Ultrastructural localization of Na-K-2Cl cotransporter in thick ascending limb and macula densa of rat kidney

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Studies using immunocytochemical methods to localize BSC-1 in the kidney have thus far been carried out only at the light microscopic level. These studies have provided strong evidence that BSC-1 is expressed throughout the TAL with predominant expression in the apical region of TAL cells, consistent with the view that BSC-1 is indeed the apical Na-K-2Cl cotransporter of the TAL (5, 10, 13). In addition, light microscopic immunolocalization studies using a rabbit polyclonal anti-BSC-1 antibody have provided strong evidence that BSC-1 is expressed in macula densa cells (see NOTE ADDED IN PROOF in Ref. 10). However, light microscopic immunocytochemistry lacks the resolution to identify specific membrane structures that contain a given integral membrane protein, pointing to a need for localization at an electron microscopic level.

Studies using scanning and transmission electron microscopy (1) have revealed that the TAL epithelium is made up of two cell types: 1) a rough-surfaced TAL cell with abundant apical microvilli, and 2) a smooth-surfaced TAL cell with a relative absence of apical plasma membrane amplification. The cortical portion of the TAL contains chiefly rough-surfaced cells, whereas the medullary portion contains a mixture of the two cell types with a slight predominance of smooth-surfaced cells (1). The smooth-surfaced cells possess an extensive population of subapical vesicles. In contrast, the rough-surfaced cells have relatively few subapical vesicles. Little is known about functional differences between the two cell types. Whether differences in BSC-1 expression or distribution might exist between these two cell types has not been investigated.

The present studies were undertaken to extend the immunolocalization of BSC-1 to an ultrastructural level addressing: 1) whether BSC-1 expression in TAL cells is limited to the plasma membrane or includes intracellular membranes; 2) whether there is a difference in BSC-1 expression or distribution in rough-versus smooth-surfaced TAL cells; and 3) whether the BSC-1 in macula densa cells is present specifically in the plasma membrane as required for a role in tubuloglomerular feedback. To do these studies, we have utilized a peptide-directed polyclonal antibody to the amino-terminal tail of BSC-1 (5).

The results demonstrated that: 1) BSC-1 is very abundant in the apical plasma membrane of TAL cells, supporting the view that BSC-1 provides the apical pathway for vasopressin-regulated NaCl transport across the TAL. 2) BSC-1 is abundant in intracellular vesicles, especially in smooth-surfaced TAL cells, raising the possibility that BSC-1 in vesicles may represent...
a reservoir of transporters to be recruited during short-term or long-term regulation of NaCl transport. 3) BSC-1 is abundant in the apical plasma membrane in macula densa cells, supporting the view that BSC-1 is involved in the tubuloglomerular feedback system.

METHODS

Preparation of Antibodies

As described in the preceding study (5), a polyclonal antiserum was raised in rabbit against a peptide corresponding to a portion of the amino-terminal tail of rat BSC-1 (amino acids 109–129, based on the sequence reported by Gamba et al. (6)). The antiserum (L224) was affinity purified for the studies described here. The specificity of the affinity-purified L224 antibody was established in the preceding study (5) by immunoblotting of crude membrane fractions and immunocytochemistry.

Membrane Fractionation

Outer medullas were dissected from rat kidneys, minced finely, and homogenized in isolation solution (250 mM sucrose and 10 mM triethanolamine, pH 7.6) containing protease inhibitors (1 µg/ml leupeptin and 0.1 mg/ml phenylmethylsulfonyl fluoride). For subcellular fractionation, sequential centrifugations of the homogenates were carried out at 1,000 g (10 min), 4,000 g (20 min), 17,000 g (20 min), and 200,000 g (60 min) (4). The resulting pellets were resuspended in isolation solution, and protein concentrations were determined by the Pierce BCA Protein Assay kit. After total protein determination, the pellets from these centrifugations were solubilized in Laemmli sample buffer. Electrophoresis of these fractions was carried out on 6% polyacrylamide, and proteins were transferred from the gels to nitrocellulose membranes as described (5). Blots were probed with anti-BSC-1 (L224) at an IgG concentration of 250 ng/ml, and labeled bands were detected by enhanced chemiluminescence (LumiGLO; Kirkegaard and Perry, Gaithersburg, MD).

