Real-time monitoring of NKCC2 endocytosis by total internal reflection fluorescence (TIRF) microscopy

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THE THICK ASCENDING LIMB (TAL) of the loop of Henle reabsorbs up to 30% of the NaCl filtered by the glomerulus and thus plays a crucial role in the maintenance of body salt and fluid homeostasis. The apical Na-K-2Cl cotransporter (NKCC2) mediates NaCl reabsorption by the thick ascending limb (TAL). The amount of NKCC2 at the apical membrane of TAL cells is determined by exocytic delivery, recycling, and endocytosis. Surface biotinylation allows measurement of NKCC2 endocytosis, but it has low time resolution and does not allow imaging of the dynamic process of endocytosis. We hypothesized that total internal reflection fluorescence (TIRF) microscopy imaging of labeled NKCC2 would allow monitoring of NKCC2 endocytosis in endosomes within the vicinity of the apical membrane, and the dynamic process resulting from a balance between endocytosis, exocytic delivery, and recycling (2, 12, 13). However, the molecular mechanisms mediating NKCC2 trafficking, the proteins involved, and the dynamic nature of these mechanisms are poorly understood. This is in part due to a lack of methods for studying NKCC2 trafficking.

Surface biotinylation of apical proteins with NHS-SS biotin (2) has been previously used to study NKCC2 endocytosis. However, this technique is limited in its time resolution and cannot capture the dynamics of endocytosis in real time (19). Optical methods have been developed that allow imaging trafficking events at the plasma membrane. For example, total internal reflection fluorescence (TIRF) microscopy generates an evanescent field of illumination of ~200 nm, allowing direct imaging of fluorescent molecules at or near the plasma membrane with high signal-to-noise ratio, and is well suited for imaging endocytic events (1). For example, TIRF microscopy has been used to study the dynamic trafficking of clathrin-coated vesicles and many membrane proteins in nonpolarized cells (28, 31, 32, 41–43). In polarized cells, TIRF microscopy has been used to image basolateral membrane dynamics (29). Imaging trafficking at the apical membrane has been challenging due to technical limitations of the TIRF configuration, which requires a glass-water interface to achieve the evanescent field. However, in recent years there have been successful attempts adapting this technique to image proteins in recycling endosomes within the vicinity of the apical membrane, and the internalization of the apical membrane sodium-phosphate cotransporter (NaPi2a) in opossum kidney (OK) cells (8, 43). We hypothesized that combining apical membrane TIRF imaging with site-specific fluorescent labeling of NKCC2 would allow monitoring of NKCC2 endocytosis with good time resolution.

Site-specific biotinylation of cell surface proteins can be achieved by tagging proteins with a biotin acceptor domain (BAD) sequence that is recognized by Escherichia coli biotin ligase (BirA). BAD is a 15-amino acid-long sequence with a linker between the carboxyl group of biotin and the amino group of the central lysine residue in the BAD domain (7, 47). This is an efficient method for biotinylating and imaging proteins in mammalian cells, and it has been used to track cells and tumors in vivo (22, 44). Here, we have developed a technique for NKCC2-specific biotinylation at the apical surface by exogenously added or coexpressed BirA for imaging...
 NKCC2 internalization by TIRF microscopy and measuring the dynamics of NKCC2 endocytosis in polarized TAL cells.

**METHODS**

**Constructs and plasmids.** The enhanced green fluorescent protein (eGFP)-NKCC2 (mouse) construct was kindly provided by Dr. Gerardo Gamba, Universidad Nacional Autonoma de Mexico (Mexico City, Mexico) (37). eGFP-NKCC2 was subcloned from pSPORT1 into a VQAd5CMV adenovirus plasmid vector (ViraQuest, North Liberty, IA) between the ApeI and NotI restriction sites. The cMyc-NKCC2 (rat) sequence (accession no. U10096.1) was commercially synthesized by Genscript (Piscataway, NJ) and subcloned into the VQAd5CMV adenovirus vector between the KpnI and NotI restriction sites. An XbaI restriction site was introduced by site-directed mutagenesis in the extracellular loop between transmembrane domains 5 and 6 of NKCC2. The XbaI site was used to introduce the BAD derived from Propionibacterium shermanii (7, 34, 44). This resulted in a VQAd5CMV-cMyc-NKCC2-BAD adenoviral construct. The ssh-BirA (secretory sequence-BirA)-IREs-mCherry construct consists of a biotin ligase fused to a yolk sac secretory sequence which targets proteins for a secretory pathway (34, 44). It was subcloned from a CSCW lentiviral vector to VQAd5CMV between the BamHI and XbaI restriction sites, resulting in the VQAd5CMV-ssBirA adenoviral construct. All clones were fully sequenced, and adenoviral particles containing the above constructs were assembled and purified by ViraQuest.

