Apical Membrane Targeting of Nedd4 Is Mediated by an Association of Its C2 Domain with Annexin XIIIb

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Abstract. Nedd4 is a ubiquitin protein ligase (E3) containing a C2 domain, three or four WW domains, and a ubiquitin ligase HECT domain. We have shown previously that the C2 domain of Nedd4 is responsible for its Ca^{2+}-dependent targeting to the plasma membrane, particularly the apical region of epithelial MDCK cells. To investigate this apical preference, we searched for Nedd4-C2 domain-interacting proteins that might be involved in targeting Nedd4 to the apical surface. Using immobilized Nedd4-C2 domain to trap interacting proteins from MDCK cell lysate, we isolated, in the presence of Ca^{2+}, a ~35–40-kD protein that we identified as annexin XIII using mass spectrometry. Annexin XIII has two known isoforms, a and b, that are apically localized, although XIIIa is also found in the basolateral compartment. In vitro binding and coprecipitation experiments showed that the Nedd4-C2 domain interacts with both annexin XIIIa and b in the presence of Ca^{2+}, and the interaction is direct and optimal at 1 μM Ca^{2+}. Immunofluorescence and immunogold electron microscopy revealed colocalization of Nedd4 and annexin XIIIb in apical carriers and at the apical plasma membrane. Moreover, we show that Nedd4 associates with raft lipid microdomains in a Ca^{2+}-dependent manner, as determined by detergent extraction and floatation assays. These results suggest that the apical membrane localization of Nedd4 is mediated by an association of its C2 domain with the apically targeted annexin XIIIb.

Key words: apical rafts • polarized epithelia • protein trafficking • ubiquitin protein ligase

Introduction

Neural precursor cell–expressed developmentally down-regulated 4 (Nedd4) is a ubiquitin protein ligase comprised of a C2 domain, three or four WW domains, and a ubiquitin protein ligase (E3) HECT domain (Kumar et al., 1992). We have previously demonstrated that an important target of Nedd4 is the apically located epithelial Na^+ channel, which binds to the WW domains of Nedd4 and is regulated by ubiquitination (Staub et al., 1996, 1997; Abriel et al., 1999).

In addition to its HECT and WW domains, Nedd4 possesses an NH_2-terminal C2 domain. The C2 domain is a protein–lipid, protein–protein interaction module originally identified in Ca^{2+}-responsive isoforms of protein kinase C (PKCαβγ)[1] (Coussens et al., 1986; Knopf et al., 1986) and later found in other proteins such as cytoplasmic phospholipase A_2 ($cPLA_2$), synaptotagmin, rasGAP, and phosphoinositide-specific phospholipase C (PLC-βγδ; for reviews see Nalefski and Falke, 1996; Ponting and Parker, 1996). Ligands for C2 domains include Ca^{2+}, phospholipids, inositol polyphosphates, and various intracellular proteins (Nalefski and Falke, 1996; Ponting and Parker, 1996; Rizo and Sudhof, 1998). Up to five conserved aspartates are believed to coordinate binding of two Ca^{2+} ions to the C2 domain (Sutton et al., 1995; Shao et al., 1996).

In addition to phospholipid binding, several C2 domains have been demonstrated to bind proteins in a Ca^{2+}-dependent or -independent manner. In most synaptotagmin isoforms, the second C2 domain binds to clathrin adaptin (AP-2) complexes with high affinity, independent of Ca^{2+} (Ullrich et al., 1994; Zhang et al., 1994). However, the first C2 domain of synaptotagmin binds to syntaxin molecules

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[1]*Abbreviations used in this paper:* GST, glutathione-S-transferase; HA, hemagglutinin; MeβCD, methyl-β-cyclodextrin; PKC, protein kinase C.

in a Ca^{2+}-dependent fashion (Li et al., 1995; Kee and Scheller, 1996; Sugita et al., 1996). Furthermore, the CaLB domain (corresponding to the ~40–amino acid core region of the C2 domain) of rasGAP was recently demonstrated to bind annexin VI in a Ca^{2+}-dependent manner (Davis et al., 1996). Annexins are a family of Ca^{2+} and lipid binding molecules and some family members (i.e., annexins I, II, IV, V, VI, and VII) have been shown to associate with membranes in a Ca^{2+}-dependent fashion (Creutz, 1992; Moss, 1997).

We have recently demonstrated a Ca^{2+}-dependent association of the C2 domain of Nedd4 with membranes and phospholipids, and that this domain is necessary for translocating Nedd4 to the plasma membrane, particularly the apical region of polarized MDCK epithelial cells in response to elevation of intracellular Ca^{2+} (Plant et al., 1997). However, this Ca^{2+}-dependent apical membrane preference of the Nedd4-C2 domain was puzzling, as it was not clear how the C2 domain could distinguish between the apical versus basolateral membranes, especially as the inner leaflet of these membranes (in MDCK cells) is homogeneous with respect to its lipid composition (van Meer and Simons, 1986). Moreover, our work has shown a lack of preference of the Nedd4-C2 domain towards charged phospholipids (Plant et al., 1997), clearly dissimilar to several other C2 domains (Davletov and Sudhof, 1993; Yamaguchi et al., 1993; Chapman and Jahn, 1994; Gawler et al., 1995). Thus, the purpose of the current study was to investigate how the Nedd4-C2 domain is preferentially mobilized to the apical region of polarized MDCK cells, and to determine whether a C2 domain-interacting protein may facilitate this Ca^{2+}-dependent apical targeting.

Using pull-down experiments with immobilized GST-Nedd4-C2 domain and MDCK II cell lysate followed by mass spectrometry (MALDI-TOF) analysis, we have identified annexin XIII as a binding partner of the Nedd4-C2 domain. We further demonstrate a Ca^{2+}-dependent association, both in vitro and in cells, of the C2 domain of Nedd4 with annexin XIIIa and b, which are epithelial-specific isoforms of the annexin family (Wice and Gordon, 1992; Fiedler et al., 1995). Annexin XIIIb is associated with apical rafts (Lafont et al., 1998). Rafts are 50–70-nm lipid microdomains enriched in glycosphingolipids and cholesterol (Simons and Ikonen, 1997; Brown and London, 1998), and play an important role in cholesterol metabolism, sorting mechanisms, and cell signaling. They also have been implicated in the pathogenesis of several diseases (Simons and Ikonen, 1997). Of particular interest is the proposed function of rafts in the delivery of proteins destined for the apical membrane of polarized MDCK cells (Simons and Ikonen, 1997). We demonstrate here that, upon induction of expression of annexin XIIIb in MDCK cells and a rise in intracellular Ca^{2+} concentration, Nedd4 is preferentially associated with the apical membrane. Moreover, we show colocalization of annexin XIIIb and Nedd4 in apical carriers and at the apical plasma membrane. We further demonstrate that Nedd4 is recruited into rafts by annexin XIIIb in the presence of Ca^{2+}. Therefore, we propose that the apical membrane targeting of Nedd4 may be mediated or facilitated by a Ca^{2+}-dependent association of its C2 domain with annexin XIIIb.

Materials and Methods

Constructs

Bacterially Expressed GST Fusion Proteins. Glutathione-S-transferase (GST) fusion proteins of the rat Nedd4-C2 domain were prepared by PCR amplification of the region of rNedd4 cDNA (amino acid residues 77–219, Staub et al., 1996) corresponding to the boundaries defined in Nalefski and Falke (1996) for a type II topology C2 domain. PCR products were subcloned with flanking BamHI and EcoRI sites into the corresponding sites in pGEX-2TK (Amersham Pharmacia Biotech). The plasmid containing the C2 insert, verified by sequencing, was used to transform the HB101 strain of *Escherichia coli*. Fusion protein production was as previously described (Plant et al., 1997).

