Use of Expression Mutants and Monoclonal Antibodies to Map the Erythrocyte Ca\(^{2+}\) Pump*

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Deletion and truncation mutants of the human erythrocyte Ca\(^{2+}\) pump (hPMCA4b) were expressed in COS-1 cells. The reactivity patterns of these mutants with seven monoclonal antibodies were examined. Of the seven, six (JA9, JA3, 1G4, 4A4, 3E10 and 5F10) react from the cytoplasmic side. JA9 and JA3 reacted near the NH\(_2\) terminus and the COOH terminus of the molecule, respectively. 5F10 and 3E10 recognized portions of the large hydrophilic region in the middle of the protein. The epitopes of 1G4 and 4A4 were discontinuous and included residues from the long hydrophilic domain and residues between the proposed transmembrane domains M2 and M3. Antibody 1B10, which reacts from the extracellular side, recognized the COOH-terminal half of the molecule. These results show that the NH\(_2\) terminus, the COOH terminus, the region between M2 and M3, and the large hydrophilic region are all on the cytoplasmic side. This means that there are an even number of membrane crossings in both the NH\(_2\)-terminal and the COOH-terminal halves. Between residues 75 and 300 there must be at least two membrane crossings, and there are at least two membrane crossings in the COOH-terminal half of the molecule.

The Ca\(^{2+}\) pump of plasma membrane extrudes Ca\(^{2+}\) from the cytosol to the extracellular space. This pump is one of the most important mechanisms used by the cell to regulate its total Ca\(^{2+}\) content (Penniston, 1983; Rega and Garrahon, 1986). This membrane-bound enzyme is related to a number of other cation transport ATPases, including the SR Ca\(^{2+}\) pump and the Na\(^+/\)K\(^+\) pump. These enzymes belong to the class of P-type ATPases, as they all have a phosphoenzyme intermediate in the enzyme cycle (Pedersen and Carafoli, 1987). The examination of the plasma membrane Ca\(^{2+}\) pump sequence by means of computer-generated hydrophobicity plots (Shull and Greeb, 1988; Verma et al., 1988) led to the proposition of topological models analogous to those of other cation transport ATPases (MacLennan et al., 1985; Brandl et al., 1986). However, structures predicted from such sequence information need confirmatory data. The proposals derived from hydropathy plots are controversial and different numbers of transmembrane a-helices have been proposed by different authors. Different proposals have also been made for the location of the NH\(_2\) terminus and the COOH terminus. In the SR Ca\(^{2+}\)-ATPase both ends were found to be on the cytoplasmic side (Matthews et al., 1989; Reichemeier and MacLennan, 1981). In the case of the Na\(^+/\)K\(^+\)-ATPase, some investigators found the COOH terminus on the extracellular side (Shull et al., 1985; Jorgensen and Andersen, 1988; Antolovic et al., 1991), while others have placed it on the outer surface of the cell (Ovchinnikov et al., 1988; Bayer, 1990). It is not clear at the present time if this variation in the proposed localization of the COOH-terminal end may be related to the ion specificity of the enzyme.

We have expressed in COS-1 cells the full length human erythrocyte plasma membrane Ca\(^{2+}\) pump hPMCA4b and a set of deletion and truncation mutants, as shown schematically in Fig. 1. Study of the reaction of these mutants with seven well characterized monoclonal antibodies has allowed us to locate the corresponding epitopes within accurately defined regions of the molecule. This information has allowed us to determine the topology of several portions of the pump, as discussed below.

EXPERIMENTAL PROCEDURES

The construction of a full-length hPMCA4b cDNA and the transfection of COS-1 cells were done as previously described (Adamo et al., 1992). Mutants coding partial fragments of the protein were obtained either using natural restriction sites present in the DNA or by introducing new restriction sites and stop codons. For this purpose, the oligonucleotides shown in Table I were used. The mutations were made using the Altered Sites Mutagenesis Kit (Promega Corp.) according to the manufacturer's instructions. The cDNA containing the desired mutations was cloned into the PMT2 vector (Kaufman, 1990) for expression in COS-1 cells (Gluzman, 1981).

