Maturation of the Epithelial Na⁺ Channel Involves Proteolytic Processing of the α- and γ-Subunits*

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The epithelial Na⁺ channel (ENaC) is a tetramer of two α, one β, and one γ-subunit, but little is known about its assembly and processing. Because co-expression of mouse ENaC subunits with three different carboxyl-terminal epitope tags produced an amiloride-sensitive sodium current in oocytes, these tagged subunits were expressed in both Chinese hamster ovary or Madin-Darby canine kidney type 1 epithelial cells for further study. When expressed alone, α (95 kDa), β (96 kDa), and γ-subunits (93 kDa) each produced a single band on SDS gels by immunoblotting. However, co-expression of αβγENaC subunits revealed a second band for each subunit (65 kDa for α, 110 kDa for β, and 75 kDa for γ) that exhibited N-glycans that had been processed to complex type based on sensitivity to treatment with neuraminidase, resistance to cleavage by endoglycosidase H, and GalNAc-independent labeling with [3H]Gal in glycosylation-defective Chinese hamster ovary cells (ltdD). The smaller size of the processed α- and γ-subunits is also consistent with proteolytic cleavage. Using α- and γ-subunits with epitope tags at both the amino and carboxyl termini, proteolytic processing of the α- and γ-subunits was confirmed by isolation of an additional epitope-tagged fragment from the amino terminus (30 kDa for α and 18 kDa for γ) consistent with cleavage within the extracellular loop. The fragments remain stably associated with the channel as shown by immunoblotting of co-immunoprecipitates, suggesting that proteolytic cleavage represents maturation rather than degradation of the channel.

The amiloride-sensitive epithelial Na⁺ channel (ENaC) is composed of three structurally related subunits, termed α-, β-, and γ-ENaC. The three subunits exhibit limited amino acid sequence identity (30–40%) but are structurally similar with two membrane-spanning domains and cytosolic amino and carboxyl termini. We and others have shown that ENaC expressed in Xenopus oocytes has a subunit stoichiometry of two α-, one β-, and one γ-subunit (1, 2), although an alternative subunit ratio has been proposed (3).

ENaC subunits assemble in the endoplasmic reticulum (ER) where they also undergo N-linked glycosylation (4–7). There are 6, 12, and 5 consensus sites (Asn-X-Ser/Thr) for N-linked oligosaccharide (N-glycan) addition present in the extracellular domains of the α-, β- and γ-subunits of mouse ENaC (mENaC), respectively. Based on apparent molecular weights on SDS-PAGE, the majority of these sites are modified during synthesis with an N-glycan core of ~3 kDa (4, 5, 7–14). N-Glycans on newly synthesized proteins are necessary for interactions with ER folding chaperones like calnexin or calreticulin, and for recognition by quality control mannosidase-like proteins prior to exit from the ER (for review see Refs. 15 and 16). N-Glycans on mature proteins generally stabilize the folded state and protect the protein core from protease digestion. Most N-glycans on glycoproteins that transit the Golgi complex during delivery to the cell surface are modified from a high mannos type to a complex type by a series of enzymatic steps. One distinguishing feature for these two types of N-glycans is whether they can be removed from the isolated protein by in vitro treatment with endoglycosidase H (Endo H). In general, N-glycans on proteins prior to their delivery to the medial Golgi complex are substrates for Endo H and N-glycans on proteins after passing this point are not substrates. However, not all N-glycans are modified during transit through the Golgi complex, and these N-glycans remain sensitive to treatment with Endo H. ENaC is apparently delivered to the cell surface via the Golgi complex because surface delivery is blocked by treatment of cells with the fungal metabolite brefeldin A that disrupts cytosolic coat assembly needed for vesicle budding throughout the Golgi complex (9, 17, 18). However, direct evidence for processing of N-glycans on ENaC to complex type is limited. Hanwell et al. (13) could not detect Endo H-resistant rat ENaC (rENaC) channels when expressed in Madin-Darby canine kidney (MDCK) cells. Prince and Welsh (12) found that human ENaC (hENaC) α- and β-subunits in a detergent-insoluble cell fraction were resistant to both N-glycanase and Endo H treatment, and they concluded that N-glycans are removed from hENaC when expressed at the surface of COS-7 cells. Valentijn et al. (19) found that all three rENaC subunits at the surface of oocytes exhibited more diffuse and significantly slower mobility on SDS-PAGE compared with the total cellular pool, consistent with acquisition of complex type N-glycans, but Endo H sensitivity of these forms was not addressed. More recently, Alvarez de la Rosa et al. (10) found Endo H-sensitive and -resistant forms of both the α- and β-subunits of xENaC in

*This work was supported in part by National Institutes of Health Grants DK53191, DK54354 (to T. R. K.), and DK54787 (to R. P. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: ENaC, epithelial Na⁺ channel; CAP, channel activating protease; CHO, Chinese hamster ovary cells; Endo H, endoglycosidase H; ER, endoplasmic reticulum; hENaC, human ENaC; N-glycanase/PNGase F, peptide N-glycanase F; MDCK, Madin-Darby canine kidney cells; mENaC, mouse ENaC; N-glycans, Asn (N-linked oligosaccharides; rENaC, rat ENaC; xENaC, Xenopus ENaC; HA, hemagglutinin.
A6 cells during pulse-chase studies, but the data were compi-
lcated by the fact that the Endo H-resistant form of the mature
α-subunit was also resistant to reducing agents.

