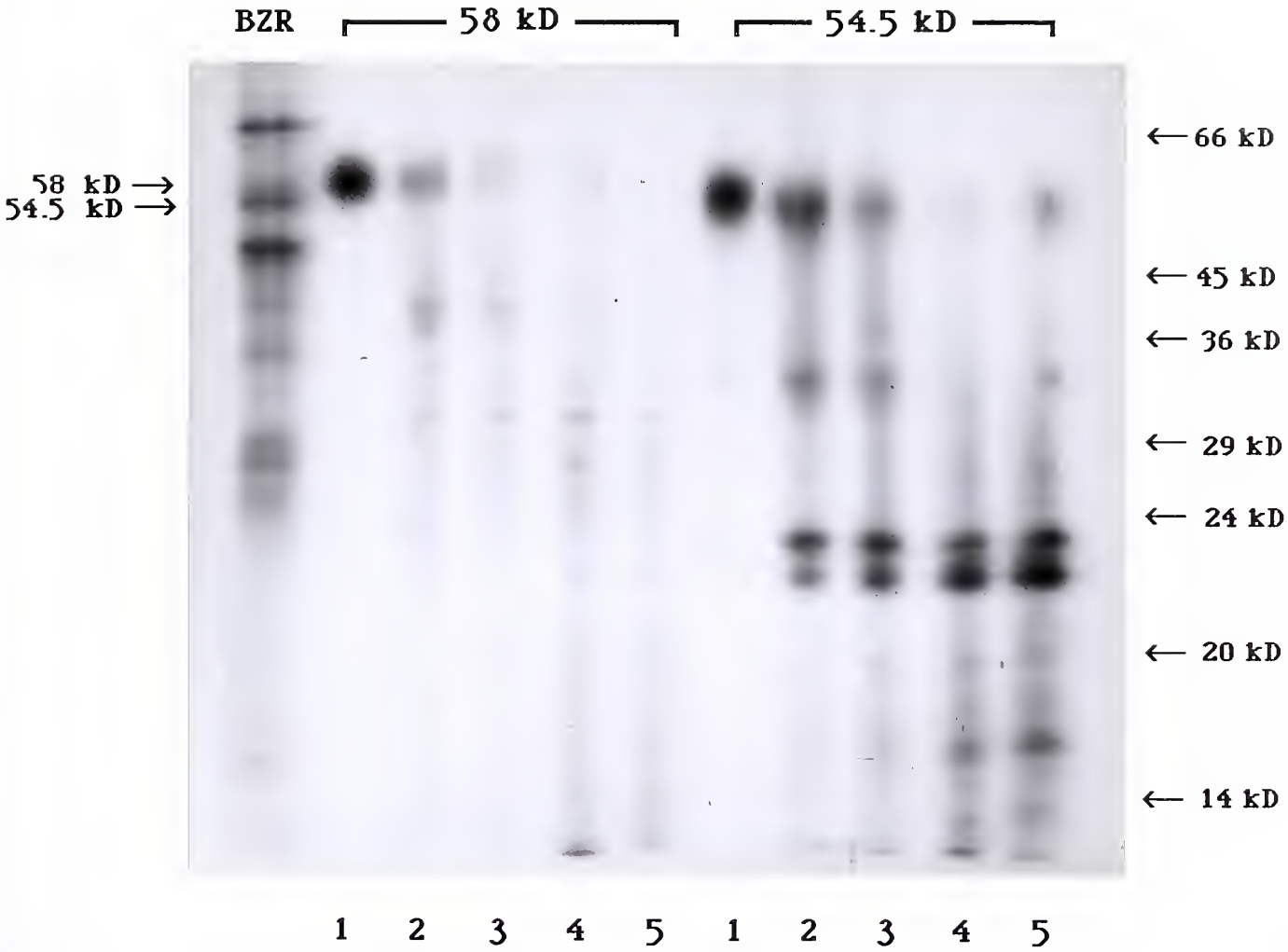

BZR
58 kD
54.5 kD
58 kD →
54.5 kD →
← 66 kD
← 45 kD
← 36 kD
← 29 kD
← 24 kD
← 20 kD
← 14 kD
1 2 3 4 5 1 2 3 4 5