Cells were cultured in 75-cm\(^2\) flasks for 48 h then washed twice with 10 ml of PBS and lysed with 200 \(\mu\)l of a solution of Nonidet P-40 1%, 50 mM Tris-HCl (pH 7.5 at 37 °C), 1 mM phenylmethanesulfonyl fluoride, and 0.9 mg/ml aprotinin. The debris was pelletted by centrifugation in a microcentrifuge for 2 min at 4 °C. Ten micrograms of protein from the extract were separated by SDS-polyacrylamide gel electrophoresis on precast 7-14% gradient gel (purchased from Bio-Rad). Proteins were transferred to Millipore Immobilon polyvinylidene fluoride membranes as described by Towbin et al. (1979). Nonspecific binding was blocked by PBS containing 1% bovine serum albumin for 1 h at 37 °C. The membranes were then incubated in antibody ascitic fluid diluted 1:500 in PBS for 1 h at 37 °C. The staining was carried out with biotinylated anti-mouse IgG and avidin-biotin peroxidase conjugate (Vectastain ABC kit). Some of the mutants shown in Fig. 1 code for proteins that may not fold correctly and, therefore, may undergo rapid proteolytic attack or may be retained in the endoplasmic reticulum to form aggregates (Haas and Wahl, 1983) which still can be recognized by the antibodies. Regard-
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Table I

Sequences of the oligonucleotides used for mutagenesis

Where changes in the nucleotide sequence of the hPMCA4 were introduced, they are indicated in capital letters. The nucleotide present in the original sequence is shown below and the restriction sites which resulted are also indicated.

| Restriction Enzyme | Oligonucleotide Sequence |
|---------------------|--------------------------|
| Miul | 5'-gtccccctccgCGtctgcgcctcga-3' |
| Miul | 5'-cacgtctccgGTtctgcggcagcagtcg-3' |
| Miul | 5'-gccatgtctccgCTtctgcgcagcagtcg-3' |
| Miul | 5'-tctgaacagccagACgCgtcagcttgccct-3' |
| Miul | 5'-gccataggggcgACgcGtcaacagagattc-3' |
| Miul | 5'-gcccatggtctcagACtctcaagtgccg-3' |
| Miul | 5'-gctgtcgtctccagctccgcgcacacagccc-3' |
| Miul | 5'-tccctcggcatcAGGtctcggccatcga-3' |
| Miul | 5'-cttctctgtcctttttttgtccttctttttctcccct-3' |
| Miul | 5'-gcgccttcacgACgctccgcttcaccagtcg-3' |
| Miul | 5'-actcctgttttttttttttcGGGtcttttttttttccct-3' |

Table II

Antibody binding to intact erythrocytes and IOVs

The preparation of IOVs used in this experiment was estimated to contain 68% of outside-out vesicles; the rest of the vesicles were right-side-out or leaky, in unknown ratio. The six antibodies directed toward the cytoplasmic face bind to IOVs and to at least some of the leaky vesicles, giving a binding of more than 68%.

| Antibody | Percent of maximum binding |
|----------|---------------------------|
| Erythrocytes | IOVs |
| 3C2 | 5 | 84 |
| JA3 | 14 | 85 |
| JA7 | 15 | 84 |
| JA8 | 27 | 85 |
| JA9 | 10 | 87 |
| 5F10 | 10 | 87 |
| 1B10 | 104 | 26 |
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Fig. 1. Diagram of the deletion and truncation mutants of the Ca\(^{2+}\) pump. The putative transmembrane segments are indicated by dark lines. The numbers in italics refer to the amino acid residues present in each mutant, according to the published sequence of hPMCA4 (Strehler et al., 1990). The initiation region of the message for the pump, coding for amino acids 1–16, was included in each construct. This is indicated by the open box.

Fig. 2. Localization in the Ca\(^{2+}\)-ATPase amino acid sequence of the JA9 and JA3 epitopes. Segments of the hPMCA4 plasma membrane Ca\(^{2+}\) pump derived from expression of the corresponding cDNA subcloned into pMT2 for expression in COS-1 cells, were immunoblotted with antibodies JA9. Lanes: 1, control (nontransfected); 2, H119; 3, Hkqm7; 4, Hiseco; 5, Hinm1; 6, Hisof. Blots were also done with antibody JA3: lanes 1, H119; 2, Hinat; 3, Hnacs; 4, Hiseco. Ten micrograms of protein from COS-1 cell homogenate was loaded in each well.

