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Differential detergent resistance of the apical and basolateral NPPases: relationship with polarized targeting

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Abbreviations: DRM, detergent-resistant membranes; GPI, glycosyl-phosphatidylinositol; NPP, nucleotide pyrophosphatase/phosphodiesterase; endo H, endoglycosidase H.

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Summary

Targeting of glycosylphosphatidylinositol-anchored proteins to the apical surface of epithelial cells involves clustering in Triton X-100-resistant membrane microdomains or rafts. The role of these microdomains in sorting transmembrane proteins is more questionable because, contrary to glycosylphosphatidylinositol-anchored proteins, apical transmembrane proteins are rather soluble in Triton X-100. They are however resistant to milder detergents like Lubrol WX or Tween 20. It has been proposed that specific membrane microdomains defined by resistance to these detergents would carry transmembrane proteins to the apical surface. We have used MDCK cells stably transfected with the apical and basolateral pyrophosphatases/phosphodiesterases NPP3 and NPP1 to examine the relationship between detergent resistance and apical targeting. The apically expressed wild-type NPP3 was insoluble in Lubrol WX whereas wild-type NPP1, which is expressed basolaterally, was essentially soluble. By using tail mutants and chimeric constructs that combine the cytoplasmic, transmembrane and extracellular domains of NPP1 and NPP3, we show that there is not a strict correlation between detergent resistance and apical targeting. Lubrol resistance is an intrinsic property of NPP3, which is acquired early during the biosynthetic process irrespective of its final destination, and depends on positively charged residues in its cytoplasmic tail.

Introduction

Detergents are amphipathic molecules that self-associate and bind to hydrophobic surfaces. Their intrinsic property forming curved micelles in aqueous solutions makes them useful for solubilizing planar biological membranes and membrane proteins by the formation of mixed micelles, often without denaturing them. Although they have proven invaluable tools for solubilizing integral membrane proteins, it has become apparent that not all detergents are equally efficient at solubilizing membranes, and that membrane proteins and lipids are differentially extracted by individual detergents (Garavito and Fergusson-Miller, 2001). These observations have provided support to the concept that membranes are not homogeneous and contain microdomains with distinct lipid and protein composition (for reviews, see Edidin, 2003; Mayor and Rao, 2004; Mukherjee and Maxfield, 2004; Pike, 2004; Simons and Vaz, 2004).

The best characterized microdomains are small entities enriched in cholesterol and sphingolipids that form liquid-ordered domains called “rafts” (Simons and Ikonen, 1997; London and Brown, 2000). These rafts domains contain a particular set of associated proteins, especially glycosyl-phosphatidylinositol (GPI)-anchored proteins and certain transmembrane proteins including the influenza viral protein hemagglutinin. Their insolubility in non-ionic detergents such as Triton X-100 at 4°C allows them to be recovered in a low-density fraction on sucrose gradients (Brown and Rose, 1992). Rafts have been involved in various cellular functions including signal transduction, immunological response and membrane trafficking. The role of rafts in membrane sorting was first hypothetized by Simons and Ikonen (1997) who proposed that raft microdomains provide a platform for intracellular sorting of apical plasma membrane proteins in epithelial cells. This hypothesis was mainly based on the observations that the glycosphingolipid glucosylceramide is preferentially targeted from the

Golgi complex to the apical membrane, and that the GPI-anchored protein alkaline phosphatase, which is apically targeted, becomes detergent-insoluble in the Golgi complex (Brown and Rose, 1992). The roles of the GPI anchor and rafts as apical determinants have been studied in details and it now appears that GPI-anchored proteins need to be stabilized in rafts by oligomerization or clustering in order to be apically sorted (Paladino et al, 2004).

However, while most GPI-anchored proteins have a preferred apical localization and are resistant to Triton X-100 extraction, many apical transmembrane proteins are soluble in Triton X-100 or only partially associated with detergent-resistant membranes (DRMs) (Danielsen, 1995). Indeed, α-helices would not easily pack into liquid-ordered domains, unless acylated (Melkonian et al., 1999; Fastenberg et al., 2003). However, apical transmembrane proteins may associate with raft microdomains by weak interactions that would not resist Triton X-100 extraction, but these interactions might nonetheless be important for sorting (Shvartsman et al., 2003). Weak interactions could be reinforced by oligomerization or clustering with sorting lectins (Fullekrug and Simons, 2004; Schuck and Simons, 2004) or by binding of the extracellular domain to a non-lectin apical sorting receptor (Marmorstein et al., 2000).

When different detergents were tested for their ability to extract transmembrane proteins, certain apical proteins proved to be soluble in Triton X-100, while insoluble in other non-ionic detergents. For example, prominin, a pentaspan apical protein localized at the tips of microvilli, is soluble in Triton X-100 but insoluble in Lubrol WX or Brij 58 (Roper et al., 2000). MDR1 is another multispanning membrane protein, which is soluble in Triton X-100 but insoluble in Lubrol WX, and whose traffic to the apical surface depends on its association with Lubrol DRMs (Aït Slimane et al., 2003). More recently, several apical proteins were shown to resist extraction by the mild detergent Tween 20 (Alfalah et al., 2005). These observations have led the authors to suggest that different types of membrane microdomains

may be used by different classes of proteins to reach the apical surface (Roper et al., 2000; Aït Slimane et al., 2003; Alfalah et al., 2005).

Our laboratory has been interested in studying the polarized targeting of two plasma membrane proteins of the ecto-nucleotide pyrophosphatase / phosphodiesterase (E-NPP) family. NPP1 and NPP3 are type II transmembrane glycoproteins. They share an overall 50% identity at the amino acid level, and yet they are differentially localized: NPP1 is at the basolateral surface and NPP3 at the apical membrane (Scott et al., 1997). We have shown that the dileucine-containing cytoplasmic amino acid sequence AAASLLAP directs NPP1 to the basolateral membrane (Bello et al., 2001), but the mechanism of apical targeting of NPP3 has not been resolved. Previous work from our laboratory showed that glycosylation is not required for apical targeting of NPP3, and that the molecule was incompletely soluble in cold Triton X-100 (Meerson et al., 2000). In this work, we have taken the nucleotide pyrophosphatases NPP1 and NPP3 as model proteins to explore the resistance to detergent extraction of typical type II transmembrane proteins and its relationship with apical/basolateral targeting.

