Epithelial sodium channels (ENaCs) are assembled in the endoplasmic reticulum (ER) from α, β, and γ subunits, each with two transmembrane domains, a large extracellular loop, and cytoplasmic amino and carboxyl termini. ENaC maturation involves transit through the Golgi complex where Asn-linked glycans are processed to complex type and the channel is activated by furin-dependent cleavage of the α and γ subunits. To identify signals in ENaC for ER retention/retrieval or ER exit/release, chimera were prepared with the interleukin α subunit (Tac) and each of the three cytoplasmic carboxyl termini of mouse ENaC (Tac-Ct) or with γ-glutamyltranspeptidase and each of the three cytoplasmic amino termini (Nt-GGT). By monitoring acquisition of endoglycosidase H resistance after metabolic labeling, we found no evidence of ER retention of any chimera when compared with control Tac or GGT, but we did observe enhanced exit of Tac-αCt when compared with Tac. ER exit of ENaC was assessed after metabolic labeling by following the appearance of cleaved α as cleaved α subunit, but not non-cleaved α, is endoglycosidase H-resistant. Interestingly ER exit of epitope-tagged and truncated (αΔ624–699-V5) with full-length βγ was similar to wild type α (+βγ), whereas ER exit of ENaC lacking the entire cytoplasmic carboxyl tail of α (αΔ613–699-V5 + βγ) was significantly reduced. Subsequent analysis of ER exit for ENaCs with mutations within the intervening sequence 613HRFRSRYWS623 within the context of the full-length α revealed that mutation αRSRYW620 to AAAAA significantly reduced ER exit. These data indicate that ER exit of ENaC is regulated by a signal within the α subunit carboxyl cytoplasmic tail.

Epithelial Na⁺ channels (ENaCs) are found in apical membranes of Na⁺-transporting epithelia that line the distal nephron, airway and alveoli, and distal colon. These channels consist of three structurally related subunits, termed α, β, and γ, with a presumed α1β1γ1 subunit stoichiometry (1, 2), although higher ordered stoichiometries have been proposed (1–3, 72). The subunits share common structure features with two membrane-spanning and adjacent extracellular hydrophobic domains (M1H1 and H2M2) separated by a large extracellular loop and cytoplasmic amino (Nt) and carboxyl (Ct) termini (2, 4, 5). ENaCs have important roles in the regulation of extracellular fluid volume, blood pressure, and airway surface liquid volume.

ENaC subunits are thought to assemble in the endoplasmic reticulum (ER) where they undergo Asn-linked glycosylation and form disulfide bridges (5–10). Quality control of ENaC subunit folding and assembly involves interactions with both Hsc70 and the small heat shock protein αA-crystallin (11, 12). There is also clear evidence that ENaC transits the Golgi complex during its maturation. Expression of active channels at the plasma membrane is blocked by treatment of cells with the fungal metabolite brefeldin A that disrupts assembly of cytosolic coats that are required for intra-Golgi transport (13, 14). Endoglycosidase H (Endo H)-resistant and neuraminidase-sensitive forms of ENaC have been described, consistent with Asn-linked glycan processing in the Golgi complex (10, 15, 16). Normal ENaC activity is dependent on cleavage by furin at two sites in the α extracellular loop and one site in the γ extracellular loop (17–19). Furin cleavage of the α subunit releases an inhibitory 26-mer peptide (20). Processing of the γ subunit by furin and prostasin releases an additional 43-mer inhibitory peptide (21). Furin is a well characterized proprotein convertase that is localized primarily in the trans-Golgi network (TGN), although it shuttles between the TGN and plasma membrane, and prostasin is a glycosylphosphatidylinositol-anchored serine protease likely found on the cell surface (22, 23).

ENaC assembly clearly begins in the ER as immature nonprocessed forms of all three subunits are found in co-immunoprecipitates (10). Co-expression of αβγENaC with chimera designed to properly orient either the α extracellular loop, the two membrane-spanning and adjacent extracellular hydrophobic domains (αM1H1 or αH2M2), or α cytoplasmic domains blocks both surface expression of ENaC and amiloride-sensi-
ER Exit Signal in ENaC

tive sodium currents in *Xenopus* oocytes, indicating that subunit folding, channel assembly, and channel exit from the ER are interdependent (24). Although ER exit signals for transmembrane cargo that interact with the prebudding coat protein complex II (COPII) (Sar1p, Sec23p, and Sec24p) are diacidic motifs, dibasic motifs, short hydrophobic motifs, or combinations of these, the signals for ER exit of multitransmembrane proteins and/or multisubunit proteins are more complex using a hierarchy of arginine-based ER retention signals and diverse ER exit (release) signals (for reviews, see Refs. 25–27). ENaCs released from the ER reach the cell surface by both transit through the Golgi complex and by a non-conventional route that bypasses glycan processing and furin-dependent activation in the Golgi complex and TGN (28, 29). Non-processed ENaC can be activated by proteolytic cleavage at the cell surface by serine proteases such as exogenous trypsin or endogenous elastase or prostasin (21, 30–33). Thus, factors that regulate the assembly, retention, and release of ENaC from the ER could represent key regulatory steps in the ability of cells to modulate Na\(^{+}\) transport.