al., 1989). In agreement with this Fig. 2 also shows that JA3 recognized a fragment containing amino acid residues 1135–1205 and that reactivity of JA3 was lost when a COOH-terminal fragment from residue 1068 to the end was removed from the protein. This demonstrates that the epitope for JA3 is localized between residues 1135 and 1205 and, therefore, confirms the cytoplasmic localization of the COOH terminus. Fig. 3 shows the reaction of different Ca\(^{2+}\) pump fragments with antibodies 1G4 and 4A4. Although these antibodies gave similar recognition patterns, they show different reactivity toward fragments generated by partial proteolysis of the purified Ca\(^{2+}\) pump, suggesting that their epitopes are not equivalent.\(^2\) These antibodies recognized two non-overlapping fragments showing intense reaction with fragments containing residues 300–350 and a weaker reaction with fragments con-

\(^2\) A. J. Caride, unpublished observations.
Fig. 3 also shows that the antibody 3E10 reacted well with fragments containing residues 1–560 and 452–1205 but it did not react with either fragments containing residues 1–724 or one containing residues 783–1205. This indicates that 5F10 binds with either fragments containing residues 1–724 or one containing residues 452–1205. It did not react with any shorter fragments, with solubilized purified ATPase, or with the smaller (less than 80 kDa) fragments generated by proteolysis (Caride et al., 1988). All of the data about 1B10’s reactivity indicate that its epitope requires a conformationally intact COOH terminus.

The lack of reactivity by the mutants Hinm5 and Hkmn7 does not show that the region represented in these constructs is not in the 1B10 epitope. Rather, it shows that these constructs do not have a conformationally intact 1B10 epitope. Nonetheless, the reactivity of 1B10 with the fragment containing residues 452–1067 indicates that at least one loop between residues 452 and 1067 must be accessible from the extracellular side of the membrane.

Effect of the Antibodies on the Ca\(^{2+}\)-ATPase Activity—Fig. 5 shows the effect of the binding of the antibodies on the Ca\(^{2+}\)-ATPase activity. In agreement with the previous reports, 5F10 did not significantly affect the Ca\(^{2+}\) pump activity. The antibody 3C2, which is functionally equivalent to JA9 (Papp et al., 1989), did not affect the Ca\(^{2+}\) pump activity either. The antibody 4A4 inhibited the Ca\(^{2+}\)-ATPase activity about 40%. This inhibitory effect is comparable with that already reported for JA9 and JA3 show that both the COOH and the NH\(_2\) termini of this pump are on the cytoplasmic side of the membrane. This contrasts with the results reported for the Na\(^+/K^+\)-ATPase, where the COOH terminus was believed to be extracellular (Ovchinnikov et al., 1988; Bayer, 1990). The results with the other four cytoplasmically directed antibodies, 1G4, 4A4, 3E10, and 5F10, demonstrate that there exists a large cytoplasmically directed domain in the middle of the primary structure of the Ca\(^{2+}\) pump. The existence of such a domain is no surprise, since most of the sites to which substrates must bind are located in this region of the pump. This domain includes the site at which the phosphorylated intermediate is formed and the site at which fluorescein isothiocyanate reacts. In addition to their reactivity with large central cytoplasmic domain, antibodies 1G4 and 4A4 react strongly with another epitope between residues 300 and 350. This demonstrates that these residues are also inside the cell, as is indicated by the double-headed arrow in Fig. 6. These residues lie between putative transmembrane domains M2 and M3.

The extracellularly oriented antibody 1B10 is clearly directed against the COOH-terminal half of the enzyme, but it

### DISCUSSION

Our understanding of the relationship between structure and function in proteins is critically dependent on knowledge about protein topology. Most of our information about the topology of membrane proteins has come from hydropathy plots of the amino acid sequence, but this kind of data only tells us where membrane-crossing regions could occur. It does not tell us whether membrane crossings actually occur in those locations. Because of this, hydropathy plots have been interpreted differently by different workers. For instance, such plots of the COOH termini of the Na\(^+/K^+\) pumps have been used as evidence for the existence of two or four or an uneven number of transmembrane helices (Kawakami et al., 1985; Shull and Lingrel, 1986; Bayer, 1990). Because of the uncertainty of hydropathy plots, additional evidence is needed to determine the topology of membrane proteins; by the use of antibodies, we have obtained independent information about the topology of the plasma membrane Ca\(^{2+}\) pump. This study has, by the use of a variety of truncation and deletion mutants of the human erythrocyte plasma membrane Ca\(^{2+}\) pump (hPMCA4b), located within accurate boundaries the epitopes of a set of antibodies. The results are summarized in Tables III and IV, which show six antibodies that are directed against the cytoplasmic face of the enzyme and one antibody (1B10) that is directed toward the outside.

