Characterization of a Membrane-Associated 3,3′,5-Triiodo-L-thyronine Binding Protein by Use of Monoclonal Antibodies

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Received April 22, 1986; Revised Manuscript Received July 22, 1986

ABSTRACT: Four mouse hybridoma cell lines have been isolated which secrete antibodies to the membrane-associated thyroid hormone binding protein (M_r 55000) from human epidermoid carcinoma A431 cells. J6 is rat specific; J2 is human and monkey specific; J8 and J9 have a wider specificity and react with similar thyroid hormone binding proteins (p55) from human, monkey, rat, and hamster. None of these antibodies reacts with mouse cells. J2, J6, and J9 are of the $IgG_{1\kappa}$ class, and J8 is an IgA_κ antibody. p55 was characterized by using these monoclonal antibodies. It is not posttranslationally processed by glycosylation, phosphorylation, or sulfation. It has a cellular degradation rate $t_{1/2} \simeq 3.2$ h. Using immunofluorescence and electron microscopic immunocytochemistry, p55 was found to be associated with the lumenal face of the endoplasmic reticulum and nuclear envelope. When cell homogenates were prepared, significant amounts of p55 were released into the 110000*g* supernatant, indicating that p55 is loosely associated with the endoplasmic reticulum and nuclear envelope.

The thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3)[1] has diverse biological effects. It promotes cell growth and development and regulates tissue differentiation. It is unclear whether all these actions are the result of a primary common event or are separately initiated from interaction of T_3 with multiple cellular sites. T_3 has been shown to influence the expression of genes for growth hormone (Spindler et al., 1982; Nyborg et al., 1984; Crew & Spindler, 1986), malic enzyme (Dozin et al., 1985), myosin heavy chain (Izumo et al., 1986), and spot 14 (Jump et al., 1984). This action is thought to be mediated through the interaction of T3 with nonhistone chromatin-associated nuclear receptors. Among the above-mentioned systems, the regulation of the growth hormone gene by T_3 is the best characterized. Evidence has been presented to show that there is a good correlation between the increase in growth hormone gene activity and the concentration of thyroid–hormone receptor complexes (Yaffe & Samuels, 1984; Nyborg et al., 1984). The regulatory site for mediating such an effect was found to locate in the 5′-flanking region of the gene (Cassanova et al., 1985; Crew & Spindler, 1986).

Extranuclear binding sites for thyroid hormone, however, have been detected on the plasma membrane (Horiuchi et al., 1982; Alderson et al., 1985; Pliam & Goldfine, 1977; Gharbi-chihi & Torresani, 1981; Krenning, 1979; Segal & Ingbar, 1982; Botta et al., 1983), on mitochondria (Sterling et al., 1984), in the cytosol (Barsano & DeGroot, 1983), and on the nuclear envelope (Surks et al., 1973; Lefebvre & Venkatraman, 1984). Recently, using the affinity labeling reagent BrAc[^{125}I]T_3, a protein with an apparent molecular weight of 55000 (p55) was specifically labeled in intact cells and plasma membrane containing fractions of 3T3 fibroblasts (Cheng, 1985), GH_3 cells, and A431 cells (Horiuchi et al., 1982; Cheng, 1983a). This protein (p55) has now been purified to apparent homogeneity (Cheng et al., 1986). We report here on the preparation of monoclonal antibodies to p55 and their use to determine the characteristics of this protein.

Experimental Procedures

Materials

[^{125}I]T_3 (2200 Ci/mmol), carrier-free [^{32}P]phosphoric acid, and [^{35}S]sulfate were purchased from New England Nuclear. CHAPS, lactoperoxidase, and gentamycin sulfate were from Sigma. Endoglycosidase H and *N*-glycanase were purchased from Genzyme Corp. (Boston, MA). [^{35}S]Methionine (1200 Ci/mmol) and Na^{125}I (14–17 mCi/μg) were from Amersham. Tunicamycin was from Calbiochem. Dulbecco's modified Eagle's medium and NCTC-109 were from Gibco. Hypoxanthine/aminopterin/thymidine was obtained from Bethesda Research Laboratory. Heat-inactivated fetal bovine serum and polyethylene glycol (M_r 1000) were from Hazleton Dutchland, Inc. Affinity-purified rabbit anti-mouse H and L chain antibodies and goat anti-rabbit H and L chain antibodies were from Jackson Immunoresearch Co. Synthesis of BrAc[^{125}I]T_3 was performed as described (Horiuchi et al., 1982). RPC-5, a monoclonal IgG_κ antibody against an unknown antigen, was obtained from Litton Bionetics, Inc.

Cell Lines. A431 cells were propagated in Dulbecco–Vogt's medium containing 10% fetal calf serum as described (Cheng, 1983a; Cheng et al., 1986). GH_3 cells were cultured as described (Horiuchi et al., 1982). Fischer rat embryo fibroblasts 3Y1 were a generous gift of Dr. Ricardo Feldman. Mouse Swiss 3T3 cells were cultured as described in Cheng (1983b). Human KB, WI-38, and monkey Vero cells were obtained from American Type Culture Collection (Rockville, MD).

Methods

Production of Monoclonal Antibodies. (I) Immunization. Three fusions (I, II, and III) using mice receiving different preparations of immunogens were carried out. Fusion II used live cells as the immunogen. GH_3 cells were washed and detached from flasks as described in Horiuchi et al. (1982). Cells (1.1×10^7 cells) were suspended in 0.5 mL of serum-free Dulbecco's modified Eagle's Medium and injected into female Balb-C mice (10–12 weeks old) intraperitoneally. After 3 weeks, mice were boosted with 7×10^6 cells. The last boost was administered 72 h before fusion. Fusion I used purified p55 as described in Cheng et al. (1986). In the last step of

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[1] Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; BrAc[^{125}I]T_3, *N*-bromoacetyl-3,3′,5-[^{125}I]triiodo-L-thyronine; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

purification, approximately 100–200 μg of 30–40% pure p55 was applied to a 7% SDS–PAGE slab gel (thickness 1 mm) according to Laemmli (1970). The p55 was located by autoradiography and the radioactive band excised. The gel slice was cut into pieces 5 mm in length and dialyzed against H_2O overnight. The pieces were lyophilized, and the dry gel was pulverized. Gel containing 20 μg of p55 in 0.25 mL of PBS was mixed with an equal amount of complete or incomplete Freund's adjuvant and injected into mice intraperitoneally. The immunization schedule was the same as described above. Fusion III used the p55 purified similarly as described above, except that SDS was replaced by 0.5 mM CHAPS in the Laemmli electrophoretic system. The preparation of immunogens and the immunization schedule were similar to that described in fusion I. Fusions were carried out as described (Richert, 1982).