As a first step toward understanding how ER exit of ENaC is regulated, we followed the acquisition of Endo H resistance for chimera made with each of the six cytosolic domains of \(\alpha\), \(\beta\), and \(\gamma\) mouse ENaC subunits. Although we found no evidence for a retention signal in any of the cytoplasmic domains, an ER exit signal was clearly present within the carboxyl terminus of the \(\alpha\) subunit. We then analyzed ER exit of ENaC containing wild type or mutant \(\alpha\) subunits and found that an ER exit signal is present within a short peptide motif of the \(\alpha\) cytoplasmic carboxyl-terminal tail.

**EXPERIMENTAL PROCEDURES**

**Vector Preparation**—Chimeras of mouse ENaC cytoplasmic domains were constructed in pcDNA5/FRT (Invitrogen) using a two-step PCR method (34). The \(\alpha\) cytoplasmic domains of the \(\alpha\) (residues 1–109), \(\beta\) (residues 1–49), or \(\gamma\) (residues 1–53) ENaC subunits were linked to the transmembrane and ectodomain of the rat \(\gamma\)-glutamyltranspeptidase (GGT) mutant T380N that lacks autocatalytic cleavage (residues 5–568) and named \(\alpha\)NT-GGT, \(\beta\)NT-GGT, and \(\gamma\)NT-GGT, respectively (35). The \(\beta\) cytoplasmic domains of the \(\alpha\) (residues 613–699), \(\beta\) (residues 555–638), or \(\gamma\) (residues 573–655) ENaC subunits were linked to the transmembrane and ectodomain of the human interleukin receptor \(\alpha\) subunit (referred to as Tac, residues 1–266) and named Tac-\(\alpha\)Ct, Tac-\(\beta\)Ct, and Tac-\(\gamma\)Ct, respectively (36). Wild type \(\alpha\) subunit with both amino-terminal HA and carboxyl-terminal V5 epitope tags (named \(\alpha\)WT, wild type \(\beta\) subunit with a carboxyl-terminal FLAG epitope tag (\(\beta\)-FLAG), wild type \(\gamma\) subunit with a carboxyl-terminal myc epitope tag (\(\gamma\)-myc), and mutant \(\gamma\)G542C were generated and characterized previously (1, 10). \(\alpha\)WT was transferred into pcDNA6, and truncation mutants \(\alpha\)624 (HA-\(\alpha\)624–699-V5) and \(\alpha\)613 (HA-\(\alpha\)613–699-V5) as well as site-specific mutations of \(\alpha\)HA-\(\alpha\)V5 named \(\alpha\)HRF (\(\alpha\)HRF\(^{515}\) to AAAAA), \(\alpha\)RSRYW (\(\alpha\)RSRYW\(^{620}\) to AAAAA), or \(\alpha\)SPG (\(\alpha\)SPG\(^{623}\) to AAAAA) were constructed using a PCR-based approach (34).

**Cell Culture and Transfections**—Clonal cultures of flip-in Chinese hamster ovary cells expressing each \(\alpha\)NT-GGT or Tac-Ct chimera were prepared by co-transfection of cells with plasmid POG44 and recombinant plasmids constructed in pcDNA5/FRT (Invitrogen) followed by clonal selection and maintenance in medium containing hygromycin (0.5 mg/ml) (37). All Chinese hamster ovary cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 (1:1) (DMEM/Ham’s F-12 medium) with 3% fetal bovine serum at 37 °C in 5% CO\(_2\). All tissue culture reagents were purchased from Invitrogen. MDCK type 1 cells were obtained from both Barry M. Gumbiner (Memorial Sloan-Kettering Cancer Center, New York, NY) and Daniela Rotin (University of Toronto, Toronto, Canada) (38). Experiments were carried out in MDCK cells from both sources, but no differences were observed. MDCK cells were maintained in DMEM with 10% fetal bovine serum as described previously (10) and transiently transfected with either \(\alpha\)WT, mutant \(\alpha\)624, or mutant \(\alpha\)613 in combination with \(\beta\)-FLAG and \(\gamma\)-myc using Lipofectamine 2000 (Invitrogen). Cells were cultured in the presence of 20 \(\mu\)M amiloride 4 h after transfection and used the following day for experiments described in Figs. 3 and 5 (10). Inclusion of amiloride in the medium after transfection improved the signal obtained in experiments using radiolabeling.

