Association with β-COP Regulates the Trafficking of the Newly Synthesized Na,K-ATPase

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Plasma membrane expression of the Na,K-ATPase requires assembly of its α- and β-subunits. Using a novel labeling technique to identify Na,K-ATPase partner proteins, we detected an interaction between the Na,K-ATPase α-subunit and the coat protein, β-COP, a component of the COP-I complex. When expressed in the absence of the Na,K-ATPase β-subunit, the Na,K-ATPase α-subunit interacts with β-COP, is retained in the endoplasmic reticulum, and is targeted for degradation. In the absence of the Na,K-ATPase β-subunit, the α-subunit does not interact with β-COP and traffics to the plasma membrane. Pulse-chase experiments demonstrate that in cells expressing both the Na,K-ATPase α- and β-subunits, newly synthesized α-subunit interacts with β-COP immediately after its synthesis but that this interaction does not constitute an obligate intermediate in the assembly of the α- and β-subunits to form the pump holoenzyme. The interaction with β-COP was reduced by mutating a dibasic motif at Lys54 in the Na,K-ATPase α-subunit. This mutant α-subunit is not retained in the endoplasmic reticulum and reaches the plasma membrane, even in the absence of Na,K-ATPase β-subunit expression. Although the Lys54 α-subunit reaches the cell surface without need for β-subunit assembly, it is only functional as an ion-transporting ATPase in the presence of the β-subunit.

The Na,K-ATPase (or the “sodium pump”) is an ubiquitous membrane protein found in the plasma membranes (PM) of most animal cells. In the majority of polarized ion-transporting epithelia, the Na,K-ATPase is abundantly expressed and is typically restricted to the basolateral surface of the PM (1, 2). The enzyme is composed of two essential subunits, α and β. The α-subunit mediates ATP hydrolysis and translocation of sodium and potassium and harbors the binding site for pump inhibiting cardiac glycosides, such as ouabain. The glycosylated β-subunit is required for enzyme maturation and localization to the PM (3, 4). Additionally, γ-subunits have been reported to modulate pump activity but are not absolutely required for functional expression of the Na,K-ATPase (5). The α- and β-subunits are synthesized separately in the ER. Their subsequent association in a 1:1 stoichiometry is a prerequisite for trafficking of the holoenzyme to the PM (6).

Passage of cargo proteins through the secretory pathway requires their packaging in vesicles. Coat proteins (COPs) encapsulate these vesicles as they form and drive their budding process. Although the exact roles of the COP complexes remain somewhat controversial, anterograde transport from the ER to the Golgi typically involves COP-II-coated vesicles. Retrograde transport from Golgi to ER and possibly anterograde passage through the Golgi cisternae (7) involve COP-I-coated vesicles. COP-I is comprised of eight polypeptides: α, β, β′, γ, δ, ε, ζ, and the small GTPase, arf-1. COP-I, but not COP-II, shows some structural homology with clathrin and its associated adaptor proteins. Membranous cargo proteins interact with COP-I by means of dibasic motifs in their cytoplasmic faces (8). Dibasic residues involved in COP interactions, typically KK, can be found anywhere within the cytoplasmic domains of a cargo protein and are not, as first thought, restricted to the C terminus. The potassium channel TASK-1 (KCNK3), for example, interacts with β-COP via an N-terminal dibasic KR motif (9) causing retention of the channel in the ER. When this dibasic motif was altered by mutation, TASK-1 no longer associated with β-COP. Instead, it associated with the 14-3-3β protein at a distant “release” site, and subsequently the channel trafficked to the PM.

We have identified β-COP as a Na,K-ATPase interacting partner. We show that in the absence of the Na,K-ATPase β-subunit, wild type α-subunit interacts with β-COP, is retained in the ER, and is subsequently degraded, at least in part, via a proteasome-dependent mechanism. This interaction with β-COP is reduced by mutating a dibasic motif at Lys54 in the Na,K-ATPase α-subunit. In this instance, a subpopulation of the mutant α-subunit is expressed at the PM alone, without a requirement for β-subunit assembly. When expressed alone, the mutant α-subunit is nonfunctional as an ion-transporting ATPase. When expressed in the presence of the Na,K-ATPase β-subunit, however, both the wild type and the Lys54 mutant

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‡1 We dedicate this paper to the memory of our friend and colleague, Dr. Jean-Daniel Horisberger (deceased April 1, 2009).

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§ The abbreviations used are: PM, plasma membrane(s); COP, coat protein; MDCK, Madin-Darby canine kidney; ER, endoplasmic reticulum.
α-subunits traffic to the PM, are fully functional, and do not interact with β-COP.