Bacterially Expressed Histidine-tagged Constructs. Hemagglutinin (HA)-tagged annexin XIIIa and b were generated by introducing the HA tag sequence (YPYDVPDYAG) at the COOH termini of annexin XIIIa or b (at amino acid residues 316 and 357, respectively; Fiedler et al., 1995) by PCR. Full-length HA-tagged annexin XIIIa and b were subcloned with flanking NdeI and XhoI sites into the pET-30b(+) bacterial expression vector, in-frame with a COOH-terminal 6xHis tag (Novagen, Inc.). The plasmid sequences were verified by sequencing and were used to transform the HB101 strain of *Escherichia coli* and proteins were produced and purified as described previously (Kanelis et al., 1998).

Mammalian Expression Vectors. HA-tagged annexin XIIIa and b were generated by introducing the HA tag sequence (YPYDVPDYAG) at the COOH termini of annexin XIIIa or b (at amino acid residues 316 and 357, respectively; Fiedler et al., 1995) by PCR and subcloning into the pRc-CMV vector (Invitrogen). The Nedd4-C2 domain (amino acid residues 77–219; Staub et al., 1996) was subcloned into pEBG mammalian expression vector in-frame with GST as previously described (Wallace et al., 1998). Myc-tagged annexin XIIIb was generated with the myc epitope (AEEQKLISEEDL), which was introduced at the COOH terminus of annexin XIIIb, and the construct, in the Lac operator vector pOPRSV-1, was stably transfected into an MDCK II variant cell line which expresses very low levels of endogenous annexin XIIIb and stably expresses the lac repressor gene (hereafter called a lac switchable MDCK II-Annexin XIIIb cell line, or MDCK(lac)-AnxXIIIb cells; Lecat et al., 2000). Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce expression of the myc-tagged annexin XIIIb (by relieving the inhibition by the constitutively active lac repressor). MDCK cells expressing the lac-switchable system have been previously characterized and shown to form functional tight monolayers (McCarthy et al., 1996).

Identification of Interacting Proteins with MALDI-TOF Mass Spectrometry

Epithelial MDCK II cells were grown to confluency in DME containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C, 5% CO_2 atmosphere. Cells were lysed with lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and 1% Triton X-100) with a protease inhibitor cocktail containing 1 mM PMSF and 10 μg/ml of each leupeptin, aprotinin, and pepstatin. The lysate was spun at 14,000 *g* (for 5 min at 4°C) to remove mitochondria and nuclei. The supernatant was incubated with purified GST-C2 fusion protein or GST alone that was immobilized on glutathione agarose beads in the presence or absence of Ca^{2+} (1 μM final concentration), 10 mM EGTA, and 1 mM $MgCl_2$ at 4°C for 1 h. The beads were sedimented at 10,000 *g* for 15 s and were washed twice with high salt HNTG (20 mM Hepes, pH 7.5, 500 mM NaCl, 0.1% Triton X-100, and 10% glycerol) followed by three washes with HNTG (same as high salt but with 150 mM NaCl). Proteins were separated on a 10% SDS-PAGE and, after electrophoresis, the gel was silver stained using a modified protocol from *Current Protocols in Protein Science* (John Wiley and Sons, Inc.). Unique bands were excised and the proteins were trypsin-digested in the gel and extracted using a protocol described previously (Shevchenko et al., 1996). Extracted peptides were purified using a microreverse phase cartridge (Michrom BioResources), and were identified using MALDI-TOF mass spectrometry with the following parameters: linear mode, 92% grid voltage, 0.150% guide wire voltage, 200-ns delayed extraction, 800-D low mass gate, and 1650 laser intensity. Samples were loaded in a matrix solution containing 20 mg/ml α-cyano-4-hydroxy-trans cinnamic acid (Sigma Chemical Co.) in 50% acetone/50% isopropanol. Masses obtained using MALDI-TOF were analyzed using the ProFound database (http://prowl.rockefeller.edu/cgi-bin/ProFound). The

masses used for the search were derived from highly resolved peaks in the 900–3,000-D range and, on average, 10–15 masses were used in the search.

In Vitro Binding Experiments

Pull-down experiments, as described above, were repeated by incubating GST-Nedd4-C2 or GST alone with lysate from MDCK II cells or from 293T cells (grown in the conditions described for MDCK II cells) transfected with HA-annexin XIIIa or b. 293T cells were transiently transfected using the Ca_2PO_4 method (Chen and Okayama, 1987), harvested, and lysed as described above for MDCK II cells. After electrophoresis on 10% SDS-PAGE, the proteins were transferred onto nitrocellulose and blotted with anti–annexin XIIIb antibodies (described previously; Fiedler et al., 1995) to detect endogenous annexin XIIIb, or with anti-HA antibodies to detect transfected HA-annexin XIIIa/b, followed by anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Boehringer Mannheim) and ECL detection (Amersham Corp.). Ca^{2+}-dependent pull-down experiments were done in an identical fashion as described above, but for Ca^{2+}-free ($-Ca^{2+}$) conditions, only 10 mM EGTA and 1 mM $MgCl_2$ were added to the lysate.

Binding experiments involving purified His-HA-annexin XIIIa/b and GST-C2 or GST alone were done as described above, in the presence of 1 μM (final) Ca^{2+} with 10 mM EGTA and 1 mM $MgCl_2$. GST and GST-C2 on beads were collected by centrifugation and, after five washes with HNTG (as described above), proteins were eluted from the beads and separated by electrophoresis on 10% SDS-PAGE. The proteins were transferred onto nitrocellulose and blotted with anti-HA antibodies to detect bound annexin XIII proteins. To calculate the optimal Ca^{2+} concentration for the annexin XIIIb-Nedd4 C2 interaction, binding experiments were performed as described above in the presence of varying Ca^{2+} concentrations (solutions buffered with $MgCl_2$ and EGTA). Western blots were developed with ECL, and the signal was collected by a CCD camera using the FluoroChem 9000 system (AlphaInnotech Corp.). The intensity of the bands was quantitated and expressed as IDVs (integrated density values) using the AlphaEase FC software. The intensities of annexin XIIIb binding to the GST-Nedd4-C2 were normalized to that of binding to GST alone, and the amount bound at the given Ca^{2+} concentrations was expressed as a percentage of the maximal amount of annexin XIIIb bound (at 1 μM Ca^{2+}). These percentages were averaged over the three experiments and plotted as mean ± SEM.

Coprecipitation Experiments

The GST-Nedd4-C2 domain (expressed in the mammalian expression vector pEGB) was transiently cotransfected into 293T cells alone or together with full-length HA-tagged annexin XIIIa or b. Before lysis, the cells were either treated or not with Ca^{2+} medium (140 mM NaCl, 6 mM KCl, 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM EGTA, 20 mM glucose, and 20 mM Hepes) together with 1 μM ionomycin to increase intracellular Ca^{2+}. Cells were lysed in lysis buffer plus protease inhibitors (as above), spun at 14,000 *g* for 5 min, and the supernatant was incubated at 4°C for ~30 min with glutathione agarose beads to precipitate the transfected GST-Nedd4-C2 domain. The beads were washed (five times) with HNTG as above, proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-GST antibodies to detect Nedd4-C2, or with anti-HA antibodies to detect tagged annexin XIIIa or b.