Figure 10

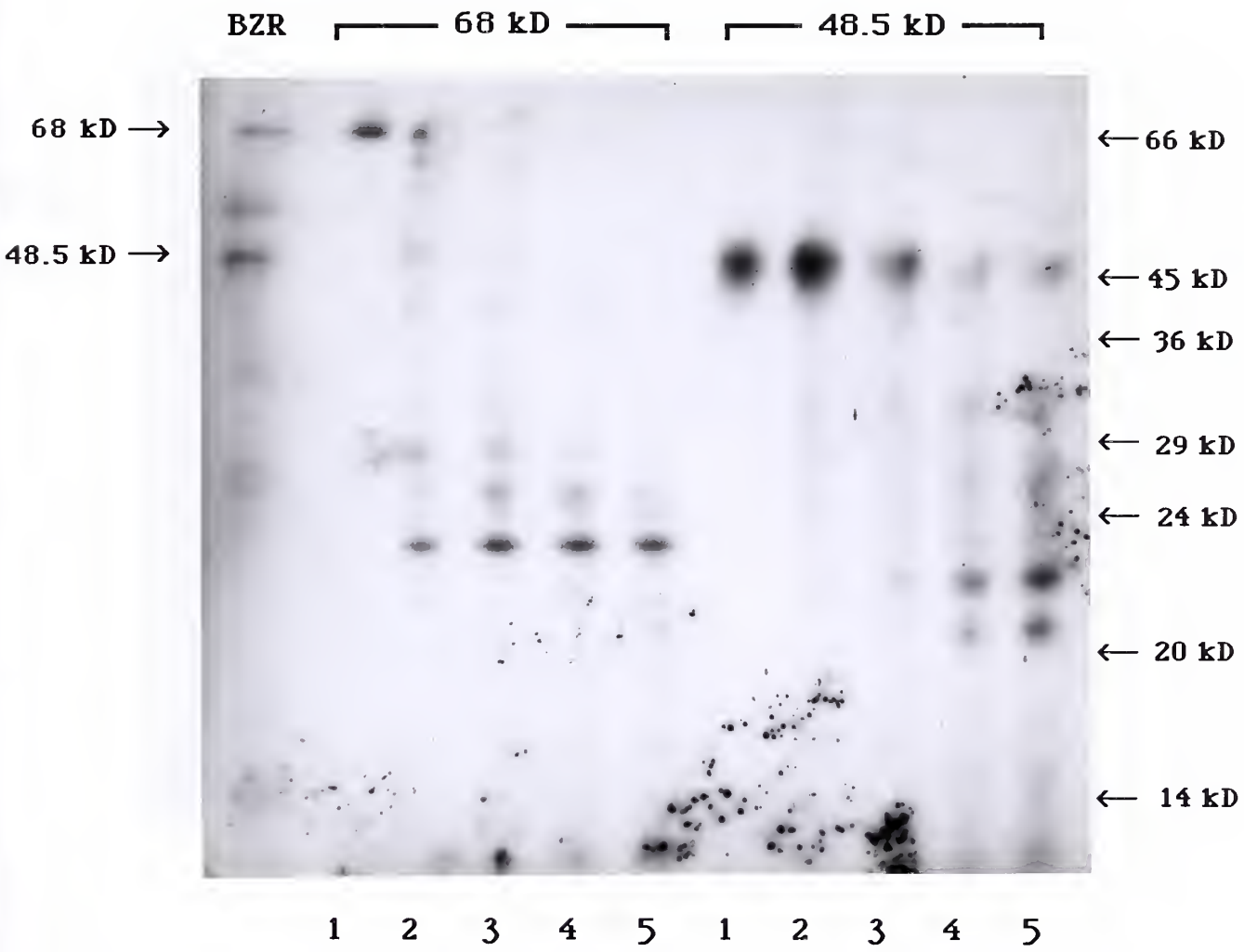