Results

Solubility properties of NPP1 and NPP3 in cold non-ionic detergents

Previous studies have shown that only a small fraction of NPP3 was recovered in the low density fractions when Triton X-100 lysates were analyzed using a flotation equilibrium sucrose density gradient at 4°C (Meerson et al., 2000). We decided to explore this observation further using additional detergents, and compare the detergent solubility properties of NPP3 with those of the basolateral protein NPP1. Stably transfected MDCK cells, which express NPP3-WT or NPP1-WT at the apical and basolateral surfaces respectively (Fig. 2A, B) were lysed in Triton X-100 at 4°C. Fractionation of the lysates on sucrose gradients, and analysis of the fractions by immunoprecipitation and immunoblotting showed that a small amount (~8%) of NPP3-WT was associated with low density detergent-resistant complexes (fractions 3 to 6) (Fig. 2C), whereas NPP1-WT was exclusively found in the higher density region (fractions 8-12) (Fig. 2D). NPP3 insolubility was markedly higher when the cells were lysed in Lubrol WX or Brij 58 instead of Triton X-100: 75% of the protein was associated to Lubrol DRMs and 59% to Brij DRMs. By contrast NPP1-WT remained almost entirely soluble with these two detergents (93% and 85% respectively). Similar patterns were observed when the detergent concentration was increased to 1% (not shown).

Lubrol insolubility depends on the presence of cholesterol

The results presented above show that NPP3 is characterized by a strong association with Lubrol DRMs and a weak association with Triton DRMs. It has been shown that raft-associated proteins become more Triton-soluble when rafts are destabilized by removal of cholesterol (Keller and Simons, 1998). In order to determine if insolubility of NPP3 in Lubrol WX is also dependent on cholesterol, MDCK-NPP3 cells were labeled with ^{3}[H]cholesterol,

then treated with methyl-β-cyclodextrin for 30 minutes. Cells were then lysed in Lubrol WX or Triton X-100 and the lysates were resolved on sucrose gradients. Analysis of cholesterol content showed that methyl-β-cyclodextrin treatment removed ~ 48% of total cellular cholesterol (not shown). In the gradient fractions, methyl-β-cyclodextrin removed nearly 50% of cholesterol from Lubrol DRMs and ~ 40% from Triton DRMs (Fig. 3C). After methyl-β-cyclodextrin treatment and Lubrol lysis, the distribution of NPP3 spread over all the fractions of the gradient, and its association to DRMs was significantly reduced (Fig. 3A). By contrast, the small fraction of NPP3 associated with Triton DRMs was not significantly affected (Fig. 3B).

Apical expression is not correlated with DRM association

Differential solubility of NPP1 and NPP3 in detergents suggested that it might be linked to the apical/basolateral localization of the proteins. Therefore, we analyzed the detergent solubility of a NPP3 construct to which the basolateral signal of NPP1 was added (NPP3-AAASLLAP), and of NPP1 constructs in which the basolateral signal was removed or inactivated (NPP1-Δ34 and NPP1-AA) (Fig. 1) (Bello et al., 2001). NPP3-AAASLLAP was expressed at the basolateral surface (Fig. 4A). Nevertheless, this construct retained solubility properties essentially identical as NPP3-WT. It was still weakly associated with Triton DRMs and almost entirely associated with Lubrol DRMs (Fig. 4D). NPP1-Δ34, in which the first 34 N-terminal amino acids including the dileucine signal were deleted, and NPP1-AA in which the leucines 31 and 32 were changed to alanines, were mainly expressed at the apical surface (Fig. 4B, C). Both constructs were still completely soluble in Triton X-100, like NPP1-WT. However, the amount associated with Lubrol DRMs was increased from 8% for the basolateral wild-type protein to ~ 40% for the apically targeted mutants (Fig. 4E, F).

Resistance of NPP3 to Lubrol extraction is acquired before reaching the cell surface, independently of targeting

A central question concerning apical/basolateral targeting is whether association with specific membrane microdomains is the basis of segregation of apical and basolateral proteins along the biosynthetic pathway. It is therefore important to determine whether NPP3 becomes resistant to Lubrol extraction during the biosynthetic process or once it has reached the cell surface. Furthermore, the construct NPP3-AAASLLAP is especially interesting to study because it is targeted basolaterally while retaining a detergent solubility profile similar to that of NPP3-WT, which is sorted to the apical surface. Previous experiments in the laboratory have shown that NPP3 is mainly targeted directly to the apical surface of MDCK cells (Meerson et al., 2000) To determine at which step of the maturation process they became resistant to Lubrol extraction, cells expressing NPP3-WT or NPP3-AAASLLAP were metabolically labeled with [^{35}S]Met/Cys, then the lysates were fractionated on sucrose gradients and sensitivity to endoglycosidase H (endo H) was examined. Preliminary pulse-chase experiments indicated that NPP3 acquired endo H resistance between 15 and 30 minutes of chase (not shown). Cells were therefore continuously labeled for 1 hour, so that all intermediate glycosylated forms would be detected. Fig. 5 shows the processing of NPP3-WT and NPP3-AAASLLAP. In both cases, the endo H-resistant molecules were almost entirely found in the floating insoluble fractions together with some of the endo H-sensitive pool, while the soluble fractions were entirely endo H-sensitive. Since N-glycans become resistant to digestion by endo H in the medial cisternae of the Golgi apparatus, Lubrol resistance was acquired in an early Golgi compartment, the medial-Golgi at the latest. The fact that NPP3-WT and NPP3-AAASLLAP became resistant at the same stage of the maturation process indicates that acquisition of Lubrol resistance is not directly correlated with sorting to apical transport vesicles in the trans-Golgi.

