Epitope topology of Na,K-ATPase α subunit analyzed in basolateral cell membranes of rat kidney tubules

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Abstract For topological analysis of integral membrane protein in situ, we used a novel immunoelectron microscopic technique, SDS-digested freeze-fracture replica labeling (SDS-FRL), and oligopeptide-specific antibodies to clarify the sidedness of Na,K-ATPase α subunit epitopes in basolateral cell membranes of kidney tubules. Unfixed tissue slices from rat kidney outer medulla were frozen with liquid helium, freeze-fractured, and replicated. After digestion with SDS to solubilize unfractured membranes and cytoplasm, the platinum/carbon replicas, along with attached cytoplasmic and exoplasmic membrane halves, were processed for immunocytochemistry. Immunogold labeling using antibodies against the N-terminus (Gly^{1}–His^{13}), Leu^{815}–Gln^{828} and the C-terminus (Ile^{1002}–Tyr^{1006}) was superimposed on the images of the electron microscope protoplasmic fracture face of the basolateral plasma membranes, thus demonstrating cytoplasmic locations of these epitopes. On the contrary, SDS-FRL showed specific binding of Asn^{889}–Gln^{903} to cross-fractured basolateral plasma membranes suggesting that this epitope is located on the extracellular side of the membrane.

Key words: Na,K-ATPase; Membrane epitope; Topology; Freeze-fracture replica labeling; Immunoelectron microscopy; Oligopeptide-specific antibody

1. Introduction

Immunoelectron microscopy using antipeptide antibodies directed against epitopes of integral membrane proteins has provided information on the topological organization of the membrane protein epitopes of e.g. Na,K-ATPase [1]. This information is useful for relating the primary amino acid sequence, obtained by cDNA analysis, to the macromolecular structure of the membrane protein. However, isolation and purification of the membranes are usually required for this approach and in some cases the cytoplasmic and exoplasmic surfaces of the membranes are indistinguishable by electron microscopic techniques. In addition, immunoelectron microscopic detection of epitopes exposed on, for instance, the extracellular surfaces of gap junctions requires splitting of this structure with urea [2].

Recently, we have developed a novel freeze-fracture replica immunoelectron microscopic technique, the SDS-digested freeze-fracture replica labeling method (SDS-FRL), to clarify the two-dimensional distribution of the immunocytochemical labeling on membrane surfaces and its relationship to the distribution of images of freeze-fracture replicas [3–5]. Our studies showed that integral membrane proteins revealed as intramembrane particles (IMPs) by freeze-fracture replication, which ordinarily are indistinguishable on purely morphological basis, can be selectively labeled by SDS-FRL with specific antibodies.

In this study we have applied SDS-FRL with oligopeptide-specific antibodies to examine the epitope topology of the Na,K-ATPase α subunit in renal basolateral membranes in situ, without prior isolation and detergent-assisted purification of the membranes, and to illustrate the potential application of SDS-FRL to topological analysis of integral membrane protein.

2. Materials and methods

2.1. Tissue preparation and quick-freezing

The inner stripe of the outer medulla of rat kidneys was cut into sections less than 100 μm thick by a Microslicer (Dosaka EM Co., Kyoto, Japan). The tissue sections were collected in phosphate buffered saline (PBS), pH 7.4. The slices were quick-frozen by contact with a copper block cooled with liquid helium [6].

2.2. SDS-digested freeze-fracture replica labeling and electron microscopy

The frozen samples were fractured in a Balzers BAF 400T freeze-etch unit (Balzers Union, Liechtenstein) at −110°C, replicated by deposition of platinum/carbon (Pt/C) from an electron beam gun positioned at a 40° angle, followed by carbon applied from overhead. After thawing and washing with PBS, the pieces of Pt/C replica were transferred to 5 ml of 2.5% SDS (Sigma Chemical Co., St. Louis, MO) containing 10 mM Tris and 30 mM sucrose, pH 8.3. SDS digestion was carried out for 12 h at room temperature with vigorous stirring. After treatment with SDS, the replicas were rinsed for at least 1 h, with four or more changes of PBS, and placed on drops of 10% bovine serum albumin (BSA; fraction V, Miles Inc., Kankakee, IL) in PBS (BSA-PBS) for 30 min at room temperature. The replicas were then labeled (dilution 1:500) with rabbit polyclonal anti-oligopeptide antibodies against 4 Na,K-ATPase α subunit epitopes: the N-terminal sequence (peptides 1–13), peptides 815–828, peptides 889–903, and the C-terminal sequence (peptides 1002–1016) [1]. After labeling for 1 h at room temperature, the replicas were washed three times with PBS and incubated for 1 h at room temperature with the secondary antibody, goat anti-rabbit IgG conjugated to 10 nm colloidal gold (Janssen Pharmaceuticals, Piscataway, NJ), diluted 1:50 in BSA-PBS. After immunogold labeling, the replicas were immediately rinsed several times in PBS, fixed with 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature, washed twice with distilled water, picked up on Formvar-coated grids and examined in a JEOL 1200EX electron microscope.