(II) Screening of Hybridoma Supernatants for the Presence of Monoclonal Antibodies against p55. (A) Iodination of p55. Approximately 1 mCi of $Na^{125}I$ was added to 1 μg of the purified p55 in 50 μL of 0.5 M phosphate buffer, pH 7.6. After addition of 2 μg of lactoperoxidase in 10 μL of 0.1 M phosphate buffer (pH 7.6), H_2O_2 was immediately added to a final concentration of 7×10^{-5}% (v/v). The mixture was incubated for 60 s at room temperature. Sodium azide was added to a final concentration of 0.1% to stop the reaction. The iodinated p55 was separated from free iodide by passing the mixture through a PD-10/Sephadex G-25 column using 10 mM sodium phosphate, 0.1 mg/mL BSA, and 0.5 mM CHAPS, pH 7.6, as an eluant. The void volume was pooled, and the ^{125}I-p55 was stored frozen.

(B) Microtiter Plate Assay. Fifty microliters of the affinity-purified goat anti-mouse antibodies (40 μg/mL) was added to each well of a microtiter plate (96-well plates; Dynatec Laboratories, Inc). After incubation at 22 °C for 2 h, the wells were washed twice with 300 μL of cold PBS (Ca^{2+} and Mg^{2+} free) containing 0.1% BSA. Fifty microliters of 1% BSA in PBS was then added to each well for 1 h at 22 °C followed by two washes with cold BSA/PBS. After addition of 50 μL of hybridoma supernatant to each well for 1 h, supernatants were removed, and the cells were washed 2 times with cold BSA/PBS. Purified ^{125}I-p55 (50 μL, 1×10^6 dpm) in 0.5 mM CHAPS containing 2 mg/mL BSA was added to each well for 1 h. After similar washes as described before, the wells were cut out, and the radioactivity was determined.

(C) Immunoprecipitation. The positive supernatants from microtiter plate assays were further screened by immunoprecipitation. ^{125}I-Labeled p55 (2×10^6 dpm) was incubated with 100 μL of each hybridoma supernatant for 30 min at 4 °C. The subsequent steps were carried out as described (Hasumura et al., 1984).

(D) [^{35}S]Methionine Metabolic Labeling. The positive supernatants from the above two assays were further screened by [^{35}S]methionine metabolic labeling of cells followed by immunoprecipitation as described (Cheng et al., 1986). The results shown in Figure 1 are from a single experiment, performed on the same day and autoradiographed the same length of time.

(III) Classification of Antibodies Secreted by the Isolated Clones. The antibodies were classified according to the procedure described (Richert, 1982).

Radiolabeling of p55 in A431 Cells. (I) Pulse–Chase Experiment. A431 cells (1.5×10^6 cells/60-mm dish) were incubated with 4 mL of methionine-free dulbecco's modified Eagle's medium containing 5% fetal calf serum at 37 °C for 15 min. [^{35}S]Methionine (1 mCi/dish) was incubated with cells at 37 °C for 10 min. The medium was aspirated, and the cells were washed with 5 mL of PBS containing 4 mM unlabeled methionine. Cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, and cells were harvested at intervals of 0, 0.25, 0.5, 1, 2, 4, and 20 h. The preparation of 3 mM CHAPS extracts and immunoprecipitation with J9 were carried out as described (Cheng et al., 1986). The results shown in Figure 6 are from a single experiment, performed on the same day and autoradiographed the same length of time.

(II) Tunicamycin Experiment. A431 cells (3×10^6 cells/100-mm dish) were preincubated with 10 μg/mL tunicamycin at 37 °C for 5 h. [^{35}S]Methionine (1 mCi/dish) was added and incubated for 3 h at 37 °C. At the end of the incubation, cells were washed and harvested with a rubber policeman. Preparation of 3 mM CHAPS extracts and immunoprecipitation with J9 were carried out as described (Cheng et al., 1986).

The optimal concentration of tunicamycin was derived from preliminary studies. Tunicamycin at a concentration greater than 10 μg/mL caused cell death. Inhibition in the incorporation of carbohydrate moieties into the receptor for epidermal growth factor was used as a positive control. Under these conditions and using Ab 2913 for immunoprecipitation (Beguinot et al., 1986), receptor for epidermal growth factor migrated as bands with molecular weights of ~133 000 and ~75 000 in contrast to the intact molecule with a molecular weight of 170 000 (Mayes & Waterfield, 1984).

(III) Phosphorylation and Sulfation Experiments. A431 cells (3×10^6 cells/60-mm dish) were incubated with [^{32}P]-phosphoric acid (25 mCi/dish) in phosphate-free medium or with [^{35}S]sulfate (1 mCi/dish) in sulfate-free medium for 2, 4, 6, or 20 h. The medium was removed, and cells were washed with PBS. CHAPS extracts were prepared and immunoprecipitated as described above.

Digestion of the p55 Protein with N-Glycanase and Endoglycosidase H. The digestion of p55 using *N*-glycanase was carried out as described by Plummer et al. (1984). Iodinated p55 (1 μg; 1×10^5 dpm) in 50 mM NaCl, 10 mM phosphate, 0.17% SDS, and 10 mM β-mercaptoethanol was boiled for 3 min. After the mixture was cooled, sodium phosphate (1 M, pH 8.6), 1,10-phenanthroline hydrate, and NP-40 were added to final concentrations of 0.2 M, 10 mM, and 1.25%, respectively; *N*-glycanase was added to a final concentration of 10 units/mL and incubated at 37 °C for 18 h. The enzymatic mixture was analyzed by 10% SDS–PAGE followed by autoradiography.

For digestion of p55 with endoglycosidase H, the method described by Trimble et al. (1984) was adopted. Iodinated p55 (1 μg, 1×10^5 dpm) in 0.5 mM CHAPS, 10 mM phosphate, 50 mM NaCl, and 0.08% SDS was boiled for 3 min. After the solution was cooled, 10 μL of 0.143 M phosphate buffer (pH 6.0) was added. Endoglycosidase H and phenylmethanesulfonyl fluoride were added to final concentrations of 40 units/mL and 2 mM, respectively. The mixture was incubated at 37 °C for 20 h. The enzymatic mixture was analyzed by 10% SDS–PAGE followed by autoradiography.