**EXPERIMENTAL PROCEDURES**

**Na,K-ATPase Clones**—An HA epitope tag was inserted directly upstream of the rat α1-subunit start codon by PCR and confirmed by DNA sequencing. The HA-tagged α-subunit construct was subcloned into the pcDNA3.1 expression vector (Invitrogen). The untagged rat β1-subunit Na,K-ATPase subcloned into the pCB6 expression vector has been described previously (4). The rat α1 Na,K-ATPase has 10 intracellular dibasic motifs. Each motif was mutated by PCR-based mutagenesis to create mutants K22Q, K25Q, K28Q, K37Q, K54Q, K727Q, K774Q, R941Q, K1006Q, and R1011Q. In addition, a multiple mutant, 5M, was created in which all five of the N-terminal dibasic motifs were mutated, *i.e.* K22Q, K25Q, K28Q, K37Q, and K54Q. All of the constructs were confirmed by DNA sequencing and transfected as described below. For proteomic analysis, HA-tagged Na,K-ATPase α-subunit, described above, was additionally tagged at the N terminus with a SNAP tag (New England Biolabs) (10). The sequences of the PCR primers utilized for all mutagenesis steps, and the sequences encoding the Na,K-ATPase α-subunit constructs are available upon request. Construction of the N- and A domain GST fusion protein constructs has been described previously (11).

**Cell Culture**—COS cells were cultured in a humidified incubator under 5% CO₂ in α-minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Madin-Darby canine kidney (MDCK) cells were stably transfected with the cDNA encoding the SNAP-tagged sodium pump and selected in medium containing neomycin (G418, 5 μg/ml) and 5 μM ouabain. This concentration of ouabain will inhibit endogenous canine Na,K-ATPase, but not the transiently transfected, with or without the rat β1-subunit, into COS cells that endogenously express β-COP. Transfections were performed with Lipofectamine 2000 in 6-well dishes according to the manufacturer’s instructions (Invitrogen). COS cells were routinely grown and transfected in 6-well plates. Twenty-four hours post-transfection, the cells were lysed with 150 mM NaCl, 5 mM EDTA, pH 8.0, 50 mM Tris-HCl, and 1% Triton X-100 and precipitated with 4 μg of polyclonal β-COP antibody (Affinity BioReagents) or polyclonal FLAG antibody (Sigma). Immune complexes were incubated overnight with a 50% slurry of protein A-Sepharose (Pierce), washed three times in lysis buffer, and eluted into SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE, electrophoretically transferred onto nitrocellulose membranes (Bio-Rad), and blotted with monoclonal HA antibody (Jackson). After washing, the blots were incubated with secondary antibody (anti-mouse HRP; Jackson) and visualized using ECL (Amersham Biosciences). The intensity of bands on Western blots was determined by scanning the film followed by densitometric analysis of the bands (Image J software).

**SNAP Tag-based Pulse-Chase Analysis**—Polarized MDCK monolayers stably expressing both the SNAP-tagged Na,K-ATPase α-subunit and unlabeled Na,K-ATPase β-subunit were incubated with 1.4 μM BG-Block (New England Biolabs) in complete medium at 37 °C for 30 min to block the SNAP tag activity of pre-existing sodium pumps. The cells were washed and allowed to recover for 0 min (control) or 30 min (chase conditions) at 37 °C. The cells were then chased for an additional 0, 30, and 90 min in 150 μg/ml cycloheximide. After rinsing three times in cold PBS/+ (PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂), the cells were scraped in TN (100 mM NaCl, 50 mM Tris, pH 7.5, 1 mM DTT, protease inhibitors) and lysed by sonication for three 15-s bursts at the 50% power setting. Homogenates were incubated in 2 μM Biotin-BG for 90 min at room temperature. EDTA was added to 2 mM, and homogenates were centrifuged for 60 min at 112,000 × g to collect membranes. The pellets were resuspended in 500 μl of TEN-T (100 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton X-100), and the biotinylated proteins were recovered by precipitation with streptavidin-Sepharose (Thermo Scientific), separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes, and immunoblotted with either anti β-COP (Affinity Bioreagents) or anti-GP58 (gift of I. Mellman, Genentech).

**Cell Surface Biotinylation**—Plasma membrane expression of the α-subunit was measured using a membrane-impermeable biotinylation reagent, Sulfo-NHS-SS-Biotin (EZ-Link; Pierce), as described previously (14). The cells were grown
and transfected on semi-permeable filters (Transwell). Posttransfection, the cells were cultured overnight at 37 °C and then for 6 h at 25 °C. The incubation of cells at 25 °C was performed to slow protein synthesis and was found to help prevent protein aggregation and ER retention that may have resulted from transient overexpression. Biotinylated samples were lysed as described above (immunoprecipitation), and biotinylated proteins were recovered through incubation with streptavidin-Sepharose (Pierce). The samples were processed (i.e. washed and eluted) as described for the immunoprecipitation experiments.