The cytoplasmic tail of NPP3 confers resistance to Lubrol extraction

The above results suggested that the detergent insolubility pattern of NPP3 is an intrinsic property of the protein. In order to investigate whether it is the cytoplasmic, transmembrane or extracellular domain that makes NPP3 more resistant to detergents than NPP1, we analyzed the detergent solubility of chimeras combining different domains of NPP1 or NPP3. First, the role of the transmembrane domain was examined with the NPP1-tmNPP3 chimera which contains only the transmembrane domain of NPP3. This construct, which bears the basolateral signal of NPP1 was expressed at the basolateral surface (Fig. 6A). Despite the presence of the transmembrane domain of NPP3, the solubility pattern of this chimera was not different from NPP1-WT (Fig. 6D; compare with Fig. 2D). Addition of the extracellular domain of NPP3 in the construct NPP3-cytoNPP1 did not increase DRM association significantly (Fig. 6E). These results pointed to the cytoplasmic tail of NPP3 as being responsible for DRM association. We therefore analyzed the solubility of NPP1-cytoNPP3, which contains only the cytoplasmic tail of NPP3. This construct was expressed at the apical surface (Fig. 6C). It was preferentially associated with the Lubrol DRM fractions (Fig. 6F), indicating that the cytoplasmic domain of NPP3 confers Lubrol resistance. It was, however, poorly detected in the Triton DRM fractions, suggesting that other elements in the molecule may also contribute to the solubility pattern of NPP3.

The role of NPP3 cytoplasmic tail was further analyzed by changing certain amino acids by site-directed mutagenesis. The cytoplasmic tail, which contains 22 amino acids, is poorly conserved with NPP1 (see Fig.1). Ser^{17} lies in a consensus sequence for phosphorylation by protein kinases A and C (Kennelly and Krebs, 1991), and might therefore play a role. However, mutation of the serine into alanine did not affect Lubrol insolubility or apical targeting (not shown). The importance of Pro^{12} and the surrounding charged amino acids was

also studied. Mutation of Glu^{10}-Glu^{11} or Pro^{12} into alanines did not change NPP3 insolubility or targeting (not shown). On the other hand, a decrease of almost 50% of NPP3 Lubrol insolubility as compared to NPP3-WT (see Fig. 2C) was obtained after en-bloc mutation of Lys^{14}-Lys^{15} into alanines (Fig. 7B). Although NPP3-KK/AA remained more associated to Lubrol DRMs as compared to NPP1, it was nearly as soluble as NPP1-Δ34 or NPP1-AA. These results indicate that the lysines take part in the detergent resistance of NPP3. It must be noted that these mutations did not affect targeting of NPP3, which was still exclusively localized at the apical cell surface (Fig. 7A). Similar results were obtained when the lysines were changed to glutamate residues (not shown).

Discussion

In this work, we have used the nucleotide pyrophosphatases NPP1 and NPP3 as model proteins to explore the detergent insolubility of typical type II transmembrane proteins and the relationship with apical/basolateral targeting. These two glycoproteins were chosen because they exhibit different solubility profiles although they are structurally related, sharing 50% identity at the amino acid level. Indeed, the basolateral protein NPP1 was fully soluble in various detergents at 4°C, while the apical protein NPP3 was very poorly solubilized by Lubrol and resisted in part to Triton extraction. This result corroborates previous observations that apical and basolateral proteins differ in their detergent solubility. Typical basolateral transmembrane proteins such as the vesicular stomatitis virus G protein, the transferrin receptor, the LDL receptor or the Na^+,K^+-ATPase are fully soluble in cold Triton X-100 (Brown and Rose, 1992; Bravo-Zehnder et al., 2000; Verkade et al., 2000; Higuchi et al., 2001). By contrast, many apical transmembrane proteins are resistant, at least partially, to Triton extraction (Garcia et al., 1993; Danielsen, 1995; Aït Slimane et al., 2003), the best examples being the influenza virus proteins hemagglutinin (Scheiffele et al., 1997) and neuraminidase (Kundu et al., 1996). Furthermore, several apical transmembrane proteins that are readily soluble in Triton X-100 are resistant to milder detergents like Lubrol WX (Roper et al., 2000; Schuck et al., 2003; Aït Slimane et al. 2003), and a recent comparison showed that several apical proteins are resistant to Tween-20 extraction whereas basolateral proteins are fully solubilized (Alfalah et al., 2005).

Whether resistance to Lubrol reflects a loose association with “classical” rafts or inclusion into a subset of rafts is under debate (Schuck et al, 2003; Pike, 2004). In our experiments, DRMs prepared with Triton X-100 or Lubrol WX were similarly enriched with cholesterol (unpublished data). Association of NPP3 with Lubrol DRMs was reduced after

cholesterol depletion, indicating that NPP3 lies within membrane microdomains whose integrity depends on cholesterol. The absence of detectable change in NPP3 Triton-solubility observed after methyl-β-cyclodextrin treatment suggests that a small part of NPP3 is packed in raft cores from which cholesterol cannot be easily extracted.

The raft hypothesis suggests that membrane microdomains enriched with glycosphingolipid and cholesterol would pack together with selected proteins while other would be excluded, thus forming sorting platforms for proteins destined to the apical plasma membrane (Simons and Ikonen, 1997). This hypothesis has been difficult to prove because only few methods are available to study rafts in cells membranes, and most studies rely on the isolation of rafts on the basis of their resistance to non-ionic detergents at 4°C, and floatation at equilibrium in density sucrose gradients. The use of detergents is open to criticism because these are unlikely to reflect the exact state and composition of rafts in vivo (London and Brown, 2002; Shigomori et al., 2003). Triton X-100, which is the most widely used, perturbs pre-existing liquid-ordered domains, and probably induces coalescence or intermingling of rafts and formation of large detergent-resistant sheets (Madore et al., 1999; Heerklotz, 2002; Lichtenberg et al., 2005). On the other hand, milder detergents would poorly solubilize non raft components and would not help at discriminating raft and non raft associated membrane proteins (Schuck et al., 2002; Pike, 2004). Despite these limitations, the use of detergents is useful to study the biochemical characteristics of transmembrane proteins and their interactions with membrane lipids.

The detergent solubility properties of NPP3 were not affected by addition of the basolateral signal of NPP1 to its cytoplasmic tail, although the protein was now expressed at the basolateral surface. Therefore, insolubility of NPP3 is not directly related to its apical localization, and rather appears as being an intrinsic property of NPP3. Furthermore the basolaterally targeted NPP3-AAASLLAP construct became resistant to Lubrol extraction at

the same stage of the biosynthetic process as NPP3-WT. This result argues against implication of putative “Lubrol rafts” in driving NPP3 to the apical membrane. Alternatively, the dileucine motif of NPP1 may act as a strong signal that dictates basolateral polarity whether the protein is raft-associated or not.

