Mapping of the cardiac sodium-calcium exchanger with monoclonal antibodies

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Porzig, Hartmut, Zhaoping Li, Debora A. Nicoll, and Kenneth D. Philipson. Mapping of the cardiac sodium-calcium exchanger with monoclonal antibodies. Am. J. Physiol. 265 (Cell Physiol. 34): C748-C756, 1993.—We used a panel of monoclonal antibodies raised against the canine cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger expressed in Sf9 insect cells to analyze the immuno reactive domains and the topological organization of this membrane protein. Antibodies, which reacted strongly on Western blots of the recombinant protein, were used to screen an expression sublibrary composed of exchanger cDNA fragments. Positive clones thus indicated the expression of antibody binding sites. Linear epitopes, 16-155 amino acids in length, could be identified for four antibodies. One antibody recognized two neighboring, but nonoverlapping, sequences. All epitopes were localized to the large hydrophilic region of the exchanger connecting the putative transmembrane segments 5 and 6. The immunodominant region of the protein is a highly charged domain in the carboxy-terminal half of the hydrophilic region. Binding studies with the \(^{3}H\)-labeled high-affinity antibody R3F1 establish that the immunodominant region is located on the intracellular surface of the membrane. The same antibody was used to directly determine the membrane concentration of the exchanger in different cell types. Newborn rat heart cells contain \(\sim 6 \times 10^6\) exchanger molecules per cell. Exchanger densities in different cells seem to correlate with the Na\(^{+}\)-dependent Ca\(^{2+}\) transport activity in the corresponding membrane vesicles.

complementary DNA sublibrary; immunodominant epitopes; tissue concentration; transport activity

THE SODIUM-CALCIUM EXCHANGE protein uses the transmembrane Na\(^{+}\) gradient to catalyze a countertransport of Ca\(^{2+}\) against its electrochemical gradient. Exchange activity was first observed in excitable tissues but has also now been detected in nonexcitable cells (for reviews see Refs. 27, 31, 48). The molecular characterization of the transport protein was initiated with the cloning of the canine myocardial exchanger (24). More recently, exchangers from other species and tissues have been sequenced (human heart (14), bovine heart (1), rabbit kidney (33)). The cDNA for the canine exchanger codes for a mature protein of 938 amino acids with a nonglycosylated molecular mass of 108 kDa. The detailed functional organization of the protein within the membrane is largely unknown. However, a tentative topological model has been derived from the hydropathy plot of the cloned protein (24). It postulates 11 transmembrane helical domains, after cleavage of a leader peptide, which are connected by hydrophilic loops of variable length (29). The model locates the amino-terminus to the extracellular and the carboxy-terminus to the cytoplasmic side of the membrane. A long hydrophilic loop connecting putative transmembrane segments 5 and 6 extends from residues 218 to 737, comprising more than one-half of the total molecule. This loop is thought to face the cytosol and to contain sites involved in the functional regulation of the exchanger (18, 21, 25).

We have now constructed an expression sublibrary of the cloned exchanger cDNA, coding for fragments of the protein that range from \(\sim 30\) to 150 amino acid residues in length. These polypeptides, expressed as fusion proteins with \(\beta\)-galactosidase in Escherichia coli, were screened with a panel of specific monoclonal antibodies (MAb) to identify the antigenic determinants (2, 22, 35). MAb binding studies to the native protein were then used to probe the topological organization of the protein. Our results show that the immunodominant region is located on the long hydrophilic loop and that this region faces the cytosolic surface of the membrane.