*Madin-Darby canine kidney cell culture.* Madin-Darby canine kidney (MDCK) type 2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in DMEM-high glucose, HEPES, no phenol red (Life Technologies, Grand Island, NY), supplemented with 5% heat-inactivated FBS (Life Technologies) and a penicillin-streptomycin mix (Life Technologies). Cells were seeded in collagen-coated Transwell permeable supports (Corning, Tewksbury, MA) and incubated for 3 days at 37°C and 5% CO₂ until 90% confluent.

*Primary culture of rat TAL.* Primary culture of rat TAL was done as previously described (13) following institutional and national guidelines for the care and use of laboratory animals. All protocols involving animals were approved by the Institutional Animal Care and Use Committee at the Henry Ford Hospital. Sprague-Dawley rats, weighing 120–180 g, were used. Kidneys were perfused with 0.1% collagenase (Sigma, St. Louis, MO), and the outer medulla was dissected. Individual TAL cells were obtained from TAL suspensions after digestion of the tubules with 0.1% collagenase, 0.25% trypsin (Sigma), and 0.0021% DNase (Sigma). Single TAL cells were isolated by density gradient centrifugation in 35% Percoll (Sigma), washed, and seeded in permeable supports (Corning) coated with basement membrane extract (Trevigen, Gaithersburg, MD). Cells were grown for 4 days in DMEM (low glucose, no phenol red, no glutamine) supplemented with 1% PBS, a penicillin-streptomycin mix, and insulin-transferrin-selenium (Life Technologies) at 37°C and 5% CO₂ until 90% confluent.

*Transduction of polarized cells.* Cells were transduced by adding adenoviruses at 1 × 10⁹ plaque forming units (PFU)/ml suspended in serum-free DMEM to the basolateral compartment of the Transwells. After 2 h, serum was added to inactive viruses and the cells were incubated at 37°C for 20–24 h.

**Immunoconfocal labeling and confocal microscopy imaging.** Cells were fixed with 4% paraformaldehyde for 30 min at 4°C. Fixed cells were incubated with 5% BSA (Equitech-Bios, Kerrville, TX) in PBS (Life Technologies) for 20 min at room temperature. Antibodies (1: 100, Life Technologies) were suspended in 5% BSA-PBS. A mouse primary antibody against zonula occludens-1 (ZO-1) was added to the cells, which were incubated for 1 h at room temperature followed by addition of an Alexa Fluor 568-conjugated secondary anti-mouse antibody for 1 h. For surface NKCC2 labeling, antibodies to the extracellular epitope of NKCC2 (12) were added to both apical and basolateral compartments of live cells cooled to 4°C and incubated for 2 h, and then washed and incubated with an Alexa Fluor 488-conjugated secondary anti-rabbit antibody for 1 h at 4°C in both apical and basolateral compartments of the Transwells. Cells were then fixed and labeled for ZO-1 as mentioned above. Membranes were cut and mounted on a glass slide. Glass slides were imaged by a laser-scanning confocal microscopy system (Visitech International, Sutherland, UK). For Z-section confocal imaging, we used an Olympus FV1200 confocal system at ×60, 1.4 NA. Z-sections were obtained at 0.25-µm steps for 10-µm total Z-distance.