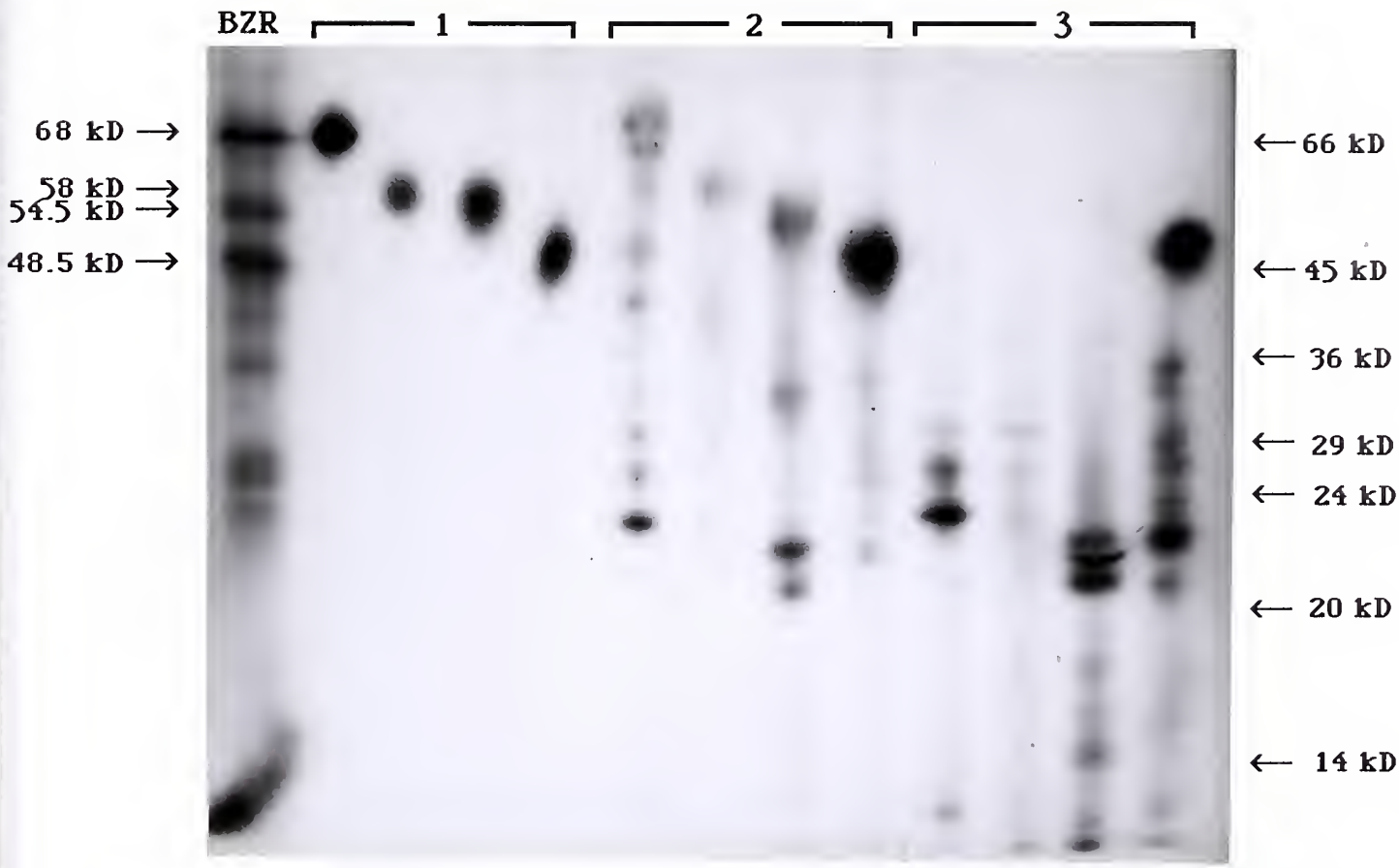