EXPERIMENTAL PROCEDURES

Preparation of Antibodies

MAb were raised to the cloned canine myocardial Na\(^{+}\)-Ca\(^{2+}\) exchanger that was expressed in Sf9 cells as previously described (19). Either crude membrane or alkaline-extracted membrane fractions (19) were used as starting material for antigen preparation. For primary immunizations, BALB/c mice received intraperitoneal injections of 100–250 \(\mu\)g membrane protein in complete Freund’s adjuvant. Booster injections were given in incomplete Freund’s adjuvant or in muramyl dipeptide. Polyethylene glycol fusion of spleen lymphocytes with NSO plasmocytoma cells and hybridoma selection was performed according to standard methods (7, 41). Hybrid cells were grown in 50% Dulbecco’s modified Eagle’s medium (DMEM)-50% Ham’s F-12 medium supplemented with 1% Nutridoma (Boehringer Mannheim) and 10% fetal calf serum. During the first 10 days after fusion, the medium also contained 2.5% Ewing sarcoma growth factor (Costar; see Ref. 12) and 100 U/ml interleukin-6 (3). Hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) (9) for MAb reacting with a partially purified preparation of equine Na\(^{+}\)-Ca\(^{2+}\) exchanger reconstituted into lipid vesicles (30). Loss of antigen during the ELISA procedure was avoided by adsorption of the vesicles to filter plates (Millipore Multiscreen HA). By screening with native rather than recombinant exchanger protein, MAb directed against specific insect proteins were eliminated. Positive clones were expanded, restested, and cloned by limiting dilution.

MAb Purification

MAb testing positive in the screening assays were isotyped using the INNO-Lia kit of Immunogenetics (Antwerp, Belgium). The MAb were then purified from culture supernatant by affinity chromatography on protein G-Sepharose (Pharmacia) columns at pH 7.6 following standard methods (9). The eluted MAb were dialyzed against isotonic KCl, pH 7.2, concentrated to 2–3 mg/ml by ultrafiltration, and stored frozen at \(-80^\circ\)C. MAb preparations were tested for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under
dissociating and nondissociating conditions followed by Coomassie blue staining. In most cases no major contaminating band could be detected. However, quantitative ELISA titration of these MAb preparations against anti-mouse and anti-bovine immunoglobulin G (IgG) and IgM in some cases revealed a significant contamination with bovine IgG originating from the fetal calf serum in the culture supernatant. Although the purities of most IgG MAb ranged between 70 and 90% of total protein, purities of IgM MAb were generally lower (3–60%).

Immunoblotting

All ELISA-positive MAb were screened for their ability to detect the exchanger protein on transblots of both the cloned exchanger from SF9 cells and the partially purified exchanger from equine hearts. After SDS-PAGE in a 7% gel, the proteins were transblotted to nitrocellulose (38). The transblots were probed with purified MAb in concentrations ranging from 50 to 200 µg protein/ml. Horseradish peroxidase-conjugated goat anti-mouse IgG (whole molecule; Sigma) was used as second antibody together with the diaminobenzidine indicator system. Only MAb showing strong immunolabeling of the cloned exchanger protein on Western blots were selected to screen the expression sublibrary for matching epitopes (see Screening of the cDNA Library for MAb Epitopes).

Immunoprecipitation

To test for immunoprecipitation of the Na⁺-Ca²⁺ exchanger by MAb, equine myocardial membranes (1 mg protein/ml) were extracted at pH 12 (28) and solubilized in a detergent buffer (containing (in mM) 70 KCl, 70 NaCl, 20 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and 5 CaCl₂, as well as 7 mg/ml 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate (CHAPS), 2 mg/ml asolectin, 10 µg/ml aproamin, 5 µg/ml pepstatin, and 5 µg/ml leupeptin). The preparation was equilibrated with individual MAb (final concentrations 20–200 µg/ml) for 2 h at 4°C. MAb-antigen complexes were subsequently adsorbed to protein G-Sepharose beads (30 min at 4°C). After sedimentation of the beads, the proteins in the supernatant were reconstituted into lipid vesicles by dialysis against sodium phosphate buffer [containing (in mM) 100 NaHPO₄, 50 NaCl, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), and 0.5 diethiothreitol] and sedimented at 200,000 g for 90 min to measure the remaining Na⁺-Ca²⁺ exchange activity. The beads were repeatedly washed and then heated to 100°C in SDS-PAGE sample buffer. The released proteins were separated by SDS-PAGE, transblotted onto nitrocellulose, and reacted with the antibody as described above.

Determination of Na⁺-Dependent Ca²⁺ Uptake

The method of Cheun and Reeves (4) was modified as follows. Sodium-loaded membrane vesicles or reconstituted lipid vesicles were suspended in KCl buffer (containing (in mM) 140 KCl, 20 3-(N-morpholino)propanesulfonic acid (MOPS), and 0.001 valinomycin, pH 7.4). Ca²⁺ uptake was initiated by adding 50 µM CaCl₂ solution (final concentration 48 µM, sp act 10 µCi/ml Ca²⁺). The reaction was stopped after 60–120 s by diluting the vesicle suspension into KCl buffer containing 5 mM EGTA but no valinomycin. Flux rates were calculated from the net ⁴⁵Ca²⁺ uptake during the first 10 s of the experiment. Unspecific uptake was measured under the same conditions but replacing KCl in the uptake medium by equimolar concentrations of NaCl.