Interestingly, the reported Endo H-resistant forms of the
α-subunit exhibit a significantly faster mobility on SDS-PAGE
(i.e. lower Mr) consistent with proteolysis (10, 12). Planas et al.
(20) report only the smaller of two forms of the α-subunit on the
surface of a mouse cell line. Masumun et al. (21) report an
aldosterone-induced shift from a larger to a smaller form of the
γ-subunit in kidneys from rats on a sodium-restricted diet.
However, there is no proof that these forms actually represent
proteolytic processing of the α- and γ-subunits. We now report
multiple lines of evidence in this present study for processing of
N-glycans on all three subunits of assembled αβγENaC to
complex-type Endo H-resistant forms. Interestingly, we find
Endo H-resistant N-glycans only on smaller forms of the α- and
γ-subunits, and we provide direct evidence that maturation of
ENaC involves proteolytic processing.

**EXPERIMENTAL PROCEDURES**

**Vector Preparation, Antibodies, Cell Culture, and Transfections—**
Carboxyl-terminal epitope-tagged mENaC subunits cDNAs (22) were
previously prepared recombinant yielding either
α(FLAG), β(FLAG), γ(FLAG), α(HA), or α(My-His6) in pcDNA3.1-
hygromycin, α(V5-His6), β(V5-His6), or γ(V5-His6) in pcDNA6-βlastici-
din and either α(My-His6), γ(My-His6), or γ(muc) in pcDNA3-neo
(G418). Double tagged α- and γ-ENaC with amino-terminal HA and
carboxyl-terminal V5 epitope tags (HA-α-V5 and HA-γ-V5) were
cloned into pcDNA3.1-hygromycin. All vectors were purchased from Invitro-
gen. The new "muc" epitope tag (SAPDTRPAPGS) was developed from
the immunodominant tandem repeat of human MUC1 (23). Immuno-
precipitation and immunoblot analysis of ENaC epitope-
tagged subunits expressed in CHO cells were carried out with mouse
anti-V5 antibody from Invitrogen, mouse anti-Myc antibody (9E10)
from Santa Cruz Biotechnologies (Santa Cruz, CA), mouse anti-HA
antibody (HA.11) from Covance (Princeton, NJ), mouse anti-FLAG an-
tibody (M2) from Sigma, or mouse anti-MUC1 antibody (BC2) from
Chemicon International (Temecula, CA). Immunoprecipitation of ENaC
epitope-tagged subunits expressed in MDCK cells was carried out with
the same antibodies, except that goat anti-V5 antibody conjugated to
agarose (NB600-087A) from Novus Biologicals (Littletown, CO) was
used for the experiment described in Fig. 7. Immunoblotting of ENaC sub-
units from MDCK cells was carried out with mouse anti-Myc antibody
(9E10) from Santa Cruz Biotechnologies, mouse anti-V5 antibody from
Invitrogen, and rat anti-HA (SF10) from Roche Applied Science. Affi-
ity-purified peroxidase-conjugated goat anti-mouse and goat anti-rat
IgG(H+L) second antibodies for all immunoblots were purchased from
Kirkegaard & Perry Laboratories (Gaithersburg, MD).

Wild type CHO and glycosylation-defective CHO (ldlD) cell lines
were cultured as described previously (24). Transient expression of
mENaC subunit cDNAs was carried out in CHO and idlD cells by
infection with recombinant vaccinia virus (vT7CP) expressing T7 RNA
denaturase, followed by transfection with expression vectors containing
T7 promoters at the 5' end of the cDNAs as described previously (25,
26). The vT7CP was a gift from Bernard Moss (NIAID, National Insti-
tutes of Health, Bethesda, MD). Transfections were carried out with
PolyFect Transfection Reagent as described by the manufacturer (Qi-
gen, Inc., Valencia, CA), and cells were used the following day. MDCK
type 1 cells were a gift from Barry M. Gumbiner (Memorial Sloan
Kettering Cancer Center, New York). MDCK cells were transfected
with mENaC cDNAs using LipofectAMINE 2000 as described by the
manufacturer (Invitrogen). Cells growing on plastic and transiently
expressing ENaC subunits were used after 2 days, whereas clonal cell
lines were selected in Dulbecco's minimum Eagle's medium and Ham's
F-12 (1:1) in a 5% CO2 incubator at 37°C with 10% fetal bovine serum
supplemented with the appropriate antibiotics (0.055 mg/ml hygromycin
from Calbiochem, 5 μg/ml blasticidin from Invitrogen, or 0.3 mg/ml
G418 from Invitrogen). Control and stably transfected MDCK cells were
plated on 24-mm diameter 0.4-μm pore size transwell permeable sup-
ports (Costar, Corning, NY) for 5 days prior to use where indicated.
The appropriate MDCK cells were transiently transfected with sulfo-NHS-SS-biotin
(Pierce) prior to extraction and recovery of biotinylated proteins with