**Immunofluorescence**—The cells were transfected on cover slips and after 24 h were washed and fixed with 4% paraformaldehyde. The cells were subsequently permeabilized with PBS containing 1 mg/ml BSA and 0.1% Triton X-100, and nonspecific binding sites were blocked with goat serum dilution buffer (33% goat serum, 40 mM NaPi pH 7.4, 800 mM NaCl, 0.6% Triton X-100). Primary antibody (monoclonal HA antibody) was prepared in goat serum dilution buffer, and secondary (anti-mouse FITC; Sigma) antibody was prepared in permeabilization buffer. The cells were visualized on a LSM 510 confocal laser scanning microscope (Zeiss). Contrast and brightness settings were chosen so that all pixels were in the linear range. The images are the product of 8-fold line averaging.

**shRNA-mediated Knockdown**—The sodium pump β-subunit sequence has not been identified for the African green monkey, from which COS cells derive. Consequently, we aligned the coding sequences from monkey (Macaca mulatta), human, dog, and rat, and selected conserved regions in designing our shRNA oligonucleotides. Target sequences were designed using the Block IT siRNA design tool (Invitrogen) and cloned into pSuper GFP (OligoEngine). The sequences used in this study were GGAAGAAATTCATCTGGAACT (human- and monkey-specific) and GCATCTTCATCGGAACTCC (cross-species specificity). For knockdown experiments, pSuper plasmids were transfected as described above.

**Electrophysiology**—Complete electrophysiological procedures have been described previously (15). Briefly, for expression in *Xenopus* oocytes, cDNAs encoding the α- and β-subunits were subcloned into the pSD5 vector (a kind gift from Kathi Geering, University of Lausanne), which has been described previously (16). Templates were linearized with FspI and capped RNAs were *in vitro* transcribed from the T7 promoter using the mMessage mMachine RNA kit (Ambion). Stage VI oocytes were injected with 8 ng of RNA and monitored for expression after 3–5 days. Two-electrode voltage clamp of *Xenopus* oocytes was used to record Na,K-ATPase activity as an outward current elicited when changing from a potassium-free to 10 mM potassium-containing extracellular solution, holding the membrane potential at −50 mV, which, under those experimental conditions, is equivalent to the ouabain-sensitive current (15).

**RESULTS**

Our first goal was to identify novel protein interacting partners of rat Na,K-ATPase stably transfected and expressed in the polarized MDCK cell line. To do this we constructed a dual-tagged Na,K-ATPase by fusing the SNAP tag at the N terminus of the HA-tagged Na,K-ATPase construct (10). The SNAP tag is a modified version of the DNA repair protein O^6^-alkylguanine-DNA alkyltransferase that can be covalently labeled with substituted groups presented as benzylguanine-based adducts (17, 18). For this study, we utilized benzylguanine substituted with biotin (BG-biotin), such that, upon labeling, the SNAP tag becomes covalently biotinylated and amenable to standard streptavidin-biotin purification techniques. The Na,K-ATPase and its associated protein partners were purified by a two-step strategy involving an immunoafinity purification using an antibody directed against the HA tag followed by specific elution with HA epitope peptide and subsequent streptavidin precipitation. We employed this two-step procedure because an initial purification exploiting the HA epitope was found both to eliminate contamination from endogenously biotinylated molecules and to deplete lysates of proteins that might nonspecifically bind to the-Sepharose resin (supplemental Fig. S1). Lysates were biotinylated with BG-biotin and incubated with anti-HA beads to immunoprecipitate the sodium pump. The majority of the sodium pump could be depleted from cell lysates by immunoprecipitation via the HA tag (supplemental Fig. S1A). The HA bead-associated sodium pump could then be eluted from the beads by competition with an HA peptide. Labeling of the SNAP tag with BG-biotin resulted in the appearance of a single band of a molecular weight that corresponds to that of the sodium pump when samples were probed with HRP-streptavidin (supplemental Fig. S1B). In addition, endogenously biotinylated proteins were also detected in unbiotinylated control samples. HA tag isolation, however, was found to deplete our samples of these contaminants (supplemental Fig. S1B). For biotinylated samples, the eluted sodium pump could be efficiently precipitated using streptavidin beads (supplemental Fig. S1A), whereas no precipitation was detected in control samples. Proteins bound to the streptavidin beads were analyzed by SDS-PAGE followed by staining with colloidal Coomassie Brilliant Blue. Numerous bands were detected in the biotinylated samples relative to control samples (supplemental Fig. S1C). Proteins that associated with the Na,K-ATPase through this two-step purification were identified through LC-MS/MS. Analysis of the LC-MS/MS data revealed an interaction of the Na,K-ATPase with coatomer protein (Fig. 1). No interaction was observed when Na,K-ATPase β-subunit, with or without the Na,K-ATPase α-subunit, were subjected to immunoprecipitation with polyclonal β-COP antibody. Subsequent Western blotting with monoclonal HA antibody (to detect transfected α-subunit) showed a clear interaction between β-COP and the Na,K-ATPase α-subunit (Fig. 1). No interaction was observed when Na,K-ATPase α- and β-subunits were co-transfected (Fig. 1A).