Construction of a cDNA Expression Sublibrary of the Cardiac Na⁺-Ca²⁺ Exchanger

A λ Zap (Stratagene) sublibrary containing 70–300-base pair (bp) fragments of the exchanger cDNA was constructed. To prepare the cDNA inserts for sublibrary construction, the cDNA insert of plasmid A4 was digested with deoxyribonuclease (DNase). pA4 codes for the entire mature exchanger protein (24). pA4 insert DNA (7 µg) was digested with DNase I (DN-EP, 0.03 ng/ml; Sigma) for 1 h at room temperature in 50 mM tris(hydroxymethyl)aminomethane (pH 7.5), 1 mM MnCl₂, and 0.1 mg/ml bovine serum albumin (BSA). The presence of MnCl₂ ensures random cutting by DNase. The reaction was stopped by the addition of EDTA, and the mixture was spun through a ChromaSpin 100 column (Clontech) to remove small DNA fragments. The product was treated with chloroform-phenol and chloroform and precipitated with ethanol. About 90% of the final DNA product was between 70 and 300 bp in length, as estimated visually on a 1.2% agarose gel. The ends of the DNA were made blunt with T4 DNA polymerase, ligated to Eco(R)NotI adapters (Pharmacia), and inserted into λ Zap arms. DNA was packaged into phage with Gigapack Gold (Stratagene) to produce a library with 225,000 independent clones. Of six random plaques, two had inserts. The library was amplified one time and used for screening as described below.

Screening of the cDNA Library for MAb Epitopes

The expression sublibrary was screened with selected antibodies using the method of Mierendorf et al. (23). After isolation of positive phage clones, pBluescript was excised from the λ Zap vector (38). The localization of the plasmid inserts with respect to the full-length exchanger sequence was determined by sequence analysis using Sequenase 2 (USB). The minimal length of a linear epitope was estimated by comparing overlapping regions of different polypeptides reacting with the same antibody.

³H-Labeling of MAb R3F1

MAb R3F1 (purity ~90%) was radiolabeled to high specific activity using N-succinimidyl [2,3-³H]propionate (Amersham) as the modifying reagent (16). The major targets of this reagent are primary amino groups of lysyl residues. At the end of the labeling reaction, MAb-bound label was separated from free reagent by Sephadex G-25 chromatography. Assuming an overall 20% loss of MAb (initial amount 250 µg) during the labeling and purification procedures, the specific activity of the final product was 210 Ci/mmol. Of the total radioactivity in the MAb stock solution, 95% could be precipitated with trichloroacetic acid. Using the method of Lindmo et al. (20), we found that ~75% of the labeled MAb was immunoreactive. The apparent membrane-binding affinity of the native MAb was found to be four-fold higher than the affinity of the radiolabeled derivative.