3. Results

The ultrastructure of the exoplasmic fracture face (E-face) and protoplasmic fracture face (P-face) of the basolateral cell membrane of the medullary thick ascending limb (mTAL) of the rat nephron as revealed by SDS-FRL was equivalent to that of conventional freeze-fracture replicas cleaned by treatment with chromic-sulfuric acid. Uniform distributions of in-

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tramembrane particles (IMPs) were observed on both the P- and E-faces, but the frequency of IMPs was much higher on the P-face than on the E-face in agreement with previous observations on isolated renal basolateral membranes [7].

The replicas of tubule cells, which were labeled with the N-terminal antibody, showed that the P-faces of the basolateral plasma membranes were densely labeled (Fig. 1, P), while the E-faces were virtually unlabeled (Fig. 1, E). The immunogold particles therefore were only bound to the protoplasmic membrane half, which remains associated with the replica after the SDS-digestion and labeling procedures. Thus, the immunocytochemical labeling was superimposed on the image of the P-face, the gold particles being readily recognized through the replica. The labeling with Leu^{815}–Gln^{828} (Fig. 3) and the C-terminus (Fig. 2) gave the same result. However, the immunogold labeling for Asn^{889}–Gln^{903} was exclusively associated with the transitional region of the fracture plane between adjacent plasma membranes (Fig. 4) and/or the cross-fractured and apposed basolateral membranes (Fig. 5).

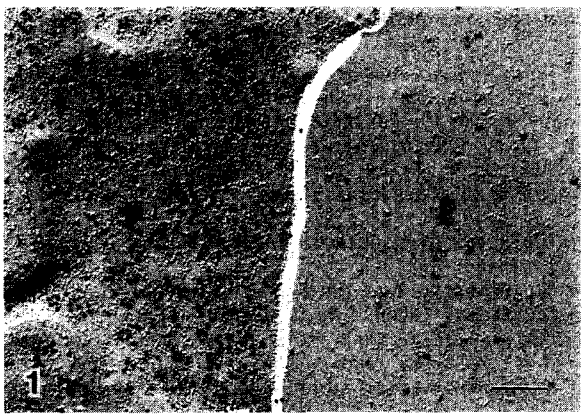

Fig. 1. Electron micrograph of rat kidney epithelial cells from medullary thick ascending limb (mTAL) processed for SDS-FRL with antibodies against the N-terminus of Na,K-ATPase α subunit. The primary antibodies were detected with 10 nm gold conjugated to goat anti-rabbit IgG antibody. The P-faces (P) of the basolateral plasma membranes of the mTAL cells are densely labeled with immunogold particles, while the E-faces (E) are virtually unlabeled. Bar = 200 nm.

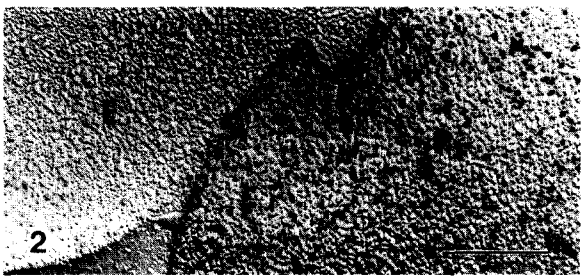

Fig. 2. Electron micrograph of rat kidney epithelial cells from medullary thick ascending limb (mTAL) processed for SDS-FRL with antibodies against the C-terminus of Na,K-ATPase α subunit. The primary antibodies were detected with 10 nm gold conjugated to goat anti-rabbit IgG antibody. The P-faces (P) of the basolateral plasma membranes of the mTAL cells are densely labeled with immunogold particles, while the E-faces (E) are virtually unlabeled. Bar = 200 nm.

