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IDENTIFICATION OF TRANSMEMBRANE DOMAINS OF THE RED CELL CALCIUM PUMP WITH A NEW PHOTOACTIVATABLE PHOSPHOLIPIDIC PROBE

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The membrane-associated regions of the human erythrocyte Ca^{2+} pump were investigated by hydrophobic photolabeling. Purified Ca^{2+} pump was reconstituted in asolectin vesicles loaded with [^{3}H]DIPETPD, a photochemical probe designed to label deeply into the hydrophobic core of the lipid bilayer (Delfino et al. *J. Am. Chem. Soc.* **115**, 3458-3474, 1993). After photolysis and SDS-PAGE analysis, a significant light-dependent labeling of the Ca^{2+} pump was found. Controlled proteolysis of the photoadduct with trypsin or protease V8 followed by SDS-PAGE and immunoblotting yielded individual labeled fragments. The labeling pattern indicated the existence of three sequential clusters of transmembrane regions, consistent with the current model for the topography of this enzyme.

The Ca^{2+} pump of plasma membrane is responsible for actively pumping Ca^{2+} at the expense of ATP hydrolysis from the cytosol to the extracellular compartment in all eucaryotic cells. This is an integral membrane protein consisting of a single polypeptide chain of Mr 135000-140000. The pump appears to comprise a small extracellular portion and large intracellular domains, which include catalytic and regulatory sites [1-3]. The ion translocation machinery should be localized in the extensive transmembrane domain of this protein. So far, knowledge of the topography of this critical region was limited to the interpretation of hydrophobicity profiles.

Reagents designed to react within the hydrophobic milieu of the lipid bilayer have helped to identify membrane-associated peptides and map the protein-lipid interface [4]. For this purpose we employ here [^{3}H]DIPETPD, a bipolar phospholipid provided with a covalently bonded chain designed to span the membrane and equipped with a centrally defined attachment point for the photolabeling group TPD (Scheme 1). Upon irradiation with uv light, the TPD function generates a very reactive carbene species that labels randomly in the lipid phase [5]. We describe here the

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Abbreviations used: DTT: 1,4-dithiothreitol; TPD: (trifluoromethyl) phenyldiazirine; [^{3}H]DIPETPD: [^{3}H]bis-phosphatidylethanolamine (trifluoromethyl) phenyldiazirine; [^{125}I]-TID: 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine; C12E10: n-dodecyl decaethylene glycol monoether; MOPS: 3-(*N*-morpholino)-propanesulfonic acid; SDS-PAGE: polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; TPCK: tosylphenylalanylchloromethyl ketone; EGTA: ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*,-tetraacetic acid; MAb: monoclonal antibody.

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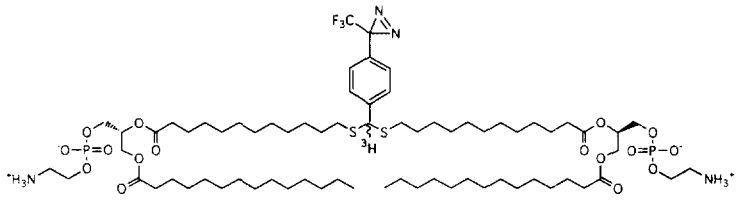


Scheme 1. [^{3}H]DIPETPD.

reaction of [^{3}H]DIPETPD with the Ca^{2+} pump of human red blood cells reconstituted in proteoliposomes, the fragmentation of the protein and the identification of labeled peptides which comprise different sections of the transmembrane domain.

MATERIALS AND METHODS

Materials - Calmodulin-agarose, strain V8 protease (type XVII-B), TPCK treated trypsin, 3,4-dichloroisocoumarin, PMSF, leupeptin hemisulfate, MOPS, HEPES, and asolectin were from Sigma Chemical Co., USA. Phosphatidylcholine was purchased from Avanti Polar Lipids, USA. [^{3}H]DIPETPD was prepared according to [5]. [^{45}Ca]Cl_2 was purchased from New England Nuclear, USA. All other chemicals were of analytical grade. Recently drawn human blood for the isolation of the Ca^{2+} pump was obtained from the Hematology Section of the Hospital de Clínicas José de San Martín (Argentina).