Western Blots. Two micrograms of the purified p55 was applied to a 5–15% SDS–polyacrylamide gradient gel. The protein was electrotransferred to nitrocellulose paper using a buffer system containing 25 mM Tris/glycine (pH 8.4) and 25% methanol. Nitrocellulose paper was blocked with 8 drops of horse serum in 20 mL of reaction buffer (25 mM Tris and 150 mM NaCl, pH 7.4) for 30 min at 22 °C. After the paper was washed with reaction mixture containing 0.1% NP 40, it

Table I: Monoclonal Antibodies against p55

fusion no.	immunogens	cloning efficiency[a]	positive clones[b]	antibody	subclass	light chain
I	purified p55 (SDS eluted)	82	3	J2	IgG_1	κ
II	live GH_3 cells	83	1	J6	IgG_1	κ
III	purified p55 (CHAPS eluted)	56	7	J8	IgA	κ
III	purified p55 (CHAPS eluted)	56	7	J9	IgG_1	κ

[a] Cloning efficiency is defined as the percent ratio of the growing clones vs. the total plated wells (i.e., 400). [b] Identification by the immunoprecipitated ^{35}S-labeled 55K protein.

was incubated with monoclonal antibodies (10 μg/mL) for 30 min at 22 °C. After being washed, biotinylated rabbit anti-mouse antibody (1:200 dilution) was incubated with the paper for 30 min. After being washed, VECTASTAIN ABC reagent (avidin–biotin complex) (Vector Laboratories Inc.) was applied, and 0.6 mM diaminobenzidine hydrochloride with 0.012% H_2O_2 in reaction buffer was used as the substrate for color development. The reaction was stopped by the addition of 10% acetic acid.

Localization of p55 by Subcellular Fractionation. A431 cells (1×10^7 cells/100-mm dish) were metabolically labeled with [^{35}S]methionine at 37 °C for 20 h. The subsequent steps were carried out at 4 °C. After being washed with PBS (Ca^{2+} and Mg^{2+} free), cells were removed from the dishes by a rubber policemen and pelleted at 250*g* for 3 min. Cells were resuspended in 2 mL of various buffers as described in Table III and homogenized (20 and 60 strokes for hypotonic and isotonic buffers, respectively) in a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1100*g* for 10 min. The postnuclear supernatant was spun at 110000*g* for 30 min. The low-speed pellet and 110000*g* pellets were extracted with 2 mL of 3 mM CHAPS. One milliliter of the extracts and the supernatant from the 110000*g* spin were immunoprecipitated with J9 (10 μg/mL) as described in Cheng et al. (1986). The intensity of the p55 band in the autoradiogram was quantified by a densitometer.

In a separate experiment, A431 cells were affinity labeled with 1.5 nM BrAc[^{125}I]T_3 at 15 °C for 60 min. Cells were fractionated similarly as described above except that aliquots of the high-speed supernatants and CHAPS extracts were applied directly to 10% SDS–PAGE without immunoprecipitation. After autoradiography, the radioactive bands were cut out, and the radioactivity was determined by a Beckman γ-8000 counter.

Immunocytochemical Localization of p55. For immunofluorescence, various cell types (summarized in Table II) were grown in 35-mm plastic culture dishes, fixed in 3.7% formaldehyde for 15 min at 23 °C, and then imcubated in 10–50 μg/mL mouse monoclonal antibody (J2, J6, J8, or J9) in the presence of 0.1% saponin/4 mg/mL normal goat globulin/PBS as previously described (Hasumura et al., 1984; Willingham, 1980). After 15 min at 23 °C, the cells were washed in saponin/PBS and further incubated in rhodamine-conjugated affinity-purified goat anti-mouse IgG (heavy + light chains) (Jackson ImmunoResearch, Avondale, PA) (50 μg/mL in saponin/normal goat globulin/PBS) for 15 min at 23 °C. After being washed, the cells were mounted under a number 1 coverslip in buffered glycerol, examined by using a Zeiss RA microscope equipped with rhodamine epifluorescence optics, and photographed by using Kodak Tri-X film with Diafine development.

For electron microscopic localization, A431 cells were fixed and processed by using the "EGS" procedure with 0.2% glutaraldehyde as previously described (Willingham, 1980). Ferritin bridge localization (Willingham, 1980) was performed by using 25 μg/mL monoclonal J9 or an equivalent amount of normal mouse globulin as a control. After embedding, thin sections were counterstained with lead citrate and bismuth subnitrate as previously described (Willingham, 1980). The normal mouse gloubulin control showed no detectable localization above background.

In some experiments, A431 cells in 35-mm plastic dishes were washed in PBS and then incubated in PBS with 0.1% saponin for 5 min at 23 °C. The cells were then fixed in 3.7% formaldehyde in PBS and gently washed and incubated with mouse monoclonal antibodies J9 (anti-p55), HB21 (anti-transferrin receptor) (Willingham & Pastan, 1985), or MC 101 (anti-Y-glycolipid antigen) (Willingham & Pastan, 1985; Le Pendu et al., 1985) in the continuous presence of saponin as described above for indirect immunofluorescence. Following a second incubation in anti-mouse rhodamine conjugate, the cells were mounted in glycerol and examined by using epifluorescence microscopy. Control cells were not incubated in saponin prior to fixation but were primarily fixed in formaldehdye before saponin exposure.

Results

Selection of Hybridoma Clones Secreting Monoclonal Antibodies to p55. Table I lists the results of three fusions using spleens from mice immunized with different preparations of immunogens. Fusion I and fusion III were from mice immunized with purified p55 prepared from human A431 cells. Fusion II used the spleen from a mouse immunized with GH_3 cells. In fusion I, the last step in the purification of p55 was SDS gel electrophoresis followed by elution of p55 using 0.1% SDS. In fusion III, p55 was purified on a gel containing CHAPS and eluted with CHAPS. Fusions I, II, and III gave 82%, 83%, and 56% of growing hybridomas, respectively. When a microtiter plate assay to screen a large number of hybridomas (see Experimental Procedures) was used, 7, 8, and 20 positive hybridomas were identified. These were further screened with metabolic labeling by [^{35}S]methionine using A431 and GH_3 cells. Three clones from fusion I, one clone from fusion II, and seven clones from fusion II were found to secrete antibodies that immunoprecipitated a 55K protein in human (fusions I and III) or GH_3 cells (fusion II). One hybridoma from fusion II (J6), one from fusion I (J2), and two from fusion III (J8 and J9) which secreted the highest titer antibodies in their media were recloned twice.