Immunofluorescence Staining and Confocal Analysis

MDCK(lac)-AnxXIIIb cells (expressing the inducible myc-tagged annexin XIIIb (McCarthy et al., 1996; Lecat et al., 2000) were grown on polycarbonate filters (0.45-μm pore size; Costar Corp.) until confluent and polarized, and induced overnight with sodium butyrate (1 mM) and IPTG (5 mM). For Ca^{2+} treatment, cells were washed twice with Ca^{2+}-free media (140 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 20 mM glucose, and 20 mM Hepes) and incubated (or not) in Ca^{2+} medium (which contains 1.1 mM Ca^{2+} as described above) with 1 μM ionomycin for 5 min at 37°C. Incubation of MDCK cells with 1 mM Ca^{2+} plus 1 μM ionomycin had been previously shown to elevate intracellular [Ca^{2+}] to ~1 μM within 2–3 min (Breuer et al., 1988). Filters were washed and fixed in methanol at −20°C for 6 min, and washed with PBS. The filters were cut from the inserts, and the cells were incubated in 0.2% fish skin gelatin (FSG; Sigma Chemical Co.) in PBS for 30 min followed by incubation overnight at 4°C with the primary antibodies diluted in PBS with 0.2% FSG (10 μg/ml of either affinity pure anti-Nedd4 or anti-AnxXIIIb antibodies). DNA was stained with 0.05 μg/ml propidium iodide for 5 min in PBS as previously described (Lafont et al., 1994). Filters were washed four times with PBS followed by an incubation with goat anti–rabbit fluorescein-conjugated antibodies (Dianova) in PBS with 0.2% FSG or 1 h at 37°C. This was followed by four washes in PBS. Cells were placed in mounting medium in PBS/glycerol (Merck) 1:1 with 0.1% NaN_3 and 100 mg/ml 1,4-diazabicyclo-2,2,2-octane. Coverslips were positioned on thin bridges cut from cellophane and sealed with nail polish. The fixed and stained cells were viewed using a Leica NTS confocal microscope. For quantitation of Nedd4 distribution, black and white confocal X,Z images of Nedd4 labeling were imported into NIH Image 1.62. Fluorescence intensities were scaled from 0 to 252 (0 being black). The mean fluorescence intensities of rectangular sections of equal sizes from the basolateral or apical region of cells were measured. The ratio of Nedd4 intensity in the apical versus basolateral area was calculated from nine induced and nine noninduced cells, and the mean were compared using a *t* test.

Detergent Extraction, Cyclodextrin Treatment, and Floatation Assays

MDCK(lac)-AnxXIIIb cells were induced to express annexin XIIIb, cells were scraped, and were processed with Triton X-100 as described previously (Lafont et al., 1998). 10 mM methyl-β-cyclodextrin was added directly onto living cells for 1 h at 37°C. The following buffers were used: TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EGTA) and TNCa buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 μM $CaCl_2$). In both cases, the buffers were supplemented with 5 mM DTT and a cocktail of protease inhibitors (CLAP: chymostatin, leupeptin, antipain, and pepstatin A; final concentration 10 μg/ml each). Samples were placed in 40% OptiPrep and overlaid with 25 and 0% OptiPrep either in TNE or TNCa. Samples were centrifuged for 4 h at 40,000 rpm in an SW 60 rotor (Beckman) at 4°C. Fractions were collected from the top, and proteins were methanol/chloroform-precipitated before separation on SDS-PAGE and immunoblotting with anti-Nedd4 antibodies (Staub et al., 1996), anti–annexin XIIIb antibodies (Fiedler et al., 1995), or anti–caveolin-1 antibodies (Santa Cruz Biotechnology, Inc.). Washed filters were incubated with ^{35}S-labeled secondary antibodies (Amersham Pharmacia Biotech) before being air dried. Each band density was measured using PhosphorImager (Fuji Photo Film Co.) and analyzed with the AIDA 2D software (Raytest isotopenmeβgeräte Gmbh).

Electron Microscopy

Double or triple immunogold labeling on ultrathin cryosections was carried out as detailed in Lafont et al. (1998), using polarized MDCK cells expressing (or not) the VSVG epitope–tagged sialyltransferase (Scheiffele et al., 1998). TGN-derived carrier vesicles were isolated from influenza-infected MDCK cells. Cells were perforated by mechanically ripping off the apical plasma membrane as previously described (Lafont et al., 1998). The buffer that was used to isolate apical exocytic vesicles contained 500 nM free Ca^{2+}. Protein A–coupled gold particles were purchased from the Department of Cell Biology, Faculty of Medicine. Samples were examined under a Zeiss transmission 10 C electron microscope.

Results

Identification of Annexin XIII as a Binding Partner for Nedd4-C2 Domain

To identify proteins that may be involved in C2-mediated targeting of Nedd4 to the apical region of polarized cells, we performed pull-down experiments with a fusion protein of the Nedd4-C2 domain. Lysates from MDCK II cells were prepared as described in Materials and Methods and incubated in the presence or absence of 1 μM Ca^{2+} (buffered with EGTA and Mg^{2+}) with immobilized GST-Nedd4-C2 domain. After separation of bound proteins by SDS-PAGE, silver staining of the gel revealed the presence of several unique bands precipitating with the C2 domain fusion protein in the presence of Ca^{2+} (data not shown). The most prominent of these bands, migrating at ~35–40 kD, was excised from the gel, purified, and ana-

PEPTIDE MASSES IDENTIFIED BY MALDI-TOF MASS SPECTROMETRY AND CORRESPONDING AMINOACIDS OF ANNEXIN XIII (trypsin cleavage):

1775.21 (5-20)	1703.0 (142-157)	1755.78 (301-316)
2783.9 (21-48)	1198.5 (191-201)	983.80 (342-350)
1365.4 (51-62)	982.04 (218-226)	
1662.2 (70-85)	1591.9 (227-240)	
1691.9 (120-134)	1748.2 (260-275)	

ANNEXIN XIIIb SEQUENCE COVERAGE:

MGNR **HSQSYSLSEGSQQLPK DIQPSAAVQPLSHPSGSGEPEAQQ PAK** AK **SHHGFDVDHDAK** KLNKACK **GMGTDEAAIIEILSSR** TSDERQQIKQKYKATYGKDLEEVFKSDLSGNFEK **TALALLDRPSE YDAR** QLQKAMKGLGTDEAVLIEILCTRTNKEIMAIKEAYQRLFDRS LESDVKADTSGNLK **AILVSLLQANR** DEGDDVDKDLAGQDAK **DLYDAGDGR WGTDELAFNEVLAK** RSHKQLRATFQAYQILIDK **DIEEAIEAETSGDLQK** AYLTLVRCAR **DQEGYFADR** LYKSMK **GTGTDEETLIHIIVTR** AEVDLQGIKAKFQEKYQKSLSDMR **SDTSGDFQK** LLVALLH

Total percent coverage: 45%
Percent coverage of peptide fragments > 900 Da: 76%

Figure 1. Identification of annexin XIII as a binding partner of the Nedd4-C2 domain. Annexin XIII, which was isolated from a canine kidney (MDCK II) cell lysate, was identified through MALDI-TOF mass spectrometry as a candidate protein interacting with GST-Nedd4-C2. The sequence of the canine isoform of annexin XIII, annexin XIIIb, was run through the PAWS program to calculate the masses of tryptic fragments from a theoretical cleavage (amino acids corresponding to tryptic fragments in brackets), and these masses were compared with the masses from MALDI-TOF analysis. 12/15 masses obtained through the MALDI-TOF analysis corresponded to the theoretical values for annexin XIIIb cleavage and represented 45% of the proteins' fragments (peptides in bold print). As only fragments >900 D were used in the search, a comparison with the masses from a theoretical trypsin cleavage of annexin XIIIb >900 D showed that the masses obtained through MALDI-TOF analysis covered 76% of the protein sequence.

lyzed by MALDI-TOF mass spectrometry. Using the ProFound program, this band was identified as annexin XIII/XIIIa (human intestine–specific annexin; Fig. 1) originally described by Wice and Gordon (1992). The canine annexin XIIIa homologue, and a 40-kD spliced variant, which contains a 41–amino acid insert at its NH_2 terminus (annexin XIIIb) were subsequently cloned by Fiedler et al. (1995), and were shown to be expressed in dog intestine and kidney epithelial (MDCK II) cells. Annexin XIIIb is 90% identical and 96% similar to human annexin XIIIa (Fiedler et al., 1995), and was found to be involved in vesicle trafficking to the apical plasma membrane in polarized MDCK cells. The annexin XIII subfamily of the annexins is the only one known to be myristoylated (Moss, 1997) and, hence, constitutively associated with membranes. Myristoylated annexin XIIIb (but not an unmyristoylated form) was shown to associate with, and function in, the formation of apical carrier vesicles from the TGN that serve in apical delivery (Lafont et al., 1998).