Binding Experiments with MAb [³H]R3F1

Intact cells. Primary cultures of intact heart cells from newborn rats or cultures from the muscle cell line H9c2 were grown in DMEM on glass cover slips (placed in polystyrol culture dishes) as described by Kokubun et al. (13). For binding assays, cover slip cultures were washed free of serum-containing medium and incubated individually (80–120 pg protein/cover slip) in DMEM on glass cover slips (placed in polystyrol culture dishes) as described by Kokubun et al. (13). For binding assays, cover slip cultures were washed free of serum-containing medium and incubated individually (80–120 µg protein/cover slip) in a total volume of 1 ml of serum-free culture medium containing 1% BSA in the presence of 0.01-2 µCi/ml radiolabeled MAb (37°C, 60 min). Unspecific binding was defined as that amount of [³H]R3F1 that could not be displaced by 100 µg/ml of unlabeled antibody. Specific and unspecific binding for each concentration were always determined in duplicate. At the end of the incubation period, the cover slip cultures were removed from the radioactive medium, washed twice for 30 s and one time for 5 min in phosphate-buffered saline (PBS) and placed directly into counting vials. After digestion in Solutron (Kontron, Zurich, Switzerland), radioactivity was determined by liquid scintillation counting.
Cell homogenate. After the glass cover slips for intact cell measurements were removed, the cells grown on the plastic growth surface between the cover slips were washed in serum-free DMEM, scraped off, homogenized in a Potter-type glass-Teflon homogenizer, and adjusted to a protein concentration between 0.3 and 3.6 mg/ml. The homogenate was then equilibrated for 60 min at 37°C in DMEM with radiolabeled MAb in the presence and absence of an excess of nonlabeled MAb as described above. To avoid vesiculation and, hence, restricted access of the antibody to its binding site, all incubation media contained 0.1% saponin. Note that any fractionation or centrifugation of the homogenate was avoided to facilitate a quantitative comparison between binding data in intact cells and homogenate on the basis of total cell protein. Bound and free MAb were separated by filtration of 0.5-ml samples of the homogenate on glass fiber filters (Whatman GF/F) presoaked with polyethylenimine (4). After Solutron digestion of the protein, radioactivity on the filters was determined by liquid scintillation counting.

Protein Determination

Protein was determined according to the method of Peterson (26) using γ-globulin as a standard. Before any processing, all cell cultures were repeatedly rinsed in isotonic NaCl solution to eliminate possible interferences from culture medium components.

Data Analysis

Sequence data from exchanger DNA fragments were aligned to the sequence of the full-length clone, using the program “bestfit” in the GCG sequence analysis package (5). Binding data were analyzed by a computerized nonlinear least-square fitting procedure (program Inplot of Graphpad Software). The program yielded values for the apparent binding affinities and maximal binding capacities that are given in the figure legends.

RESULTS

Characterization of Anti-Exchanger MAb

From two different immunization cycles using the cloned myocardial exchanger expressed in S99 cells as antigen, we isolated 36 MAb that reacted with equine myocardial membranes in an ELISA screen. Of these antibodies, 12 showed medium to strong specific immunolabeling of the expressed exchanger protein on Western blots. The immune reaction was very faint or absent with the remaining 24 MAb. Typical responses are shown in Fig. 1. Most MAb produced similar immunolabeling pat-
terns with either the cloned exchanger (Fig. 1A) or myocardial membranes (Fig. 1B). This observation pointed to a possible colocalization of the respective epitopes. Only three of the MAb yielded banding patterns distinctly different from those obtained with a majority of the MAb.

The MAb were further characterized by determining the apparent binding affinities to sarcolemmal membranes using a quantitative titration of the ELISA reaction. The titration curves for three representative MAb are shown in Fig. 2. The apparent $K_D$ values varied over two orders of magnitude from 0.04 to 3.4 $\mu$g MAb/ml. MAb R3F1 showed by far the highest affinity of all MAb tested, with a $K_D$ of $\sim$260 pM. This was also the only antibody found to precipitate the solubilized exchanger protein out of a detergent and phospholipid-containing buffer. Figure 3 shows that $\sim$80% of the exchange activity could be removed by protein G-Sepharose beads coated with MAb R3F1. The immunoprecipitated exchanger protein could be recovered from the beads by heating in SDS (Fig. 3B, lane 1). The antibody did not cause significant unspecific precipitation of membrane proteins. All bands visible in a silver-stained SDS-PAGE separation of the immunoprecipitated material (in addition to the MAb heavy-chain band at $\sim$55 kDa) seem to belong to the exchanger and have matching bands in the immunoblot (Fig. 3B, lane 2). The three distinct bands at 60-80 kDa on the immunoblot (Fig. 3B, lane 2) most likely result from proteolytic degradation and appear frequently after prolonged storage of the exchanger in a solubilized form.

Screening of the Exchanger Sublibrary for MAb Epitopes

Recombinant cDNA clones coding for exchanger antigenic determinants were isolated by MAb screening of fusion proteins induced in E. coli. We included in this screening those 12 MAb that reacted most strongly with the exchanger on Western blots. In addition, we screened with the previously described MAb C2C12 that had been used in exchanger immunolocalization studies (6).