Characterization of Monoclonal Antibodies. The monoclonal antibodies listed in Table I were examined for their immunoreactivity with the 55K thyroid hormone binding protein (p55). This was done in two ways. Human A431 or rat GH_3 cells were first affinity labeled with BrAc[^{125}I]T_3, and extracted with 3 mM CHAPS. The CHAPS extracts were immunoprecipitated by monoclonal antibodies. Figure 1B shows that the BrAc[^{125}I]T_3-labeled p55 was immunoprecipitated. It is of importance to note that J6 is rat specific (lanes 4 and 9) and J2 is human specific (lanes 3 and 8), whereas J8 and J9 react both with human (lanes 5 and 6) and with rat (data not shown). A431 cells were also [^{35}S]methionine labeled and extracted with CHAPS. Immunoprecipitation of CHAPS extracts with the monoclonal antibodies indicates that a specific major band with an apparent molecular weight of

Table II: Reactivities of Mouse Monoclonal Antibodies against p55 in Various Cultured Cells[a]

	human			monkey	rat		hamster	mouse
antibody	A431	KB	WI38	Vero	GH_3	NRF	CHO	Swiss 3T3
J2	+	+	+	+	–	–	–	–
J6	–	–	–	–	+	+	+	–
J8	+	+	+	+	+	+	+	–
J9	+	+	+	+	+	+	+	–

[a] Mouse monoclonal antibodies J2, J6, J8, and J9 were tested by indirect immunofluorescence on these various cell types using formaldehyde fixation and incubation in antibodies (10–50 μg/mL) in the continuous presence of saponin as described under Experimental Procedures. All positive patterns were consistent with a distribution in the nuclear envelope and endoplasmic reticulum.

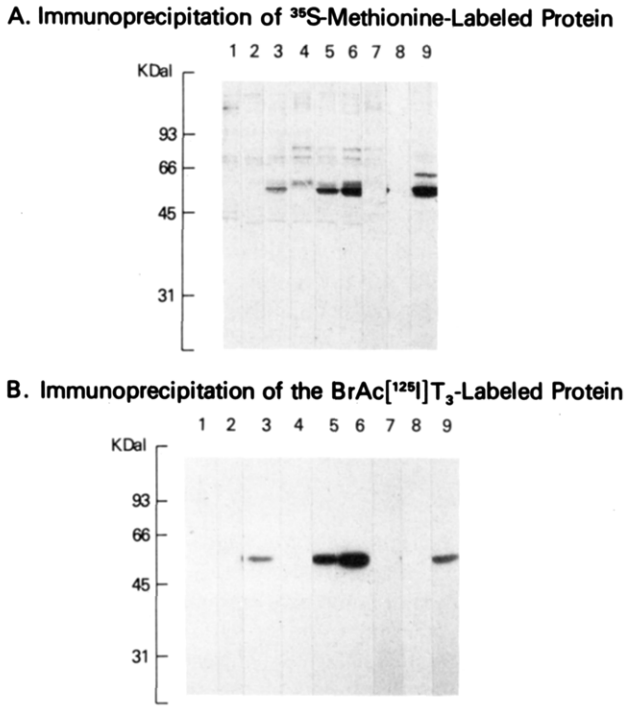

FIGURE 1: Autoradiogram of the immunoprecipitates from [^{35}S]-methionine (A) or BrAc[^{125}I]T_3 (B) labeled CHAPS cellular extracts. (A) A431 cells (5×10^6 cells/100-mm dish, lanes 1–6) or GH_3 cells (5×10^6 cells/100-mm dish, lanes 7–9) were labeled with [^{35}S]-methionine (1 mCi/dish) in methionine-free medium for 18 h at 37 °C. After being washed, cells were extracted with 4 and 1 mL of 3 mM CHAPS for A431 and GH_3 cells, respectively. CHAPS extractes were aliquoted into a 0.5-mL fraction, and 10 μg of antibodies was added. Immunoprecipitation was carried out as described under Experimental Procedures. Lanes 1 and 7, monoclonal antibody RPC5 (IgG_κ) as a control; lane 2, cloning medium (HA medium); lanes 3 and 8, J2; lanes 4 and 9, J6; lane 5, J8; lane 6, J9. (B) A431 cells (1.3×10^8 cells) or GH_3 cells (1.5×10^7 cells) were labeled with 1.5 nM BrAc[^{125}I]T_3 at 15 °C for 1 h. After being washed, A431 and GH_3 cells were extracted with 10 and 1 mL of 3 mM CHAPS. The aliquots (0.5 mL) were immunoprecipitated according to Experimental Procedures. The antibodies used for each lane were identical with those in (A).

55K was detected (Figure 1A). Again, the same species specificity was seen. To show that J2, J8, and J9 were antibodies against the purified p55, a Western blot experiment was carried out. As shown in Figure 2, the purified human p55 reacted with J2, J8, and J9 (lanes 4, 6, and 7). Consistent with the results from immunoprecipitation, J6 did not react with p55 which was purified from human A431 cells.

To determine the class and subclass of the antibodies, the clones were labeled with [^{35}S]methionine; the culture supernatants were harvested and immunoprecipitated with affinity-purified rabbit anti-mouse IgG subclass specific or anti-mouse IgA antibodies. As shown in Table I, J8 was determined to be an IgA. The rest of the clones (J2, J6, and J9) are IgG_1-secreting hybridomas. The light chains in the four antibodies all belong to the class of κ.

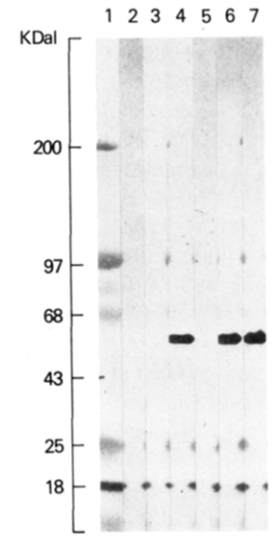

FIGURE 2: Western blots. Two micrograms of the purified p55 from A431 cells was applied to a 5–15% gradient gel. Electrotransfer of p55 to nitrocellulose paper and immunoreaction were carried out as described under Experimental Procedures. Lane 1, prestained standards; lane 2, control monoclonal antibody RPC-5; lane 3, cloning medium (HA medium); lane 4, J2; lane 5, J6; lane 6, J8; lane 7, J9.