*Steady-state surface biotinylation.* Surface biotinylation was performed using NHS-SS-biotin by a method that we described previously in primary cultures of rat TAL suspensions (13). To confirm correct targeting of the eGFP-NKCC2 construct to the apical surface and an absence of targeting to the basolateral surface, we performed apical and basolateral surface biotinylation. Cells were biotinylated at 4°C with NHS-SS-biotin (0.9 mg/ml) added to either the apical or basolateral compartment of the Transwells. To prevent access of NHS-SS-biotin to the apical surface from the basolateral compartment during basolateral surface biotinylation or vice versa, MDCK cells were pretreated with NHS-acetate (2 mg/ml) for 1 h at 4°C added to either the apical or basolateral compartment of the Transwells to mask lysine biotinylation sites (12) before the addition of NHS-SS-biotin. Cells were washed and lysed in buffer containing 150 mM NaCl, 50 mM HEPES (pH 7.5), 5 mM EDTA, 1% Triton X-100, 0.2% SDS, and protease inhibitors. Extracted proteins were incubated with streptavidin-agarose beads (Pierce Biotechnology) at 4°C overnight. Beads were washed, and biotinylated proteins were extracted by boiling in 60 µl SDS loading buffer containing 100 mM dithiothreitol and 5% β-mercaptoethanol. Proteins extracted from the beads (surface) and supernatant (intracellular) were resolved by SDS-PAGE (6% gels). NKCC2 and GAPDH were detected by Western blotting.

*Site-specific NKCC2 biotinylation and streptavidin Alexa Fluor-488 labeling.* Cells transduced to express NKCC2-BAD were serum starved for 2 h at 37°C and then treated with or without 5 mM methyl-β-cyclodextrin (MβCD; Sigma) in serum-free DMEM for 20 min. Cells were then washed with PBS containing 0.1 mM CaCl₂ and 4 mM MgCl₂ (PBS-Ca-Mg), pH 7.6. Cells were incubated with 5% PBS-Ca-Mg (pH 7.6), 0.5% β-mercaptoethanol, and an absence of targeting to the basolateral surface, we performed metabolic biotinylation (BirA expressed in cells), the VQAd5CMV-ssBirA adenovirus construct was cotransduced with NKCC2-BAD. Cells were cooled to 4°C and briefly washed first with chilled PBS-Ca-Mg (pH 7.6) and then with PBS-Ca-Mg (pH 3.0) to remove unreacted biotin. The apical surface was then incubated with Alexa Fluor 488-conjugated streptavidin (Life Technologies) at 1:400 dilution in 5% BSA/PBS-Ca-Mg (pH 7.6, 4°C). Finally, unbound streptavidin was washed away by alternate washes with PBS-Ca-Mg at pH 7.6 and 3.0.

**Apical TIRF microscopy.** The membranes in the Transwells were carefully cut and separated from the inserts and placed in a temperature-controlled chamber at 37°C facing down, such that the apical surface was facing the objective lens. Cells were covered with a sealed 0.13- to 0.17-mm-thick glass coverslip (Fisher Scientific, Waltham, MA) and transferred to an inverted scope (Nikon TE2000U, equipped with a “through the lens” TIRF module, ×100 1.45 NA TIRF lens (Nikon), in such a way that the apical membranes faced the objective lens. A blunt glass pipette mounted on micromanipulators was lowered onto the membrane from the basolateral side to push the apical membranes down and closer to the glass coverslip. Serum-free DMEM was added to the basolateral side of the chamber, and cells were equilibrated at 37°C for 10 min before imaging. A region of interest was selected, and the 488-nm excitation laser was angled until
reflection was observed on the glass coverslip. In some cases, images were acquired continuously once per second to observe real-time endocytosis. In other cases, images were acquired once per minute at a 1,024 ms frame rate for 20 min. Images were acquired at 1,024 × 1,024-pixel resolution with an EM-enhanced cooled CCD (Photometrics Cascade II 1024 EMCCD). To calculate the rate of endocytosis, each image was deconvolved using AutoQuant software (Media Cybernetics, Rockville, MD) and analyzed with Metamorph software (Molecular Devices, Sunnyvale, CA). The Granularity module of Metamorph was used to automatically count the number of puncta (singular: punctum) present on the TIRF field over time. Background fluorescence intensity was measured from a region on the TIRF field with no puncta.