Immunocytochemistry and Immunoelectron Microscopy

Immunocytochemistry was performed as previously described (3, 14, 19). Kidneys from six rats were perfusion fixed with 4% paraformaldehyde or with 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Tissue blocks were prepared from the inner stripe and the outer stripe of the outer medulla as well as from the cortex. The blocks were postfixed in the same fixative for 2 h, infiltrated for 30 min with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen.

Immunohistochemistry. For light microscopy, semithin (0.8–1 µm) cryosections were preincubated with PBS containing 0.05 M glycine and 0.1% skimmed milk powder (30 min) and incubated overnight at 4°C with anti-BSC-1 antibody (L224, 1–3 µg/ml IgG) in PBS containing 0.1% skimmed milk powder and 0.3% Triton X-100. The labeling was visualized by use of horseradish peroxidase-conjugated secondary antibody (P448, 1:100; DAKO, Copenhagen, Denmark).

Immunoelectron microscopy. For immunoelectron microscopy, three different methods were used as follows: 1) ultrathin (80 nm) cryosections were preincubated with PBS containing 0.05 M glycine and 0.1% skimmed milk powder (30 min) and incubated overnight at 4°C with anti-BSC-1 antibody (L224, 1–3 µg/ml IgG) in PBS containing 0.1% skimmed milk powder and 0.3% Triton X-100. The labeling was visualized by use of horseradish peroxidase-conjugated secondary antibody (P448, 1:100; DAKO, Copenhagen, Denmark).

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Fig. 1. BSC-1 immunoblots of rat outer medulla. A: immunoblot using 1 or 3 µg of membrane fraction (17,000 g pellet) from inner stripe of outer medulla probed with affinity-purified anti-BSC-1 antibody (L224) or with preimmune IgG from the same rabbit. B: individual lanes were loaded with membrane fractions prepared by sequential centrifugation at progressively higher speeds. Immunoblots were probed with anti-BSC-1. BSC-1 is abundant both in fractions enriched for intracellular vesicles (200,000 g) and plasma membranes (17,000 g).

Fig. 2. Immunolocalization of BSC-1 in inner stripe of outer medulla. A and B: abundant BSC-1 labeling is associated with all thick ascending limb (TAL) segments with no labeling of other structures. BSC-1 labeling is associated with apical plasma membrane domains (arrows). C: in addition to the strong BSC-1 labeling of the apical plasma membrane domain (arrows), a distinct labeling is also associated with intracellular vesicle domains in a subpopulation of cells (arrowheads). D: immunolabeling control using nonimmune IgG revealed no labeling. Magnifications: A, ×490; B, C, and D, ×980. T, thick ascending limb; C, collecting duct; D, descending thin limb, V, vascular structure.
0.05 M Tris with 0.1% Triton X-100, pH 7.4) containing 0.05 M glycine and 0.1% borohydride and incubated overnight at 4°C with anti-BSC-1 antibody (L224, 1–3 µg/ml IgG) in TBST with 0.2% skimmed milk powder or 0.2% bovine serum albumin. The labeling was visualized using goat anti-rabbit gold (10-nm particles; BioCell Research Laboratories, Cardiff, UK) in TBST with 2% bovine serum albumin and 5 mg/ml polyethylene glycol (PEG, mol wt 20,000).