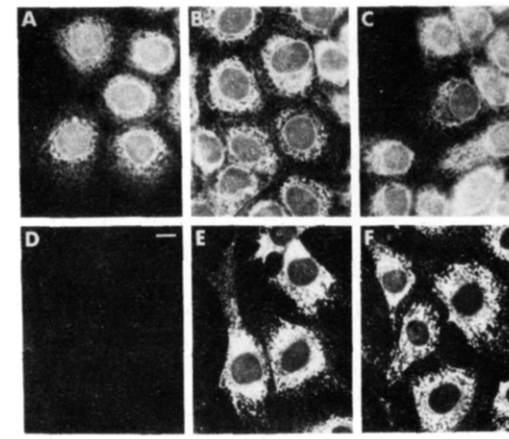

FIGURE 3: Immunofluorescence localization of p55 using monoclonal antibodies. A431 human carcinoma cells (A–C) and 3Y1 normal rat fibroblasts (D–F) were fixed with formaldehyde and incubated with monoclonal antibodies J2 (A and D), J8 (B), J6 (E), or J9 (C and F) in the presence of saponin as described under Experimental Procedures. The indirect immunofluorescence images show that A431 cells react with monoclonals J2, J8, and J9, giving a similar nuclear envelope–endoplasmic reticulum pattern. In the flattened rat fibroblastic cells 3Y1, monoclonal antibody J2 shows no localization (D), whereas monoclonals J6 and J9 give similar nuclear envelope–endoplasmic reticulum patterns. (Magnification, 260×; bar = 10 μm.)

Localization of p55 by Immunofluorescence. For light microscopic immunofluorescence, various cell types were fixed and incubated with the different monoclonal antibodies to p55 in the continuous presence of saponin as described under Experimental Procedures. These resutls are summarized in Table II. All positive reactions appeared as a nuclear en-

velope–endoplasmic reticulum pattern (Willingham & Pastan, 1985). Selected examples are shown in Figure 3. A431 cells (Figure 3A–C) and rat (3Y1) fibroblasts (D–F) are shown after incubation with different monoclonal antibodies. Monoclonal J2 reacts only with human and monkey cells (Table II) and shows localization in A431 (Figure 3A) but not with rat fibroblasts (Figure 3D). The reaction of monoclonal J8 with A431 cells is shown in Figure 3B. Monoclonal J6 reacts with rat fibroblasts (Figure 3E) but not with A431 cells (not shown). Monoclonal J9 reacts with both A431 cells (Figure 3C) and rat cells (Figure 3F).

These experiments were all performed by using primary formaldehyde fixation followed by incubations in the presence of saponin. This protocol is important because p55, like some other membrane proteins, is extractably by detergents after formaldehyde fixation (Goldenthal et al., 1985). No localization of p55 was seen within the nucleus itself. This was determined not only in saponin-treated cells, in which the nucleus is not well permeabilized, but also with cells primarily fixed in acetone or methanol. Primary fixation in these organic solvents does not maintain morphologic structures well, but these solvents allow antibodies to gain access to the nuclei and mitochondria and leave sufficient p55 to allow detection in the endoplasmic reticulum. No localization was detected in mitochondria.

In a previous paper (Cheng et al., 1986), we employed a rabbit polyclonal antibody to p55, which was not affinity purified, to determine its cellular location. Because of possible trace contaminants in the polyclonal antibody, we could not be certain whether or not small amounts of p55 might be found on the plasma membrane. With these monoclonal antibodies to p55, we were now able to examine whether p55 is present on the plasma membrane. When formaldehyde-fixed cells were permeabilized in the saponin and then exposed to antibody, p55 could not be detected associated with the plasma membrane. Furthermore, when living A431 cells or GH_3 cells were incubated at 4 °C with either J2 or J9 for A431, or J6 or J9 for GH_3, and then fixed, no detectable surface reactivity was found by immunofluorescence (results not shown; see below).

Electron Microscopic Localization of p55. Electron microscopic localization of p55 using monoclonals J9 and J2 was performed as described under Experimental Procedures using the ferritin bridge localization method (Willingham, 1980). Figure 4 shows the results using J9. Specific localization was found in the lumen of the endoplasmic reticulum and the lumen of the nuclear envelope. Figure 4B shows a tangential section of endoplasmic reticulum that emphasizes that the localization seen appears intimately associated with the inner surface of the membrane. No localization was found on the plasma membrane, in the Golgi system, inside the nucleus itself, or within mitochondria. The determinant reactive with both J2 and J9 clearly resides on the lumenal side of the endoplasmic reticulum membrane. If p55 were present on the cell surface, the determinant would be expected on the exterior of the cell. Therefore, the lack of detection on the surface of living cells shown using immunofluorescence indicates the absence of the protein from the plasma membrane. No p55 was detected in lysosomes.

Extractability of p55 from Permeabilized Cells. To help determine whether p55 is an integral membrane protein, immunofluorescence experiments were carried out by using a different protocol. A431 cells were incubated in saponin for 5 min prior to fixation with formaldehyde and examined for the presence of p55, transferrin receptor (using MC HB21),