Four of the 13 MAb were able to react with fusion peptides from the exchanger sublibrary (Table 1). The epitope for each antibody was deduced from the minimum overlap of MAb-positive cDNA clones. Despite extensive screening, only a single positive clone could be identified for MAb C2C12. This clone carried an unusually long insert (463 bp, corresponding to amino acids 372-525; average insert size was 70-300 bp). We attempted to define further the C2C12 recognition site by reacting C2C12 with fusion proteins containing amino acids 372-445 or 471-525. The antibody reacted with neither fusion protein. The lack of shorter C2C12-reactive clones suggests that smaller fragments of the exchanger do not possess the proper conformation for the binding of MAb C2C12. In Fig. 4 we indicated the position of the epitopes on the topographical model of the exchanger suggested by Nicoll and Philipson (25).

All MAb binding sites are located on the hydrophilic region that connects the putative transmembrane domains 5 and 6. Overlapping epitopes were observed for
three MAbs (R2F10, R3F1, R12E6) on the carboxy-terminal half of this region. A highly charged sequence containing a cluster of four glutamic acid residues and three basic residues (residues 656–662) forms the heart of this region. In fact, the determinant for MAb R2F10 could be traced to a peptide only encompassing residues 649–705. Although both polypeptide sequences apparently contributed to the high binding affinity for this antibody, each alone interacted strongly enough to allow detection on immunoblots.

Several observations suggested that the three MAbs (R2F10, R12E6, and R3F1), despite recognizing partially overlapping epitopes, originated from different clones. 1) In cross-reaction studies, R2F10 and R12E6 both recognized epitope b but not epitope a of R3F1 (cf. Table 1). 2) R12E6 and R3F1 belong to different immunoglobulin classes (IgM and IgG, respectively) and differed in binding affinity by more than two orders of magnitude (Fig. 2). 3) The frequency of finding positive clones in the exchanger sublibrary was higher for R2F10 than for R12E6 (one in 3 \times 10^4 vs. one in 12 \times 10^4 clones).

### Topological Localization of MAb Binding Sites

Are the MAb epitopes on the large hydrophilic loop of the exchanger protein located on the intra- or extracellular surface of the membrane? This loop is placed intracellularly in the model of Nicoll et al. (24), but this tentative topology has not been confirmed. We examined the sidedness of antibody binding using radiolabeled MAb R3F1.

In a first set of experiments, we measured the binding of MAb [\(^3\)H]R3F1 (0.03–2 \mu g/ml) either to monolayer cultures on glass cover slips or to cell homogenates prepared from the remaining cells of the same tissue culture. Unspecific binding in the presence of 100 \mu g/ml unlabeled antibody was a linear function of MAb concentration and reached 15% of specific binding at 0.1 and 38% at 2 \mu g/ml MAb in homogenate preparations. It varied between 4 and 12% in intact cells. Specific binding was well fit by a saturation isotherm assuming binding with a 1:1 stoichiometry to a single homogeneous population of binding sites. In a representative experiment shown in Fig. 5, the binding affinity (K\(_D\) value) and maximal binding capacity for the homogenate, extrapolated from nonlinear least-square fits to the data points, were determined as 0.18 \mu g/ml [95% confidence interval (CI) 0.15–0.21] and 81 ng/mg protein [95% CI 58–104], respectively. The corresponding parameter estimates for intact cells (0.83 \mu g/mg protein, 95% CI 0.35–1.3; and 345 ng/mg protein, 95% CI 328–361), respectively. The corresponding parameter estimates for intact cells (0.83 \mu g/ml, 95% CI 0.35–1.3; and 345 ng/mg protein, 95% CI 58–104) were subject to a large error, because the binding curve is far from saturating even at the highest MAb concentration used. This curve probably describes a linear (intracellular?) uptake process rather than binding to
an extracellular saturating site. It should be noted that no cellular proteins were removed from the homogenate preparation. Therefore the homogenate binding data are quantitatively comparable with the ones for intact cells. Hence the increase in specific binding after homogenization suggests an intracellular location for the majority of binding sites.