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**Sodium Channel Expression in Xenopus Oocytes—** Wild type and
epitope-tagged ENaC subunit cRNAs were prepared using the mMES-
SAGE mACHINE kit (Ambion, Inc., Austin, TX). The mENaC sub-
unit cRNAs (1 ng per subunit) were injected into oocytes, and electro-
physiological measurements were performed after 24 h as described
previously (27). Physiological measurements were performed after
24 h on a rotating wheel with the appropriate mouse monoclonal anti-
epitope antibody and Protein G immobilized on Sepharose 4B beads
defaulting on Sepharose 4B beads from Sigma. Goat anti-V5 conjugated to agarose was used for immuno-
precipitations described in Fig. 7. Where indicated, subunits were dis-
associated with SDS from Bio-Rad, prior to immunoprecipitation, by
scraping into buffer (100 mM NaCl, 50 mM triethanolamine, pH 8.6,
5 mM EDTA) prior to addition of SDS to 0.5% and heating as described
previously (28). Immunoprecipitates were pelleted by centrifugation
in a microcentrifuge, and beads were washed once with 1% Triton X-100
in HEPES-buffered saline (HBS, 150 mM NaCl, 10 mM HEPES, pH 7.4),
one with 0.01% SDS in HBS, and once with HBS. Beads were heated
in sample buffer containing fresh β-mercaptoethanol (0.14 M β-mercapto-
ethanol) for 3.5 min at 90°C prior to recovery of the supernatant
for SDS-PAGE as described previously (24). Molecular weights (Mr) of
proteins were estimated using Precision Protein Standards from Bio-
Rad. Proteins on the gels were electrophoretically transferred to
0.45-μm pore size Immobilon-NC membrane as described by the
manufacturer (Millipore, Bedford, MA) and blocked with 5% non-fat dry
milk (Food Club, Topco Association, Inc., Skokie, IL) in blotting buffer
(100 mM NaCl, 20 mM NaH2PO4, 80 mM Na2HPO4, pH 7.5). Membranes
were incubated in blotting buffer with 1% non-fat dry milk and anti-
epitope monoclonal antibody either for 2.5 h at room temperature
or overnight at 4°C, followed by peroxidase-conjugated second antibody
for 1 h at room temperature. After incubation with antibodies, mem-
branes were washed with multiple changes of blotting buffer. Peroxi-
dase on the blot was detected and visualized using Western Lighting
Chemiluminescence Reagent from PerkinElmer Life Sciences as di-
rected by the manufacturer with Kodak BioMax MR film from Eastman
Kodak Co.

**Enzymatic Treatment of Immunoprecipitated ENaC—** ENaC was
immuno-purified from either stable or transiently transfected cells as
described above. Immunoprecipitates of ENaC were treated with either
endo-β-N-acetylglucosaminidase or endo-β-N-glycanase/PNGase F from
Calbiochem, or N-glycans/PNGase F from New England Biolabs
(Beverly, MA) as directed by the supplier.

**Metabolic Labeling and Immunoblot Analysis of idlD Cells—**
Tran-
siently transfected idlD cells expressing ENaC subunits in 25-mm
dishes were incubated overnight with 25 μCi of [3H]galactose
([3H]Gal, 60 Ci/mmol from ICN, Irvine, CA) before extraction with lysis
buffer (or SDS-buffer (28)) and immunoprecipitation of subunits as
described for SDS-PAGE. Proteins were electrophoretically transferred
to nitrocellulose as already described for immunoblotting, and
[3H]ENaC subunits were visualized on the dried nitrocellulose using a
Bio-Rad PhosphorImager TR screen. Where indicated, the nitrocel-
 lulose was subsequently subjected to immunoblot analysis. Unlabeled
GalNac (100 mM) was added to the culture media with the [3H]Gal
where indicated. In some cases, transiently transfected idlD cells
carrying ENaC subunits were incubated overnight with unlabeled Gal
(10 mM), GalNac (100 mM), both sugars, or no sugars prior to solubi-
 lization and immunoblot analysis of mENaC immunoprecipitates as de-
scribed above.

**RESULTS**

Peptide sequences representing epitope tags were added to the
carboxyl terminus of the three subunits of mouse ENaC using standard recombinant DNA techniques. As described
under "Experimental Procedures," a new epitope tag was
designed from the immunodominant sequence of the human
MUC1 tandem repeat and named muc.5 To determine whether
core-expression of epitope-tagged ENaC subunits resulted in as-

ImmunoPure Immobilized Streptavidin (Pierce) as described previously (25).
mENaC Processing

Fig. 1. Co-immunoprecipitation of αβγENaC subunits using anti-epitope antibodies. Individual epitope-tagged mouse ENaC subunits or all three epitope-tagged subunits were transiently expressed in CHO cells and immunoprecipitated (IP) with subunit-specific anti-epitope monoclonal antibodies (Ab) as indicated below the gel. Immunoblotting (IB) was carried out with antibodies as indicated. The major band for each subunit is shown with α in each case, whereas a second faster migrating α and γ band and a second slower migrating β band appear only when all three subunits are expressed (see < in each case). A background band due to the anti-Myc antibody (*) is found in all lanes of the immunoblot for γ. Estimated molecular weights of the major and minor bands are indicated at the right of each panel based on SDS gel mobility of Bio-Rad Precision Protein standards. Similar co-immunoprecipitation data were obtained for several combinations of epitope-tagged subunits. Results for analysis of αγ (Myc), β(V5), and γ(Myc) expression is shown for the anti-α-FLAG) and anti-β(V5) immunoblot,

co-assembly of functional amiloride-sensitive sodium channels, various combinations of α-, β-, and γ-subunit cRNAs were injected into oocytes: α(HA) β(V5) γ(FLAG), α(Myc) β(V5) γ(muc), α(FLAG) β(FLAG) γ(V5), and α(HA) β(V5) γ(Myc). Amiloride-sensitive sodium currents measured in oocytes by two-electrode voltage clamp the following day indicated that all combinations of epitope-tagged subunits produce functional sodium channels with amiloride-sensitive whole cell currents in the range of 7–27 μA/oocyte, similar to whole cell currents we have observed with wild type ENaC (27, 29). Because these data indicate that the epitope-tagged αβγENaC subunits produced functional amiloride-sensitive sodium channels, they were used to study ENaC assembly and processing.