**Radiolabeling, Immunoprecipitation, and Immunoblotting**—Confluent cultures of flip-in cell lines expressing chimera were grown in 25- or 35-mm dishes (6- or 12-well clusters). Cells were washed once with 1 ml of Dulbecco’s modified Eagle’s medium lacking methionine and cysteine (ICN, Costa Mesa, CA) and starved for Met and Cys in 1 ml of the same medium for 1 h before addition of 50–100 \(\mu\)Ci of \(^{35}\)S Met/Cys (MP Biomedicals Inc., Irvine, CA) for 15 min as described previously (39). Labeled cells were chased in DMEM/Ham’s F-12 medium (with 3% fetal bovine serum) containing excess Met (2.5 mm) for the times indicated. Cells were washed with 1 ml of phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 15.2 mM \(\text{Na}_2\)HPO\(_4\), 1.47 mM KH\(_2\)PO\(_4\), 0.5 mM MgCl\(_2\), and 0.7 mM CaCl\(_2\)) and extracted into 0.15 ml (12-well) or 0.3 ml (6-well) of lysis buffer (0.4% sodium deoxycholate, 1% Nonidet P-40, 63 mM EDTA, and 50 mM Tris-HCl, pH 8) for 20 min at room temperature (10). Protease inhibitor mixture set III from Calbiochem was added to the lysis buffer as directed by the manufacturer. Extracts were centrifuged for 7 min at 14,000 rpm in a centrifuge at 4 °C to remove insoluble material. Supernatants were incubated overnight at 4 °C on a rotating wheel with either mouse monoclonal anti-Tac antibody (anti-CD25, Ancell, Bayport, MN) or goat anti-GGT antibody (40) and protein G conjugated to Sepharose beads (Zymed Laboratories Inc., San Francisco, CA).

MDCK cells expressing \(\alpha\)\(\beta\)\(\gamma\)ENaC (using either double tagged \(\alpha\)WT, \(\alpha\)624, \(\alpha\)613, \(\alpha\)HRF, \(\alpha\)RSRYW, or \(\alpha\)SPG) were starved for Met and Cys for 30 min, pulse-labeled with \(^{35}\)S Met/Cys for 20 min, and chased in DMEM (with 10% fetal bovine serum) containing excess Met (2.5 mCi) for varying times. After extraction in lysis buffer and removal of insoluble material by centrifugation, 10% SDS was added to a final concentration of 2.5% to dissociate the channel into individual subunits. Samples were incubated at room temperature for 20 min and diluted with lysis buffer to reduce the SDS concentration to 0.73% prior to immunoprecipitation of the \(\alpha\) subunit (95-kDa
unsealed and 65-kDa cleaved forms) with mouse monoclonal anti-V5 antibodies (Serotec, Oxford, UK) and protein A conjugated to Sepharose beads (Zymed Laboratories Inc.). Immunoprecipitates were recovered by brief centrifugation and washed once with 1 ml each of 1% Triton X-100 (Roche Applied Science) in HEPES-buffered saline (Ref. 10; 150 mM NaCl and 10 mM HEPES, pH 7.4), 0.01% SDS in HEPES-buffered saline, and finally HEPES-buffered saline, before elution into SDS sample buffer. In some cases, samples were treated overnight with or without Endo H (0.04 unit, Roche Diagnostics) or peptide N-glycanase-F (500 units, New England Biolabs, Beverly, MA). Proteins were recovered by heating the immunoprecipitates for 3.5 min at 95 °C in 30 μl of SDS sample buffer containing fresh 0.14 M β-mercaptoethanol. Samples were subjected to SDS-PAGE (Criterion Tris-HCl precast 4–15% acrylamide gradient gels from Bio-Rad) and transferred to nitrocellulose (0.45-μm pore size Immobilon-NC membrane from Millipore, Bedford, MA). Radioactive protein bands were imaged and quantitated using a Molecular Imager and Quantity One software (Bio-Rad). Immunoblotting of proteins on nitrocellulose was carried out with anti-V5 antibodies from Serotec as described previously (10).

**Measurement of ENaC Exocytosis and Endocytosis in Xenopus Oocytes**—ENaC exocytosis and endocytosis rates were determined as described previously (41, 42) from oocytes injected with cRNAs for wild type β, mutant γG542C, and α with (αWT) or without (α613) its carboxyl cytoplasmic tail. None of the constructs had epitope tags. 24–36 h after injection, channels at the cell surface were blocked with 1 mM MTSET for 4 min, resulting in a covalent modification of the channel that causes an ~80% reduction of current. After removal of MTSET from the bath, Na+ current was measured by two-electrode voltage clamp at −100 mV every 30 s for 10 min to monitor current recovery. The initial rates of reappearance of amiloride-sensitive currents were determined from the linear portion of the current recovery curve (0–2 min).

ENaC endocytosis rates were determined from oocytes injected with cRNA for wild type β, mutant γG542C, and α with (αWT) or without (α613) its carboxyl cytoplasmic tail. 24–36 h after injection, amiloride-sensitive currents were determined by two-electrode voltage clamp before and after 2, 4, and 6 h of incubation with 5 μM brefeldin A. Amiloride-sensitive currents were expressed relative to the initial amiloride-sensitive current, and data for the current declines were compared.