**Statistics.** Results are expressed as means ± SE. One-way ANOVA was used to determine differences between means in treatments. \( P < 0.01 \) was considered significant.

**RESULTS**

**Heterologous NKCC2 can be expressed in polarized MDCK cells.** Expression of full-length transmembrane proteins such as NKCC2 in polarized epithelial cells has proven challenging. While N-terminal tagged eGFP-NKCC2 (full-length) has been expressed in nonpolarized cells such as OK cells (48), very few investigators have succeeded in expressing full-length NKCC2 in polarized cells (15). To determine our ability to express a full-length NKCC2 clone in polarized epithelial cells and study its correct apical targeting, we first tested whether N-terminal eGFP-tagged NKCC2 could be expressed in polarized MDCK cells after transduction with adenoviruses. For this, MDCK cells were grown to confluence on collagen-coated permeable support wells and transduced with eGFP-NKCC2 adenoviruses. After 20–24 h, cells were fixed and labeled for the tight junction protein ZO-1. Figure 1A shows a representative image of MDCK cells in which green fluorescence indicates expression of eGFP-NKCC2 in the same plane as ZO-1, indicated by red fluorescence. To confirm apical targeting of the eGFP-NKCC2 construct and lack of basolateral targeting, MDCK cells transduced with eGFP-NKCC2 were labeled with antibodies that bind surface NKCC2 (directed to the extracellular loop between transmembrane domains 5 and 6) on both the apical and basolateral compartments of the Transwells. Apical tight junction protein ZO1 was also labeled, as described in METHODS. \( X-Y \) and \( Y-Z \) confocal reconstruction of polarized MDCK cells show that surface NKCC2 was only localized to the apical surface in the same plane as ZO-1. No labeling was observed in the lateral or basal membranes (Fig. 1B).

To confirm that heterologously expressed eGFP-NKCC2 reached the apical surface in MDCK cells, we performed biotinylation of apical or basolateral surface proteins of MDCK

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Fig. 1. Apical expression of enhanced green fluorescent protein (eGFP)-Na-K-2Cl cotransporter (NKCC2) in polarized Madin-Darby canine kidney (MDCK) cells. A: immunofluorescence labeling of zonula occludens-1 (ZO-1; red) indicating polarization of MDCK cells grown in collagen-coated permeable supports. Green fluorescence indicates expression of eGFP-NKCC2 in MDCK cells. B: \( X-Y \) and \( Y-Z \) confocal reconstruction of MDCK cell monolayer transduced with eGFP-NKCC2 fluorescently labeled with surface NKCC2 antibody (green) and ZO-1 (red) indicating correct targeting to the apical surface and absence of basolateral targeting of eGFP-NKCC2. White dotted line in \( Y-Z \) image represents the basolateral plane in the MDCK cell monolayer. C: representative Western blot indicating the presence of eGFP-NKCC2 in the apical surface fraction and absence of eGFP-NKCC2 in the basolateral surface fraction of polarized MDCK cells subjected to surface biotinylation (\( n = 3 \) independent preparations).

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cells transduced with eGFP-NKCC2. Expression of eGFP-NKCC2 detected with an antibody against GFP was observed at 180–190 kDa, only in the apical surface fraction but not in the basolateral surface fraction (Fig. 1C). Taken together, these data indicate correct apical targeting of the eGFP-NKCC2 construct to the apical surface and absence of basolateral targeting in polarized MDCK cells.