Newly synthesized Na,K-ATPase subunit proteins are unable to depart the ER until they assemble into the heterodimeric αβ holoenzyme (6). Because β-COP and the COP I complex are
thought to be involved in ER retention, we next wished to determine the fate of the Na,K-ATPase α-subunit when it is expressed by itself and retained in the ER. Toward this end, we investigated whether α-subunit retained in the ER is degraded by the proteasome. Incubation of transfected cells with the proteosomal inhibitor, lactacystin, at 10 mM for 2 h prior to cell lysis increased the extent of the detectable interaction between β-COP and the Na,K-ATPase α-subunit (Fig. 1B). These data suggest that a portion of the β-COP-associated pool of Na,K-ATPase α-subunit is subjected to proteasome-mediated degradation.

Identification of the Na,K-ATPase α-subunit Sequence Domain That Interacts with β-COP—The Na,K-ATPase α-subunit possesses two major and structurally discrete cytoplasmic domains (20, 21). The large loop between the fourth and fifth transmembrane segments forms the NP domain, which participates directly in both the nucleotide binding and phosphorylation steps of sodium pump catalysis. The A, or actuator, domain receives contributions from both the N-terminal tail and the loop between transmembrane segments two and three.

Based upon its homology to the Ca-ATPase, whose structure has been solved in several conformational states (22), the NP and A domains are thought to undergo large scale movements with respect to one another during the reaction cycle of the sodium pump. To begin to assess the sequence domains of the Na,K-ATPase α-subunit that participate in the interaction with β-COP, we made use of GST fusion proteins that incorporate either the NP domain or the A domain of the Na,K-ATPase α-subunit (11). The NP domain fusion protein includes the entire sequence of the cytosolic loop that connects transmembrane helices four and five. Because the A domain is composed of two noncontiguous stretches of amino acid sequence, the GST-A domain construct was created by attaching the α-subunit N-terminal tail (residues 1–85) through a random coil linking sequence composed of (Gly-Gly-Gly-Gly-Ser)2 to the sequence derived from the M2-M3 loop (residues 137–280). The fusion proteins encoded by these constructs were purified from bacterial lysates on glutathione-Sepharose, and the-Sepharose-linked fusion proteins were subsequently incubated with lysates prepared from MDCK cells. The bound proteins were recovered by centrifugation, separated by SDS-PAGE, and analyzed by Western blotting using a polyclonal antibody directed against the β-COP protein. As can be seen in Fig. 2A, the A domain fusion protein interacts with and pulls down β-COP, whereas no β-COP is associated with glutathione-Sepharose beads carrying either the NP domain fusion protein or GST alone. These data suggest that the interaction between β-COP and the Na,K-ATPase α-subunit involves the domains that comprise the A domain of the pump.

Because β-COP interacts with cargo proteins through dibasic motifs, we next mutated all such dibasic motifs in the Na,K-ATPase α-subunit. The mutated α-subunits were expressed by transfection in COS cells, which were lysed and subjected to immunoprecipitation with the antibody directed against β-COP. Compared with wild type α-subunit, the α-subunit mutants K54Q and 5M (which includes the K54Q mutation) showed a much reduced interaction with β-COP (Fig. 2B). All other α-subunit mutants interacted with β-COP to a similar extent, as did the wild type α-subunit protein. Consistent with the results of fusion protein experiments presented in Fig. 2A, the Lys134 residue that appears to be critically required for the interaction between the sodium pump and β-COP is located in the N-terminal tail of the α-subunit and is thus contained within the structural A domain of the α-subunit.

Time Course of the Interaction between the Newly Synthesized Na,K-ATPase and β-COP—To depart from their sites of synthesis in the
endoplasmic reticulum and begin their transit to the plasma membrane, the two Na,K-ATPase subunit polypeptides must first assemble to form the heterodimeric sodium pump holoenzyme. Our data indicate that the interaction between the Na,K-ATPase α-subunit and β-COP involves only the pool of α-subunits that has not assembled with Na,K-ATPase β-subunit. These observations suggest the interesting possibility that newly synthesized unassembled Na,K-ATPase α-subunit interacts transiently with β-COP, which contributes to its retention in the ER. According to this model, subsequent assembly with the Na,K-ATPase β-subunit could directly or indirectly lead to the release of the Na,K-ATPase α-subunit from its association with β-COP, permitting it to traffic to the cell surface. It is also possible that the β-COP-associated pool of unassembled α-subunit is unavailable for assembly with the Na,K-ATPase β-subunit and is instead targeted for degradation. If the first possibility were correct, then we would expect a pulse-chase experiment to reveal that the Na,K-ATPase α-subunit can be co-precipitated with β-COP most extensively at a time shortly after its synthesis is completed and that this interaction will wane according to the same time course that is associated with the assembly of the α- and β-subunits and their subsequent delivery to the Golgi complex. In contrast, if the β-COP-associated pool of newly synthesized Na,K-ATPase α-subunit is not available for assembly with the Na,K-ATPase β-subunit, then we expect that the interaction between the α-subunit and β-COP will persist beyond the completion of holoenzyme assembly.