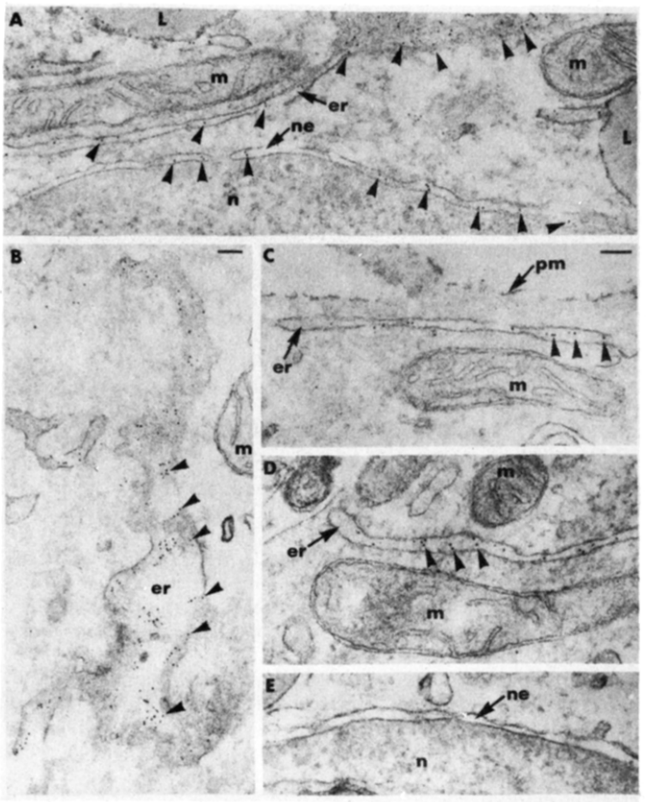

FIGURE 4: Electron microscopic immunocytochemical localization of p55. A431 cells were fixed and processed for electron microscopic immunocytochemistry using monoclonal antibody J9 and the "EGS" method and "ferritin bridge" labeling as described under Experimental Procedures. Panel A demonstrates the localization of p55 found in the nuclear envelope (ne) and endoplasmic reticulum (er) (arrowheads = ferritin cores). A tangentially sectioned portion of the endoplasmic reticulum (er) is shown in (B), demonstrating the large amount of p55 detected in the lumen of the endoplasmic reticulum. Panel C shows that essentially no localization above background was found on the plasma membrane (pm), whereas a portion of endoplasmic reticulum (er) located just beneath the plasma membrane shows localization of p55 in its lumen. Panel D shows a segment of endoplasmic reticulum demonstrating that essentially all of the ferritin (arrowheads) is located on the lumenal side of the endoplasmic reticulum membrane. Panel E shows a region of the nuclear envelope from a control sample incubated with normal mouse globulin in place of mouse monoclonal J9. [Magnification (A, B) 31500×, (C–E) 40500×; bars = 0.1 μm; L = lipid droplet, m = mitochondrion, pm = plasma membrane, er = endoplasmic reticulum, ne = nuclear envelope; lead citrate/bismuth subnitrate counterstains.]

or Y-antigen glycolipid (MC 101). Both the transferrin receptor and Y-antigen glycolipid are known to be integral membrane elements. When cells were primarily fixed in formaldehyde prior to saponin exposure (Figure 5A), the typical endoplasmic reticulum–nuclear envelope pattern of p55 was easily seen. However, if the cells were extracted with saponin prior to fixation (Figure 5B), all of the detectable p55 had disappeared, unlike the integral membrane components detected by HB21 (Figure 5C) or MC 101 (Figure 5D).

Localization of p55 by Subcellular Fractionation. The subcellular distribution of p55 was also evaluated by homogenization and fractionation of [^{35}S]methionine-labeled A431 cells. Tables III lists the distribution of p55 in 1100*g* pellet and in the 110000*g* supernatant and pellet (microsomal fraction) after cells were homogenized in different buffers. A large amount of p55 (20–60%) was in the 1100*g* pellet, which contained nuclei, large sheets of plasma membrane, and remnants of endoplasmic reticulum attached to the nuclear envelope and to the unbroken cells; 20–30% of the cells were unbroken as determined by phase-contrast microscopy. Except

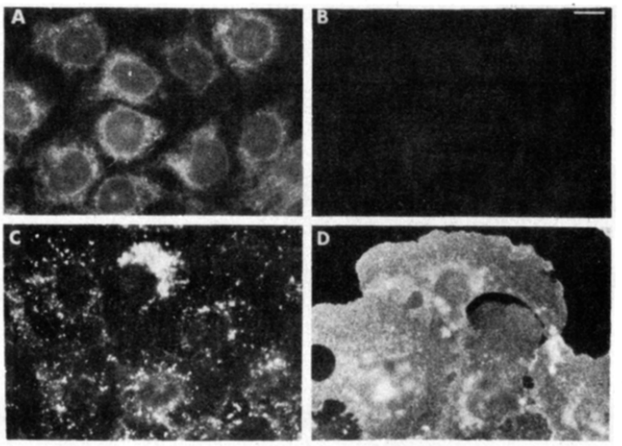

FIGURE 5: Extraction of p55 from permeabilized cells. A431 cells were treated with 0.1% saponin in PBS for 5 min at 23 °C and then fixed with formaldehyde (B–D); other cells were fixed first in formaldehyde prior to saponin treatment (A). These fixed cells were then exposed to monoclonal antibodies J9 (A, B), HB21 (C), or MC 101 (D). This was then followed by anti-mouse IgG conjugated to rhodamine. Panel A shows the typical pattern of endoplasmic reticulum–nuclear envelope shown for p55 using J9. If the cells were exposed first to saponin prior to fixation, the p55 pattern was absent (B). Similar treatment of cells using HB21, an antibody to the integral membrane protein portion of the transferrin receptor (C), or using MC 101 (D), an antibody to an integral membrane glycolipid determinant, showed that these integral membrane components were not extracted. Thus, this experiment suggests that p55 is not an integral membrane component. (Magnification, 405×; bar = 10 μm.)

Table III: Subcellular Distribution of p55 Determined by Fractionation[a]

homogenization buffer[b]	[35S]methionine-labeled p55 (% of total)		
	CHAPS extracts of 1100g pellet[c]	CHAPS extract of 110000g pellet	110000g supernatant
0.15 M NaCl/ 1 mM $CaCl_2$	47	9	44
0.15 M NaCl/ 10 μM $CaCl_2$	54	9	37
0.15 M KCl/ 1 mM $CaCl_2$	36	12	52
0.15 M KCl/ 10 μM $CaCl_2$	53	11	36
0.24 M sucrose/ 1 mM EDTA	60	21	19
1 mM EDTA	22	24	54

[a] A431 cells (1×10^7 cells/100-mm dish) were metabolically labeled with [35S]methionine at 37 °C for 20 h. After cells were washed, they were homogenized in 2 mL of buffers as stated in the table. Preparation of CHAPS extract and immunoprecipitation were carried out as described under Experimental Procedures. [b] All buffers contained 5 mM Hepes and 1 mM $MgCl_2$, pH 7.3. [c] The pellet contained 20–30% of unbroken cells. The [35S]methionine-labeled p55 in unbroken cells has been subtracted from the total p55 in the 1100g pellet.

for the buffer containing 0.24 M sucrose in which a nearly equal distribution of p55 was found in the 110000g pellet and supernatant, the remaining buffers all resulted in 2–4-fold more p55 in the supernatant than in the pellet. Subcellular fractionation using BrAc[^{125}I]T_3-labeled A431 cells gave similar results.