If resistance to Lubrol extraction is an intrinsic property of the apical NPP3, it must be conferred by a specific sequence that is not shared by NPP1. Using NPP1/NPP3 chimeras, we showed that resistance of NPP3 to Lubrol extraction was given by its cytoplasmic domain. Detergent resistance may be influenced by lipid modifications, especially palmitoylation, which would target proteins to DRMs through partitioning of saturated acyl chains into liquid-ordered domains (Melkonian et al., 1999). Palmitoylation of NPP3 is unlikely because there is no cytoplasmic cysteine close to the transmembrane segment that could be palmitoylated. On the other hand, we found that mutation of a pair of lysines significantly increased NPP3 solubilisation by Lubrol. The localization of these lysines, at positions -8 and -9 from the transmembrane domain, i.e. close to the membrane, suggests that they may interact with the lipid bilayer. A few peptide motifs that mediate DRM association have been identified in the cytoplasmic domain of proteins. A basic peptide sequence RHRRR present in the membrane-proximal cytoplasmic domain of CD4 was identified as critical for partitioning to Triton DRMs (Popik and Alce, 2004). A less charged motif LIRW, located in close vicinity to the membrane was also identified in the MAL/VIP17 proteolipid (Puertollano and Alonso, 1998). The nature of the interacting molecules has not been identified. In the case of NPP3, it is possible that the positively charged cytoplasmic tail can interact directly with anionic lipids in the inner membrane leaflet. Alternatively, the cytoplasmic domain of NPP3 may associate with membrane lipids indirectly, by binding to DRM associated proteins.

The cytoplasmic tail of NPP1 does not bear a pair of lysines like that of NPP3, and NPP1 does not have intrinsic affinity for DRMs. Nevertheless, the NPP1-AA and NPP1-Δ34

constructs, in which the basolateral signal was removed, and which were apically sorted, were ~40% resistant to Lubrol extraction, which is less than NPP3 but more than NPP1-WT. It has been already observed that invalidation of basolateral signals from a basolateral protein results in its apical localization (Matter and Mellman, 1994; Fiedler and Simons, 1995). Based on these observations, it has been suggested that basolateral proteins bear cryptic apical sorting signals. Removal or invalidation of the dileucine motif in the NPP1-AA and NPP1-Δ34 constructs may unmask determinants of raft affinity. Alternatively, basolateral proteins lacking basolateral signal may be excluded from basolateral vesicles and embedded in apical vesicles by default. Since apical vesicles are enriched with detergent resistant lipids, such proteins would become more resistant to detergent extraction.

Materials and methods

Materials

Triton X-100, Brij-58 and methyl-β–cyclodextrin were from Sigma (St-Quentin-Fallavier, France). Lubrol WX, cell culture reagents, and PBS were from Invitrogen SARL (Cergy-Pontoise, France). Horseradish peroxidase-conjugated species-specific secondary antibodies, Protein-A and Protein-G Sepharose, ECL Plus Western blotting detection reagents, [^{3}H]cholesterol and [^{35}S]methionine/cysteine (Promix) were from Amersham Biosciences (Saclay, France). Fugene 6, endoglycosidase H and protease inhibitors were from Roche Diagnostics (Basel, Switzerland). FITC-conjugated species-specific donkey anti-mouse IgG antibodies were from Interchim (Lyon, France).

Constructs and mutagenesis

A schematic representation of the constructs is shown on Fig. 1. The engineering of pCI-neo vectors encoding rat NPP3-WT, mouse NPP1-WT, NPP1-Δ34, NPP3-AAASLLAP, NPP1-CytoNPP3, and NPP3-CytoNPP1 has been published (Bello et al., 2001; Meerson et al., 2000). The NPP1-TmNPP3 construct was generated by extraction of the sequence corresponding to the cytoplasmic domain of NPP1 and the transmembrane domain of NPP3 from the NPP3-cytoNPP1 construct, using the Nhe1 and BamH1 restriction sites. This sequence was subcloned into the NPP1-cytoNPP3 construct using the same restriction sites. Amino acids point or double mutations of the cytoplasmic domain of NPP3 at K^{14}, K^{15}, E^{10}, E^{11}, P^{12} and S^{17} were introduced using the QuikChange mutagenesis kit (Stratagene Europe, Amsterdam Zvidoost, The Netherlands). All constructs were verified by automated sequencing.

Cell culture and transfection

MDCK II cells were grown at 37°C in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a 5% CO_2/air atmosphere. Cells were transfected using Fugene 6 and stable transfectants were selected with 800 µg/ml G418 as described (Bello et al., 2001). Before the experiments, cells were treated overnight with 10 mM sodium butyrate, in order to increase the cytomegalovirus promoter transcriptional activity.

Detergent extraction, flotation gradients, and immunoprecipitation

The detergent solubility assay was performed as described by Brown and Rose (1992). MDCK cells were seeded at $2x10^3$ cells/cm^2 in 100-mm dishes and grown for 8 days, then washed and lysed on ice for 30 minutes in 2 ml TNE buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) containing 0.5 % (w/v) of either Triton X-100, or Lubrol WX or Brij-58, in the presence of a protease inhibitor cocktail. The detergent to protein mass ratio was 3:1. Lysates were passed through a 23G gauge needle to shear the DNA, mixed with 80% sucrose to a final concentration of 40% sucrose, transferred into Ultraclear tubes (Beckman Coulter France S.A, Villepinte, France) and overlaid with 4 ml of 35% sucrose and 3.5 ml of 5% sucrose prepared in TNE buffer. The flotation gradient was spun at 38,000 rpm (175,000x*g*) for 18 hours at 4°C without brake in a Beckman SW 41 rotor. Twelve fractions of 1 ml were harvested from the top of the gradient and brought to 1% Triton X-100 in the presence of antiproteases. The pellets were resuspended in 1 ml TNE-1% Triton and protease inhibitors. All fractions were sonicated and warmed to 37°C for 30 minutes to solubilize detergent-resistant membranes. The different constructs were immunoprecipitated from each fraction with Protein-A or Protein-G Sepharose beads coated respectively with B10 or IR518 monoclonal antibodies (Scott et al., 1997). After washing in TNE buffer 1% Triton X-100 and

TNE buffer, samples were processed for SDS-PAGE. Immunoblotting was performed as previously described (Scott et al., 1997) using a rabbit polyclonal anti-NPP3 antibody or the 4H4 monoclonal antibody and the ECL Plus detection kit. Bands were scanned and quantified using Image J software. Fractions 3 to 6 were considered as DRM fractions and fractions 7 to 12 as soluble fractions. Pellets did not contain significant amount of either NPP1 or NPP3.