The α-, β-, and γ-ENaC cDNAs were transiently expressed in CHO cells to determine whether the specific antibodies against the epitope tags could be used to immunoprecipitate individual subunits or co-immunoprecipitate subunits from assembled channels. The day following transfection, cells were extracted with lysis buffer, and ENaC subunits were immunoprecipitated with anti-epitope antibodies. Immunoprecipitates were subjected to immunoblot analysis as shown in Fig. 1. When expressed as individual subunits, no cross-reactivity between antibodies was observed. When all three subunits were co-expressed, subunit co-immunoprecipitation was observed indicating that channel assembly had occurred. For example, a band migrating at 95 kDa was observed when γ was expressed alone and immunoprecipitated with anti-γ antibodies. No 95-kDa band was found on the anti-α-immunoblot in immunoprecipitates using anti-β or anti-γ antibodies. However, the 95-kDa band was observed on the anti-α-immunoblot when αβγENaC was expressed in CHO cells, and cell extracts were immunoprecipitated with either anti-α, anti-β, or anti-γ antibodies. A second faster migrating band (65 kDa) was found in the α-immunoprecipitate when all three subunits were co-expressed but not when αENaC was expressed alone (described in more detail below). The 65-kDa form of the α-subunit co-immunoprecipitated with the β-subunit but not with γ-subunit. In a similar manner, a 93-kDa band was immunoprecipitated by anti-γ antibodies but not anti-α or anti-β antibodies when γ was expressed alone. The minor sharp band at 75-kDa in the γ-immunoprecipitate represents the non-glycosylated γ-subunit because it was resistant to treatment with either Endo H or N-glycanase that releases N-glycans from glycoproteins (data not shown). A second more diffuse band at 75 kDa was found when all three subunits were co-expressed, and both the 93-kDa and diffuse 75-kDa bands co-immunoprecipitated with the α- and β-subunits. Broad diffuse bands for glycoproteins on SDS-PAGE usually result from microheterogeneity in N-glycan processing.

A unique pattern of expression was observed for the β-subunit (Fig. 1). When expressed alone, the β-subunit produced a single 96-kDa band, but when expressed as αβγENaC, a second slower migrating 110-kDa band was observed for β. The 96- and 110-kDa bands were also co-immunoprecipitated with the α-subunit. Interestingly, the 110-kDa form of the β-subunit was not immunoprecipitated with the γ-subunit. The 110-kDa band was consistently observed on immunoblots only when all three subunits of ENaC were co-expressed, so it seemed likely that it represented processing of β-subunit glycans on the assembled channel during its transit to the cell surface. Two types of glycoprotein processing that occur within the Golgi complex that slow protein mobility on SDS-PAGE are modification of N-glycans from high mannose to complex type and addition of O-glycans. To determine whether glycan processing was responsible for the slower migrating β-subunit band, αβγENaC was expressed in a glycosylation-defective mutant of CHO cells (IdlD) that lacks the epimerase to convert UDP-GlcNAc to UDP-Gal/ManNAc to UDP-Gal/GalNAc (30). This mutation effectively prevents synthesis of all mucin-type O-glycans with a GalNAc-Ser/Thr core as well as the formation of complex-type N-glycans that involves addition of Gal to terminal non-reducing GlcNAc residues. However, supplementing the media with Gal and/or GalNAc will reverse, or partially reverse, the phenotype of the epimerase deficiency in IdlD cells (30). As shown in Fig. 2 (left panel), only the 96-kDa β-subunit band was found in extracts of IdlD cells by immunoprecipitation and immunoblotting when the β-subunit was expressed alone or as αβγENaC. Addition of Gal to the culture media, with or without GalNAc, resulted in expression of a doublet for the β-subunit with the appearance of the 110-kDa β-subunit band. The 110-kDa band was not observed when the media were supplemented with GalNAc alone. Because the presence of the 110-kDa band was not dependent on the presence of GalNAc in the media, this indicates that its appearance results from processing of N-glycans on the β-subunit of αβγENaC to complex type and not from addition of O-glycans. This was confirmed by finding that addition of [3H]Gal to the media of IdlD cells expressing αβγENaC, but not the β-subunit alone, produced a [3H]-labeled 110-kDa band when immunoprecipitates were analyzed after SDS-PAGE using a PhosphorImager (Fig. 2, right panel). Labeling of the β-subunit in αβγENaC with [3H]Gal was not GalNAc-dependent indicating that [3H]Gal was being added to
FIG. 2. Processing of glycans on the β-subunit of αβγENaC is galactose-dependent. Either control CHO cells (C) or glycosylation-defective CHO mutant cells (D, Id1D cells lacking the UDP-Gal/GalNAc epimerase) transiently expressing all three ENaC subunits (α(Myc), β(V5), and γ(muc)) or the β(V5) subunit alone were incubated overnight with either (left panel) no sugars (−), Gal (G), GalNAc (N), both Gal and GalNAc (G−N), or (right panel) [3H]Gal with (N) or without (−) GalNAc, as indicated, prior to immunoprecipitation (IP) with anti-β(V5) monoclonal antibody. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and then cut for either (left) immunoblotting (IB) or (right) analysis of [3H]-labeled bands with a PhosphorImager. The film from the immunoblot and the scan of the radioactive gel were re-aligned as shown using the Bio-Rad Precision Protein standards (indicated at the right of the gel in kDa) because the 100-kDa band migrates between the faster (96 kDa) and slower (110 kDa) migrating β bands. Note that three [3H]-labeled bands are found only when all three subunits are expressed, one migrating slower than the 100-kDa standard, and two bands migrating near the 75-kDa standard.

N-glycans and not to O-glycan GalNAc-Ser/Thr cores. Two additional [3H]-labeled bands were also noted in the β-subunit immunoprecipitates migrating near the 75-kDa protein standard on SDS-PAGE. These [3H]-labeled bands were also observed only when all three subunits were co-expressed. Because bands of similar size (65 and 75 kDa) were observed for both the α- and γ-subunits, respectively, by immunoblotting upon αβγENaC expression (see Fig. 1), additional experiments were carried out to determine whether these [3H]-labeled bands were processed forms of the α- and γ-subunits.