The high number of binding sites in the homogenate does not prove binding to functional exchangers. Part of the binding could have been due to the presence of a precursor polypeptide in intracellular membranes. Therefore, in additional experiments, we have determined MAb R3F1 binding to purified equine myocardial membranes (Fig. 6). Compared with cell homogenate, this preparation was enriched ~10-fold in sarcolemmal marker proteins (β-adrenoceptors, L-type calcium channels) and Na⁺-Ca²⁺ exchange activity. Specific binding exceeded homogenate binding by a factor of ~10, whereas the background of nonspecific binding remained very low (<10% at 1.5 μg/ml MAb). We conclude from this result that MAb R3F1 binding capacity measures the amount of functional exchanger present in the sarcolemmal membrane.

**Binding Sites for MAb R3F1 in Noncardiac Tissues**

The binding characteristics for MAb R3F1 suggested that it might be generally suitable as a highly specific ligand to study the abundance and regulation of Na⁺-Ca²⁺ exchange in various tissues. No such ligand is presently available. We tested this possibility in two different cell lines, H9c2, a muscle cell line derived from embryonic rat heart cells (11), and PC12 rat pheochromocytoma cells (8). No published data on Na⁺-Ca²⁺ exchange in these two cell lines seemed to be available. The results of these experiments are shown in Figs. 7 and 8. Separate binding curves for intact cells and homogenate were obtained only for the muscle cell line. Intact PC12 cells did not adhere sufficiently to the growth surface and would have been lost from the cover slip in the course of the experiment. Homogenate from both cell lines clearly showed a saturating component of MAb binding. However, the maximal binding capacity for H9c2 and PC12 cells reached only ~10 and 5%, respectively, of the corresponding value in cardiac cells. Again, binding to intact cells was very low. Because the presence of Na⁺-Ca²⁺ exchange activity had not been established for these cells, we measured sodium-activated ⁴⁵Ca²⁺ uptake as a functional equivalent for the exchanger. In view of the expected low activity, the flux studies were performed in a partially purified membrane vesicle preparation rather than in intact cells. The results (Figs. 7 and 8, insets) confirm the presence of a small component of Na⁺-dependent Ca²⁺ influx. The apparent initial rates for H9c2 and PC12 membrane vesicles (0.24 and 0.14 nM Ca²⁺-mg protein⁻¹·10 s⁻¹, respectively) reached <3% of the corresponding value (11.4 nM·mg protein⁻¹·10 s⁻¹) in cardiac membrane vesicles. However, the method of membrane preparation that may significantly affect the calcium transport rates was not identical in all cases.
Therefore, on the basis of these data alone, a reliable quantitative comparison of the flux rates is not yet possible.

**DISCUSSION**

We have identified the main immunogenic region of the cardiac Na\(^{+}\)-Ca\(^{2+}\) exchanger and mapped it to an intracellular portion of the long hydrophilic loop connecting the putative transmembrane domains 5 and 6. Our findings, although compatible with the tentative model suggested by Nicoll et al. (24), constitute important information on the topological organization of the exchanger that is not derived from often ambiguous hydrophathy analysis. Our study was subject to the same limitations that were noted in previous attempts at mapping membrane proteins with MAb. 1) Out of a fairly large panel of MAb raised against the native protein, only a small minority recognized linear continuous sequence epitopes as generated from our expression sublibrary. A majority of MAb probably detected epitopes elsewhere on the surface of the protein that were discontinuous with respect to the primary sequence (17, 39, 42). 2) The immune response appeared to be dominated by a single domain that interacted with most of the high-affinity MAb (37, 40).

What distinguishes the immunodominant region from the rest of the protein? Earlier immunological studies with other proteins have shown that linear epitopes are formed by sequences exposed on the surface of an antigenic protein, preferentially in rather mobile domains of the polypeptide chain. Antigenicity and mobility in native proteins are well correlated for continuous (but not for discontinuous) epitopes (37, 42). A relatively small number of amino acid residues usually contribute the bulk of the binding energy in linear epitopes. In contrast, discontinuous epitopes frequently occupy a rather large surface area of the protein (17). The strongly charged residues (residues 656-662) that are present in three of five defined epitopes make a very good candidate for such a highly antigenic core region. This is confirmed by the structure of the epitope for MAb R2F10, consisting of only 15 amino acid residues, grouped around the cluster of charged residues. On the other hand, the rather extended epitope for MAb C2C12 indicates that linear high-affinity determinants can be formed outside the core region of the immunodominant region but require the contribution of many residues. The peak of the insert size distribution in our library ranged between 100 and 300 bp. The scarcity of 400- to 500-bp inserts may explain why only a single epitope of this kind could be identified.