Metabolic labeling and immunoprecipitation

Cells were starved for 1 hour in serum-free, methionine/cysteine-free medium, then metabolically labeled for 1 hour in depletion medium supplemented with 3.5 mCi of [^{35}S]methionine/cysteine (Promix). After 4 washes with cold PBS+ on ice, cells were processed for detergent extraction, flotation and immunoprecipitation as described above. Immunoprecipitates from fractions 3 to 6 were pooled to make the DRM fraction and the proteins from fractions 8 to 12 were pooled to make the soluble fraction. DRM and soluble fractions were adjusted to 50 mM sodium citrate pH 5.5, and divided in two portions for incubation with or without 2 µl endoglycosidase H (5 U/ml). Samples were incubated overnight at 37°C under agitation, and processed for SDS-PAGE and fluorography as described previously (Meerson et al., 2000).

Cholesterol radiolabeling and depletion

MDCK cells were incubated with 1 µCi/ml [^{3}H]cholesterol for 20 hours in complete culture medium, then treated with 10 mM methyl-β–cyclodextrin under agitation for 30 minutes at 37 °C, in serum-deprived medium. Cells were then processed for detergent extraction and analysis of NPP3 distribution on flotation gradients. The amount of cholesterol in the gradient fractions was quantified by counting aliquots of the fractions by liquid scintillation in a LS 6000 SC scintillation counter (Beckman Coulter France SA, Villepinte, France).

Immunofluorescence and confocal microscopy

Cells were grown to confluence for 8 days on Transwell polycarbonate filter units (0.4 µm pore size) (Costar Corp., Cambridge, MA, USA). Filter-grown cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature, permeabilized with saponin and processed for immunostaining as described (Bello et al., 2001). Confocal imaging was acquired with a LEICA TCS SP2 Laser Scanning Spectral system attached to a DMR inverted microscope with a 63/1.4 objective. Image processing was performed with the use of the on-line “Scan Ware” software. Digital images were processed with Photoshop and Image J softwares (NIMH, Maryland, USA).

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Figure Legends

Fig. 1. Schematic representation of wild-type proteins, mutants and constructs. The transmembrane and extracellular domains of NPP3 and NPP1 are drawn in black and white respectively. The amino acid sequences of the cytoplasmic tails are given in single letter code. Amino acids concerned by site-directed mutagenesis are underlined. In the NPP3-AAASLLAP construct, the sequence AAASLLAP of NPP1 was inserted after the second amino acid at the N-terminus of NPP3. Constructs are not drawn to scale.

Fig. 2. NPP3 and NPP1 differ with respect to their solubility in cold non-ionic detergents. A-B, scanning confocal microscopy pictures of MDCK cells stably transfected with NPP3 (A), or NPP1 (B) cDNAs. NPP3 and NPP1 are labeled with specific monoclonal antibodies followed by secondary FITC-conjugated antibodies. Projections (xy) and xz views are shown. Bars, 10 µm. C-D, DRM association of NPP3 and NPP1. MDCK-NPP3 and MDCK-NPP1 cell homogenates were extracted with 0.5% Triton X-100, or 0.5% Lubrol WX, or 0.5% Brij 58 at 4°C, and separated on sucrose gradients. The distribution of NPP3 (C) and NPP1 (D) was analyzed by immunoblotting after immunoprecipitation from each fraction. Typical immunoblots are shown. The diagrams represent quantifications of the DRM-associated protein (fractions 3-6) (DRM) and soluble protein (fractions 7-12) (Sol) from the blots. Results are means ± SD of at least three independent experiments.

Fig. 3. Effect of cholesterol depletion on DRM association of NPP3. MDCK cells stably transfected with NPP3-WT cDNA were labeled with [^{3}H]cholesterol for 20 hours, then treated (MβCD) or mock-treated (mock) with 10 mM methyl-β-cyclodextrin for 30 minutes before detergent extraction. A, B**,** Distribution of NPP3 along the sucrose gradient fractions after

solubilization with Triton X-100 (A) or Lubrol WX (B). Cell homogenates were detergent-extracted and separated on sucrose gradients and the distribution of NPP3 was analyzed as in Fig. 2. The diagrams represent quantifications of the DRM-associated (fractions 3-6) (DRM) and soluble proteins (fractions 7-12) (Sol). C, Cholesterol content of Lubrol WX and Triton X-100 DRMs. The amount of cholesterol in the gradient fractions was quantified by counting the radioactivity of pooled aliquots of the DRM and soluble fractions by liquid scintillation.

Fig. 4. Apical localization does not correlate with DRM association. A-C, scanning confocal microscopy pictures of MDCK cells stably transfected with the cDNAs of indicated constructs. Cells were treated as in Fig. 2. Bars, 10 μm. D-F, DRM association of NPP3-AAASLLAP (*D*), NPP1-Δ34 (E), and NPP1-AA (F). Cell homogenates were extracted and separated on sucrose gradients and the distribution of the proteins was analyzed as in Fig. 2. Typical immunoblots are shown. Diagram values are means ± SD of at least three independent experiments.

Fig. 5. Resistance of NPP3 to Lubrol extraction is acquired early, independently of targeting. MDCK cells stably expressing NPP3-WT or NPP3-AAASLLAP were labeled for 1 hour with [^{35}S]Met/Cys. Cells were lysed with Lubrol WX and separated on sucrose gradients. Each fraction was subjected to immunoprecipitation with anti-NPP3 antibody, then immunoprecipitates corresponding to the DRM and soluble fractions (Sol) were separately pooled, and treated (+) or not treated (-) with endo H, separated by SDS-PAGE electrophoresis and analyzed by fluorography. The mature (1), high-mannose (2), and deglycosylated (3) forms are indicated by arrows. The mature forms of both NPP3-WT and NPP3-AAASLLAP (band 1) migrate slightly faster after endo H treatment because not all glycan chains are complex glycosylated (Maurice et al., 1994).