**RESULTS**

**Identification of an ER Exit Signal in the Carboxyl Cytoplasmic Tail of αENaC**—To identify potential ER retention or exit signals present in the cytoplasmic domains of ENaC subunits, chimeras were designed to individually characterize functional sequences within the Nt or Ct cytoplasmic tails of the α, β, and γ subunits. To maintain the correct orientation of the tails with respect to the ER membrane, Nt and Ct were attached to the transmembrane and ectodomains of the type 2 transmembrane glycoprotein γ-glutamyltranspeptidase (Nt-GGT) and the type 1 transmembrane glycoprotein interleukin receptor α subunit referred to as Tac (Tac-Ct), respectively. Because both GGT and Tac ectodomains exhibit Asn-linked glycosylation (35, 36), the rate of chimera exit from the ER was measured in pulse-chase experiments by following the acquisition of Endo H resistance. Glycoproteins with Asn-linked glycans are sensitive to cleavage by Endo H prior to reaching the medial Golgi complex, and most glycans become resistant to cleavage after Asn-linked glycans are trimmed by mannosidase II in that compartment (for reviews, see Refs. 43 and 44). To measure ER exit of the proteins, Chinese hamster ovary flip-in cell lines stably expressing GGT (residues 1–568), Tac (residues 1–266), or one of the six chimeras were pulse-labeled for 15 min with [35S]Met/Cys and chased for 10–30 min. GGT, Tac, and the six chimeras were immunoprecipitated from cell extracts with anti-ectodomain antibodies, and duplicate samples were treated with or without Endo H and subjected to SDS-PAGE. Radioactive bands with altered mobility after Endo H treatment due to removal of Asn-linked glycans represented immature chimera (from markings to <), whereas bands that did not shift after Endo H treatment represented mature chimera with processed Asn-linked glycans (arrow) (see Fig. 1, A–D and F–I). The mature forms of the chimera also exhibited slower mobility than the immature forms consistent with processing of Asn-linked glycans to more complex types, and their levels increased with time of chase consistent with a precursor-product relationship. We observed a significant increase in maturation (ER exit) of Tac-αCt when compared with Tac (20 and 30 min of chase, p < 0.001) and a small but significant increase in maturation of Tac-γCt when compared with Tac (30 min, p < 0.05) (Fig. 1E). The increase in maturation of Tac-βCt when compared with Tac was not statistically significant. There was no apparent difference in the rate of maturation between GGT and any of the Nt-GGT chimeras (Fig. 1J). Therefore, there appeared to be a strong ER exit signal present in the cytoplasmic carboxyl-terminal tail of αENaC but no ER retention signal in any of the six cytoplasmic tails.

**Only a Small Fraction of the α Subunit Is Assembled into a Channel That Transits the Golgi Complex and TGN**—We previously showed that the α and γ subunits of αβγENaC complex are cleaved by the resident TGN protease furin during transit through the Golgi complex where Asn-linked glycans are terminally processed to complex type (28). Processing of subunits within an individual channel is an all-or-none event, and only the cleaved forms of the α (65-kDa) and γ (75-kDa) subunits exhibit resistance to Endo H cleavage (28). Therefore, we measured ER exit of properly assembled αβγENaC by following the appearance of the cleaved α subunit (65 kDa). MDCK cells transiently co-expressing HA-α-V5 (αWT), β-FLAG, and γ-Myc were pulse-labeled for 30 min with [35S]Met/Cys and chased for 0–6 h. After cell lysis, ENaC subunits were dissociated in SDS prior to immunoprecipitation of the α subunit with anti-V5 antibodies to obtain both the non-cleaved 95-kDa and cleaved 65-kDa forms (Fig. 2A). The data revealed a half-life for the non-cleaved α subunit of 1.5–2 h and a longer half-life of >4 h for the cleaved α subunit (Fig. 2B). The levels of non-cleaved and cleaved forms observed between 4 and 6 h of chase were consistent with the steady state levels observed by immunoblotting (see Fig. 2B, inset) (10). Thus, the majority of the non-cleaved α subunit is likely degraded in the ER, whereas ~20% is found in channels that exit the ER (see residual levels at 4–6 h
ER Exit Signal in ENaC

FIGURE 1. A chimera with the cytoplasmic carboxyl terminus of αENaC exhibits an enhanced rate of exit from the endoplasmic reticulum. Chinese hamster ovary (CHO) flip-in cells stably expressing native Tac (A); chimeras of the Tac ectodomain and transmembrane domain with the cytoplasmic carboxyl-terminal domains of α (Tac-αCt) (B), β (Tac-βCt) (C), or γ (Tac-γCt) (D) subunits of ENaC or GGT (F); or chimeras of the GGT ectodomain and transmembrane domain with the cytoplasmic amino-terminal domains of α (αNT-GGT) (G), β (βNT-GGT) (H), or γ (γNT-GGT) (I) subunits of ENaC were metabolically labeled with [35S]Met/Cys for 15 min and chased for the times indicated. Immunoprecipitates were treated (+) or not treated (−) with Endo H prior to SDS-PAGE. The mobility of the immature and Endo H-sensitive bands before (Š) and after (**) Endo H treatment is noted in each case, and background bands in F–I are marked with an asterisk (*). Mature bands are indicated by an arrow. Bands in Endo H-treated lanes were quantified, and the percentage of Endo H-resistant protein (mean and S.E. from multiple experiments) is plotted for control and Tac chimera (E; n = 2 at 10 min, n = 3 at 20 and 30 min) or control and GGT chimera (H; n = 3–7) as indicated. Statistically significant differences were found at 20 and 30 min between Tac and Tac-αCt (**, p < 0.001) and at 30 min between Tac and Tac-γCt (*, p < 0.05).