Posttranslational Modification and Turnover of p55. Localization studies indicate that the 55K protein is found in the endoplasmic reticulum and nuclear envelope. To determine if p55 is posttranslationally modified, A431 cells were pulsed with [35S]methionine for 10 min followed by a chase with unlabeled methionine for 0.25, 0.5, 1, 2, 4, and 20 h. Figure 6 shows the autoradiogram of an SDS gel of the immunoprecipitates using J9. No specific radioactive band with a molecular weight higher than 55K was detected at all the time

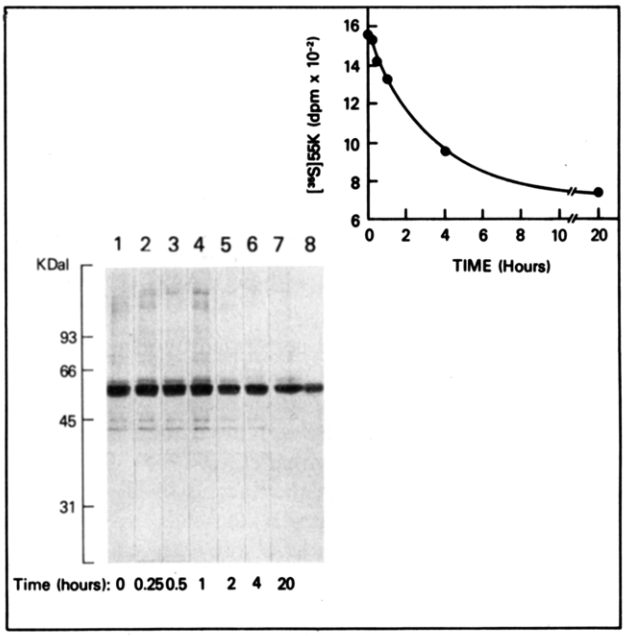

FIGURE 6: Autoradiogram of the immunoprecipitates from a pulse–chase experiment. A431 cells (1.5×10^6 cells/60-mm dish) were incubated with [35S]methionine (1 mCi/dish) in 4 mL of serum-free medium at 37 °C for 10 min. The dishes were cooled to 4 °C and washed with 5 mL of PBS containing 4 mM unlabeled methionine. The cells were then incubated in serum-containing medium for 0 (lane 1), 0.25 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 4 (lane 6), and 20 (lane 7) h. Preparation of CHAPS extracts and immunoprecipitation with J9 (10 μg/mL) were carried out as described. Lane 8 is the purified p55 standard. Inset: The radioactive bands at various time points were quantified by densitometry, and the decay curve was plotted by computer fitting using the equation $y = A + Be^{-\kappa t}$ where y is the total activity at time t, A is the activity at 20 h, B is the activity at time t, κ is the rate constant, and $t_{1/2} = 0.693/\kappa$.

points examined. These results indicate that within the detection sensitivity of the size change in a 10% gel, p55 is not processed. The band from each time point was quantified by densitometry, and the decay curve was plotted. As shown in the inset in Figure 6, the majority of p55 is degraded with a half-life of approximately 3.2 h. At the 20-h point, medium was collected and assayed for its content of p55 by immunoprecipitation. No detectable p55 was found in the medium; thus, p55 is not secreted from the endoplasmic reticulum.

To confirm further that p55 is not posttranslationally modified by glycosylation, two additional experiments were carried out. A431 cells were pretreated with tunicamycin for 5 h and labeled with [35S]methionine for 3 h. After immunoprecipitation of the cellular lysate with J9, only one radioactive 55K protein band was detected in cellular lysates regardless of whether cells were pretreated with tunicamycin or not (data not shown), indicating p55 is probably not glycosylated. Furthermore, purified p55 was iodinated and treated with *N*-glycanase, endoglycosidase H, or a mixture of neuraminidase and mixed glycosidases. No reduction in the molecular weight of p55 was detected in a 10% SDS gel (data not shown). For the digestion experiments, ovalbumin and human thyroxine binding globulin were treated similarly as positive controls, and human thyroxine binding prealbumin was used as a negative control. Under the experimental conditions, the enzymes removed the carbohydrates from both ovalbumin (M_r 45 000) and human thyroxine binding globulin (M_r 56 000) as evidenced by the appearance of protein bands with lower molecular weight, ~34 000 and ~43 000, respectively. Moreover, codigestions of ovalbumin and p55 were also carried out under the same conditions. Again, the sugar

chains were removed from ovalbumin, but no change in the molecular weight of p55 was seen. The results from these three experiments indicate that the p55 is not likely to be a glycosylated protein.

To determine if p55 is a phosphorylated or sulfated protein, A431 cells were treated with [^{32}P]phosphoric acid or [^{35}S]-sulfate for up to 20 h. After immunoprecipitation with J9 and SDS–PAGE, no incorporation of ^{32}P or ^{35}S was detected (data not shown). These results show that p55 probably is not a phosphorylated or sulfated protein.

Discussion

The present study describes the development of monoclonal antibodies to the membrane-associated thyroid hormone binding protein p55. These antibodies were then used to determine the cellular localization of p55. As previously demonstrated with a polyclonal antibody, p55 was localized to the endoplasmic reticulum and nuclear envelope (Cheng et al., 1986). None could be detected on the plasma membrane, within the nucleus, or in other cellular structures. Thus, p55 appears to be located in the endoplasmic reticulum and probably carries out its function in that location.

Initial characterization of the membrane-associated p55 showed that it is acidic with a p*I* of 5.1 (Cheng et al., 1986). p55 was further characterized by using monoclonal antibodies. Pulse–chase experiments showed that p55 has a degradation rate ($t_{1/2}$) of approximately 3.2 h. p55 does not appear to be posttranslationally modified by either glycosylation, phosphorylation, or sulfation. In homogenates, most of the p55 is found associated with the nuclear fraction and membrane-containing fractions, although up to 54% can be released into the 110000*g* supernatant. The fact that p55 is readily released on homogenization suggests that p55 is not an integral protein and that its association with membranes is not extremely tight. Further evidence that p55 is not an integral membrane protein comes from the fact that it is readily extractable from cells by saponin. Furthermore, all the antibodies to p55 so far isolated react only with determinants on the lumenal face of the endoplasmic reticulum.