As shown in Fig. 3A, immunoprecipitation of either the α-, β-, or γ-subunit of ENaC from Id1D cells transiently expressing αβγENaC and labeled overnight with [3H]Gal produced the same three [3H]-labeled bands. [3H] Labeling of the three bands was not dependent on the presence of GalNAc, consistent with N-glycan processing of these glycoproteins. Because it is clear that all three ENaC subunits will co-immunoprecipitate after extraction of cells in lysis buffer (see Fig. 1), immunoprecipitation was carried out after dissociation of ENaC subunits with SDS. As shown in Fig. 3B, immunoprecipitation of individual ENaC subunits after cell extraction in SDS produced a single [3H]-labeled band (65 kDa for the α-subunit, 110 kDa for the β-subunit, and 75 kDa for the γ-subunit) corresponding to the three [3H]-labeled proteins that co-immunoprecipitated when cells were extracted in standard lysis buffer under non-dissociating conditions (see Fig. 3A).

Additional experiments were carried out to confirm that the [3H]-labeled bands observed for the α-, β-, and γ-ENaC subunits represented forms with processed N-glycans. Either immunoprecipitates from CHO cells transiently expressing αβγENaC (Fig. 4) or streptavidin precipitates from surface-biotinylated MDCK cells stably expressing αβγENaC (Fig. 5) were incubated with either neuraminidase, endoglycosidase H (Endo H), or PNGase F (N-glycanase) to characterize the nature of the N-glycans on each band. Neuraminidase cleaves sialic acid from either O-glycans or complex type N-glycans. Endo H cleaves only high mannose type N-glycans, whereas N-glycanase cleaves both high mannose and complex N-glycans from glycoproteins. As shown in Fig. 4, neuraminidase treatment of the α-subunit immunoprecipitate from CHO cells altered the mobility of only the 65-kDa band (see the slight difference between the bands on each side of the asterisk). The 95-kDa α-subunit band was equally sensitive to treatment with Endo H and N-glycanase yielding a band at 85 kDa. However, the 65-kDa α-subunit was somewhat resistant to treatment with Endo H yielding a band at 60 kDa, whereas treatment with N-glycanase yielded a band at 50 kDa. These data indicate that the 65-kDa band represents a form of the α-subunit with some of its N-glycans processed to the complex type, whereas all the N-glycans on the 95-kDa band are apparently high mannose type and unprocessed.

Treatment of the β-subunit immunoprecipitates with neuraminidase altered the 110-kDa band but not the 96-kDa band (Fig. 4, see asterisk). The 110-kDa band was also partially resistant to cleavage with Endo H yielding a band at 100 kDa, whereas N-glycanase treatment produced a band at 71 kDa indicating that most of the N-glycans were complex type. However, the 96-kDa band had only high mannose type N-glycans because it was completely sensitive to Endo H treatment producing the same 71-kDa band as does treatment with N-glycanase.

Treatment of the γ-subunit with various enzymes reveals a complex pattern (Fig. 4). Neuraminidase treatment produced a slight alteration in the mobility of the 75-kDa band of the γ-subunit (see asterisk), whereas treatment with Endo H shifted the 93-kDa band to 75 kDa. Because N-glycanase treatment also shifted the 93-kDa band to 75 kDa, this indicates that all the N-glycans on the 93-kDa subunit are high mannose type. However, N-glycanase treatment also revealed new bands at 60 and 55 kDa consistent with the presence of mostly complex type N-glycans on the 75-kDa form of the γ-subunit.
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The epitope-tagged α-, β-, and γ-subunits of ENaC expressed on the apical surface of stably transfected MDCK cells were also characterized by neuraminidase, Endo H, and N-glycanase treatment. Cells were grown on permeable supports for 5 days prior to treatment of the apical surface with sulfo-NHS-SB-streptavidin-conjugated beads, treated with enzymes as indicated, and subjected to immunoblot analysis to characterize the forms of ENaC at the cell surface. As shown in Fig. 5, both the 95- and 65-kDa forms of the α-subunit, and the 110-kDa form of the β-subunit, and only the 93-kDa form of the γ-subunit were found on the cell surface. In general, the signal obtained for the γ-subunit by immunoblotting was faint compared with the immunoblots for the α- and β-subunits. This low signal is likely because of the inefficiency of the anti-Myc antibody in detecting the γ(Myc) that was expressed in the stable cell line making it difficult to visualize minor forms of the γ-subunit such as the 75-kDa band. However, the 75-kDa form of γ was found in cell extracts of transiently transfected MDCK cells (see below in Fig. 7). Treatment of immunoprecipitates with neuraminidase, Endo H, and N-glycanase yielded results quite similar to that observed in CHO cells, where only the 65-kDa form of the α-subunit, and the 110-kDa form of the β-subunit exhibit N-glycans that were processed to more complex type as shown primarily by differential mobilities after treatment with either Endo H or N-glycanase.