Fig. 6. The cytoplasmic domain of NPP3 is responsible for Lubrol-DRM association. A-C, scanning confocal microscopy pictures of MDCK cells stably transfected with cDNAs of the indicated constructs. Cells were treated as in Fig. 2. Bars, 10 μm. D-F, DRM association of NPP1-TmNPP3 (D), NPP3-CytoNPP1 (E), and NPP1-CytoNPP3 (F). Cell homogenates were extracted and floated on sucrose gradients and the distribution of the proteins was analyzed as in Fig. 2. Typical immunoblots are shown. Diagram values are means ± SD of at least three determinations.

Fig. 7. Mutation of lysines 14-15 reduces affinity of NPP3 to Lubrol DRMs. A, scanning confocal microscopy picture of MDCK cells stably expressing NPP3-KK/AA. Cells were labeled with specific monoclonal anti-NPP3 antibody followed by secondary FITC-conjugated antibody. Bar, 10 μm. B, DRM association of NPP3-KK/AA. Cell homogenates were extracted and separated on sucrose gradients and the distribution of the proteins was analyzed as in Fig. 2. Typical immunoblots are shown. Diagram values are means ± SD of two determinations.

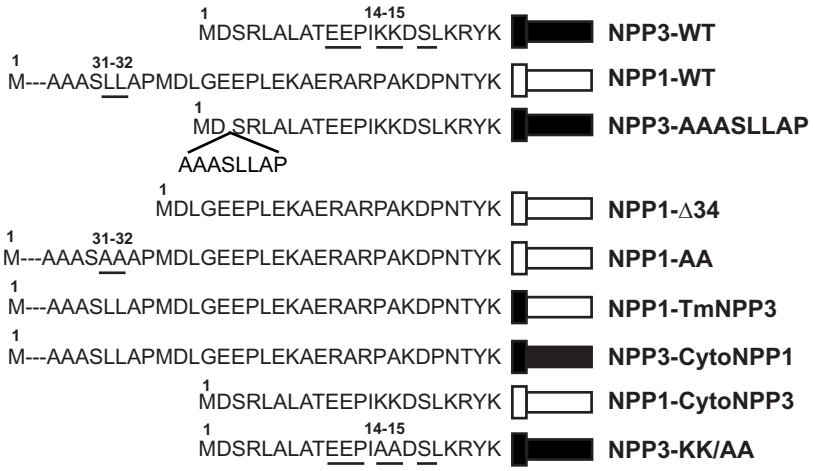

1
14-15
MDSRLALATEEPIKKDSLKRYK
NPP3-WT
1
31-32
M---AAASLLAPMDLGEEPLEKAERARPAKDPNTYK
NPP1-WT
1
MDSRLALATEEPIKKDSLKRYK
NPP3-AAASLLAP
AAASLLAP
1
MDLGEEPLEKAERARPAKDPNTYK
NPP1-Δ34
1
31-32
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1
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NPP1-TmNPP3
1
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NPP3-CytoNPP1
1
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14-15
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NPP3-KK/AA

Figure 1

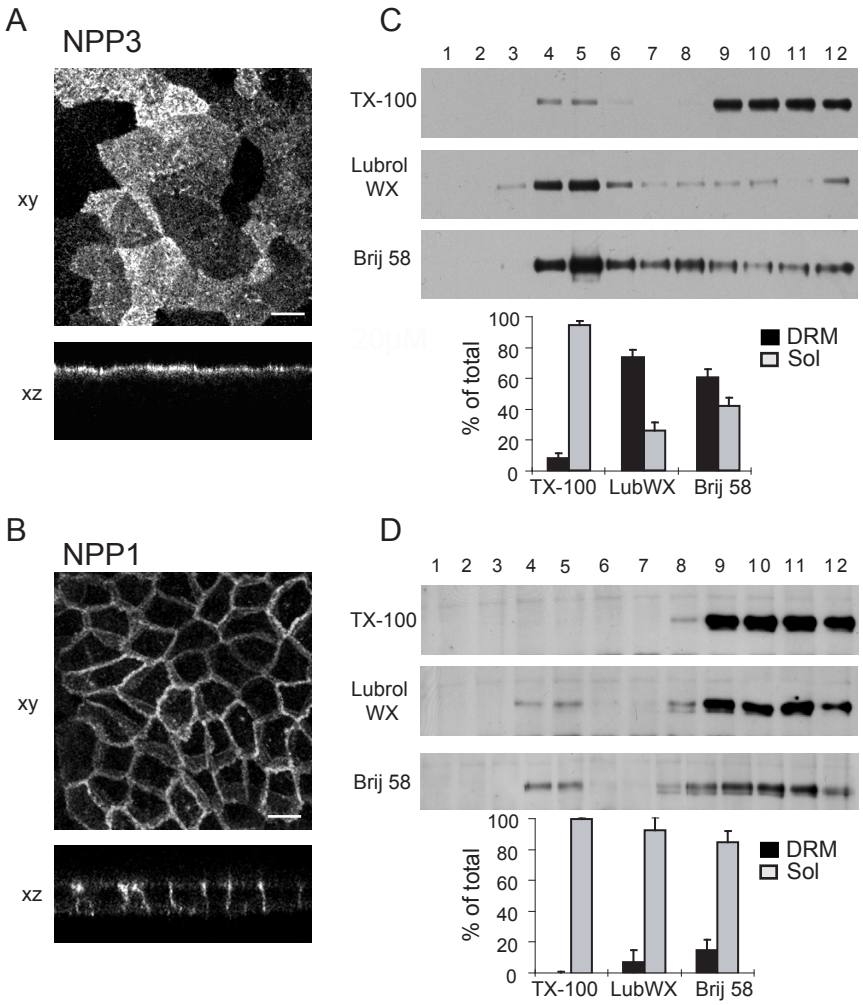

A
NPP3
xy
xz
B
NPP1
xy
xz
C
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
Brij 58
% of total
100
80
60
40
20
0
DRM
Sol
TX-100
LubWX
Brij 58
D
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
Brij 58
% of total
100
80
60
40
20
0
DRM
Sol
TX-100
LubWX
Brij 58