Taken together, these data provide considerable information about the topology of the plasma membrane Ca\(^{2+}\) pump. The results with antibodies JA9 and JA3 show that both the COOH and the NH\(_2\) termini of this pump are on the cytoplasmic side of the membrane. This contrasts with the results reported for the Na\(^+/K^+\)-ATPase, where the COOH terminus was believed to be extracellular (Ovchinnikov et al., 1988; Bayer, 1990). The results with the other four cytoplasmically directed antibodies, 1G4, 4A4, 3E10, and 5F10, demonstrate that there exists a large cytoplasmically directed domain in the middle of the primary structure of the Ca\(^{2+}\) pump. The existence of such a domain is no surprise, since most of the sites to which substrates must bind are located in this region of the pump. This domain includes the site at which the phosphorylated intermediate is formed and the site at which fluorescein isothiocyanate reacts. In addition to their reactivity with the large central cytoplasmic domain, antibodies 1G4 and 4A4 react strongly with another epitope between residues 300 and 350. This demonstrates that these residues are also inside the cell, as is indicated by the double-headed arrow in Fig. 6. These residues lie between putative transmembrane domains M2 and M3.

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### TABLE III

| Mutant   | Amino acid residues | JA9 | JA3 | IG4 | 4A4 | 3E10 | 5F10 | 1B10 |
|----------|---------------------|-----|-----|-----|-----|------|------|------|
| H119     | 1–1205              | +   | +   | +   | +   | +    | +    | +    |
| Hinn1    | 1–16, 75–1205       | -   | -   | +   | +   | +    | +    | -    |
| Hinn7    | 1–16, 358–1205      | +   | +   | +   | +   | +    | +    | +    |
| Hinat    | 1–16, 452–1205      | -   | +   | +   | +   | +    | +    | -    |
| Hinnm5   | 1–16, 783–1205      | -   | -   | -   | -   | -    | -    | -    |
| Hineco   | 1–16, 1135–1205     | -   | +   | +   | +   | +    | +    | -    |
| Hkmn7    | 1–300, 452–1205     | +   | +   | +   | +   | +    | +    | +    |
| Hmsce    | 1–1067              | +   | -   | +   | +   | +    | +    | +    |
| Hisce    | 1–724               | +   | +   | +   | +   | +    | +    | -    |
| Haval    | 1–560               | +   | -   | +   | +   | +    | +    | -    |
| Hespt    | 1–350               | +   | -   | +   | +   | +    | +    | -    |
| Hisof    | 1–84                | +   | -   | -   | -   | -    | -    | -    |

The recognition of the mutant by the antibody is indicated by +, absence of reactivity is indicated by −, blank space indicates reactivity not tested.
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is not possible to define its reactivity more closely than this. This is presumably because its epitope requires the cooperation of more than one portion of the primary structure. Shorter versions of the pump do not show reactivity with this antibody even though they may contain one portion of its epitope. Despite this difficulty in more exactly locating the epitope of 1B10, its reactivity with the COOH-terminal half demonstrates that at least one loop from the COOH-terminal half must be exposed to the outside of the cell. In addition to the studies in this paper, a recent study has shown that another antibody (1E4) reacts extracellularly between putative transmembrane regions M1 and M2 (Feschenko et al., 1992).

Taken altogether, these data demonstrate that there are an even number of transmembrane crossings in the whole molecule, in the NH\(_2\)-terminal half of the molecule, and in the COOH-terminal half of the molecule. In each half of the molecule, there is at least one pair of transmembrane crossings, since antibody 1E4 reacts with an extracellular domain in the NH\(_2\)-terminal portion of the molecule and 1B10 in the COOH-terminal portion. In the amino-terminal half, membrane crossings must occur in the vicinity of the putative transmembrane domains M1 and M2, since the cytoplasmic and extracellular locations of regions at both ends of these domains have been identified. It is probable that M3 and M4 also cross the membrane, although no antibody against the extracellular loop between these two domains has yet been found. Similarly, in the COOH-terminal half of the molecule there could be 2, 4, or 6 membrane crossings. Fig. 6 retains the model with 10 total transmembrane regions which was first introduced by MacLennan for the sarcoplasmic reticulum Ca\(^{2+}\) pump (Brandl et al., 1986). Clearly, this model is consistent with all of the data presented here, and our data have added considerably to the evidence supporting a model like this.