The smaller size of the processed α- and γ-subunits when compared with the unprocessed α- and γ-subunits is consistent with proteolytic cleavage of the full-length subunits. Experiments were carried out to test this possibility by placing an epitope tag at both the amino (HA) and carboxyl (V5) terminus of the α- and γ-subunits (HA-α-V5 and HA-γ-V5, respectively) to identify the putative fragments. As shown in Figs. 6A and 7A, transient expression of HA-α-V5 (with or without β and γ) in either CHO or MDCK cells, respectively, produced the full-length 95-kDa α-subunit that was visible by either anti-HA or anti-V5 immunoblotting of the channel immunoprecipitated with either anti-HA or anti-V5 antibody. However, the 65-kDa band was visible only on anti-V5 immunoblots from cells expressing HA-α-V5 (with β and γ), whereas a new 30-kDa band

![Image](https://example.com/image.png)

**Fig. 4.** Complex N-glycans are present on a fraction of the α-, β-, and γ-subunits when αβγENaC is transiently expressed in CHO cells. Individual subunits from extracts of CHO cells transiently expressing epitope-tagged subunits of ENaC (α(HA), β(V5), and γ(FLAG)) for analysis of the α- and β-subunits, and α(FLAG), β(FLAG), and γ(V5) for analysis of the γ-subunit) were immunoprecipitated from cell extracts with anti-epitope antibodies and subjected to immunoblotting with the same antibodies after SDS-PAGE. Where indicated, recovered subunits were treated (+) with neuraminidase (N.ase), endoglycosidase H (Endo H), or PNGase-F (N-glyc) as described under Experimental Procedures. Untreated samples (−) were incubated under the same conditions but without enzymes. The values in kDa estimated for ENaC subunits are noted at the left of the immunoblots. The asterisk denotes the band for each subunit that shifts slightly after neuraminidase treatment. Open arrowheads (>) and closed arrowheads (≥) are included to indicate (track) the changes in mobility of the processed and unprocessed bands for each subunit, respectively, with (−) and without (+) enzyme treatment. Background bands due to antibodies (Ab) are indicated to the left of each panel. Models for each subunit are presented at the right. The number of N-glycans (N or N−) is based on consensus sites (Asn-X-Ser/Thr), and the number of processed N-glycans (<N-Gal-SA>) are estimated by the change in mobility when treated with Endo H; α and β were partially resistant, and γ may be fully resistant to Endo H treatment. The peptide cleavage sites are estimated from data presented in Figs. 6 and 7.
was visible only on anti-HA immunoblots. Neither the 65-kDa nor the 30-kDa band was present when HA-α-V5 was expressed without β and γ (see Fig. 7A). Interestingly, the V5-tagged 65-kDa band and the HA-tagged 30-kDa band co-immunoprecipitated indicating that the cleaved fragments remained stably associated within the channel (compare the side-by-side anti-HA and anti-V5 immunoprecipitates on each immunoblot in Fig. 6B). The sharp 75-kDa band observed in immunoprecipitates from cells expressing HA-γ-V5 in the absence of α and β represents the non-glycosylated γ-subunit described previously in the discussion of Fig. 1. Interestingly, a 22-kDa band was visible in anti-V5, but not anti-HA, immunoprecipitates of the γ-subunit when subjected to anti-V5 immunoblotting (see asterisk in Fig. 6B), indicating that this fragment from the carboxyl terminus does not co-immunoprecipitate with the channel. The 22-kDa fragment is also present when HA-γ-V5 is expressed with or without the α- and β-subunits, although a corresponding unique amino-terminal HA-tagged fragment was not observed. These data are most consistent with the 22-kDa fragment arising from a second cleavage of the γ-subunit within the extracellular domain just before the M2 domain near the carboxyl terminus (see model b in Fig. 6B). This second cleavage would leave the extracellular domain without an epitope tag (predicted to be 53 kDa), and not visible by immunoblotting, while effectively decreasing the level of the 75-kDa band. Loss of the carboxyl-terminal epitope tag on the γ-subunit would also explain why the processed forms of α- and β-subunits are not co-immunoprecipitated by the anti-γ epitope antibody in Fig. 1.

**DISCUSSION**

**N-Glycans Are Processed on All Three Subunits of ENaC—**

We have shown that N-linked glycans on a sub-population of all three subunits of mouse ENaC are processed to complex type when expressed in either CHO or MDCK cells and that this processing occurs only when all three subunits are co-expressed. N-Glycan processing also seems to be linked to cleavage of the α- and γ-subunits. Our evidence for N-glycan processing is based on several criteria including altered mobility of the subunits on SDS-PAGE, sensitivity to neuraminidase treatment, resistance to Endo H treatment, and incorporation of [3H]Gal into N-glycans on ENaC. Although metabolic labeling with [3H]Gal is usually quite inefficient in normal cells, ldlD cells have no internal pool of the UDP-Gal precursor because they lack the epimerase that normally produces UDP-Gal/GalNAc from UDP-Glc/GlcNAc (30). Thus, [3H]Gal added to the ldlD cell culture media is readily incorporated into glycoproteins through the formation of UDP-[3H]Gal. Under these conditions, [3H]Gal is used exclusively for terminal processing of N-linked glycans because there is no UDP-GalNAc present to initiate O-linked glycans on Ser or Thr residues. Because we observed labeling of all three subunits of ENaC in ldlD cells with [3H]Gal in the absence of GalNAc and no increased incorporation of [3H]Gal in the absence of GalNAc, this is most consistent with processing of ENaC N-glycans to complex types rather than addition of O-linked glycans. [3H]Gal incorporation was not observed when α, β, or γ were expressed individually (data not shown) (shown for β-ENaC in Fig. 2) indicating that only channels assembled with all three subunits are processed in the Golgi complex where enzymes for N-glycan processing reside.