Site-specific NKCC2 biotinylation and apical labeling for TIRF microscopy imaging. Once we confirmed that NKCC2 reaches the apical surface in polarized MDCK cells, we developed a method to selectively label NKCC2 at the apical surface and monitor its endocytosis. To selectively biotinylate NKCC2, we engineered adenoviruses to deliver a modified NKCC2 construct containing a BAD in the extracellular loop between transmembrane domains 5 and 6 (NKCC2-BAD). MDCK cells were transduced with NKCC2-BAD (Fig. 2A), and surface biotinylation was achieved biochemically by adding the exogenous bacterial biotin ligase BirA, biotin, and ATP for 15 min to the apical bath (22). To fluorescently label biotinylated NKCC2 on the apical membrane, we incubated the apical surface with Alexa Fluor 488-conjugated streptavidin (Fig. 2B). After labeling, we imaged apical membranes by TIRF microscopy in a temperature-controlled chamber as shown in Fig. 2C and described in METHODS. TIRF microscopy is based on the formation of an evanescent field that allows excitation of fluorophores only in the immediate vicinity (100–200 nm) of the glass coverslip (30, 46). Thus, to maximize the apical membrane area in proximity to the glass coverslip to be visualized, we gently pushed down the collagen-coated membrane with a glass pipette. As shown in Fig. 2D, we were able to visualize NKCC2 at the apical surface of MDCK cells. We observed a heterogeneous pattern of distribution where apical surface NKCC2 was restricted to discrete domains referred to here as puncta (singular: punctum). To ensure that only NKCC2 was biotinylated, we performed two negative controls in parallel with every experiment. First, MDCK cells transduced with NKCC2-BAD were incubated in the absence of BirA but in the presence of biotin followed by streptavidin labeling. In the second negative control, MDCK cells were not transduced with NKCC2-BAD but treated with BirA plus biotin and then labeled with streptavidin (data not shown). Negative controls showed an absence of nonspecific labeling in MDCK cells (Fig. 2E). These data indicate that addition of the BAD domain to NKCC2 results in specific biotinylation of NKCC2 at the apical surface of polarized MDCK cells, and this allows fluorescent labeling and TIRF imaging of apical NKCC2.

Visualization of individual NKCC2 endocytic events on the apical surface of MDCK cells in real time. Once we were able to image apical surface NKCC2 by TIRF microscopy in MDCK cells, we monitored the disappearance of an individual punctum in real time as an indication of endocytosis. We placed the cells at 37°C to allow endocytosis, and we acquired images every second by TIRF as described in METHODS. We tracked an individual punctum by defining a region of interest, as shown in Fig. 3A. We quantified the fluorescence intensity within the region of interest and observed a rapid decrease in fluorescence, reaching background fluorescence intensity within 1 s (Fig. 3B). We took this to represent a single endocytic event. The occurrence of several endocytic events resulted in a decrease in the total number of surface NKCC2 puncta on the TIRF field over time. This was quantified with the Granularity module of Metamorph. The number of surface puncta was analyzed over a period of 20 min. We observed that after 20 min, 23.5 ± 6.1% of the initial number of NKCC2 puncta had disappeared from the cell surface (Fig. 4A). This represents an endocytic rate of 1.18 ± 0.16%/min under baseline conditions. To ensure that the disappearance of NKCC2 puncta from the surface was due to endocytosis, we pretreated cells with 5 mM MβCD, a cholesterol-chelating agent previously shown to completely block endocytosis of NKCC2 (2). We observed that MβCD completely prevented the decrease in NKCC2 puncta from the TIRF field of view, and consequently the number of surface puncta did not change significantly over 20 min (Fig. 4A). The rate of NKCC2 endocytosis in the presence of MβCD was 0.14 ± 0.06%/min (P < 0.01 MβCD treated vs. untreated) (Fig. 4B). These data indicate that the decrease in the number of surface puncta observed under baseline conditions was due to endocytosis.