of chase). Less than half of the channels that exit the ER are subsequently processed during transit of the Golgi complex, whereas the remainder reaches the cell surface without processing within the Golgi complex, consistent with our previous studies (29).

The ER Exit Signal Observed in Tac-αCt Is Functional within αβγENaC—To determine whether a functional ER exit signal is present in the carboxyl terminus of the α subunit during expression of αβγENaC, MDCK cells were transiently transfected with wild type β-FLAG and γ-Nmyc and either wild type α (αWT, 699 residues) or mutant α truncated at residue 674 (HA-αΔ675–699-V5), 649 (HA-αΔ650–699-V5), 623 (HA-αΔ624–699-V5), or 612 (HA-αΔ613–699-V5). Transfected MDCK cells were pulse-labeled for 20 min with [35S]Met/Cys and chased for 0 or 2 h. In preliminary experiments, ENaC with either αWT or α subunit truncated at residue 674, 649, or 623 showed similar levels of expression at 0 and 2 h of chase, and similar levels of cleavage after a 2-h chase. Only ENaC with the α subunit truncated at residue 612 had reduced cleavage after a 2-h chase despite normal levels of non-cleaved α at 0 and 2 h of chase. Subsequent experiments were focused on characterization of the features in the α subunit cytoplasmic tail that resulted in different rates of maturation for ENaCs with α subunits truncated at residue 623 (HA-αΔ624–699-V5, named α624) or 612 (HA-αΔ613–699-V5, named α613).

We consistently observed a band migrating slower than the non-cleaved α624 and α613 subunits after a 2-h chase (see asterisk (*) in Fig. 3, B and C, respectively). A corresponding band was not observed for αWT (Fig. 3A). We hypothesized that the decreased mobility of the non-cleaved α subunit was due to terminal processing of Asn-linked glycans from high mannose to complex type in the absence of furin cleavage. To test this possibility, αWT, α624, and α613 immunoprecipitates were treated with either Endo H or peptide N-glycanase-F. Endo H removes only high mannose type Asn-linked glycans, whereas peptide N-glycanase-F removes all Asn-linked glycans regardless of their processing. Prior to treatment with Endo H or peptide N-glycanase-F, we observed the slower migrating band in α624 and α613 immunoprecipitates from t = 2-h chase that was absent at t = 0 (see asterisk (*) in Fig. 3, D and E). Interestingly we found an identical banding pattern for both α624 and α613 after treatment with Endo H or peptide N-gly-
ER Exit Signal in ENaC

ENaC Lacking the α Carboxyl Cytoplasmic Tail Has Both Reduced Delivery and Retrieval from the Plasma Membrane, Resulting in Normal Levels of Surface Expression—Although we observed a significant decrease in ENaC cleavage when the α subunit was truncated to 612 residues (α613), consistent with decreased ER exit, we observed previously that ENaC containing this mutant α had activity similar to that of wild type ENaC when expressed in Xenopus oocytes (41). This could result from concurrent changes in both cell surface delivery and endocytosis. To test this possibility, the rates of ENaC delivery to the cell surface and endocytosis from the cell surface were determined (12). Channel delivery to the cell surface was determined in Xenopus oocytes injected with cRNAs encoding wild type β, mutant γ542C, and either αWT or α613. The mutation γ542C is located in the mouth of the channel pore where modification by the water-soluble sulfhydryl-reactive reagent MTSET blocks ~80% of the current originating from ENaC at the cell surface (45). Recovery of amiloride-sensitive current subsequent to MTSET washout can be attributed to the delivery of new, unblocked channels to the cell surface. As shown in Fig. 4A, we observed a 40% inhibition of the initial rate of current recovery in oocytes expressing ENaC with α613 compared with those expressing ENaC with αWT (p < 0.05), consistent with the 60% decrease in ER exit determined by pulse-chase experiments (Fig. 3F).