Purification of the Ca^{2+}pump from human erythrocytes - Calmodulin-depleted erythrocyte membranes were prepared as described in [6]. Ca^{2+} pump was isolated by the calmodulin affinity chromatography procedure [7]. The enzyme was stored in 0.05 % C12E10, 0.1 % asolectin, 20 mM MOPS-KOH (pH 7.4 at 4°C), 2 mM EDTA, 2 mM $CaCl_2$, 2 mM DTT and 300 mM KCl. Fractions were selected according to their specific ATPase activity [8]. Protein concentration was determined according to [9].

Reconstitution of the purified Ca^{2+}pump into lipid vesicles loaded with [^{3}H]DIPETPD- Ca^{2+} pump was reconstituted according to an adaptation of the cholate-dialysis method [10]. [^{3}H]DIPETPD was purified before use by TLC as described in [5]. 70-200 μCi of [^{3}H]DIPETPD (1 μCi per ml of toluene: ethanol, 1:1) were added to 140-400 μg of phosphatidylcholine. The solvent was evaporated under a stream of nitrogen under constant stirring to leave a thin film at the bottom of the tube. Traces of solvent were removed under high vacuum. The film was resuspended in 80 mM sodium cholate and sonicated for 10 min at 20°C. Purified Ca^{2+}pump (60-100 μg) was then added and the composition was adjusted to 130 mM KCl, 20 mM MOPS-KOH (pH 7.4 at 4°C, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM EDTA, 2 mM DTT, 0.05 % C12E10, 0.1 % asolectin, 50-150 μCi/ml [^{3}H]DIPETPD and 20 mM sodium cholate. The mixture was incubated for 10 min at 4°C and dialyzed three times for 6 h at 4°C against 650 ml of 130 mM KCl, 20 mM MOPS-KOH (pH 7.2), 1 mM $MgCl_2$, 50 μM $CaCl_2$ and 2 mM DTT.

Photolysis - Samples of proteoliposomes loaded with [^{3}H]DIPETPD were placed in a quartz cuvette and irradiated for 10 min at 366 nm, with a Desaga UVIS setup (C. Desaga, Germany).

Tryptic digestion of the Ca^{2+} pump - After photolysis, labeled proteoliposomes were partially digested with trypsin under conditions modified from [11] in 130 mM KCl, 20 mM MOPS-KOH (pH 7.2), 1 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 2 μg/ml trypsin and 20 μg/ml Ca^{2+}pump at 4°C. After 30 min, hydrolysis was stopped by the addition of 300 μM PMSF and 100 μM leupeptin hemisulfate.

Protease V8 digestion of the Ca^{2+} pump - After photolysis, labeled proteoliposomes (100 μg of Ca^{2+}pump per ml) were dialyzed against 50 mM HEPES-KOH (pH 7.2), 5 mM $MgCl_2$, 4 mM $CaCl_2$, 1 mM EDTA, 2 mM DTT, then supplemented with 0.8 % SDS and partially digested with V8 protease (200 ng per ml) at 25°C. The reaction was stopped after 30 min by the addition of 3,4-dichloroisocoumarin [12] up to a final concentration of 25 μM.

Lipid extraction - Labeled proteoliposomes, containing either intact or proteolysed Ca^{2+} pump, were delipidized according to [13] with some modifications. Samples were washed three times with 1 vol. of

chloroform:methanol (3:1) in the presence of 0.2 % aqueous SDS. The organic phase was separated after centrifugation for 3 min at 12000 x g. Traces of organic solvent were evaporated under a stream of nitrogen, before the remaining aqueous phase was dried under vacuum.

Polyacrylamide gel electrophoresis - Samples were incubated at room temperature for 5-10 min in sample buffer. SDS-PAGE (10 % T and 2.6 % C) was carried out by the Tris/tricine method [14]. Staining of peptide bands was carried out according to [15]. The gel was dried and cut into slices. Radioactivity present in each slice was determined by liquid scintillation counting as described in [16]. A background correction was performed by subtracting the average radioactivity for gel slices containing no peptidic material.

Peptide identification- After SDS-PAGE, peptides were transferred to nitrocellulose paper (pore size 0.2 μm) as described in [17] and identified by reaction with a set of specific monoclonal antibodies [18].