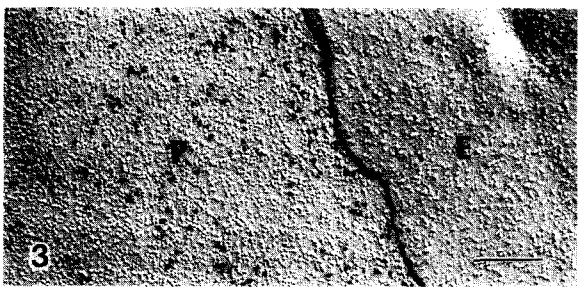

Fig. 3. Electron micrograph of rat kidney epithelial cells from medullary thick ascending limb (mTAL) processed for SDS-FRL with antibodies against Leu^{815}–Gln^{828} of Na,K-ATPase α subunit. The primary antibodies were detected with 10 nm gold conjugated to goat anti-rabbit IgG antibody. The P-faces (P) of the basolateral plasma membranes of the mTAL cells are densely labeled with immunogold particles, while the E-faces (E) are virtually unlabeled. Bar = 200 nm.

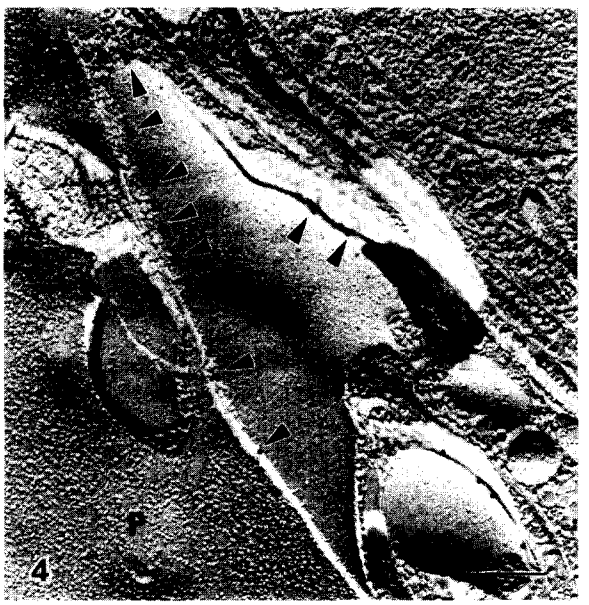

Fig. 4. Electron micrograph of rat mTAL cells processed for SDS-FRL with antibodies against Asn^{889}–Gln^{903}. The immunogold particles are exclusively associated with the transitional region of the fracture plane between adjacent plasma membranes (arrowheads). Bar = 200 nm.

4. Discussion

During freeze-fracture membranes are split along an internal plane between terminal methyl-groups in the center of the bilayer, and integral membrane proteins partition asymmetrically between the fracture faces (Fig. 6a,b). This study shows that immunocytochemical analysis of the split membrane halves yields direct information on the transmembrane distribution of components.

The present observation that immunolabeling against the N-terminal site (sequence 1–13) was restricted to the P-faces of the basolateral plasma membranes is consistent with much previous evidence that the N-terminus is present on the cytoplasmic side of the membranes [1,8–11]. Also the C-terminus, which was initially located on the extracellular side of isolated membranes [12,13] has more recently been demonstrated on the cytoplasmic side of the isolated membranes [1,8,14,15].

SDS-FRL revealed that the P-faces of the basolateral plasma membranes were densely labeled with antibodies both to the N- and the C-terminus as well as Leu ^{815}–Gln^{828}, while the E-faces were not labeled. The immunolabeling of Asn^{889}–Gln^{903} was restricted to the transitional region of the fracture plane between adjacent plasma membranes. These findings demonstrate, first, that the Na,K-ATPase α subunit partitions with the inner (cytoplasmic) half of the cell membrane during freeze-fracture and are available for labeling (Fig. 6d, open circles), which implies that the epitopes of the N-terminus (Gly^{1}–His^{13}), C-terminus (Ile^{1002}–Tyr^{1016}) and Leu^{815}–Gln^{828} are exposed on the cytoplasmic side of the basolateral membrane. Second, the labeling pattern with the antibody against Asn^{889}–Gln^{903} suggests that this epitope is present on the extracellular side (Fig. 6d, filled circles). These results support the existence of an M5/M6 loop [9] and the presence of one transmembrane segment between Leu^{815}–Gln^{828} and Asn^{889}–Gln^{903} in the Na,K-ATPase α subunit of basolateral membranes in intact mTAL cells, consistent with our previous report on isolated membranes [1] and some immunological data reported by others [16,17].