Annexin XIIIa/b and Nedd4-C2 Domain Interact In Vitro and in Living Cells

To verify the interaction of annexin XIII with the Nedd4-C2 domain, we repeated the pull-down experiments (used for the initial identification of annexin XIII) by incubating immobilized GST-Nedd4-C2 fusion protein with lysate from MDCK II cells (expressing endogenous annexin XIIIb) or from 293T cells transfected with HA-tagged annexin XIIIa or b, in the presence of Ca^{2+}. After precipitation, SDS-PAGE, and transfer to nitrocellulose, the proteins were detected by immunoblotting with either anti–annexin XIIIb antibodies to detect endogenous annexin XIIIb, or with anti-HA antibodies to detect transfected annexin XIIIa and b. Our results show that immobilized GST-Nedd4-C2 domain (but not GST alone) was able to precipitate annexin XIIIa and b (35- and 40-kD

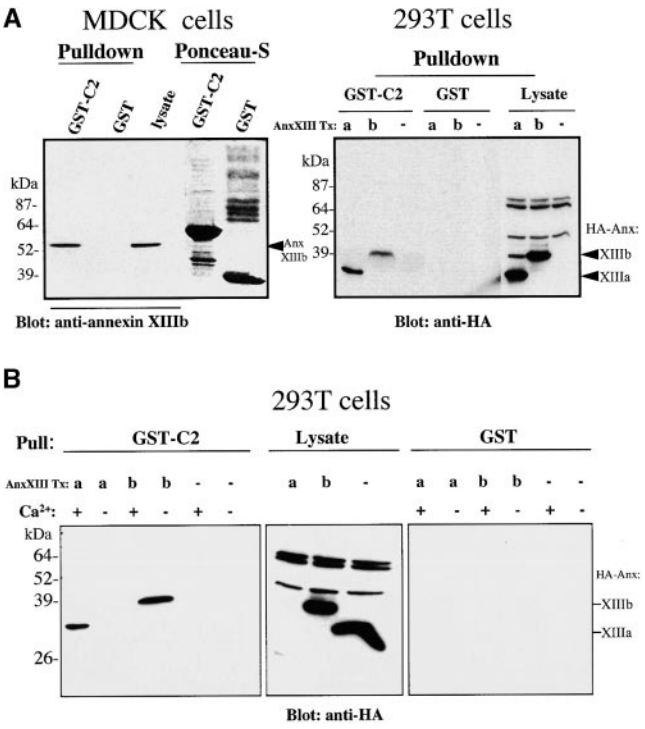

Figure 2. Ca^{2+}-dependent coprecipitation of Nedd4-C2 domain and annexin XIIIa and b. (A) The C2 domain of Nedd4, expressed as a GST fusion protein (GST-C2), or GST alone, was incubated with lysate from MDCK II cells (left, MDCK cells) or with lysate from 293T cells transiently transfected with HA-tagged annexin XIIIa or b (right, transfected (Tx) 293T cells, XIIIa or XIIIb) in the presence of Ca^{2+}. GST or GST-C2 was precipitated by incubation with glutathione agarose beads. Coprecipitated proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti–annexin XIIIb antibodies (left), to detect endogenous annexin XIIIb, or with anti-HA antibodies (right), to detect transfected HA-tagged annexin XIIIa and b. Aliquots of the lysate were also analyzed for levels of expression of either endogenous annexin XIIIb or transfected annexin XIIIa and b (a and b) together with untransfected lysate (−). Total GST or GST-C2 proteins used in the pull-down experiments are shown (Ponceau S). (B) Identical experiments to those described in A were performed with 293T cells transiently transfected with HA-annexin XIIIa and b (a and b), except in the presence (+) or absence (−) of Ca^{2+}. Western blots were performed as described for A using an anti-HA antibody.

bands, respectively) from either MDCK II cells or from transfected 293T cells (Fig. 2 A), suggesting the Nedd4-C2 domain can bind annexin XIII. These interactions were dependent on the presence of Ca^{2+}, as chelating the free Ca^{2+} in the binding buffer with EGTA (-Ca^{2+}), abrogated binding of annexin XIIIa or b to the Nedd4-C2 domain (Fig. 2 B).

To determine if the interaction between annexin XIII and the Nedd4-C2 domain could take place in living cells, a mammalian GST-Nedd4-C2 expression construct was transfected into 293T cells together with HA-annexin XIIIa or b. Before harvesting, the cells were either treated or not with the Ca^{2+} ionophore ionomycin, plus Ca^{2+}, to increase cytosolic Ca^{2+} concentrations. Cells were lysed and the C2 domain was precipitated from cellular lysate by incubation with glutathione agarose beads. As shown in Fig. 3, annexin XIIIa and b coprecipitated with the Nedd4-C2 domain only when the cells had been Ca^{2+}-treated. The annexin XIIIa and b constructs expressed alone did not precipitate with the beads (Fig. 3). These results show that the C2 domain of Nedd4 binds annexin XIIIa and b in a Ca^{2+}-dependent manner.

To test whether the interaction between the C2 domain and the annexin isoforms was direct, purified GST-C2 or GST alone was incubated with purified His-HA–tagged annexin XIIIa or b in the presence of Ca^{2+} as described above. As shown in Fig. 4 A, the interaction appeared to be direct, as the full-length annexins bound to the GST-C2 on beads but not to the GST alone. The optimal Ca^{2+} concentration for the Nedd4-C2/annexin XIIIb interaction was 1 μM (Fig. 4 B), which was the concentration used in all our experiments. Fig. 4 B also shows a second (suboptimal) binding peak at 100 nM Ca^{2+}, likely reflecting the presence of two binding sites with different affinities for Ca^{2+} in the C2 domain as previously reported (Shao et al., 1996).

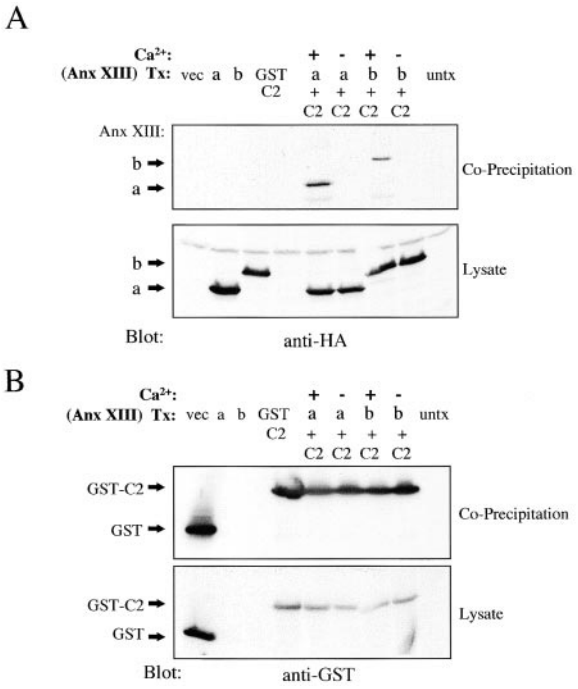

Figure 3. Ca^{2+}-dependent coprecipitation of Nedd4-C2 domain and annexin XIIIa and b from mammalian cells. The Nedd4-C2 domain (C2) expressed as a GST fusion protein (in the mammalian expression vector pEBG) was transiently cotransfected with HA-tagged annexin XIIIa or b (a and b) in pRC-CMV into 293T cells. Transfected cells were either treated (+) or not (−) with Ca^{2+}/ionomycin to raise intracellular Ca^{2+} levels. Cells were lysed and the lysate was incubated with glutathione agarose beads to precipitate GST-Nedd4-C2 and associated proteins. Proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-HA antibodies (A), to detect coprecipitated HA-tagged annexin XIIIa and b, or with anti-GST antibodies (B), to detect GST-Nedd4-C2. Precipitation and immunoblotting of annexin XIIIa (a) or annexin XIIIb (b), or GST-C2 (GST-C2) expressed alone, used as controls, are shown as well. Bottom panels show aliquots of the lysate that were analyzed for protein expression of either the HA-tagged annexin XIIIs (A) or GST-Nedd4-C2 (B). Tx, transfected; untx, untransfected.