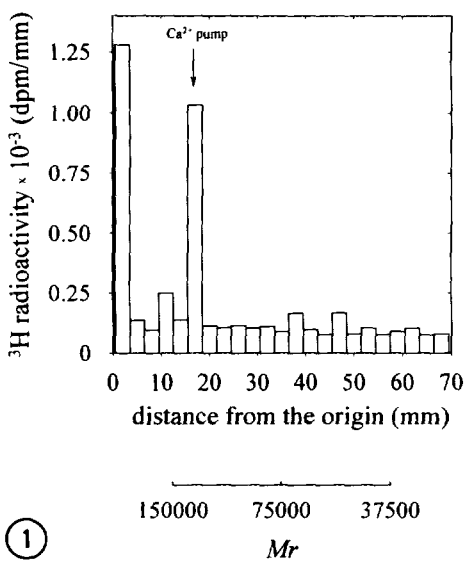
RESULTS AND DISCUSSION

Coreconstitution of the Ca^{2+} pump and [^{3}H]DIPETPD into lipid vesicles and SDS-PAGE analysis. [^{3}H]DIPETPD can be effectively incorporated into lipid vesicles following a variety of procedures [5,19]. Here, detergent dialysis of a mixture of purified Ca^{2+} pump and [^{3}H]DIPETPD dissolved in sodium cholate rendered proteoliposomes at a low phospholipid: protein molar ratio (800:1), to minimize the competing reaction of the probe with fatty acyl chains of phospholipids. The functional integrity of the Ca^{2+} pump in this preparation was ascertained by the uptake of $^{45}Ca^{2+}$ into the lumen of the vesicles in the presence of ATP [20].

[^{3}H]DIPETPD coincorporated with functional Ca^{2+} pump into lipid bilayers photolyzes readily by irradiation at 366 nm (in our setup, the typical half life of the TPD function was lower than 1.5 min). We then replaced phospholipids present in the sample by SDS through the combined addition of an organic phase of chloroform:methanol and an aqueous solution of SDS. In this fashion, the photoadduct with the Ca^{2+} pump was freed from labeled lipids, while keeping the protein always in contact with amphiphiles, so as to minimize the irreversible aggregation of the protein, specially when the sample is concentrated before SDS-PAGE.

Figure 1 shows the radioactivity profile of an SDS-PAGE analysis of intact Ca^{2+} pump labeled with [^{3}H]DIPETPD. Incorporation of label occurs to a band of Mr 135000-140000, which corresponds to the molecular mass of this protein. All MAbs tested reacted with this band. Radioactivity at the origin (between the stacking and running gels) arises from residual amounts of aggregates of the Ca^{2+} pump, as ascertained also by immunoblotting. A parallel non-irradiated sample showed insignificant incorporation of label. Thus, effective incorporation of radiolabel to the enzyme is demonstrated, pointing to the existence of transmembrane regions capable of being labeled with [^{3}H]DIPETPD.

Tryptic digestion of the Ca^{2+} pump, SDS-PAGE analysis and identification of labeled peptides. In order to locate these membrane-associated regions, we adopted controlled proteolysis conditions which lead to defined peptide products. After trypsin digestion, the following fragments were detected to be labeled with the probe: Mr 76000-85000 (A), 52000 (B), 43500 (C), 33500-35000 (D) and 22000 (E) (Figure 2). Identification of each fragment was achieved by reaction with specific MAbs whose corresponding epitopes in the human isoform 4b of the Ca^{2+} pump (hPMCA4b) [21] were mapped [18]. These are JA9, 5F10 and JA3, which recognize segments from residue 17 to 75, 724 to 783 and 1135 to 1205, respectively (Figure 5). Fragment A reacts with 5F10, fragments B and C with JA3 and fragments D and E with JA9, therefore, peptides D and E should include the N-terminus of the protein, peptides B and C should include



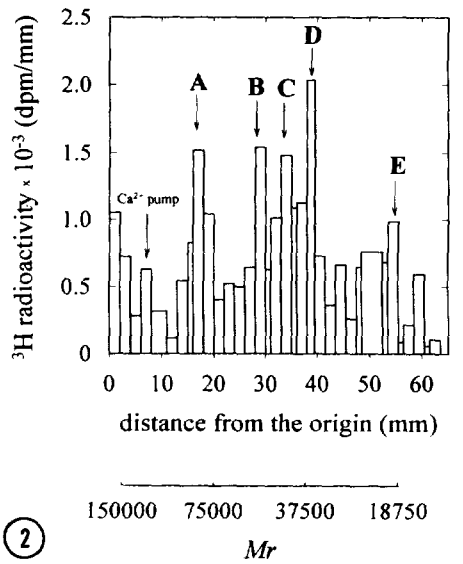
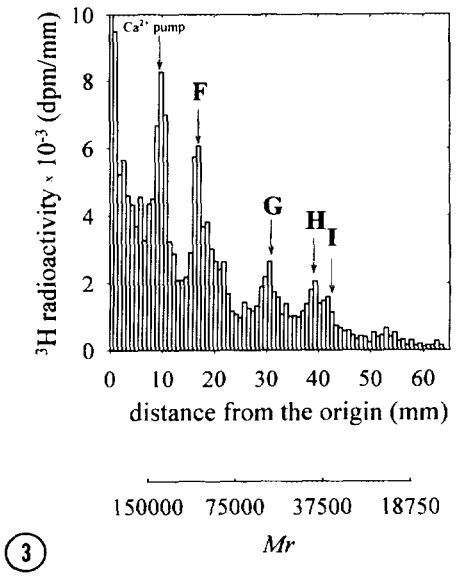