To test these possibilities, we took advantage of the properties of the SNAP tag in combination with pulse-chase protocols to capture specific temporally defined cohorts of Na,K-ATPase α-subunit protein and to assess the extent to which these cohorts are interacting with the β-COP protein at various intervals following their biosynthesis. In the first experiment, a confluent monolayer of MDCK cells stably expressing SNAP-tagged Na,K-ATPase α-subunit was incubated for 30 min at 37 °C with the BG block compound, which is membrane-permeable and becomes covalently and irreversibly bound to any SNAP tags that are present in the cell. Following the BG block step, the cells were washed and returned to 37 °C for a 30-min “pulse” incubation, during which a new cohort of unblocked SNAP-tagged Na,K-ATPase α-subunit was synthesized. After 30 min, cycloheximide was added to the incubation medium to prevent further protein synthesis and hence terminate the pulse period. A “chase” incubation continued in the presence of cycloheximide for 90 min at 37 °C. At various intervals following the blocking step (0, 30, 60, and 120 min), the cells were harvested by scraping, lysed by sonication, and incubated with the BG-biotin compound to label the cohort of pump proteins that was synthesized during the pulse interval. The membranes were recovered by ultracentrifugation to remove unreacted BG-biotin compound and solubilized in a detergent-containing buffer. The biotinylated population of Na,K-ATPase α-subunit and any associated proteins were recovered by streptavidin precipitation, separated by SDS-PAGE, and analyzed by Western blotting using an antibody directed against β-COP. The detection of β-COP in this Western blot thus indicates that β-COP was associated at that time point with Na,K-ATPase α-subunit that was synthesized during the pulse interval.

To test the efficacy of this BG-biotin pulse-chase protocol, we first probed the blots of the streptavidin precipitates with an antibody directed against the Na,K-ATPase β-subunit. As would be expected, immediately after the block and prior to any pulse incubation, no Na,K-ATPase β-subunit is detected in the streptavidin precipitate (Fig. 3A, Block). At the end of the 30-min pulse period, the newly synthesized Na,K-ATPase α-subunit co-precipitates with a 45-kDa protein that corresponds to the endoplasmic reticulum form of the of the Na,K-ATPase β-subunit protein (Fig. 3A, 0 min Chase). Over the course of the ensuing chase incubation, the 45-kDa ER form of the Na,K-ATPase β-subunit matures into a 55-kDa post-Golgi form of the protein (Fig. 3A, 30 and 90 min Chase). Quantification of these data indicates that the amount of the ER form of the β-subunit detected during the pulse period is ~35-fold greater than that detected in the blocked control cells (Fig. 3B). During the chase, ER-β-subunit levels decrease rapidly, reaching a level corresponding to 2-fold that seen in blocked control cells after 90 min of chase. Mature β-subunit protein levels increase by 7-fold during the pulse, indicating that some of the newly synthesized β-subunit protein has reached the Golgi as early as 30 min post-block. Glycosylated β-subunit levels increase by 26-fold through the course of 90 min of chase (Fig. 3B). This time course agrees quite closely with that established by a number of investigators using traditional metabolic labeling-based pulse-chase approaches (6). Thus, the SNAP tag technique used in combination with BG-biotin allows us specifically to recover a temporally defined cohort of Na,K-ATPase α-subunit and its associated proteins at various defined intervals after its synthesis.

Probing the streptavidin-recovered proteins with an antibody directed against β-COP reveals that the β-COP protein is specifically bound to the newly synthesized BG-biotin-labeled Na,K-ATPase α-subunit at the end of the 30-min pulse period (Fig. 3C, 0 min Chase). Under these conditions, we observed a 4.4-fold increase in β-COP binding in material recovered from pulse-labeled cells relative to that detected in material from blocked control cells (Fig. 3D). This association is markedly reduced (1.7-fold decrease) and essentially absent (2.6-fold decrease) after 30 and 90 min of chase incubation (Fig. 3D, 30 and 90 min Chase). These data demonstrate that the Na,K-ATPase α-subunit interacts with β-COP soon after its synthesis, and this interaction terminates relatively rapidly. Taken together with the data presented in Fig. 1A, these results suggest that a pool of newly synthesized Na,K-ATPase α-subunit that has not yet assembled with the Na,K-ATPase β-subunit interacts with β-COP, whereas it is still resident in the membranes of the ER.