In summary, our results predict that the immunodominant region of the exchanger extending from residues 560 to 705 is neither heavily folded nor otherwise immobilized. Nevertheless, the split epitope for MAb R3F1 may reflect some substructuring of this domain. Possibly the residues within the gap are not exposed on the surface of the protein.

**Topological Organization of the Long Hydrophilic Loop Within the Exchanger Protein**

Before our study, direct experimental evidence for an intracellular or extracellular location of the long hydrophilic loop was lacking. Analogies to the structural models of other transport proteins (15, 34) favored an intracellular location. The amino-terminus of this loop contains a potential calmodulin binding site (residues 219-238) that should be located on the cytoplasmic side if it is to interact with calmodulin. However, a direct effect of calmodulin on the exchanger has not been reported. A peptide with the sequence of this potential calmodulin binding site (XIP) was shown to inhibit transmembrane Na\(^{+}\)-Ca\(^{2+}\) exchange by acting on the cytoplasmic side (18). However, the exact binding site for XIP has not yet been defined. The loop also contains a potential calmodulin-like Ca\(^{2+}\) binding site. However, it is not clear whether this site is indeed involved in the functional regulation of the exchanger by intracellular Ca\(^{2+}\) (25). Immunolocalization studies suggest that an analogous hydrophilic loop of the retinal Na\(^{+}\)-Ca\(^{2+}\)-K\(^{+}\) exchanger is exposed on the inside of the membrane, but there is little sequence similarity between these two transport proteins (32). Our labeling studies with MAb R3F1 now clearly establish an intracellular location of the immunodominant region. Because most of the MAb epitopes are clustered on the carboxy-terminal half of the long hydrophilic loop, there is still a theoretical possibility of an outward-facing sequence on the amino-terminal half of the loop. At present such an arrangement is not supported by experimental data. Earlier fluorescence labeling studies of the exchanger using MAb C2C12 seemed to suggest an extracellular binding site (6). Judging from the results of our binding experiments, however, an apparent signal from extracellular MAb could be due to intracellular uptake of a small fraction of the label. In principle, the observed extracellular binding of MAb R3F1 could also have resulted from a cross-reaction with the external domain of a low-abundance membrane protein not related to the exchanger. We cannot definitely exclude this possibility but consider it unlikely. A major contribution from a second binding site would have caused a significant deviation of the data points from the one-site binding isotherm in purified and permeabilized membrane vesicles (Fig. 6).

The high-affinity MAb R3F1 provides an excellent ligand for the quantitative evaluation of membrane con-
centration and tissue distribution of the cardiac-type exchanger protein. On the basis of our measurements (2.2 \times 10^6 cells/mg protein; mean maximal binding capacity from 4 cover slip cultures at 37°C, 365 ng MAb/mg protein), we calculate that intact cultured rat heart cells express \sim 600,000 exchanger molecules/cell. Assuming a surface area of \sim 800 \mu m^2 for a cultured neonatal cell (\sim 20 \times 40 \mu m), this value would correspond to a density of 750 exchanger molecules per micrometer squared. From the measurements of charge movements in guinea pig sarcolemma, Hilgemann et al. (10) estimated a density of 400 exchangers/\mu m^2. The agreement between the two estimates, derived from entirely different approaches, is quite good. In addition, it supports the view that most of the exchanger molecules expressed in the cell membrane are functional at any given time. The high specific activity of the ligand (which could be further enhanced by using iodine rather than \textsuperscript{3}H as radiolabel) facilitates the binding affinities may be useful in the search for isoforms of the exchanger. In addition, analysis of MAb binding affinities may be useful in the search for isoforms of the exchanger. Cardiac preparations that we have tested (dog, rat, horse) all showed similar binding affinities for \textsuperscript{[3]H}R3F1, whereas the corresponding values for PC12 and H9c2 cells appeared significantly higher or lower, respectively. Further studies are required to reveal what changes in the epitope conformation may cause these differences.

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ANTIYOD MAPPING OF THE CARDIAC NA\textsuperscript{+}-CA\textsuperscript{2+} EXCHANGER

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