Metabolic biotinylation yields specific labeling of NKCC2-BAD in MDCK cells. Our data indicate that surface NKCC2-BAD can be successfully biotinylated biochemically by adding exogenous BirA to the extracellular bath. However, this method may be associated with variability in tagging NKCC2 with biotin due to various steps involved, such as addition of BirA and ATP to the extracellular bath. Also, ATP in the reaction mixture can cause purinoceptor activation (11, 21). To improve this method, we performed metabolic biotinylation, which consists of expressing the biotin ligase (BirA) via transduction of cells with a secretory form of the enzyme (ssBirA) (7, 34, 38, 40). The presence of the secretory signal (ss) ensures that expressed biotin ligase will be in contact with proteins in the trans-Golgi and the secretory pathway. This allows biotinylation of proteins with endogenous biotin without the need of exogenously adding BirA and biotin. To test whether surface NKCC2-BAD can be labeled by metabolic biotinylation, we cotransduced MDCK cells with NKCC2-BAD and ssBirA. After 24 h, cells were then cooled to 4°C and directly labeled with Alexa Fluor 488-conjugated streptavidin. To ensure that NKCC2 biotinylation and labeling were specific, we included a negative control in parallel with every experiment in which MDCK cells were transduced with only NKCC2-BAD but not with ssBirA and then incubated them with labeled streptavidin. We observed apical surface NKCC2-BAD biotinylation as indicated by surface puncta obtained by TIRF imaging (Fig. 5A). No signal was observed in the negative control, which indicated specific biotinylation and labeling of NKCC2-BAD by coexpressed ssBirA (Fig. 5B). These data indicate that metabolic biotinylation can be used as a more convenient alternative to biochemical biotinylation for labeling of apical surface NKCC2-BAD to study trafficking.

Studying NKCC2 endocytosis in rat TAL cells. To test whether apical surface TIRF imaging can be used to study endocytosis in a cell model that more closely resembles the native TAL, we generated primary cultures of rat TAL as we previously described (13). Primary cultures of rat TAL cells were transduced with adenovirus expressing NKCC2-BAD and ssBirA to perform metabolic biotinylation as described in METHODS. Figure 6A shows a representative image of NKCC2 puncta observed at the apical surface of rat TAL cells after labeling with Alexa Fluor 488-conjugated streptavidin. In par-
Fig. 2. Imaging of apical surface biotinylated NKCC2 in MDCK cells by total internal reflection fluorescence (TIRF) microscopy. A: schematic representation of the expression of the NKCC2-biotin acceptor domain (BAD) on apical membranes of MDCK cells (left). The BAD was inserted in the extracellular loop between the transmembrane domains 5 and 6 of NKCC2 (right). B: representation of the procedure for specific biotinylation of surface NKCC2-BAD and labeling with streptavidin Alexa Fluor 488. MDCK cells expressing apical NKCC2-BAD were incubated with biotin and ATP for 15 min at room temperature (RT) in the presence of biotin ligase BirA to catalyze the covalent biotinylation of the extracellular BAD in NKCC2. Next, cells were cooled to 4°C and 1:400 streptavidin Alexa Fluor 488 was added. C: diagrammatic representation of the temperature-controlled chamber set up for live cell imaging with TIRF microscopy. Cells were retrieved by cutting the collagen-coated permeable supports and placed in a temperature-controlled chamber at 37°C with the apical membranes in close contact with the glass coverslip. The chamber was inverted to face the objective lens of the TIRF microscope, and cells were gently pressed against the coverslip with a glass coverslip. D: representative TIRF image of MDCK cells expressing biotinylated NKCC2-BAD at the apical surface labeled with Alexa Fluor 488-conjugated streptavidin (green). Surface NKCC2-BAD was biotinylated in the presence of biotin ligase as described above. E: negative control without biotin ligase. Images were acquired at ×2 magnification and laser exposure of 10 s. Scale bar = 5 μm.
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Fig. 3. Visualization of single endocytic event at the apical membrane of MDCK cells by TIRF. A: snapshots of TIRF images taken every 1 s. Individual puncta were encircled in regions of interest (red circle), and fluorescence intensity was measured on every frame. B: quantification of the fluorescence intensity (measured in arbitrary fluorescent units) decreased in intensity exponentially within 1 s and reached background fluorescence, representing an individual punctum undergoing internalization.

allel to every experiment, negative controls were performed in which MDCK cells were transduced with only NKCC2-BAD but not with ssBirA to ensure specific biotinylation and labeling with Alexa Fluor 488-conjugated streptavidin. As shown in Fig. 6B, no labeling was observed in negative control cells. These data indicate that NKCC2-BAD can be specifically labeled at the apical surface of rat TAL primary cultures to study its endocytosis in real time. Thus we monitored the disappearance of surface NKCC2 puncta from the apical membrane of rat TAL cells over 20 min at 37°C. We observed that the number of surface NKCC2 puncta gradually decreased over time (Fig. 6C). The calculated rate of decrease in number of surface NKCC2 puncta representing the rate of constitutive NKCC2 endocytosis was 1.09 ± 0.08%/min. Taken together, these data indicate that single-molecule biotinylation of surface NKCC2-BAD and apical TIRF imaging can be used to monitor NKCC2 endocytosis in TAL cells.