Ca^{2+}-dependent Distribution of Nedd4 in MDCK Cells Expressing Annexin XIIIb

A MDCK II variant expressing very low levels of endogenous annexin XIII and stably transfected with inducible myc-tagged annexin XIIIb (MDCK(lac)-AnxXIIIb cells; Lecat et al., 2000), was used to determine whether annexin codistributes with endogenous Nedd4 in response to Ca^{2+}.

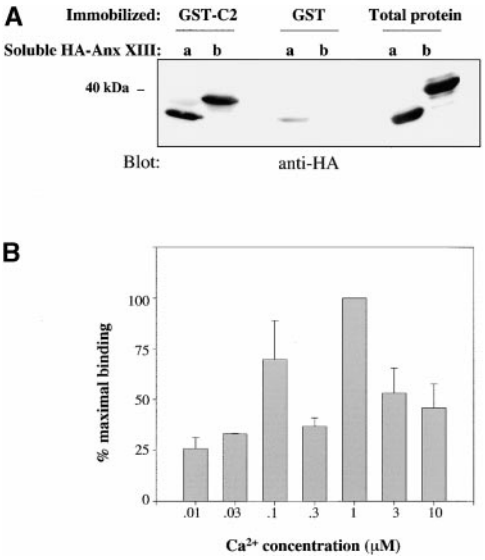

Figure 4. In vitro (direct) binding of annexin XIIIa and b and Nedd4-C2 domain. (A) Purified and immobilized GST-Nedd4-C2 (GST-C2) or GST alone (~5 μg each) was incubated with purified HA-annexin XIIIa (a) or b (b; 5 μg each) in the presence of 1 μM Ca^{2+} in the binding solution. Western blots to detect bound annexin XIIIa or b were performed using an anti-HA antibody as described in Fig. 2 above. (B) Optimal Ca^{2+} concentration for in vitro binding between annexin XIIIb and GST-Nedd4-C2 domain. The experiment described in A was repeated only in the presence of the indicated concentrations of Ca^{2+} in the binding solution. The amount of HA-annexin XIIIb bound to the GST-C2 domain was quantified using the FluoroChem 9000 system and AlphaEase FC software. Bars represent mean ± SEM of three independent experiments (expressed as a percentage of maximal binding that was observed at 1 μM Ca^{2+}).

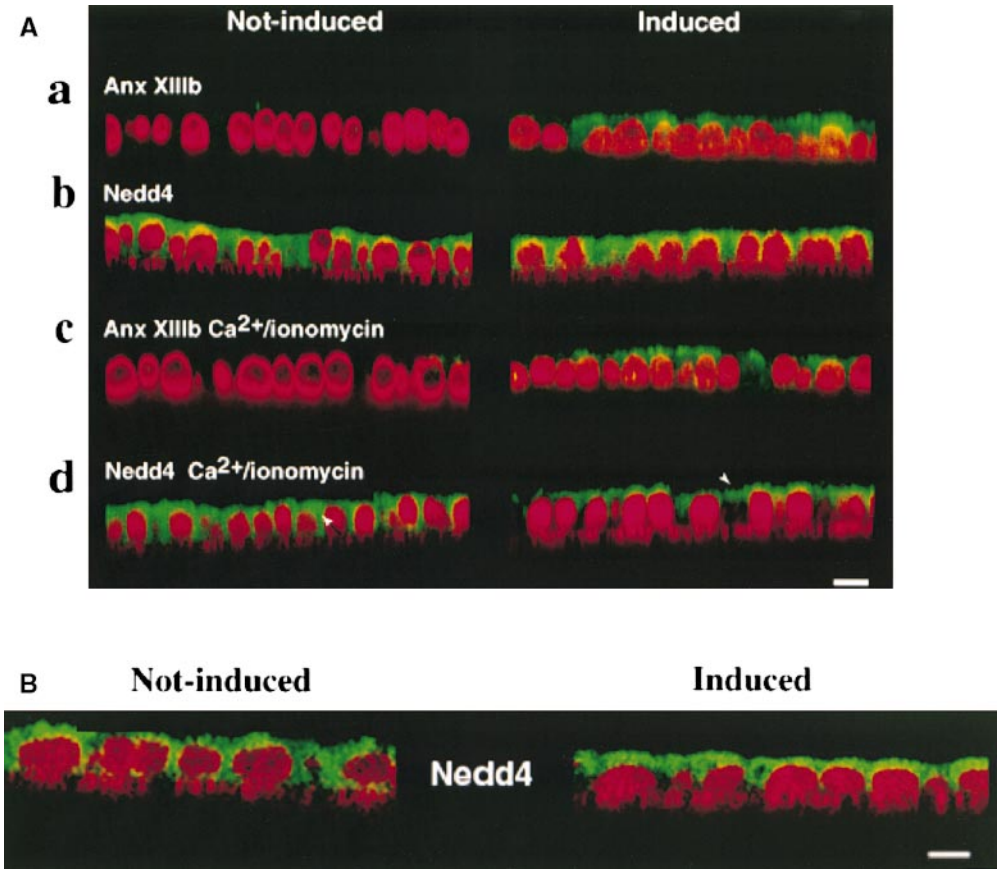

Figure 5. Ca^{2+}-dependent apical distribution of Nedd4 after induction of annexin XIIIb expression in MDCK(lac)-AnxXIIIb cells. (A) Filter-grown polarized MDCK(lac)-AnxXIIIb cells were either not induced or induced to express annexin XIIIb, and were either untreated (a and b) or treated with ionomycin/Ca^{2+} to raise intracellular Ca^{2+} levels (c and d). Cells were processed for immunofluorescence labeling using either anti–annexin XIIIb antibodies (Anx XIIIb) or anti-Nedd4 antibodies (Nedd4), both shown in green. Nuclei were stained with propidium iodide (red). X,Z confocal sections are shown. (B) Cells not induced or induced to express annexin XIIIb were preextracted with 0.01% saponin and further processed for Nedd4 immunostaining and propidium iodide staining as above. X,Z confocal sections are shown. Arrowheads in panel (A, d) point to cytosolic (left) and apical (right) regions. Bars, 15 μm.

Immunofluorescence analysis using anti–annexin XIIIb and anti-Nedd4 antibodies revealed that, in cells that were not stimulated with Ca^{2+}/ionomycin and in which annexin XIIIb expression was not induced, Nedd4 was detected in both apical and basolateral compartments (Fig. 5 A, a and b). The distribution of Nedd4 was not dramatically modified by the induction of myc-tagged annexin XIIIb expression. However, after Ca^{2+}/ionomycin treatment, Nedd4 was redistributed mainly to the apical compartment (Fig. 5 A, c and d). These results are compatible with the previously reported Ca^{2+}-dependent membrane association of Nedd4 (Plant et al., 1997). Furthermore, this distribution pattern became more obvious when cells were preextracted with mild saponin treatment to remove the cytosolic pool of Nedd4 (Fig. 5 B). Quantitation of the amount of Nedd4 at the apical versus basolateral compartments (after Ca^{2+}/ionomycin treatment), based on fluorescence intensity, revealed an apical/basolateral ratio of 2.63 ± 0.28 (mean ± SEM, $n = 9$) before induction, and 3.69 ± 0.40 ($n = 9$) after induction of annexin XIIIb expression ($P = 0.049$). Thus, these results demonstrate that after a rise in intracellular Ca^{2+} levels, Nedd4 accumulates at the apical membrane in cells in which annexin XIIIb expression is induced. This can be explained by an increase in the number of binding sites for Nedd4 because of annexin XIIIb overexpression. Therefore, in the presence of Ca^{2+}, Nedd4 staining is mostly apical, where annexin XIIIb is localized.