ENaC endocytosis was assayed in Xenopus oocytes by measuring the decay of amiloride-sensitive currents after treatment of cells with brefeldin A, a drug that inhibits the formation of transport vesicles that mediate protein transport from the ER to the Golgi complex (46). Oocytes were injected with cRNAs for wild type β, mutant γ542C, and either αWT or α613. After 24–36 h, each group of oocytes was incubated with 5 μM brefeldin A, and the current was measured every 2 h (Fig. 4B). After the 6-h time point, amiloride was added to determine whether a leak had developed over the course of the experiment. We

G, αWT and α613 were co-expressed with wild type βγ and immunoprecipitated after a 15-min pulse and 1–6-h chase. Levels of αWT and α613 are plotted relative to levels at 1 h of chase (n = 4, no statistical difference in half-lives between 1 and 4 h of chase).
observed that the current resulting from expression of ENaC containing G613 decayed more slowly than current resulting from expression of wild type ENaC. There was significantly more observed that the current resulting from expression of ENaC containing G613 decayed more slowly than current resulting from expression of wild type ENaC. There was significantly more activity (p < 0.05) found at 2 and 6 h after addition of brefeldin A when ENaC lacked the cytoplasmic carboxyl terminus of α, causing the apparent half-life to shift from 2 to 3 h. Together the data indicate that ENaC lacking the carboxyl cytoplasmic tail of α had both reduced delivery to and reduced retrieval from the plasma membrane resulting in levels of surface expression that were similar to levels of WT ENaC.

An ER Exit Signal Is Present within a Short Peptide Motif of the α Carboxyl Cytoplasmic Tail—As we observed normal ER exit for ENaC with an 11-residue α carboxyl cytoplasmic tail (α624) but significantly reduced ER exit for ENaC lacking an α carboxyl cytoplasmic tail (α613), we examined whether an ER exit signal was present in the sequence 613HRFRSRYW623. Mutations were prepared in three tracts within the context of the full-length α subunit: αHRF615 to AAAA, αRSRYW620 to AAAA, and αSPG623 to AAAA. The motif αHRF615 is similar to the HLF exit signal found in the NMDA receptor (47), and a consensus site (RXRSS(S/T)) for serum and glucocorticoid kinase phosphorylation is present at αRSRYW620. As shown in Fig. 5, when ER exit of ENaCs containing αWT or α subunits mutated at αHRF615, αRSRYW620, or αSPG623 were analyzed, we found that only mutation of αRSRYW620 to AAAA signifi-
analytically inhibited ER exit when compared with WT ENaC ($p < 0.001$). Together our data indicate that an ER exit signal is present within the sequence $\alpha$RSRYW$^{620}$ adjacent to the second membrane-spanning domain of $\alpha$.

**DISCUSSION**

We tested the hypothesis that motifs within the cytoplasmic domains of ENaC regulate its traffic from the ER. By following the acquisition of Endo H resistance for chimeras prepared with one of the six cytoplasmic domains of the $\alpha$, $\beta$, and $\gamma$ subunits of ENaC, we found a notable and statistically significant increase of ER exit for Tac-$\alpha$Ct. Consistent with these data, ER exit of ENaC was significantly inhibited by either loss of the entire carboxyl cytoplasmic tail of the $\alpha$ subunit or mutation of a short peptide sequence near the $\alpha$ subunit transmembrane domain. Thus we identified a signal within the tract $\alpha$RSRYW$^{620}$ that regulates the transport of ENaC within the early secretory pathway.

The trafficking of multimeric cargo proteins within the secretory pathway is regulated by the folding dynamics of the nascent subunits, their assembly, and their interaction with the protein trafficking machinery. Folding and assembly are coupled to transport through regulated presentation of peptide motifs or codes that yield low affinity binding sites to the transport machinery involved in anterograde traffic or retention and retrieval of proteins in the early secretory pathway. Folding and exit of glycoproteins from the ER is tightly coupled to quality control to ensure that only properly folded proteins are released (for review, see Refs. 43, 44, and 48–50). In general glycoproteins undergo multiple cycles of interaction with chaperones such as calnexin and calreticulin that are governed by reversible glucosylation of N-glycans and direct protein folding. Chaperones such as BiP bind and protect exposed hydrophobic patches within the folding proteins to direct folding. These interactions are governed by cycles of ATP binding and hydrolysis. Unfolded proteins are removed from the folding cycle and are targeted for proteasomal degradation in a process referred to as ER-associated degradation. In recent studies we established that ENaC is a *bona fide* ER-associated degradation substrate (12).