DISCUSSION

Current techniques to study NKCC2 trafficking are limited to biochemical- and immunolabeling-based procedures that provide a snapshot of a dynamic process but lack the time resolution achieved by live cell imaging. TIRF microscopy has become a widespread technique for visualization of dynamics of molecules at or near the cell surface in real time. This method has yielded crucial and detailed real-time information about clathrin-coated vesicle trafficking at the plasma membrane (28, 31, 32, 41–43). TIRF-based methods have several advantages over biochemical techniques. For example, in the case of poliovirus entry into cells by clathrin-mediated endocytosis, TIRF microscopy has been utilized not only to visualize initial entry but also visualize and characterize its behavior later in the endocytic pathway, including genome release (9).

We have previously shown that constitutive NKCC2 endocytosis is a crucial process in maintaining baseline NaCl absorption by the TAL because inhibition of endocytosis enhanced surface NKCC2 and increased chloride absorption by TAL (2). This highlights the importance of understanding the endocytic machinery that regulates NKCC2 retrieval from the apical membrane in TAL. However, traditional surface biotinylation techniques currently used to study NKCC2 endocytosis are limited because they do not provide information in real time. Moreover, traditional surface biotinylation using NHS-SS-biotin reacts and biotinylates accessible lysine residues in all surface proteins, not just NKCC2. Thus it does not discriminate NKCC2 from other surface proteins in the TIRF field. Therefore, the engineered NKCC2-BAD construct provides the advantage of selective NKCC2 biotinylation by BirA to study NKCC2 endocytic events at the plasma membrane of polarized MDCK cells and TAL in real-time by TIRF microscopy. To perform live cell imaging for obtaining the rate of endocytosis, we imaged the apical surface of MDCK cells and rat TAL primary cells at 37°C every second over 20 min. We observed that NKCC2 distribution was heterogeneous as well as individual fluorescent entities (referred to as puncta) in the TIRF field. Moreover, traditional surface biotinylation using NHS-SS-biotin and NHS-SM-biotin inhibited NKCC2 endocytosis in the past. When used correctly, MβCD is one of the most potent inhibitors of endocytosis, but it is of limited use in experiments lasting more than 60 min, as it depletes both intracellular and plasma membrane cholesterol. We previously showed that MβCD blocks NKCC2 endocytosis by depleting membrane cholesterol and not by other mechanisms (2). We also showed that MβCD blocks clathrin- and lipid raft-mediated endocytosis in TAL (3). Upon pretreatment with MβCD, we observed that the number of initial puncta remained constant over time. This suggests that TIRF microscopy of labeled NKCC2 allows imaging of single endocytic

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events with a time resolution of 1 s. This time resolution allows for minimal or no photobleaching of Alexa Fluor dyes during the 20 min of image acquisition. We found that a resolution time shorter than 1 s is possible by labeling NKCC2 with fluorescent quantum dots because they are brighter and more photo stable. However, quantum dots present the problem of fluorescent intermittency or blinking (data not shown). This is a discontinuous and random emission of light from fluorescent sources due to random switching between bright fluorescent periods (ON state) and dark, nonemissive periods (OFF state) (25). Due to this phenomenon of blinking, disappearance of fluorescence can be mistaken for an endocytic event. Therefore, in our experiments we used Alexa Fluor dyes covalently linked to streptavidin to avoid blinking. The resolution time was restricted to one second at the camera configurations used.