We next wished to determine whether this β-COP-associated pool of newly synthesized Na,K-ATPase α-subunit is a biosynthetic intermediate in the formation of the sodium pump αβ holoenzyme complex. We repeated the pulse-chase protocol using shorter pulse and chase intervals to assess whether a precursor-product relationship can be detected between the pool of α-subunit that is engaged with β-COP and the population of α-subunit that assembles with the Na,K-ATPase β-subunit. In the experiment depicted in Fig. 3 (E–H), the pulse interval was reduced to 10 min, and samples were harvested after chase intervals of 0, 10, 20, and 40 min. As can be seen in Fig. 3E,
assembly between the Na,K-ATPase α- and β-subunits appears to be extremely fast and is complete by the end of this much shorter pulse period. Indeed, the quantity of Na,K-ATPase β-subunit that co-precipitates with the newly synthesized α-subunit does not increase over the course of the 40-min chase. Quantification of our blots demonstrates that levels of the ER form of the β-subunit begin to decrease immediately after the pulse period (Fig. 3F, compare 0, 10, 20, and 40 min of chase), which is accompanied by a reciprocal increase in mature β-subunit levels during this time course. Similar to the results shown in Fig. 3D, we observed an association between β-COP and the α-subunit immediately following the biosynthesis of the α-subunit. This interaction persisted throughout most of the chase and only began to diminish late in the chase when the ER-associated pool of α-subunit was depleted (Fig. 3, G and H). Together with the data from Fig. 3 (A and B), this suggests that αβ assembly is complete shortly after synthesis, and there is no contribution from the β-COP-associated pool. Instead, association with β-COP appears to constitute a dead end destination for newly synthesized α-subunit that has failed to assemble with β-subunit and that will ultimately be targeted for destruction, as suggested by the data depicted in Fig. 1B.

Plasma Membrane Expression of Na,K-ATPase Mutants—If the association between the Na,K-ATPase α-subunit and β-COP causes the unassembled α polypeptide to be retrieved to and retained in the endoplasmic reticulum and thus prevents it from reaching the plasma membrane, then blocking or disrupting the β-COP interaction might be expected to permit α-subunit to escape the endoplasmic reticulum without need for assembly with the Na,K-ATPase β-subunit. To test this possibility, we used cell surface biotinylation to examine the surface expression of the wild type and mutated α-subunit proteins described in Fig. 2.

Biotinylation was performed on cells using the membrane-impermeant NHS-ss-biotin reagent. Successful biotinylation, therefore, demonstrates protein expression at the PM. In the absence of the β-subunit, only minimal Na,K-ATPase α-subunit was...
**β-COP Controls Na,K-ATPase Exit from ER**

**FIGURE 4.** Plasma membrane expression of Na,K-ATPase α-subunit in the absence of the β-subunit is increased for the K54Q mutant. Wild type and mutant HA-tagged α-subunits were expressed by transfection in COS cells. Cell surface proteins were labeled with a membrane-impermeant biotin reagent and subsequently isolated with streptavidin-agarose beads and subjected to Western blotting with anti-HA antibody to detect the Na,K-ATPase α-subunit. The blots were scanned, and band intensities were measured by densitometry and normalized to PM expression in cells transfected with α-subunit alone. The data represent the means ± S.E. (*n* = 3).

Expressed at the PM (Fig. 4). This small amount possibly reflects the presence of endogenous β-subunit in the COS cells, resulting in the trafficking of a small amount of the transfected α-subunit protein to the PM. When co-transfected with β-subunit, PM expression of Na,K-ATPase α-subunit doubled. If β-COP association is necessary to mediate retention of the α-subunit in the ER when it is expressed without the β-subunit, we would expect that mutations that perturb the β-COP interaction would result in an α-subunit that is able to access the PM independent of the presence of the β-subunit. Fig. 2 demonstrates that the K54Q and 5M α mutants do not interact with β-COP. Based on this finding we wondered whether these mutated α-subunit proteins could get to the PM in the absence of exogenous β-subunit expression. Biotinylation revealed increased PM expression of K54Q and 5M α-subunits, relative to wild type α-subunit, when these proteins were expressed in the absence of the β-subunit (Fig. 4). No other mutants showed increased cell surface expression of the α-subunit in the absence of exogenous β-subunit protein.

To complement the biotinylation data, immunofluorescence analysis was performed on transfected cells. Wild type Na,K-ATPase α-subunit alone showed no detectable labeling at the PM. The α-subunit was expressed at high levels but showed a distribution consistent with localization in the ER. As expected, co-transfection with the Na,K-ATPase β-subunit increased PM expression of the α-subunit. PM labeling of the Na,K-ATPase was also apparent in cells transfected with K54Q and 5M α-subunit mutants, even in the absence of co-transfection with the Na,K-ATPase β-subunit (Fig. 5A).