BZR
68 kD
48.5 kD
68 kD →
48.5 kD →
← 66 kD
← 45 kD
← 36 kD
← 29 kD
← 24 kD
← 20 kD
← 14 kD
1 2 3 4 5 1 2 3 4 5

Figure 11


BZR
1
2
3
68 kD →
58 kD →
54.5 kD →
48.5 kD →
← 66 kD
← 45 kD
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← 29 kD
← 24 kD
← 20 kD
← 14 kD

Figure 12

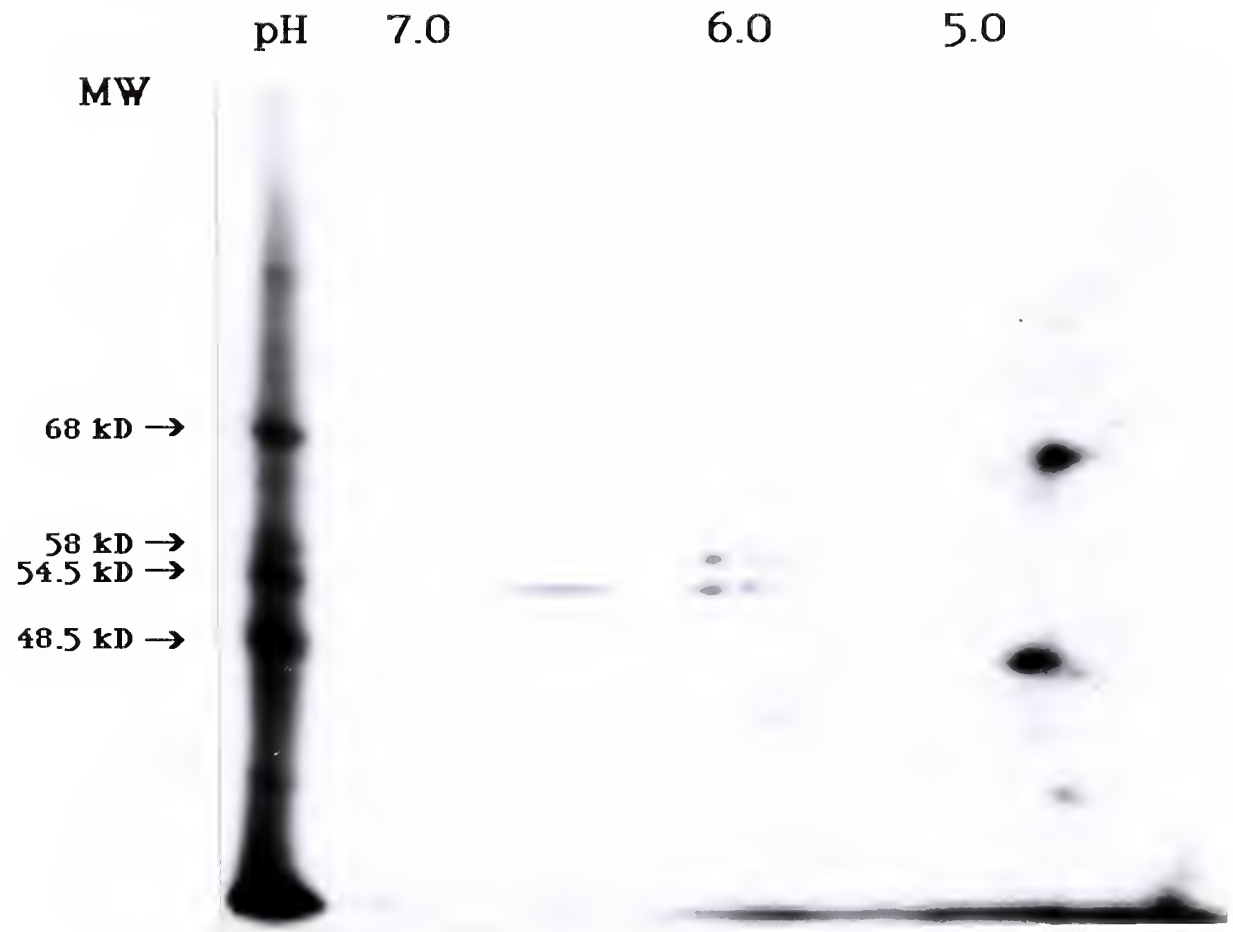
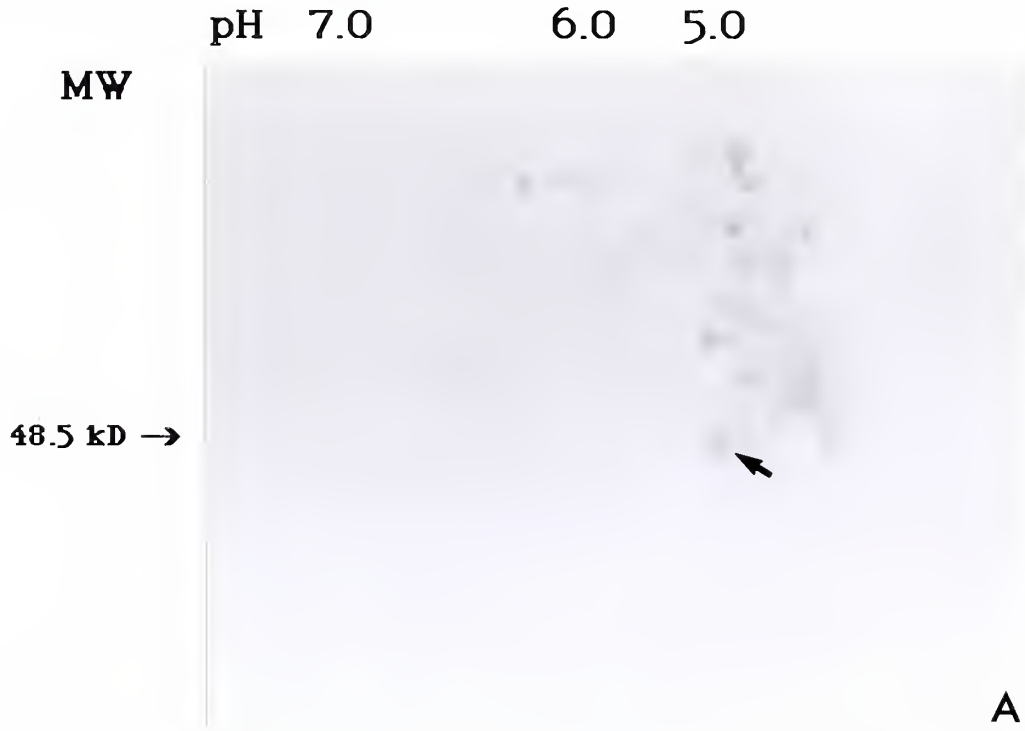