To follow the recruitment of Nedd4 to the apical membranes, we quantitated the amount of Nedd4 associated with membranes upon annexin XIIIb overexpression using either the MDCK(lac)-AnxXIIIb cells or adenovirus-infected cells (Lecat et al., 2000). We used immunogold electron microscopy staining on ultrathin cryosections and counted the number of gold particles associated with either annexin XIIIb or Nedd4 on surfaces located within 500 nm from the apical plasma membrane. As shown in Table I, after annexin XIIIb overexpression, using either the MDCK(lac)-AnxXIIIb cells or adenovirus-infected cells, we found that the number of annexin XIIIb–associated gold particles/μm^2 increased significantly (*t* test, $P < 0.05$), as did the number of Nedd4-associated gold particles/μm^2. Interestingly, the ratios of annexin XIIIb and Nedd4 associated with the apical membrane, both before and after annexin XIIIb overexpression, were of similar magnitude (e.g., annexin XIIIb was elevated 1.7-fold and Nedd4 1.6-fold after overexpression of annexin XIIIb using adenoviruses). These results suggest that the more annexin XIIIb present on apical membranes, the more Nedd4 is recruited there. Moreover, our data show that a large amount of Nedd4 colocalized with annexin XIIIb (Table I).

Subcellular Localization of Nedd4 in the Apical Pathway in MDCK Cells

We investigated, in greater detail, the distribution of both Nedd4 and annexin XIIIb in the apical compartment using

Table I. Quantitative Distribution of Nedd4 Associated with Apical Membranes as a Function of Annexin XIIIb Expression

	Total surface area	N	Gold particles/μm^2		Percent colocalization
			Annexin XIIIb	Nedd4	
	μm^2				
Not induced	26.3	10	7.2 ± 1.3	1.3 ± 0.3	61.0 ± 10.4
Induced	29.0	10	11.1 ± 1.1	2.8 ± 0.3	66.9 ± 3.3
Not infected	27.7	10	7.8 ± 0.9	1.6 ± 0.2	62.7 ± 9.3
Infected	28.3	10	13.5 ± 0.9	2.6 ± 0.3	61.3 ± 5.3

Double immunogold staining of annexin XIIIb and Nedd4 was performed as described in Materials and Methods. Experiments were performed with either MDCK(lac)-AnxXIIIb cells allowing inducible expression of annexin XIIIb, or adenovirus-infected MDCK cells expressing annexin XIIIb after infection. Membrane surfaces within 500 nm from the apical plasma membrane were analyzed. N, No. of cells analyzed. Colocalization was considered positive when a gold particle labeling Nedd4 was >30 nm apart from a gold particle labeling annexin XIIIb. Mean ± SEM are given.

cryo immunogold labeling. We observed colocalization (>90%) of Nedd4 with annexin XIIIb at the apical plasma membrane and in tubulovesicular structures underneath the apical surface (Fig. 6 A), suggesting they were apical carriers en route to the surface. Large (>200 nm) membranous structures resembling endosomes were also immunodecorated for both proteins (Fig. 6 B). In some cases, these large structures contained internal membranes reminiscent of multivesicular bodies, confirming their endosomal nature (Verkade, P., unpublished data). We also examined whether Nedd4 was associated with TGN membranes by testing its colocalization with the TGN resident enzyme sialyltransferase (Fig. 6 C). We did not find significant colocalization of Nedd4 and sialyltransferase, whereas we could observe clear colocalization of annexin XIIIb with this TGN marker, as previously reported (Lafont et al., 1998).

To directly visualize the localization of Nedd4 in exocytic apical carriers, we studied its distribution in TGN-derived vesicles isolated from influenza-infected MDCK cells using immunogold electron microscopy. As shown in Fig. 7, Nedd4 was present on the isolated vesicles identified by the presence of the apical marker hemagglutinin. Annexin XIIIb (5 nm) was also found associated with the apical carriers as previously reported (Lafont et al., 1998). Collectively, these results suggest that the binding of Nedd4 to annexin XIIIb occurs at an early step before the arrival at the apical plasma membrane, but after the carriers have budded off the TGN.

Annexin XIIIb Expression Enhances the Association of Nedd4 with Rafts

Since annexin XIIIb was shown previously to be associated with rafts in apical carriers (Lafont et al., 1998), we analyzed whether Nedd4 was associated with rafts as well. We first examined whether Nedd4 could be found floating in association with detergent-insoluble material. To this end, we performed quantitative analysis using the MDCK(lac)-AnxXIIIb cells that express myc-tagged annexin XIIIb in an inducible manner. In these cells, which express low levels of endogenous annexin XIIIb, Nedd4 was hardly detectable in the top fraction of the OptiPrep gradient (2% was recovered in the first top three fractions), indicating that it was not associated with light membranes that are resistant to Triton extraction (Fig. 8 A). The low amount of Nedd4 found floating most probably represents the binding to endogenous annexin XIIIb. We investigated whether the inducible expression of annexin XIIIb had an effect on the association of Nedd4 with the detergent-resistant membranes. After MDCK (lac)-AnxXIIIb, cells were induced to express annexin XIIIb, the relative amount of Nedd4 detected at the top of the gradient was clearly increased (23% recovered in the first top three fractions; Fig. 8 B). This suggests that annexin XIIIb could recruit to, or facilitate the association

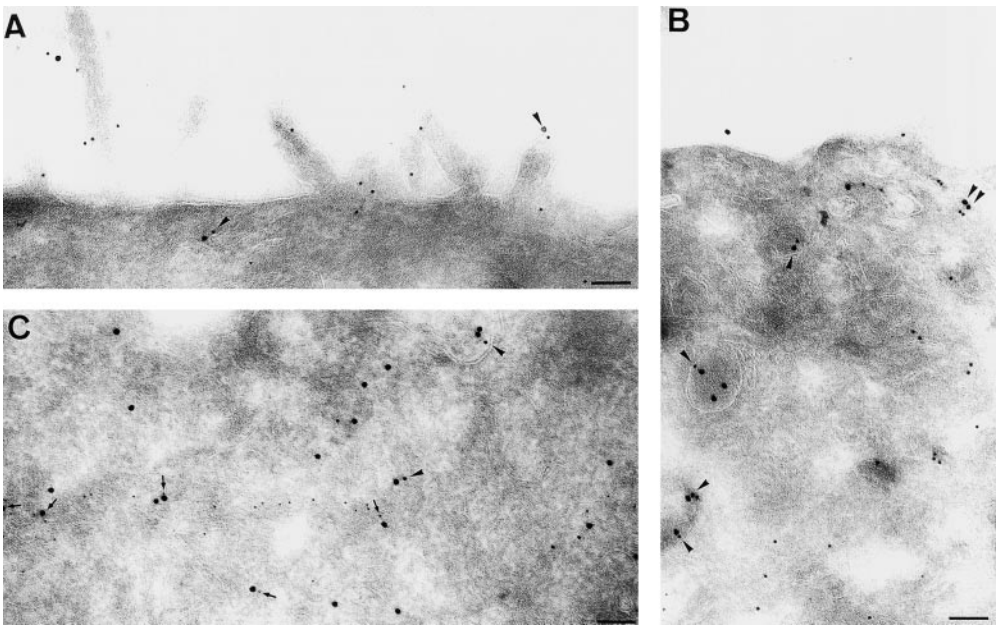

Figure 6. Colocalization of annexin XIIIb and Nedd4 in MDCK cells. Double immunostaining on ultrathin cryosections of MDCK cells showing colocalization of Nedd4 (15 nm gold) and annexin XIIIb (10 nm gold) at the apical surface (A) and in internal membrane structures of large size (B). Note, in B, the presence of membranes in the immunodecorated structures. (C) Triple labeling showing localization of sialyltransferase (5 nm gold), Nedd4 (10 nm gold), and annexin XIIIb (15 nm gold). Arrowheads depict colocalization of Nedd4 and annexin XIIIb; small arrows point to colocalization of annexin XIIIb and sialyltransferase. Bars, 100 nm.