Our experiments in which the wild type Na,K-ATPase α-subunit was transfected alone showed no detectable labeling at the PM, suggesting that endogenous β-subunit expression is not sufficient to mediate PM delivery. However, it is possible that endogenous expression of the Na,K-ATPase β-subunit might account for some of the plasma membrane delivery observed with the K54Q and 5M α-subunit mutants. To conclusively rule out this possibility, shRNA constructs were designed to target and knock down β-subunit expression. Because the coding sequence for COS cell β-subunit is not known, we selected two target sequences against conserved regions of β-subunit cDNAs and simultaneously transfected both shRNAs into COS cells. Quantitative RT-PCR analysis of transfected cells indicates that endogenous β-subunit mRNA levels are reduced by 95 ± 1.3% upon transfection with these plasmids. In accordance with this finding, transfection of our knockdown constructs were found to abrogate cell surface delivery of the wild type Na,K-ATPase α-subunit when co-transfected with β-subunit (Fig. 5B). Interestingly, cell surface expression of the K54Q and 5M mutants were not affected in cells co-transfected with shRNAs, indicating that β-subunit expression is not required for their delivery to the PM (Fig. 5C).

**Electrophysiological Recordings of Na,K-ATPase**—Our data show that by mutating the dibasic motif responsible for interaction with β-COP, the unassembled K54Q mutant α-subunit successfully traffics to the PM. To address whether unassembled K54Q was functional when expressed at the PM, electrophysiological recordings were made in *Xenopus* oocytes (15). Stage VI *Xenopus* oocytes do not express the endogenous Na,K-ATPase β-subunit, making them an ideal expression system in which to test our mutants (23). Oocytes were induced to express the α-subunit constructs by microinjection of mRNA. Cell surface Na,K-ATPase activity was measured by two-electrode voltage clamp and defined as the potassium-stimulated component of the transmembrane current. In *Xenopus* oocytes expressing wild type Na,K-ATPase α-subunit alone, no ATPase-generated currents were recorded. In contrast, when mRNA encoding the β-subunit was co-injected with the α-subunit, ATPase-generated currents were recorded, indicative of functional Na,K-ATPase at the PM. In *Xenopus* oocytes co-expressing the K54Q α-subunit and the β-subunit, currents were similar to those obtained for the wild type α-subunit (Fig. 6). Interestingly, when mRNA encoding the K54Q α-subunit was injected in the absence of β-subunit mRNA, no ATPase-generated currents were recorded. Because our data suggest that K54Q can reach the PM when it is expressed alone (Figs. 4 and 5), we conclude that the α-subunit alone is non-functional as a Na,K-ATPase at the PM. However, because we
have yet to directly visualize surface expression of K54Q in oocytes, it remains possible that the mutant α-subunit is not efficiently delivered to the plasma membrane in this system.

**DISCUSSION**

A large body of evidence indicates that assembly of the sodium pump heterodimer is required to generate a functional Na,K-ATPase that is capable of departing the ER and reaching its site of functional residence at the cell surface. We report here that in the absence of its β-subunit, the α-subunit of the Na,K-ATPase interacts with the coat protein, β-COP, a component of the COP-I complex. The extent of this interaction is increased in the presence of lactacystin, a proteosome inhibitor. Taken together, these data suggest that, when synthesized in excess of the β-subunit, the α-subunit is retained in the ER via a COP-I-dependent mechanism and subsequently degraded, at least in part, by the proteosome. However, in the presence of sufficient (or excess) Na,K-ATPase β-subunit, the α-subunit associates with the β-subunit and does not interact with COP-I, and the assembled αβ heterodimer progresses to the PM. Eliminating the dibasic motif at position Lys54 (mutants K54Q and 5M) was sufficient to prevent the interaction between the α-subunit and COP-I. In keeping with this loss of the capacity for COP-I interaction, these mutated α-subunits exhibited increased expression at the PM, as judged by cell surface biontinylation and immunofluorescence, even in the absence of the β-subunit.