RESULTS

Immunoblotting

Figure 1A shows an immunoblot run with membrane fractions prepared by differential centrifugation from rat outer medullary homogenates and probed with the L224 anti-BSC-1 antibody. There was a single broad band at an apparent molecular mass of ~161 kDa. Controls using preimmune IgG showed no bands (Fig. 1A). Furthermore, preadsorption of the anti-BSC-1 antibody with an excess of the immunizing peptide eliminated the 161-kDa band (5). As shown in Fig. 1B, the BSC-1 band was abundant in both the 17,000 g and the 200,000 g membrane fractions enriched for plasma membranes and intracellular vesicles, respectively. The band was not seen in the final supernatant from the 200,000 g spin, which contained predominantly cytosolic proteins.

Immunocytochemistry

The cellular distribution of BSC-1 was examined by the immunoperoxidase labeling technique at a light microscopic level (Fig. 2). In thin cryosections of kidney outer medulla, there was abundant labeling in the apical and subapical regions of TAL cells (Fig. 2, A and B). Note that there was considerable cell to cell variability in the extent of subapical labeling with little subapical labeling in some cells and extensive intracellular labeling in others (arrowheads, Fig. 2C). There was no labeling of basolateral regions of TAL cells. There was no detectable labeling in other cell types including thin descending limbs, collecting ducts, or vasa recta. Incubation with preimmune IgG (Fig. 2D) or affinity-purified antibody preadsorbed with an excess of immunizing peptide (not shown) revealed absence of labeling.

Immunoelectron Microscopy

To determine the subcellular localization of BSC-1 in medullary TAL and cortical TAL cells, immunoelectron microscopy of ultrathin cryosections, Lowicryl HM20 sections, or LR-White sections was carried out. These methods gave identical labeling patterns, whereas the labeling efficiency was higher using LR-White. Figure 3 shows immunogold labeling in rough-surfaced TAL cells in the renal cortex. [The cortical TAL has been shown to have a strong predominance of rough-surfaced cells (1).] BSC-1 labeling was especially prominent in the apical plasma membrane of these cells (Fig. 3, arrows). In these TAL cells, a modest amount of BSC-1 labeling was associated with cytoplasmic vesicles.

Figure 4 shows BSC-1 labeling in the medullary TAL, which has a mixture of rough- and smooth-surfaced cells. Here, typical rough-surfaced (Fig. 4, right) and smooth-surfaced (Fig. 4, left) cells are shown. As shown in Fig. 4, the rough-surfaced cells have a labeling pattern similar to the rough-surfaced cells in the cortex with strong labeling of the apical plasma membrane, but also a modest amount of labeling of subapical vesicles. As is also illustrated in Fig. 4, the smooth-surfaced cells also show abundant expression of BSC-1. Previous studies have demonstrated an increased number of subapical vesicles in smooth-surfaced cells relative to rough-surfaced cells (1). Our observations are consistent with that conclusion and furthermore show that this subapical vesicle population is heavily labeled by the BSC-1 antibody, consistent with the...
presence of a large intracellular reservoir of BSC-1 in smooth-surfaced TAL cells. In addition, the smooth-surfaced cells, like the rough-surfaced TAL cells, showed abundant labeling of the apical plasma membrane (Fig. 4). Immunolabeling controls were negative (not shown).

Figure 5 shows another example of BSC-1 labeling of a smooth-surfaced TAL cell of the inner stripe of the outer medulla. Abundant BSC-1 labeling is associated with apical plasma membrane (arrows) and small intracellular vesicles (arrowheads) of this smooth-surfaced TAL cell (arrows). Magnification, ×24,500.
outer medulla. Extensive labeling of both intracellular vesicles (arrowheads) and apical plasma membranes (arrows) can be appreciated.