Fig. 1. **SDS-PAGE analysis of the Ca^{2+} pump labeled with [^{3}H]DIPETPD.** After photolysis of a proteoliposome preparation containing Ca^{2+}-pump and [^{3}H]DIPETPD, the sample was delipidized and analyzed by SDS-PAGE. See the Materials and Methods section for details of the procedures.

Fig. 2. **SDS-PAGE analysis of the tryptic digest of Ca^{2+} pump labeled with [^{3}H]DIPETPD.** Labeled proteoliposomes were partially digested with trypsin. After delipidization of the sample, peptides were separated by SDS-PAGE. See the Materials and Methods section for details of the procedures. Arrows indicate the main proteolytic fragments.

the C-terminus and peptide A would correspond to a major central portion of the Ca^{2+} pump (Figure 4). Fragment A may arise by enzymatic cleavage between known sites T1 (or T2) and T3 (or T4) [11,1] (Figure 5), giving rise also to fragment D. However, peptides B, C and E would require the existence of additional cleavage sites, one before T1-T2 and two between T1-T2 and T3-T4. Our results agree well with those of Zvaritch et al. [11] who identified by limited peptide sequencing fragment A, which includes the phosphorylation site, and fragment D, which comprises the calmodulin binding site. In addition, fragments B and C may correspond to those peptides of Mr 53000 and 46000 reported by Emelyanenko et al. [22], both of which include the calmodulin binding site. Our results also agree with those of Zurini et al. [23], who described tryptic fragments of Mr 81000 and 33500 (quite likely peptides A and D), which react with([^{125}I]-TID), a generic hydrophobic probe of membranes [24].

Protease V8 digestion of the Ca^{2+} pump, SDS-PAGE analysis and identification of labeled peptides. Digestion of the Ca^{2+} pump with endoprotease V8 produced the following labeled fragments: Mr 96000 (F), 60000 (G), 37000 (H) and 36000 (I) (Figure 3). Fragments G and I could not be obtained unless at least 0.5% SDS was present in the incubation mixture. With this addition, the low V8 protease: Ca^{2+} pump ratio (500:1, w/w) reported by Wang et al. [25] was enough to yield the pattern observed. Fragments H and I could be cleanly separated by SDS-PAGE, and only the former reacted with JA9. Fragments F and G reacted with both 5F10 and JA3, and fragment I showed no reaction with any of the three MAbs tested. We conclude that peptide H includes the N-terminus of the protein and both peptides F and G include the C-terminus, the former also comprising a major central portion of the protein (Figure 4). Two



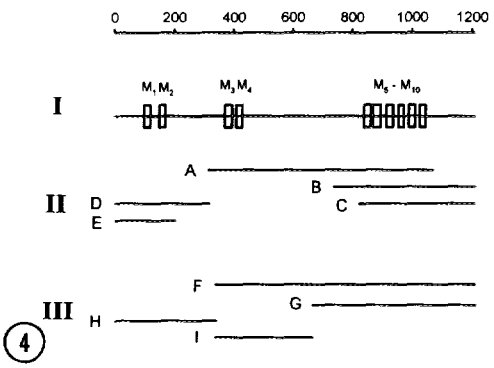


Fig. 3. **SDS-PAGE analysis of the V8 protease digest of Ca^{2+} pump labeled with [^{3}H]DIPETPD.** Labeled proteoliposomes were partially digested with V8 protease. After delipidization of the sample, peptides were separated by SDS-PAGE. See the Materials and Methods section for details of the procedures. Arrows indicate the main proteolytic fragments.