Our present study defined a short peptide sequence near the $\alpha$ subunit transmembrane domain that regulates the trafficking of ENaC from the ER. Importantly truncation of the $\alpha$ subunit carboxyl terminus did not lead to reduced stability or enhanced ENaC degradation (Fig. 3G). Therefore both trafficking and functional effects that we characterized using truncated forms of the channel are unlikely to be a result of compromised folding in the ER. We therefore focused our attention on identifying possible signals that regulate the mobilization of ENaC within the secretory pathway. Signals for retrieval of ER-resident proteins are best characterized for the carboxyl terminus of soluble (-KDEL) and type 1 transmembrane proteins (-K(X)KXX) although type 2 transmembrane proteins likely exhibit basic retention motifs as well (25–27). These retrieval motifs are recognized by COP1 coat proteins in the ERGIC and Golgi complex for retrograde delivery to the ER. There are now many examples of ER retention (or retrieval) signals within individual subunits of multimeric (and multispansing) transmembrane proteins that subsequently acquire steady state expression outside of the ER. In general, these signals are cytoplasmic arginine-based motifs and conform to the sequence $\Phi$/$\Psi$/R$/\Psi$/R which $\Phi$/$\Psi$ is an aromatic or bulky hydrophobic residue, and they are buried or masked when subunits assemble to form multimeric complexes (26). The mechanisms for release of assembled complexes are quite variable and can also involve masking of motifs upon protein assembly as observed for Kir6.1/6.2 and SUR1 subunits of the $K_{\text{ATP}}$ channel or the $\gamma$-aminobutyric acid, type B, R1 receptor (51). There are several examples where masking of retention signals is linked to the presentation of an exit (or release) signal. For example, in the case of the NMDA receptor, an Arg-based retention motif is found in the NR1 subunit, whereas a cytoplasmic exit motif with the sequence HLF in the NR2 subunit is exposed in the tetrameric receptor and is required for receptor exit from the ER (47). Importantly both ER exit and retention motifs have been identified within cytoplasmic domains of the NMDA receptor subunits using a reporter protein fused to the cytoplasmic domains from various NMDA receptor subtypes (47, 52). Using this same approach, we observed no retention of any of the six chimeras containing the cytoplasmic domains of the $\alpha$, $\beta$, and $\gamma$ subunits of ENaC when compared with controls even though the sequences FRSR$^{618}$ in the carboxyl terminus of $\alpha$ and RRRR$^{561}$ in the carboxyl terminus of $\beta$ fit the consensus motif for an Arg-based ER retention signal, $\Phi$/$\Psi$/R$/\Psi$/R$/\Psi$/R$/\Psi$/R$/\Psi$/R. However, we did find significantly increased ER exit of Tac-$\alpha$Ct when compared with Tac alone, which led us to analyze the cytoplasmic carboxyl tail of the $\alpha$ subunit within the context of $\alpha$R$\beta$ENaC.

We reported previously that during ENaC maturation the $\alpha$ and $\gamma$ subunits were cleaved by furin during transit of the TGN and that only the cleaved subunits exhibited Endo H resistance (10, 29, 53). Therefore, we followed cleavage of the $\alpha$ subunit as a measure of $\alpha$$\beta$$\gamma$ENaC exit from the ER. In these pulse-chase studies we found that the cleaved form of ENaC was quite stable ($t_{1/2} > 4$ h) when compared with the immature (non-cleaved) form of the $\alpha$ subunit ($t_{1/2} \sim 1.5–2$ h). A similar short half-life of 1–2 h for the non-cleaved $\alpha$ subunit was reported by other groups using both immunoblotting and pulse-chase protocols (15, 54–57), whereas the greater stability of the cleaved form was more consistent with the reported stability of amiloride-sensitive sodium currents after cycloheximide treatment of MDCK type I cells expressing murine ENaC (57). We reported previously that there are two distinct pools of ENaC at the cell surface: one pool of channels where all subunits have N-glycans processed to complex type and with $\alpha$ and $\gamma$ cleaved and another pool where all subunits are uncleaved (29). We also reported that only channels with fully cleaved $\alpha$ and $\gamma$ subunits are normally active (19). The uncleaved pool of channels is likely delivered to the cell surface by an alternative pathway that bypasses the Golgi complex, and these channels represent a readily available pool for activation by proteolytic cleavage at the cell surface (28, 32, 58). Interestingly we now observed that proteolytic processing of the $\alpha$ subunit of ENaC was significantly reduced only when the entire cytoplasmic carboxyl tail domain was absent in mutant $\alpha$613, consistent with reduced exit from the ER and transit of the TGN (Fig. 3). We observed
normal proteolytic processing of α when the tail was truncated to 11 amino acids as compared with the full-length 87 amino acids. Therefore, we focused on identification of an ER exit motif within the 11 residues HRFRRSRYWSPG immediately adjacent to the transmembrane domain.

To identify an ER exit signal within αHRRSRYWSPG, mutations were made in three different tracts: αHRR to AAA, αRSRYW620 to AAAA, and αSPG623 to AAA. The tract HRF615 at the boundary of the α subunit transmembrane domain is similar to the HLF release signal found in the NR2 sensus sequence PPP/H9252/H9251 ER Exit Signal in ENaC.

Interestingly we found that mutations αHRR615 to AAA and αSPG623 to AAA within the context of the full-length cytoplasmic tail had no effect on α subunit cleavage, whereas mutation αRSRYW620 to AAAAA reduced α subunit cleavage by 40%. In separate experiments, we found that ENaC with the α subunit lacking its carboxyl tail (α613) had a 40% reduction in the rate of delivery to the cell surface in Xenopus oocytes (Fig. 4A), consistent with the 60% reduction we observed in the rate of ER exit (Fig. 3). A variety of ER exit motifs have been characterized to date. These signals include diacidic motifs (D/E)x(D/E)), dibasic motifs ((R/K)x(R/K)), short hydrophobic motifs (FF, FY, LL, or YY), a combination of motifs (e.g. FCYENE), or multiple cooperating signals (as for ERGIC-53) in the cytoplasmic domains of proteins. These signals are recognized by the COPII prebudding complex that includes members of the Sec24p family of proteins, Sec23, and Sar1p (25–27). Direct binding of exit signals and critical reading of the manuscript and Paul A. Poland for technical assistance.