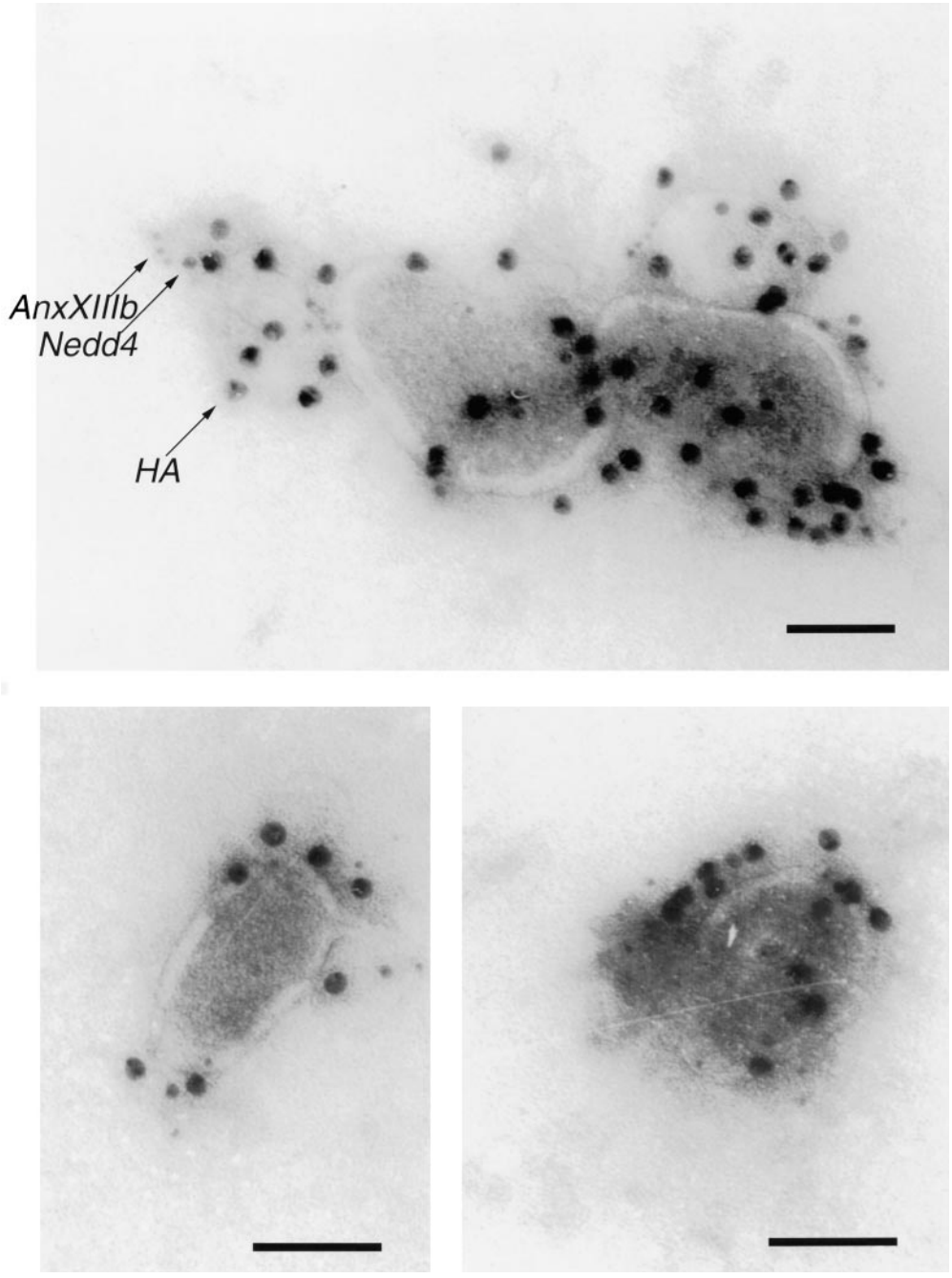

Figure 7. Nedd4 localization in apical exocytic carriers. Colocalization of HA (15 nm gold), Nedd4 (10 nm gold), and annexin XIIIb (5 nm gold) in isolated apical carriers. Bars, 60 nm.

of, Nedd4 with detergent-insoluble membranes. Annexin XIIIb overexpression did not alter the level of expression of Nedd4. To determine whether Nedd4 was raft-associated, rafts were disorganized using the cholesterol-sequestering drug MeβCD. The floatation patterns of both Nedd4 and annexin XIIIb were dramatically perturbed after MeβCD treatment, with virtually no proteins recovered in the top fractions (Fig. 8 C). Finally, the raft association of Nedd4 was examined in the absence of Ca^{2+} (Fig. 8 D). Under such conditions, as previously reported (Lafont et al., 1998), annexin XIIIb was still associated with apical rafts (27% recovered in the first top three fractions). However, Nedd4 was almost undetectable in the light fraction (6% in the first top three fractions), demonstrating that the association of Nedd4 with rafts is Ca^{2+}-dependent, which is consistent with our previous membrane binding results (Plant et al., 1997). Caveolin-1, which is a protein constitutive of, and tightly bound to, apical rafts (Scheiffele et al., 1998), was used as a positive control for raft association in these experiments.

Discussion

One of the distinguishing features of polarized epithelial cells is the compartmentalization of their plasma membrane into distinct apical and basolateral domains. These specialized domains are maintained by tight junctions and are characterized by differences in lipid and protein com-

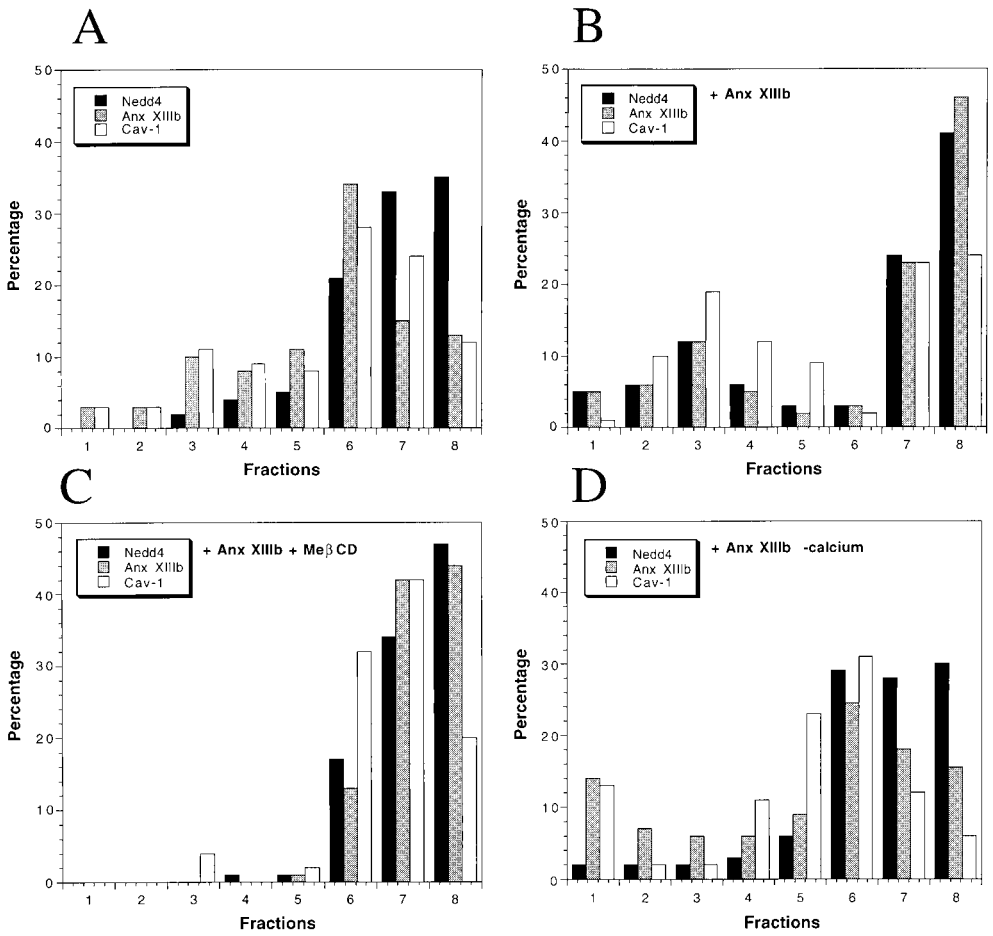

Figure 8. Ca^{2+}- and annexin XIIIb–dependent raft association of Nedd4. Control noninduced MDCK(lac)-AnxXIIIb cells (A) or cells induced overnight to express annexin XIIIb (B) were scraped, Triton X-100–extracted on ice, placed in 40% OptiPrep, and overlaid with 25 and 0% OptiPrep in Ca^{2+}-containing (TNCa) buffer. Fractions were collected from the top (fraction 1), and proteins were precipitated and analyzed by SDS-PAGE and Western blotting with either anticaveolin (Cav-1), anti–annexin XIIIb (Anx XIIIb), or anti-Nedd4 (Nedd4) antibodies. After incubation with ^{35}S-labeled secondary antibody, filters were washed and the intensity of bands was quantitated as described in Materials and Methods. (C) Induced cells were treated with the raft-disruptive agent, MeβCD, before scraping. (D) Induced cells were scraped, detergent-extracted, and floatation assays were performed in Ca^{2+}-free (TNE) buffer. A representative of six experiments is shown.