[3H]Gal incorporation into the 110-kDa form of the β-subunit was predicted because N-glycan processing usually slows the migration of glycoproteins on SDS gels; only the 96-kDa band was found when the β-subunit was expressed alone whereas the doublet of 96- and 110-kDa was readily observed on immunoblotting only when all three subunits were expressed (Figs. 3 and 4). Alvarez de la Rosa et al. (10) also reported two forms of the β-subunit in A6 cells at 115- and 100-kDa that were sensitive to N-glycanase treatment, but only the 115-kDa form was resistant to Endo H treatment. However, we were surprised to find two additional [3H]Gal-labeled bands around 75 kDa in
our immunoprecipitates for the \( \beta \)-subunit. Because additional bands for the \( \beta \)- and \( \gamma \)-ENaC subunits were also found around 75 kDa by immunoblotting, we addressed the possibility that \( \alpha \)-glycans on all three subunits were processed. In fact, we did find that immunoprecipitation with antibodies to either \( \alpha \)-, \( \beta \)-, or \( \gamma \)-ENaC revealed the same three \( [3H] \)Gal-labeled bands, and that dissociation of the channel subunits with SDS prior to immunoprecipitation revealed only one unique \( [3H] \)Gal-labeled band for each subunit. Based on immunoblotting of immunoprecipitates treated with various enzymes, we confirmed that only the 65-kDa \( \alpha \)-, 110-kDa \( \beta \)-, and the 75-kDa \( \gamma \)-subunit exhibit processed N-glycans.

**Maturation of ENaC Involves Cleavage of \( \alpha \)- and \( \gamma \)-Subunits**—There is considerable evidence in the literature indicating that the cleaved 65-kDa form of \( \alpha \)-ENaC that we identified represents the mature form of the subunit in vivo. For example, Planès et al. (20) found two forms of the endogenous ENaC \( \alpha \)-subunit in rat lung alveolar epithelial type II cells at 85 and 65 kDa on SDS-PAGE, but only the 65-kDa form of \( \alpha \)-ENaC was found at the cell surface by biotinylation and subsequent immunoblotting with a rabbit polyclonal antibody. Alvarez de la Rosa et al. (10) also reported two forms of the endogenous \( x \)ENaC \( \alpha \)-subunit at 85 and 65 kDa on SDS-PAGE after immunoprecipitation from \( [35S] \)Met/Cys-labeled A6 cells using rabbit polyclonal antibodies prepared against glutathione S-transferase fusion proteins containing \( \alpha \)-subunit residues around and including the M2 domain. The authors also found these two forms of the \( \alpha \)-subunit by transient overexpression of \( \alpha \beta \gamma \)ENaC in A6

![Diagram of ENaC processing](image-url)
cells. In pulse-chase studies, the 85-kDa form of the α-subunit appeared to be the precursor of the 65-kDa form, and only the 65-kDa form was resistant to Endo H treatment (10). However, the 65-kDa form disappeared when cells were treated with a combination of dithiothreitol followed by iodoacetamide in the media, and the authors (10) concluded that the 65-kDa form represented the 85-kDa form with disulfide bonds that were resistant to reducing agents. When we treated our ENaC immunoprecipitates with dithiothreitol to break disulfide bonds and then with iodoacetic acid to block all of the sulfhydryl groups, we found no change in either the immunoblotting pattern or the [3H]Gal-labeling pattern on SDS-gels (data not shown). Instead, we found clear evidence for cleavage of the full-length mouse ENaC α-subunit by characterization of an α-subunit construct with epitope tags at the amino (HA) and carboxyl (V5) terminus. Cleavage in the extracellular domain close to the first membrane-spanning domain (M1) accounts for the appearance of the 30-kDa HA-tagged fragment and the 65-kDa V5-tagged fragment when HA-α-V5 is co-expressed with the β- and γ-subunits.

There is also evidence in the literature indicating that the cleaved form of γ-ENaC represents the mature form of the subunit in vivo. Masilamani et al. (21) reported an aldosterone-induced shift in the molecular weight of the γ-subunit of endogenous rENaC in rat kidney in response to dietary NaCl restriction. After 10 days on a sodium-restricted diet, there was a decrease in the 85-kDa form and an increase in a 70-kDa form of γENaC by immunoblotting with a rabbit polyclonal antibody prepared against residues within the cytosolic carboxyl terminus. The authors noted that a proteolytic cleavage in the early portion of the extracellular loop of the γ-subunit by an aldosterone-induced protease such as CAP could account for the appearance of the 70-kDa form of γENaC (21). This possibility is supported by our present data with the γ-subunit of mENaC.
epitope-tagged at both the amino (HA) and carboxyl (V5) terminus. Cleavage in the extracellular domain closest to the first membrane-spanning domain (M1) would explain the co-immunoprecipitation of an 18-kDa HA-tagged fragment and a 75-kDa V5-tagged fragment. Interestingly, we also found a 22-kDa V5-tagged fragment from the carboxyl terminus of HA-γV5 when expressed with or without α and β. Because this 22-kDa fragment of γ was not found in the anti-HA immunoprecipitate by immunoblotting with anti-V5 antibody, it apparently does not remain associated with a corresponding HA-tagged fragment (predicted to be 71 kDa). In fact, we have never observed a 71-kDa HA-tagged fragment from cells expressing HA-γV5 by immunoblotting with anti-HA antibodies. Instead it seems that the V5-tagged 22-kDa fragment of γ is likely to result from cleavage of the V5-tagged 75-kDa fragment of HA-γV5, releasing a 53-kDa fragment of the extracellular loop without an epitope tag (see Fig. 6B, model b). This second cleavage of HA-γV5 would explain the absence of an HA-tagged 71-kDa fragment and the very low levels of V5-tagged 75-kDa fragment that we found upon expression of αβγENaC. Finally, loss of the carboxyl-terminal epitope tag from the γ-subunit would explain why γ-immunoprecipitates do not co-immunoprecipitate the processed forms of the α- and β-subunits shown in Fig. 1. However, finding co-immunoprecipitation of the processed γ in anti-α- and anti-β-immunoprecipitates indicates that the cleavage of the γ carboxyl terminus could represent a degradation step.