Figure 2

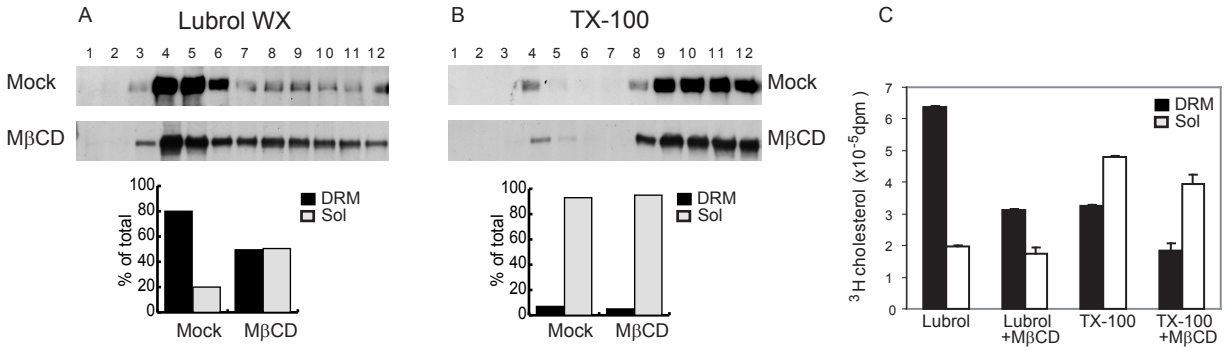

A
Lubrol WX
1 2 3 4 5 6 7 8 9 10 11 12
Mock
MβCD
100
80
60
40
20
0
% of total
DRM
Sol
Mock
MβCD
B
TX-100
1 2 3 4 5 6 7 8 9 10 11 12
Mock
MβCD
100
80
60
40
20
0
% of total
DRM
Sol
Mock
MβCD
C
7
6
5
4
3
2
1
0
3H cholesterol (x10-5dpm)
DRM
Sol
Lubrol
Lubrol +MβCD
TX-100
TX-100 +MβCD

Figure 3

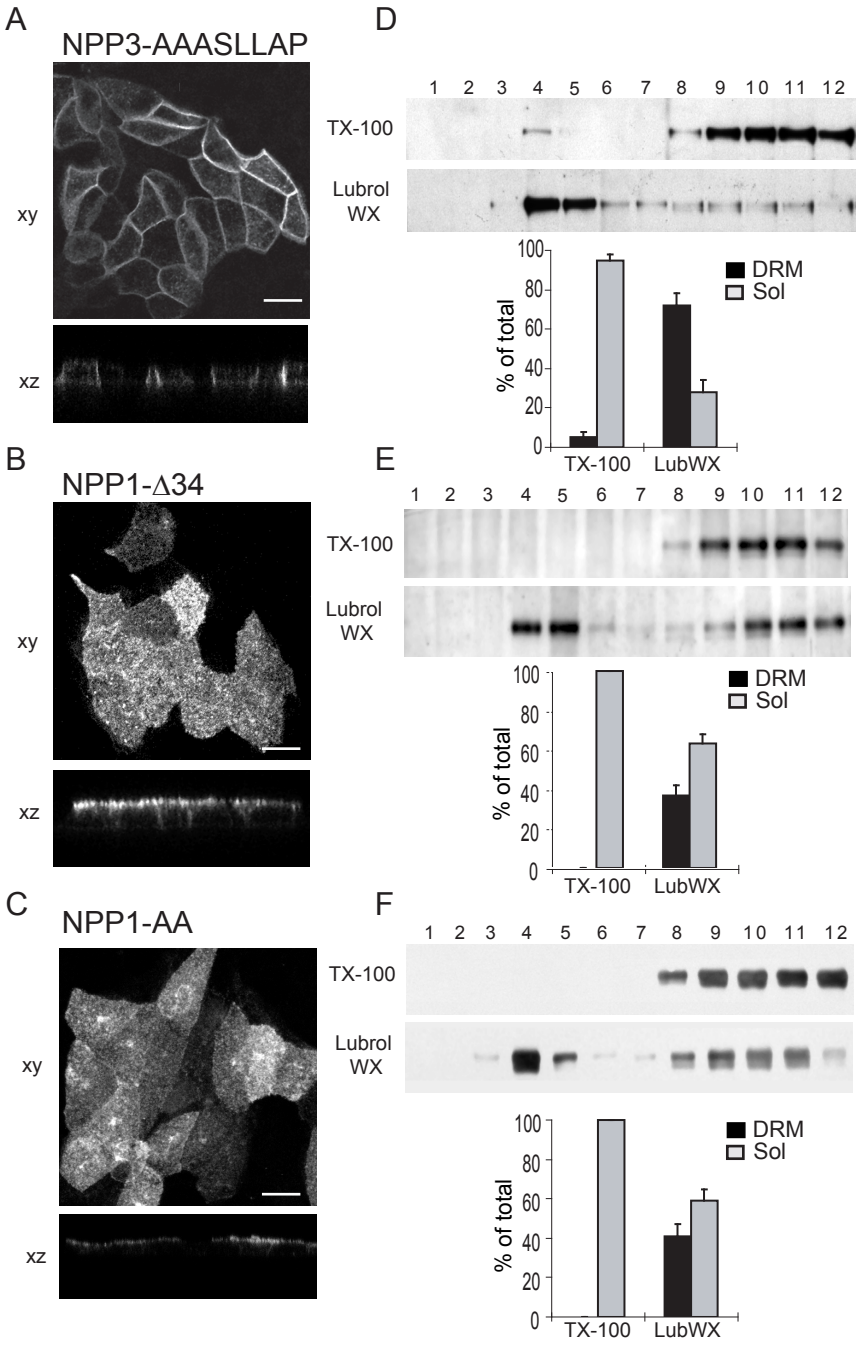

A
NPP3-AAASLLAP
xy
xz
B
NPP1-Δ34
xy
xz
C
NPP1-AA
xy
xz
D
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
DRM
Sol
% of total
100
80
60
40
20
0
TX-100
LubWX
E
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
DRM
Sol
% of total
100
80
60
40
20
0
TX-100
LubWX
F
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
DRM
Sol
% of total
100
80
60
40
20
0
TX-100
LubWX