Fig. 4. **Proteolytic map of the Ca^{2+} pump showing labeled fragments after photoreaction with [^{3}H]DIPETPD.** The upper rule shows the aminoacid sequence position. I: Boxed regions along the line indicate the location of putative transmembrane segments M_1 through M_{10}. II: Tryptic peptides A through E. III: Protease V8 peptides F through I. Positive identification of each peptide was achieved by reaction with specific monoclonal antibodies.

cleavage sites V1 and V2 account for the peptide pattern observed (Figure 5), which is consistent with that described by Wang et al. [25]. Peptide I would includes the phosphorilation site.

Mapping the membrane-embedded domains of the Ca^{2+} pump. Hydrophobic photolabeling of integral membrane proteins with [^{3}H]DIPETPD has proven to be strictly confined to those peptides in direct contact with the lipid bilayer. Moreover, due to restricted mobility of the fatty diacyl chain, labeling will most likely occur around the middle plane of the lipid bilayer [5,19,26]. The distribution of label among individual polypeptide fragments might, in principle, be correlated with topographical features of these segments. According to [3], up to 10 hydrophobic (putative membrane-interacting) segments should exist located at positions: 98-119 (M1), 149-169 (M2), 370-394 (M3), 407-429 (M4), 839-862 (M5), 869-891 (M6), 914-937 (M7), 955-973 (M8), 989-1010 (M9) and 1026-1045 (M10). These segments are clustered around three distinct regions along the sequence: the first comprising M1 and M2 (hydrophobic cluster 1: HC1), the second comprising M3 and M4 (HC2) and the third comprising M5 through M10 (HC3) (Figure 4).

The distribution of labeled peptides presented in this paper provides evidence for the existence of all three hydrophobic clusters in this protein and supports the current model for the topography of this enzyme in membranes [1] (Figure 5). Specifically, peptides D, E and H comprise HC1, peptide I comprises HC2, peptides B, C and G comprise HC3 and peptides A and F comprise both HC2 and HC3. The existence of HC1 has been recently ascertained by a MAb recognizing an epitope in the first extracellular loop of the enzyme connecting M1 to M2 [27].

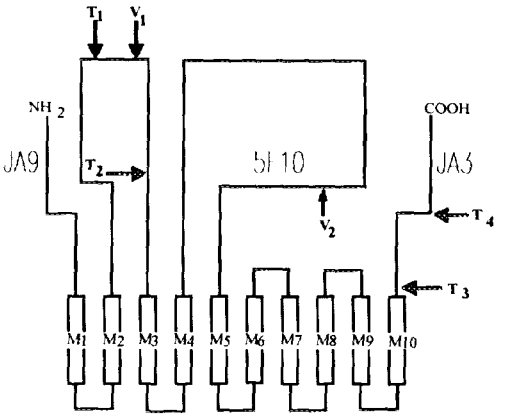


Fig. 5. **Proposed model for the transmembrane organization of the plasma membrane Ca^{2+} pump.** Previously known tryptic cleavage sites are marked T_1 through T_4. V_1 and V_2 represent protease V8 cleavage sites. Regions in the molecule that react with monoclonal antibodies are also indicated.

At this first level of analysis, i.e. by digestion of the enzyme into fragments ranging between Mr 20000 and 100000, no preference for the reaction of [^{3}H]DIPETPD with a given cluster was readily apparent, in agreement with the expectation for a purely topographical reagent which should not show any chemical selectivity for reaction with particular functional groups. Evidence for this is the significant incorporation of label into peptides comprising HC1 or HC2, where the putative transmembrane sequences are dominated by aliphatic side-chain aminoacids. Indeed, carbene-generating probes based on the TPD function label peptides regardless of the aminoacid composition, however, some chemical selectivity has been described for the reaction of carbenes with Cys, Met and Trp [4]. HC3 bears many of these residues, including some which may lie deeply buried within the membrane. This fact, compounded by the larger number of hydrophobic stretches in this cluster, would suggest a higher level of incorporation of label for peptides including HC3 (e.g. peptide G). Confirmation of this should await a more accurate peptide quantitation, in order to yield reliable estimates of specific radioactivity.

Current efforts focus on (a) the preparative isolation and subdigestion of peptides A-I to yield peptides of Mr<20000, and (b) a full enzymatic "trimming" of the extramembraneous domains of the enzyme, in order to circumscribe membrane-spanning segments more precisely.

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