**BSC-1 in Macula Densa Cells**

To investigate the distribution of BSC-1 in macula densa cells, serial cryosections of kidney cortex were subjected to immunoperoxidase labeling for BSC-1. As demonstrated in Fig. 6, a distinct BSC-1 labeling was associated with macula densa cells (arrows in Fig. 6, A–C). (Macula densa cells can be easily recognized as tall cells with apical nuclei adjacent to glomeruli.) In addition to the labeling of macula densa cells, a distinct labeling of TAL cells (arrowheads in Fig. 6, A–C) was present in tubule cells positioned opposite to the macula densa cells. Distal convoluted tubules (Fig. 6, A and B) were unlabeled as were proximal tubules (Fig. 6, A–C) and cells in the glomerulus (Fig. 6, A–C).

Immunoelectron microscopy confirmed the abundant expression of BSC-1 in macula densa cells (Fig. 7). The inset of Fig. 7 demonstrates the area presented at higher magnification in the main panel in Fig. 7. Abundant labeling of macula densa cells was seen of the apical plasma membrane and occasionally also of intracellular vesicles (Fig. 7). The neighboring TAL cells (Fig. 7) also displayed extensive labeling of BSC-1.

**DISCUSSION**

In this study, we have determined the subcellular distribution of the absorptive isoform of Na-K-2Cl cotransporter (termed “BSC-1” or “NKCC-2”) in rat kidney. To achieve this goal, we raised a polyclonal antibody to BSC-1 by immunizing rabbits with a synthetic peptide corresponding to a 21-amino acid region predicted to be located within the amino-terminal cytoplasmic tail of rat BSC-1 (5). This sequence is distinct from any region of the secretory bumetanide-sensitive Na-K-2Cl cotransporter (2, 22). Using the BLAST sequence-comparison computer program, we could find no significant overlap with any other known eukaryotic protein. Supporting the view that the antibody is specific for BSC-1 in the kidney, immunoblots revealed a single band of apparent molecular mass 161 kDa, consistent with the expected size of the glycosylated protein. This band was absent when the antibody was preadsorbed with the immunizing peptide (5) or when preimmune IgG was substituted (present study). In addition, the band was not seen in colon or parotid gland, sites of strong expression of the secretory isoform of Na-K-2Cl cotransporter (5). Differential centrifugation studies (Fig. 1B) revealed the presence of BSC-1 in the 17,000 g membrane fraction as well as in the 200,000 g membrane fraction prepared from rat outer medullary homogenates (Fig. 1B). The 200,000 g fraction of the outer medulla has been shown to be devoid of proteins associated strictly with the plasma membrane (4) and is thought to contain predominantly intracellular vesicles. Indeed, immunoelectron microscopy showed abundant BSC-1 labeling in intracellular vesicles of medullary TAL cells, particularly those manifesting a smooth apical surface (see below).

Both immunocytochemistry at a light microscopic level (Fig. 2) and immunoelectron microscopy (Fig. 3) demonstrated a predominant apical localization of BSC-1 in TAL cells, consistent with prior observations.
Fig. 7. Immunogold localization of BSC-1 in LR-White sections of macula densa. Abundant BSC-1 labeling is associated with apical plasma membrane (arrows) of macula densa cells (MD) and TAL cells. Magnification, ×44,000. Inset: overview showing MD cells and TAL cells. Regions indicated by MD are shown at higher power in main panel from adjacent section. Magnification, ×3,500.
(5, 10). This localization, which was observed in both the medullary and cortical TAL, supports the conclusion by Gamba et al. (6) that the BSC-1 cDNA clone corresponds to the apical bumetanide-sensitive Na-K-2Cl cotransporter previously characterized in physiological studies (7, 8).