Evidence for Activation of ENaC by Proteolysis—There is evidence in the literature that extracellular proteolysis of ENaC by serine proteases is associated with increased amiloride-sensitive currents, but a specific cleavage of ENaC has not been reported (31–36). In general, this protease activation is mimicked by extracellular trypsin and inhibited by the serine protease inhibitor aprotinin. Bridges et al. (36) also observed that amiloride-sensitive short circuit currents in human bronchial epithelial cells were inhibited by the Kunitz-type serine protease inhibitor BAY 39-9437. However, we found that addition of aprotinin to the media of ld1D cells expressing αβγENaC did not alter the pattern of [3H]Gal labeling of the three subunits, and aprotinin addition to the media of polarized MDCK cells did not alter either the appearance of the α-subunit pattern at the apical cell surface or the level of amiloride-sensitive sodium current (data not shown). We also found that exclusion of the protease inhibitor mixture from the cell extraction buffer did not alter the resulting pattern by immunoblotting or metabolic labeling (data not shown). Vallet et al. (33) used a functional complementation assay in Xenopus oocytes expressing xENaC to screen an A6 cell cDNA library and cloned a channel-activating protease (xCAP1) that stimulated the activity of ENaC 2–3-fold by increasing the open probability of the channel. The mouse homologue, mCAP1, and two additional serine proteases, mCAP2 and mCAP3, have been subsequently cloned by reverse transcriptase-PCR from the mouse cortical collecting duct cell line (mPCkCCD14) (34, 35). All three exhibit parallel patterns of tissue expression with αENaC, except that mCAP2 is not expressed in kidney; and all three proteases activate ENaC when co-expressed in oocytes. However, ENaC activation by mCAP1 and mCAP2 is blocked by preincubation with aprotinin, whereas activation by mCAP3 is insensitive to aprotinin (35). Thus, the possibility does exist that an aprotinin-insensitive CAP is also present in CHO and MDCK cells and accounts for the cleaved forms of the α- and γ-ENaC subunits that we observed in the present studies. Guipponi et al. (31) have also cloned the human CAP2 homologue TMPRSS3 that is localized to the ER rather than to the cell surface. Proteolytic activation of TMPRSS3 correlates with activation of ENaC when co-expressed in oocytes (31), and the action of an intracellular protease of this type could represent a possible mechanism for the biosynthetic processing of ENaC that we observe.

Cleaved Forms of ENaC Are Found on the Cell Surface—Proteolytic processing of ENaC subunits is not restricted to degradation in lysosomes or by proteasomes (19, 37), as processed subunits are clearly expressed at the plasma membrane. Jovov et al. (38) previously identified trypsin-sensitive sites within the cytoplasmic carboxyl termini of the β- and γ-subunits. However, our results suggest that these trypsin-sensitive sites are not targeted by proteases when channels are expressed in CHO or MDCK cells. Whether CAPs (39) or other proteases participate in the post-translational processing of ENaC subunits remains to be determined. There is evidence for proteolytic processing of other channels. For example, several groups have reported that voltage-gated calcium channel α-subunits are processed by proteases (40, 41). Certain pore-forming bacterial toxins, such as aerolysin, are activated by proteases (42). Aquaporin 2 channels are processed in endosomes by calpain, although this processing is thought to facilitate aquaporin 2 degradation (43).

Both our data presented here in a heterologous system (Fig. 5) and the data of Alvarez de la Rosa et al. (10) with endogenous xENaC in A6 cells indicate that both the processed and unprocessed forms of the α- and β-subunits are found at the cell surface. Weisz et al. (9) also found a doublet of the β-subunit on the surface of A6 cells although Planèes et al. (20) found only the processed α-subunit of xENaC at the surface of rat lung alveolar epithelial type II cells. We found co-immunoprecipitation of both the processed and unprocessed forms of the three subunits in total cell lysates (Fig. 1–3) and processed and unprocessed forms of the α- and β-subunits at the cell surface (Fig. 5), indicating that there is heterogeneity in the processing of subunit N-glycans in the channels.

Not all N-glycans on a single glycoprotein are necessarily processed to complex type, but it is highly unusual to find the all-or-none processing of N-glycans on cell surface glycoproteins that we observed with ENaC. One mechanism that could explain the heterogeneous processing of the N-glycans on all three ENaC subunits is that this processing is linked to cleavage of the α- and γ-subunits within the tetramer. We have found that inhibition of N-glycan processing with the ER α-mannosidase-1 inhibitors deoxymannojirimycin or kifunensine does not block proteolytic processing of either the α- or γ-subunit of ENaC,2 but it is possible that N-glycan processing is dependent on cleavage of the α- and γ-subunits. It is unclear from our present data whether all subunits within a single channel/tetramer are processed (or alternatively, not processed), or whether individual subunits within a channel complex are differentially processed. Whereas both extracellular (and intracellular) proteases such as CAPs, and protease inhibitors such as aprotinin and BAY 39-9437, have been reported to modulate ENaC activity, it is still unclear whether CAPs actually cleave ENaC subunits and whether the primary site of action is extracellular. Future experiments are needed to better define the temporal relationships of the many steps in αβγENaC processing as well as the subcellular site(s) of proteolytic cleavage. The ability to inhibit discrete steps in ENaC proteolytic processing will provide a means to determine whether ENaC cleavage regulates the functional properties, the subunit stoichiometry, or the membrane trafficking and turnover of the channel.

Acknowledgment—We thank Dr. Ora Weisz for providing helpful suggestions.