Figure 13



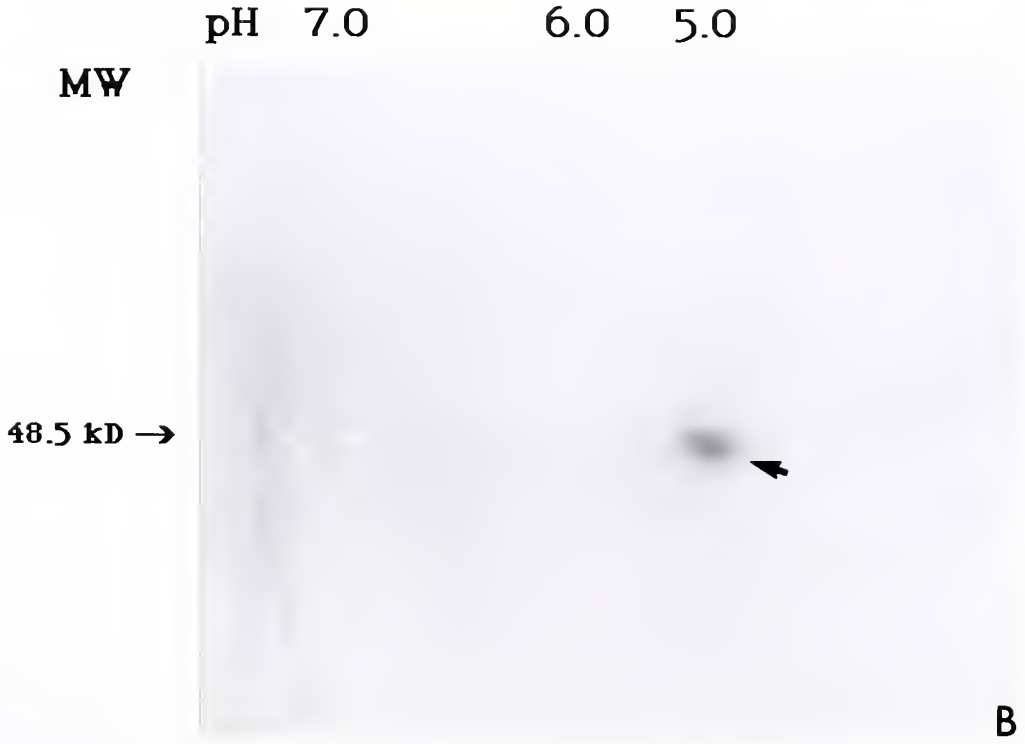
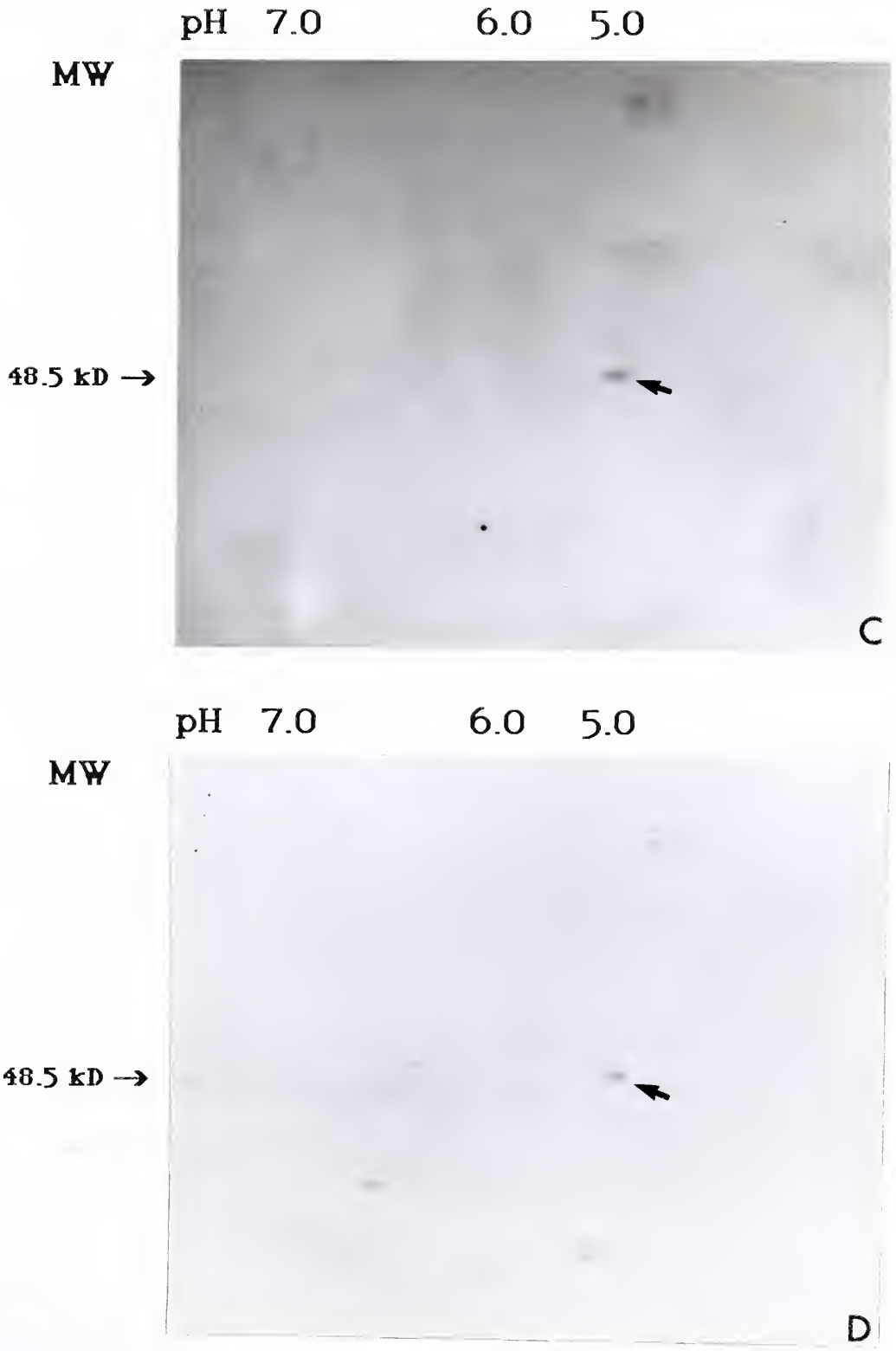


Figure 14



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