We also observed that puncta are heterogeneously distributed in the apical surface mainly confined to clusters or discrete domains. The reason for such a heterogeneous distribution is not clear but may be due to homo-oligomerization of NKCC2, which has been suggested by previous cross-linking and immunoprecipitation studies (10). A similar distribution pattern was observed by confocal imaging of TAL cells after labeling apical surface NKCC2 with an antibody against an extracellular epitope (13). However, other reasons may be the cause of the heterogeneous pattern, such as usage of tetravalent streptavidin.

**Fig. 4.** Inhibition of endocytosis with a cholesterol-chelating agent (methyl-β-cyclodextrin; MβCD) prevented punctum internalization. A: cumulative change in the number of surface puncta over 20 min of live cell imaging by TIRF under baseline conditions and treatment with 5 mM MβCD. B: quantification of the average rate of endocytosis. Baseline rate of NKCC2-BAD endocytosis averaged 1.2 ± 0.1%/min (n = 6). Treatment with 5 mM MβCD decreased the rate of NKCC2-BAD endocytosis to 0.14 ± 0.06%/min (n = 5). *P < 0.01 vs. baseline.

**Fig. 5.** Metabolic apical surface biotinylation of NKCC2-BAD by cotransduction with secreted form of biotin ligase (ssBirA). A: representative TIRF image of apical membranes of MDCK cells expressing NKCC2-BAD fluorescently labeled with streptavidin Alexa Fluor 488 (green) after metabolic biotinylation. Cells were cotransduced with NKCC2-BAD and ssBirA. B: negative control transduced with NKCC2-BAD alone (no ssBirA). Scale bars = 5 μm.

**Fig. 6.** Visualization of NKCC2 endocytosis in primary cultures of thick ascending limbs of the loop of Henle (TALs) by TIRF microscopy. A: representative TIRF image of apical membranes of rat TAL primary culture expressing biotinylated NKCC2-BAD labeled with streptavidin Alexa Fluor 488. Metabolic biotinylation was achieved by cotransduction with ssBirA. B: negative control consisting of transduction with NKCC2-BAD alone (no ssBirA). Scale bars = 5 μm. C: cumulative decrease in the number of surface puncta over 20 min of TIRF imaging of apical membranes of TAL primary cultures. Constitutive rate of endocytosis was 1.09 ± 0.08%/min. *P < 0.01 vs initial number of puncta at time 0.
vidin-conjugated dyes, possibly leading to multimerization of the monobiotinylated NKCC2-BAD molecules. Nevertheless, the rate of endocytosis in rat TAL cells that we calculated by TIRF is 1.09 ± 0.08%/min, which is similar to that calculated by surface biotinylation methods in native TAL using NHS-SS-biotin (0.98 ± 0.17%/min) (2). While it is not clear why surface NKCC2 is located in clusters, our data suggest that labeling with streptavidin did not affect the rate of endocytosis.

Concerns are always raised in cell models with exogenous overexpression of the protein under study. We tested whether changes in concentration of the virus used for transduction had any impact on the rate of endocytosis. We found that changes in concentration of the virus did not affect the rate of endocytosis of NKCC2 (data not shown). These data suggest that overexpression does not seem to affect the dynamics of NKCC2 endocytosis.

We finally conclude that our method of specifically tagging NKCC2 is appropriate for studying its endocytosis in real time and can be extended to other proteins. Single-molecule biotin-tagging of proteins has several potential applications. The specificity of BAD-mediated biotinylation has been exploited by researchers who have used it for protein detection, labeling, protein purification, and immobilization (17, 18, 24, 26, 27). It has also been applied to drug targeting (5, 35) and viral gene therapy-targeting strategies (4, 14, 16, 39). Characterization of protein-protein interactions is crucial in understanding the biological role of a protein in the cell. Methods like the yeast two-hybrid have been very useful but usually present with a fair percentage of false positives. Selective precipitation of biotin-tagged proteins with streptavidin yields more biologically relevant protein-protein interactions (19, 20, 45), which can then be identified by mass spectrometry.

We conclude that site-specific biotinylation allows for specific labeling of NKCC2 at the cell surface to visualize its endocytosis in live cells by TIRF microscopy of apical membranes in polarized TAL cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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