The present studies support previous conclusions that there are two morphologically different cell types in the medullary TAL with rough and smooth apical plasma membrane appearances (1). Functional studies by Lmai and colleagues (23, 26) in TAL showed that there are two cell populations with different electrophysiological properties corresponding to rough- and smooth-surfaced morphologies. Recent studies localizing the potassium channel ROMK along the nephron showed that some TAL cells were heavily labeled while others showed little or no labeling (16, 25). In the present studies, immunoelectron microscopy employing the immunogold technique in ultrathin cryosections showed a different localization of BSC-1 in the smooth and rough cell types. Both cell types display substantial labeling of the apical plasma membrane. However, as originally demonstrated by Allen and Tisher (1), smooth-surfaced TAL cells possess numerous subapical vesicles that, as we have demonstrated here (Figs. 4 and 5), were heavily labeled by the BSC-1 antibody. Although intracellular vesicles were also labeled by the BSC-1 antibody in rough-surfaced cells, such BSC-1-laden vesicles appear to be much less abundant in these cells. An important issue that remains to be addressed is: What is the relationship between the distributions of ROMK and BSC-1, and how does this correlate with the functional properties of the two cell types?

The regulation of renal water excretion occurs through coordinated effects of vasopressin on collecting ducts to increase water and urea permeability and on the TAL of Henle’s loop to increase active NaCl absorption (11). The action of vasopressin on the TAL is in part due to direct stimulation of the activity of the apical Na-K-2Cl cotransporter (17). Enhancement of active NaCl absorption in the medullary TAL by vasopressin is believed to augment the counter current multiplication process that concentrates NaCl in the renal medullary interstitium and therefore would be expected to increase maximum urinary concentrating ability. The mechanism by which vasopressin acutely increases Na-K-2Cl cotransporter activity of the TAL has not yet been directly investigated. Given the finding of abundant expression of BSC-1 in intracellular vesicles of smooth-surfaced TAL cells, it appears possible that the NaCl entry across the apical plasma membrane may be increased as a result of vasopressin-stimulated insertion of these vesicles into the apical plasma membrane, similar to the process demonstrated for the vasopressin-regulated water channel aquaporin-2 (14, 18). Further studies will be required to address this hypothesis.

Micropuncture studies have demonstrated that furosemide and bumetanide inhibit tubuloglomerular feedback in the rat, indicating that one or more loop diuretic-sensitive transporters are expressed in the macula densa cells (12, 24). Consistent with that view, Obermuller et al. (20) demonstrated the presence of mRNA coding for the absorptive form of the Na-K-2Cl cotransporter in macula densa cells by in situ hybridization. Our results confirm the expression of BSC-1 in the macula densa cells and demonstrate its localization in the apical plasma membrane of these cells. An earlier immunolocalization study using a different anti-BSC-1 antibody also indicated apical localization of BSC-1 in macula densa cells (see NOTE ADDED IN PROOF in Ref. 10). The finding of BSC-1 in macula densa cells is not surprising based on the efficacy of loop diuretics in increasing salt excretion. If loop diuretics did not block tubuloglomerular feedback, then they would be expected to substantially decrease glomerular filtration rate and a much more modest natriuresis would be expected.

In conclusion, the finding that BSC-1 is heavily expressed in the apical plasma membrane of TAL cells provides support for the view that BSC-1 is responsible for the apical entry of Na and Cl in the process of active NaCl reabsorption across the TAL cells. The subcellular distribution of BSC-1 differs between rough- and smooth-surfaced TAL cells with greater abundance in intracellular vesicles in smooth-surfaced TAL cells than in rough-surfaced TAL cells. The presence in both the apical plasma membrane and intracellular vesicles raises the possibility that BSC-1 in vesicles may represent a reservoir of transporters to be recruited during short-term or long-term regulation of apical NaCl transport. Furthermore, BSC-1 is strongly expressed in the apical plasma membrane of the macula densa cells, consistent with the proposed role for BSC-1 in the tubuloglomerular feedback.

We thank Hanne Weiling and Else-Merete Løcke for expert technical assistance. We thank Dr. Maurice Burg for advice during the course of this study and for carefully reading the manuscript.

This work was supported by the intramural budget of the National Heart, Lung, and Blood Institute, as well as the Karen Elise Jensen Foundation, the Novo Nordisk Foundation, and the Danish Medical Research Council.

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Received 30 April 1998; accepted in final form 3 September 1998.

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