Figure 4

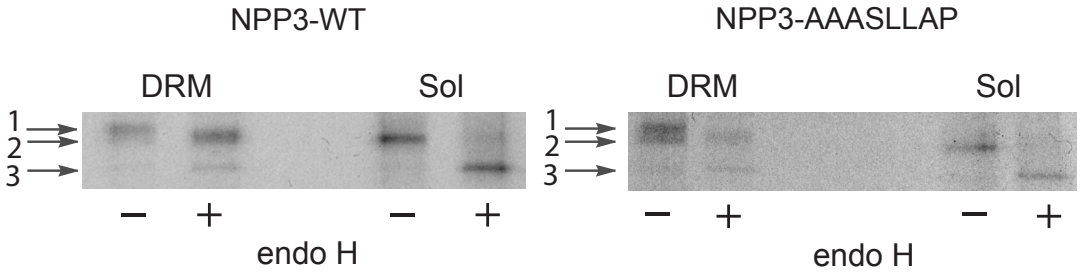


Figure 5

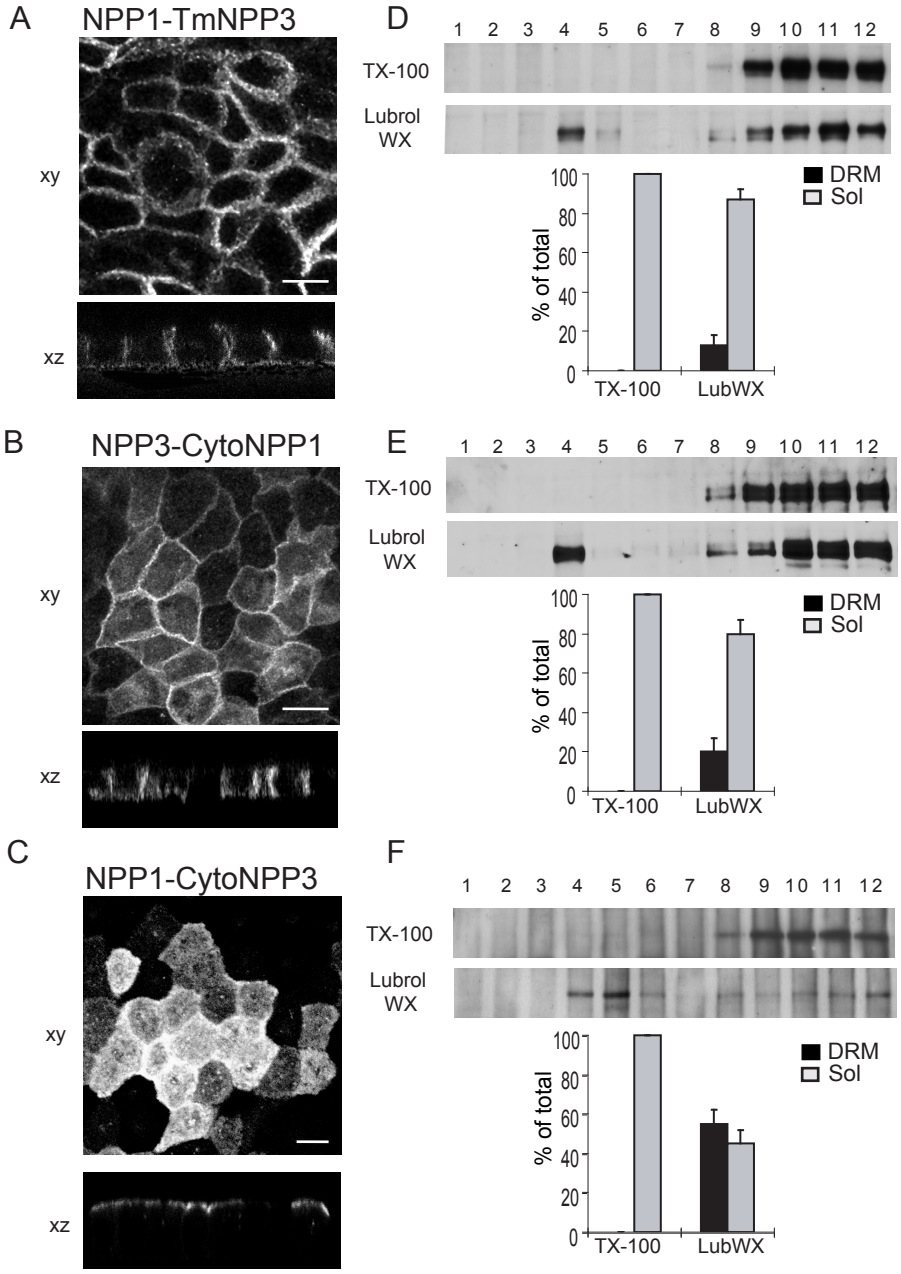

A
NPP1-TmNPP3
xy
xz
B
NPP3-CytoNPP1
xy
xz
C
NPP1-CytoNPP3
xy
xz
D
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
DRM
Sol
% of total
100
80
60
40
20
0
TX-100
LubWX
E
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
DRM
Sol
% of total
100
80
60
40
20
0
TX-100
LubWX
F
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
DRM
Sol
% of total
100
80
60
40
20
0
TX-100
LubWX

Figure 6

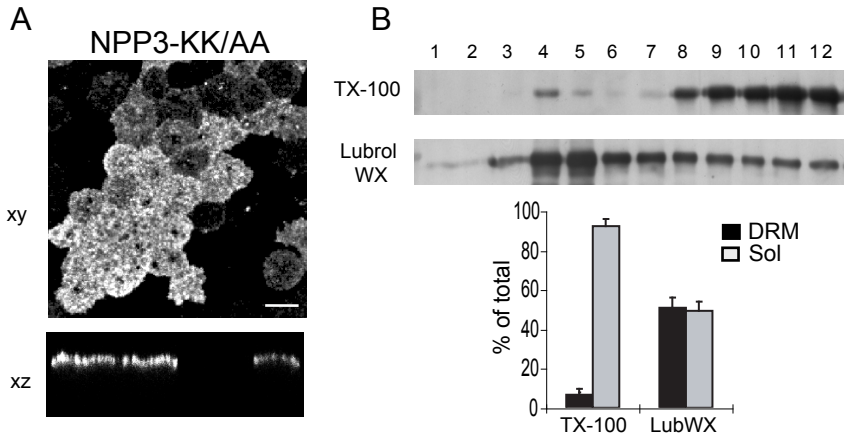

A
NPP3-KK/AA
xy
xz
B
1 2 3 4 5 6 7 8 9 10 11 12
TX-100
Lubrol WX
100
80
60
40
20
0
% of total
DRM
Sol
TX-100
LubWX

Figure 7