Three different preparations of immunogens were used to immunize mice. From Figure 1 and Table II, it is clear that the antibodies derived from these fusions exhibit species specificity in that none of them recognizes p55 from mouse cells. Even though J2, J8, and J9 were prepared against the human protein, they probably recognize different epitopes. These species specificities and epitope variants will be useful in mapping the antigen recognition sites and in structure–function relationship studies of p55.

Using the same amount of affinity-purified J2 and J9 to immunoprecipitate identical amounts of [^{35}S]methionine-labeled cellular lysate, 10 times more of the p55 was immunoprecipitated by J9 then by J2. These results suggest that J9 has a higher affinity for p55 than J2. J9 was derived from fusion III (see Table I) which used CHAPS-treated purified p55 as an immunogen. Compared to fusion I, which used SDS-treated purified p55 as an immunogen, fusion III has a positive clone yield which is 2-fold more than that in fusion I (Table I). This suggests that CHAPS retains the immunogenic structure of p55 better than SDS. This same observation has previously been made in p55 from GH_3 cells in which immunogenicity and binding activity were preserved best by CHAPS (Hasumura et al., 1984).

A number of issues are not clear about the relationship of p55 to T_3. As shown in this paper, p55 is selectivity localized to the endoplasmic reticulum and nuclear envelope; however, we cannot rule out that a specialized altered conformation of p55 exists in other sites, such as the plasma membrane, that are not detectable by these different antibodies. p55 has been detected by labeling intact cells with BrAc[^{125}I]T_3 at 15 °C where endocytosis does not occur. This result suggested p55 might be the plasma membrane receptor for T_3. However, the current localization studies suggest that another protein may be involved in T_3 binding to cells. What then is the explanation for the labeling of an internal protein by bromoacetyl-T_3? Because bromoacetyl-T_3 has different solubility properties than T_3, it may enter cells more readily than T_3 by directly penetrating the plasma membrane. An example of this exists for the β-adrenergic receptor where some lipid-soluble analogues of propanolol can enter cells whereas hydrophilic analogues, such as CGP-12177, cannot (Staehelin et al., 1983; Hertel et al., 1983).

Finally, the monoclonal antibody studies show that p55 is highly conserved between species and is present in high amounts in many types of cells. These findings suggest that p55 may have an important role in the endoplasmic reticulum. The highly selective binding of p55 to T_3 suggests that p55 may play some role in T_3 action.

Registry No. T_3, 6893-02-3.

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Molecular Mechanisms of Band 3 Inhibitors. 1. Transport Site Inhibitors†

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Received February 13, 1986; Revised Manuscript Received August 25, 1986

ABSTRACT: The band 3 protein of red cells is a transmembrane ion transport protein that catalyzes the one-for-one exchange of anions across the cell membrane. ^{35}Cl NMR studies of Cl^- binding to the transport sites of band 3 show that inhibitors of anion transport can be grouped into three classes: (1) transport site inhibitors (examined in this paper), (2) channel-blocking inhibitors (examined in the second of three papers in this issue), and (3) translocation inhibitors (examined in the third of three papers in this issue). Transport site inhibitors fully or partially reduce the affinity of Cl^- for the transport site. The dianion 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS) and the arginine-specific reagent phenylglyoxal (PG) each completely eliminate the transport site ^{35}Cl NMR line broadening, and each compete with Cl^- for binding. These results indicate that DNDS and PG share a common inhibitory mechanism involving occupation of the transport site: one of the DNDS negative charges occupies the site, while PG covalently modifies one or more essential positive charges in the site. In contrast, ^{35}Cl NMR line broadening experiments suggest that 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) leaves the transport site partially intact so that the affinity of Cl^- for the site is reduced but not destroyed. This result is consistent with a picture in which DIDS binds near the transport site and partially occupies the site.

Band 3 is a 95-kDa single polypeptide chain that spans the red cell membrane and catalyzes the transmembrane one-for-one exchange of anions [for review see Macara and Cantley (1983) and Knauf (1979)]. The exchange of Cl^- for HCO_3^-—the latter produced by hydration of CO_2—is essential for CO_2 respiration; in fact, band 3 is the most abundant transport protein in the red cell, and the ion flux through band 3 is higher than through any other pathway (Knauf, 1979; Falke et al., 1984a). Recently, substantial progress has been made in elucidating both the structure and mechanism of this ion transport protein. Chemical labeling and proteolysis experiments have demonstrated the existence of at least seven transmembrane segments in the monomer (Jenning & Nicknish, 1984), and the gene sequence suggests as many as 13 transmembrane segments (Kopito & Lodish, 1985). Each monomer acts as an independent catalytic unit in anion transport (Macara & Cantley, 1983), although the monomer is associated in dimers and tetramers in the membrane (Nakashima et al., 1981). ^{35}Cl NMR studies of anion transport sites (Falke et al., 1984b; Falke & Chan, 1985), as well as transport studies [Gunn & Fröhlich, 1979; reviewed by Macara and Cantley (1983)], indicate that the catalytic unit alternates between two conformations, one in which a single transport site faces the internal compartment, and another in which a single transport site faces the external compartment. The Cl^- binding and dissociation reactions at both the inward- and outward-facing transport site conformations are rapid compared to the translocation of the bound Cl^- between these conformations (Falke et al., 1985a), and the translocation of an empty site is negligibly slow. The slowness of empty site translocation explains the observation that net transport of Cl^- is 10^4 times slower than the one-for-one exchange of two Cl^- ions in opposite directions [reviewed by Knauf (1979)]. Thus, the transport cycle catalyzed by band 3 is relatively well understood; however, the molecular nature of the structural changes that occur during the transport cycle is unknown.

Inhibitors of the transport cycle provide a practical approach to structure elucidation. A large number of organic inhibitors of the transport machinery have been described. In the series of three papers presented here, we investigate the molecular mechanisms of some of these inhibitors by using ^{35}Cl NMR

† Contribution No. 7343. This research was supported by Grant GM 22432 from the National Institute of General Medical Sciences, U.S. Public Health Service.
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0006-2960/86/0425-7888$01.50/0