**FIGURE 6.** The K54Q Na,K-ATPase is not functional in the absence of the β-subunit. Electrophysiological recording of Na,K-ATPase-generated currents (in nA) in *Xenopus* oocytes showed no activity for wild type α-subunit expressed in the absence of β-subunit. In the presence of β-subunit, wild type α Na,K-ATPase generated currents consistent with PM expression of a functional Na,K-ATPase. K54Q Na,K-ATPase α-subunit expressed with the β-subunit produced pump current, but no currents were detected when it was expressed alone, implying that the pool of unassembled K54Q α-subunit that reaches the plasma membrane is nonfunctional. The data represent the means ± S.E (n = 3).
β-subunit. We propose that COP-I binding serves as a component of the quality control machinery of the cell, recognizing unassembled Na,K-ATPase α-subunit and returning it to the ER until it is either liberated through assembly with the β-subunit or targeted for degradation. Consistent with this view, it has been suggested that mutant cystic fibrosis transmembrane conductance regulators are recognized as aberrant through the binding of protein partners to dibasic (di-arginine) motifs and that COP-I may be involved in this process (24). The role of COP-I in mediating subsequent degradation of aberrant multimeric proteins has also been reported for unassembled subunits of the nicotinic acetylcholine receptor, which are recognized by COP-I and subsequently ubiquitinated prior to degradation (25).

The data gathered in the pulse-chase experiment presented in Fig. 3 demonstrate that, at least under the circumstances of our experiments, the complex between the newly synthesized α-subunit and β-COP does not constitute an obligate biosynthetic intermediate en route to the assembly of the α-subunit with the Na,K-ATPase β-subunit to form the pump holoenzyme. Our data indicate that αβ assembly is very rapid and is complete very soon after the conclusion of subunit protein synthesis. The association between Na,K-ATPase α-subunit and β-COP persists long after subunit assembly has reached completion. It seems likely, therefore, that assembly with β-COP targets the α-subunit that has failed to assemble with β-subunit for retention in the ER and ultimate degradation in the proteasome, as evidenced by the increase in the quantity of α-subunit that can be co-immunoprecipitated with β-COP in the presence of lactacystin. It is not possible to determine from our analysis whether the complex between the α-subunit and β-COP is functionally irreversible or whether it is instead a dynamic association, from which the α-subunit can be liberated to assemble productively into holoenzyme if a pool of unassembled β-subunit were available.

Our data indicate that the α-subunit will bind β-COP when it is expressed in excess of the β-subunit polypeptide. It is worth wondering whether this situation might ever exist in a physiological system. In poorly differentiated carcinoma cell lines, β-subunit expression is down-regulated by the Snail transcription factor (26). Furthermore, in cardiac myocytes, the β-subunit is regulated independently of the α-subunit by changes in external K (27). Both scenarios, and presumably others as well, could potentially result in the wild type α-subunit being synthesized in excess of the β-subunit protein.

The results presented here cannot be interpreted to suggest that there is a direct competition between β-COP and the Na,K-ATPase β-subunit. The association between the Na,K-ATPase α- and β-subunits has been shown to involve motifs residing in the extracellular loop between TM domains 7 and 8 of the α-subunit (28). In addition, the recent high resolution structures of the Na,K-ATPase complex reveal critical contacts between the seventh and tenth transmembrane helices of the α-subunit and the membrane spanning segment of the β-subunit (20, 21). In contrast, we find that β-COP binds to the intracellular N-terminal domain of the α-subunit. Thus, we hypothesize that assembly between the α-subunit and the β-subunit induces a global change in the conformation of the α-subunit, which inhibits or reverses the binding of the α-subunit to β-COP. Previous work has demonstrated that αβ assembly results in dramatic changes in the trypsin sensitivity of the α-subunit (23), suggesting that β-subunit binding produces a substantial effect on the conformation of the α-subunit.

It is clear from Figs. 4 and 5 that in the absence of the β-subunit, PM expression of the K54Q α-subunit is greater than that of the wild type α-subunit protein under these circumstances. However, PM expression levels do not approach those seen when the wild type α-subunit is co-expressed with the Na,K-ATPase β-subunit. This suggests that other factors and interactions also contribute to the ER retention or PM expression of the Na,K-ATPase. For example, the interaction of Na,K-ATPase with BiP, an immunoglobulin heavy chain binding protein and member of the heat shock 70 protein family, may be involved in retaining unassembled sodium pump α-subunit in the ER. The Na,K-ATPase α-subunit and BiP have previously been shown to interact (29). This interaction was correlated with the ER retention of overexpressed α-subunit in Xenopus oocytes and was reversed by expression of the β-subunit. Interestingly, BiP was also identified in our proteomic screen of Na,K-ATPase α-subunit interacting partners (data not shown).

In conclusion, Na,K-ATPase α-subunit associates with β-COP, a component of the COP-I complex. This association can cause the retention of the α-subunit in the ER and results in its subsequent degradation. Na,K-ATPase α-subunit that has assembled with the β-subunit does not interact with β-COP and is not subject to retention in the ER. The association between the α-subunit and β-COP is probably not an obligate step in the mechanism that governs the assembly and trafficking of the Na,K-ATPase but rather marks for ER retention and ultimate destruction those α-subunits that fail to productively assemble with β-subunit to form holoenzyme. Thus, this association may constitute an important quality control mechanism through which the cell regulates this physiologically critical multimeric transport enzyme.

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