The antibodies studied here include samples both of simple epitopes composed of contiguous residues (JA9, JA3, 3E10, and 5F10) and of epitopes which are composed of regions which are separated from one another on the primary structure (1G4, 4A4, and 1B10). In the latter cases, the epitopes, while assembled from amino acid sequences separated in the primary structure, probably come together on the surface of the molecule when the polypeptide chain folds to form the native protein (Laver et al. 1990). Of the second group of antibodies, 1G4 is particularly interesting because we have already studied its inhibition of the Ca\(^{2+}\) pump (Caride et al., 1989). This antibody reacted strongly with a fragment which, in the proposed topological model, would form a minor cytoplasmic loop between the transmembrane helices 2 and 3. This region must contain the residues containing most of the binding energy for the antibody. The antibody also recognized more weakly the large cytoplasmic region of the central part of the molecule. This suggests that in the native protein, these two regions interact closely, and it is presumably the binding of 1G4 to both of these structures that inhibits the enzyme. It is worthwhile to note that, in a recent study, Harris et al. (1991) have also shown evidence of the interaction between the first two hydrophilic loops of the yeast plasma membrane H\(^+\)-ATPase during the hydrolysis of the aspartyl-phosphate intermediate. It is interesting to note that the 1G4 inhibition was found to be noncompetitive with ATP in the high affinity ATP site, and it has also been shown to be concomitant with the decrease in the steady-state level of \(E \rightarrow P\) of 50%. This inhibition has been explained assuming that the 1G4 binding favors the E2 conformation of the enzyme (Caride et al., 1989). It is plausible that the region containing the epitope

**Fig. 4.** Localization of the 5F10 and 1B10 epitopes. 5F10, lanes: 1, H119; 2, Hmace; 3, Hkqat; 4, Hin76; 5, Hesp.

**Fig. 5.** Effect of monoclonal antibodies on the ATPase activity from purified erythrocyte Ca\(^{2+}\)-pump. The Ca\(^{2+}\)-ATPase activity was estimated as described under "Materials and Methods." The reaction media contained the indicated monoclonal antibody or nonimmune IgG. The epitope for the antibodies are localized in the stretch of amino acid residues indicated.

**Fig. 6.** Model proposed for the transmembranous organization of the plasma membrane Ca\(^{2+}\) pump. D represents the phosphate acceptor site. The regions of the molecule which react with antibodies are indicated.

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**TABLE IV**

Localization of the epitopes for antibodies in the hPMCA4 primary sequence

| Antibody | Amino acid residues |
|----------|---------------------|
| JA9      | 17–75               |
| JA3      | 1135–1205           |
| IG4      | 300–350 and 452–783 |
| 4A4      | 300–350 and 452–783 |
| 3E10     | 452–560             |
| 5F10     | 724–783             |
| 1B10 (outside) | 452–1067            |
for 1G4 undergoes large motions during the E2 → E1 transition and that the attachment of the antibody would slow down such motions, producing a concurrent decrease in the pump's activity.

It has been proposed that the calmodulin-binding domain of the Ca²⁺ pump interacts with a region located just downstream of the phosphate acceptor site causing the inhibition of the pump in the absence of calmodulin (Palchetti et al., 1991). The epitope of antibody 3E10 lies very near the sequence they indicated as the receptor site for the calmodulin-binding domain with its site on the molecule when 3E10 is bound there instead. It has been previously reported that this was the case, and this fragment could have restricted interaction of the inhibitory calmodulin-binding domain with its site on the molecule when 3E10 is bound there. It was supposed to interact in a bending mode with the phosphorylation site (MacLennan et al., 1985). A recent mutagenesis analysis of the functional importance of this region was carried out in the sarcoplasmic reticulum Ca²⁺ pump (Vilsen et al., 1991) and showed that at least half of the mutations introduced in this region did not significantly affect Ca²⁺ transport. Thus, although this region is highly conserved, neither the binding of the antibody 5F10 to the "hinge" region of the hPMCA, nor many of the mutations introduced in the homologous region of the SR Ca²⁺ pump inhibited this pump.

The location we have found for the epitope of antibody JA9 is very near the NH₂ terminus and differs from that previously published (Papp et al., 1989). The previous study put the epitope of JA9 at least 10 kDa away from the amino terminus. This localization of the JA9 epitope was based on the assumption that a 125-kDa fragment of the pump had 10 kDa removed from the amino terminus. There was no definitive evidence that this was the case, and this fragment could have resulted from removal of 10 kDa from the COOH terminus. If this were the case, then the previous data would agree with our current assignment.

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