position, which are the foundations of the functional specificity of each region (Rodriguez-Boulan and Powell, 1992). The differences in composition of the domains are generally believed to originate in the TGN, where classes of proteins and lipids are included or excluded from microdomains destined for a specific membrane (Rodriguez-Boulan and Nelson, 1989; Simons and Ikonen, 1997). For example, sphingolipids and cholesterol are preferentially packaged into platforms or rafts within the TGN and targeted to the apical membrane (Simons and Ikonen, 1997; Brown and London, 1998). These transportable segments of membrane associate with specific classes of proteins that are either destined for the apical region of polarized epithelia or play an important role in raft formation.

We have shown previously that increases in intracellular Ca^{2+} prompted a redistribution of Nedd4 from the cytoplasm to the plasma membrane region of polarized epithelia, in particular, the apical region of polarized MDCK cells (Plant et al., 1997). In this study, we have established a relationship between the ubiquitin protein ligase Nedd4 and the apical raft-associated annexin XIIIb. Annexin XIIIb, which is associated with the membrane in the absence of Ca^{2+}, likely because of its myristoylation, has been shown to associate with, and proposed to be involved in, the formation of apical rafts in the TGN (Lafont et al., 1998). As such, its distribution within the cell is predominantly TGN, along the apical route in tubulovesicular structures and at the apical membrane (Lafont et al., 1998). It is likely that the Ca^{2+}-dependent association of Nedd4 with annexin XIIIb forms the basis for the partitioning of Nedd4 to the apical region of polarized MDCK cells. The finding that Nedd4 binds to, and localizes to, the same membrane compartments as annexin XIIIb in response to increases in intracellular Ca^{2+} (Fig. 5) is in agreement with the observed distribution of Nedd4 in apical carriers, at the apical plasma membrane, and in endosomal compartments, as demonstrated by immunogold electron microscopy (Figs. 6 and 7). Furthermore, overexpression of annexin XIIIb caused an increased association of Nedd4 with apical rafts, an interaction that was ostensibly Ca^{2+}-dependent because it was abrogated by the addition of the Ca^{2+} chelator EGTA.

The exact mechanisms involved in mediating this Ca^{2+}-dependent interaction between annexin XIIIb and the Nedd4-C2 domain are unknown. Structural studies of the C2 domain have shown that the coordination of the Ca^{2+} ions is provided, in part, by the side chains of the conserved aspartates (corresponding to Asp 172, 178, 230, 232, and 238 of synaptotagmin; Sutton et al., 1995; Shao et al., 1996) and in synaptotagmin and PKCα, mutation of all or several of these residues to asparagine affect the Ca^{2+}-dependent membrane binding properties of the C2 domain (Sutton et al., 1995; Medkova and Cho, 1998). In the Nedd4-C2 domain, four of the five aspartate residues (Asp 95, 101, 153, and 161) are conserved. This finding, in addition to the fact that not all C2 domains possess the full

complement of aspartates, yet still display Ca^{2+}-dependent membrane association (Nalefski et al., 1994; Gawler et al., 1995), may further reflect differences in functional specificity of the different C2 domains as well as variability in the mechanisms of Ca^{2+} coordination and Ca^{2+} affinity.

The C2 domain of Nedd4 also binds to annexin XIIIa, which is targeted to the apical membrane of MDCK cells, but is also found in basolateral rafts (Lecat et al., 2000), where it is engaged in a pathway that remains to be characterized. The higher content of annexin XIII at the apical compartment because of the presence of both annexin XIIIa and b could explain the preferential apical distribution of Nedd4. Based on the observations of Nedd4-C2 domain binding to annexin XIII (this study), and of ras-GAP-CaLB domain binding to annexin VI (Davis et al., 1996), we hypothesize that other C2 domain–containing proteins may interact with other annexins.

In addition to our demonstration of the role of the Nedd4-C2 domain in Ca^{2+}-dependent plasma membrane targeting in polarized epithelia (Plant et al., 1997, and this study), recent reports have proposed other functions for this domain, not necessarily mutually exclusive with the role in targeting we have demonstrated. For example, it has been shown that after caspase activation, during the onset of apoptosis, Nedd4 is cleaved at the NH_2 terminus, clipping off its C2 domain (Harvey et al., 1998). This cleavage may result in reduced stability of Nedd4 or affect the ability of Nedd4 to localize properly within the cell to bind its physiological targets (Harvey et al., 1998). Interestingly, the C2 domain of the *Saccharomyces cerevisiae* homologue of Nedd4, Npi1/Rsp5p, was shown recently to be important for endocytosis of the Gap1 permease (Springael and Andre, 1998). Although the C2 domain of Rsp5p is dispensable for survival in yeast (unlike the HECT domain), and is not required for ubiquitination of Gap1 per se, this finding extends the function of the C2 domain of E3 enzymes to include endocytosis of ubiquitinated substrates. In fact, the C2B domain of synaptotagmin is implicated in the regulation of endocytosis by associating with the adaptin AP-2 complex. Whether the C2 domain of Rsp5p functions in a similar manner is yet to be determined. Finally, interaction between the C2 domain of Nedd4 and the adaptor molecule Grb10, primarily via a phosphotyrosine-independent association with the Grb10-SH2 domain, has been recently described (Morrione et al., 1999). This interaction was Ca^{2+}-independent, and did not result in ubiquitination of Grb10, suggesting that it may, instead, serve to localize Nedd4 to targets of Grb10 such as the insulin receptor or the IGF-I receptor. Indeed, ubiquitination of the IGF-I receptor was recently demonstrated (Sepp-Lorenzino et al., 1995; Plant, P.J., A. Morrione, and D. Rotin, unpublished).

The ultimate consequence of Nedd4 targeting to the plasma membrane, and particularly to the apical region, is proximity to specific substrates. Such targeting would facilitate binding of Nedd4 to these substrates via its WW domains, subsequent substrate ubiquitination and likely endocytosis and degradation. If intracellular Ca^{2+} was to affect delivery of Nedd4 to its cellular targets, Ca^{2+} would be expected to affect the stability or activity of the Nedd4 targets. Indeed, whole-cell patch clamp studies of MDCK cells heterologously expressing ENaC, a known target of Nedd4 (Staub et al., 1996; Abriel et al., 1999), have revealed an inhibition of amiloride-sensitive Na^+ current through ENaC in response to the elevation of intracellular Ca^{2+} (Ishikawa et al., 1998). We do not know yet whether Nedd4 is involved in this inhibition. Likewise, the relationship of ENaC with rafts remains to be analyzed.

The finding that Nedd4 is associated with apical rafts raises the possibility that ubiquitination may play a role in regulating the dynamics of rafts. Phosphorylation has been already proposed as one parameter that controls the partitioning of proteins between raft and nonraft domains (Brown and London, 1998). Finally, as both Nedd4 and annexin XIIIb were detected in endosome-like structures, it would be interesting to examine the possible role of ubiquitination on the recycling of internalized raft-associated proteins.

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