The process of fracture of integral membrane proteins probably depends on the relative expression and anchoring of these proteins at either surface, their possible associations into oligomeric forms, and the number and disposition of transmembrane peptide chains. Thus, for sidedness analyses of integral membrane protein epitopes using SDS-FRL, attention must be paid to the partition of the integral membrane proteins during freeze-fracture. Integral membrane proteins that partition with the inner (cytoplasmic) half of the membrane, can be labeled with antibodies against cytoplasmic sites, as shown in this study, while extracellular sites are unavailable for labeling. The latter sites are partially exposed in cross-fractured membranes, as here observed for the epitope Asn^{889}–Gln^{903}. By contrast, proteins heavily expressed at the outer membrane surface tend to partition with the outer half of the membrane. In this case, the proteins can be labeled with antibodies against extracellular (exoplasmic) sites, and the labeling is restricted to the E-faces [3].

SDS-FRL using site-directed antibodies is expected to make its greatest contribution as a conceptually different means to examine the topological analysis of integral membrane proteins. In addition, this approach has the advantage of localizing specific peptide antibody epitopes to either the cytoplasmic or exoplasmic surfaces of intracellular membranes of intact, untreated cells, without isolation and purification procedures.

Fig. 5. Electron micrograph of rat mTAL cells processed for SDS-FRL with antibodies against Asn^{889}–Gln^{903}. The immunogold particles are exclusively associated with the cross-fracture basolateral membranes. Bar = 200 nm.

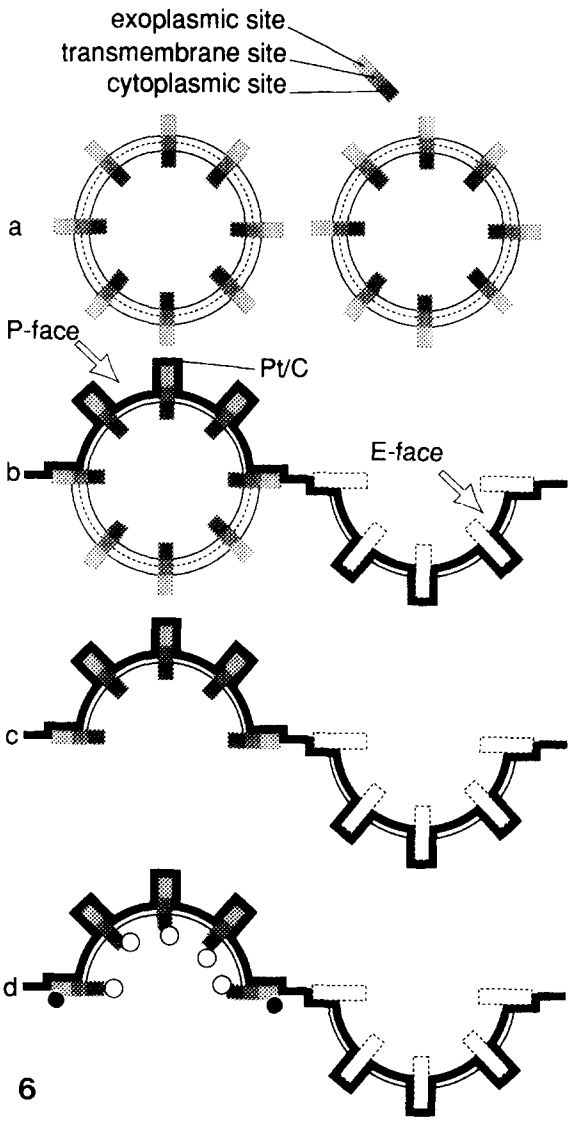

Fig. 6. Interpretation of the results obtained from SDS-FRL with peptide-specific antibodies. (a) Cells are frozen. (b) Freeze-fracturing and platinum/carbon shadowing (Pt/C) reveal the exoplasmic fracture face (E-face) and the protoplasmic fracture face (P-face) of the cell membrane. (c) SDS dissolves the unfractured membrane and cell components, but does not extract the split membrane halves. (d) SDS-FRL using antibody recognizing the cytoplasmic site of the integral membrane protein reveals the labeling on the P-faces (open circles). On the other hand, SDS-FRL using antibody recognizing the exoplasmic site reveals labeling associated with the transitional region of the fracture plane between adjacent plasma membranes and/or the cross-fractured membranes (filled circles).

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