ER Exit Signal in ENaC

Together the data suggest that the absence of the α subunit cytoplasmic tail in α613 does not directly affect the stability or cleavage of ENaC by furin in our assay system but rather that absence of the α tail reduces ENaC furin cleavage by removing an important ER exit signal for the channel within the sequence αRSRYW620 and reducing transit of the trans-Golgi network during delivery to the cell surface.

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Note Added in Proof—Jasti et al. (72) recently published the crystal structure of ASIC1 showing that it is a homotrimer. As ENaC and ASIC are both members of the degenerin family of channels with high amino acid homology, ENaC may also be an αβγ heterotrimer.

REFERENCES

1. Kosari, F., Sheng, S., Li, J., Mak, D.-O. D., Foskett, J. K., and Kleyman, T. R. (1998) J. Biol. Chem. 273, 13469–13474
2. Firsov, D., Gautschi, I., Meriellat, A. M., Rossier, V. C., and Schild, L. (1998) EMBO J. 17, 344–352
3. Eaton, D. C., Chen, J., Ramosevac, S., Matalon, S., and Jain, L. (2004) Proc. Am. Thorac. Soc. 1, 10–16
4. Canessa, C. M., Meriellat, A. M., and Rossier, B. C. (1994) Am. J. Physiol. 267, C1682–C1690
5. Snyder, P. M., McDonald, F. J., Stokes, J. B., and Welsh, M. J. (1994) J. Biol. Chem. 269, 24379–24383
6. Firsov, D., Robert-Nicoud, M., Gruender, S., Schild, L., and Rossier, B. C. (1999) J. Biol. Chem. 274, 2743–2749
7. Sheng, S., Maarouf, A. B., Bruns, J. B., Hughy, R. P., and Kleyman, T. R. (2007) J. Biol. Chem. 282, 20180–20190
8. Renard, S., Linguiglesia, E., Voilley, N., Lazdunski, M., and Barbry, P. (1994) J. Biol. Chem. 269, 12981–12986
9. Adams, C. M., Snyder, P. M., and Welsh, M. J. (1997) J. Biol. Chem. 272, 27295–27300
10. Hughy, R. P., Mueller, G. M., Bruns, J. B., Kinlough, C. L., Poland, P. A., Harkleroad, K. L., Carattino, M. D., and Kleyman, T. R. (2003) J. Biol. Chem. 278, 37073–37082
11. Goldfarb, S. B., Kashlan, O. B., Watkins, J. N., Suadl, L., Yan, W., Kleyman, T. R., and Rubenstein, R. C. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 5817–5822
12. Kashlan, O. B., Mueller, G. M., Qamar, M. Z., Poland, P. A., Ahner, A., Rubenstein, R. C., Hughy, R. P., Brodsky, J. J., and Kleyman, T. R. (2007) J. Biol. Chem. 282, 28149–28156
13. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) J. Cell Biol. 116, 1071–1080
14. Shimkets, R. A., Lifton, R. P., and Canessa, C. M. (1997) J. Biol. Chem. 272, 25537–25541
15. Alvarez de la Rosa, D., Li, H., and Canessa, C. M. (2002) J. Gen. Physiol. 119, 427–442
16. Prince, L. S., and Welsh, M. J. (1998) Biochem. J. 336, 705–710
17. Kemedy, A. E., Kleyman, T. R., and Eaton, D. C. (1992) Ann. J. Physiol. 263, C825–C837
18. Palmer, L. G., and Frindt, G. (1996) J. Gen. Physiol. 107, 35–45
19. Sheng, S., Carattino, M. D., Bruns, J. B., Hughy, R. P., and Kleyman, T. R. (2006) Am. J. Physiol. C285–C287
20. Carattino, M. D., Sheng, S., Bruns, J. B., Pilewski, J. M., Hughy, R. P., and Kleyman, T. R. (2006) J. Biol. Chem. 281, 18901–18907
21. Bruns, J. B., Carattino, M. D., Sheng, S., Maarouf, A. B., Weisz, O. A., Pilewski, J. M., Hughy, R. P., and Kleyman, T. R. (2007) J. Biol. Chem. 282, 6153–6160
22. Jean, F., Thomas, L., Molloy, S. S., Liu, G., Jarvis, M. A., Nelson, J. A., and Thomas, G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2864–2869
23. Adachi, M., Kitamura, K., Miyoshi, T., Narikoshi, T., Iwashita, K., Shiraishi,
