Role of the glycosyl-phosphatidylinositol anchor in the intracellular transport of a transmembrane protein in Madin-Darby canine kidney cells

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Abstract

In order to compare the trafficking of proteins with different membrane anchors, we have constructed and expressed three different recombinant forms of neutral endopeptidase (NEP) in MDCK cells. The wild type form of NEP (WT-NEP) is attached to the plasma membrane by a single N-terminal membrane spanning domain, whereas the glycosylphosphatidylinositol-anchored form of the protein (GPI-NEP) contains a C-terminal GPI anchor. A double anchored form of NEP (DA-NEP) was also constructed, that contains both the original N-terminal membrane spanning domain and a C-terminal GPI anchor. We show here that WT-NEP, GPI-NEP and DA-NEP, which are all apically targeted in MDCK cells, behave differently when subjected to Triton X-100 solubilisation: despite the presence of the transmembrane anchor DA-NEP behaves as a GPI-anchored protein. This suggests that the GPI anchor of DA-NEP is dominant over the transmembrane anchor of the native protein to determine its pattern of solubility in Triton X-100. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Polarised epithelial cells are characterised by the existence of tight junctions that join adjacent cells and separate their plasma membrane into distinct apical and basolateral domains. This cell polarity is necessary to the diverse functions of secretion, absorption, and ion transport in epithelia [1,2]. In addition to its specific protein composition, the apical membrane is also enriched in sphingolipids, especially glycosphingolipids [3]. This particular lipid composition serves as a protection against harsh environmental conditions.

Madin-Darby canine kidney cells (MDCK) pro-
vide a useful model for the study of proteins and lipids trafficking in epithelial cells. In these cells, apical and basolateral membrane proteins are confined to separate transport vesicles that emerge from the TGN to reach the appropriate membrane domain [1,4].

Membrane proteins that are exposed to the cell surface can be anchored in the plasma membrane either by one or more hydrophobic amino acid segments or a glycosyl-phosphatidylinositol (GPI) anchor [5–7]. In the latter case, proteins have been shown to be targeted to the apical membrane of epithelial cells [8] in a vast majority of epithelial cell lines, including MDCK cells. During their sorting to the apical membrane, it has been suggested that GPI-anchored proteins associate in the Golgi apparatus with domains or rafts that are enriched in glycosphingolipids, sphingomyelin and cholesterol [9,10]. In MDCK cells these domains can be isolated by virtue of their low density and their insolubility in Triton or CHAPS at low temperature [11,12]. From the TGN, these glycolipid rafts are thought to bud into vesicles that are stabilised by intermolecular hydrogen bonds and carry the GPI-anchored proteins to the apical surface [10,13]. In MDCK cells these rafts were shown to be distinct from the basolaterally directed vesicles [4].

Here we wanted to determine the importance of the GPI anchor for Triton X-100 solubility and transport to the cell surface. For that purpose we compared the behaviour of variants of neprilysin (EC 3.4.24.11, neutral endopeptidase, NEP) differing by their membrane anchor. NEP is a major component of the brush border membrane of epithelial cells of the kidney proximal tubule and intestine (reviewed in [14]). In its native state wild-type NEP (WT-NEP) is a type II membrane protein anchored by a hydrophobic segment located at its N-terminus. GPI-anchored NEP (GPI-NEP) is a constructed chimaeric form of NEP lacking the original N-terminus hydrophobic segment, and attached to the cell membrane by a C-terminus GPI anchor [15]. Double anchored NEP (DA-NEP) contains both the N-terminal transmembrane anchor of WT-NEP and a C-terminal GPI anchor [16,17]. DA-NEP constitutes a unique protein model, which allows monitoring directly the effect of a GPI anchor when added to a transmembrane protein. This strategy can thus distinguish between the effects of the removal of the transmembrane anchor and those of the GPI addition, contrary to previous reports on the targeting of a chimaeric GPI anchored protein [18].

As expected, the three chimaeric enzymes were targeted to the apical membrane of MDCK cells. They also were shown to have an enzymatic activity comparable to that of the native membrane-bound NEP purified from kidney brush border membranes [15,16,19,20]. Moreover, at the steady state, WT-NEP was recovered in fractions of sucrose gradients corresponding to Triton X-100-soluble proteins, whereas GPI-NEP and DA-NEP partitioned in light fractions together with Triton X-100-insoluble proteins. In pulse-chase studies WT-NEP, GPI-NEP and DA-NEP were initially found as soluble proteins. Both GPI-NEP and DA-NEP acquired Triton-insolubility only after a chase time corresponding to transfer of newly synthesised molecules to the Golgi apparatus. When chase incubation was repeated at 20°C to prevent targeting of newly synthesised molecules to the cell surface, GPI-NEP and DA-NEP were still recovered as Triton X-100-insoluble proteins. Our results suggest that Triton insolubility is acquired only when GPI-anchored forms of NEP reach an intracellular compartment tentatively identified here as the Golgi apparatus.

2. Experimental procedures

2.1. Expression vectors and cell lines

Construction of the vectors pReCMVGPINEP and pReCMVDANEPE encoding respectively GPI-NEP and the DA-NEP is described in [16]. They were transfected into MDCK cells using the CaPO₄ precipitation method [21]. The neomycin analogue G418 was used to select pools of resistant MDCK cells. The isolation of MDCK cells expressing GPI-NEP or DA-NEP was then performed with a Fluorescence-Activated Cell Sorter as described in [22]. Construction of the stable cell line expressing WT-NEP is described in [22].

2.2. Culture of MDCK cells

MDCK cells were cultured as described in [22]. All
the experiments on transfected cells were performed within 50 subsequent passages. Before each experiment, cells were incubated overnight in medium containing 10 mM sodium butyrate to enhance the production of NEP.

2.3. Immunofluorescence

The guinea pig polyclonal antibody raised against rabbit NEP was described earlier [23]. The antibody recognises WT-NEP, GPI-NEP and DA-NEP. The monoclonal anti-NEP antibody 2B12 recognises an

![Images of WT-NEP, GPI-NEP, and DA-NEP localization in MDCK cells.](image)

Fig. 1. Localisation of transfected NEP in MDCK cells: vertical optical sections. MDCK cells expressing either WT-NEP, GPI-NEP or DA-NEP, as indicated at the top of the figure, were grown on filters to form a polarised monolayer, fixed, and stained immunocytochemically for NEP, using antibodies that recognise both forms of the protein, or for E-cadherin. Scale bar: 20 μm. (A) The cells were labelled with a monoclonal anti-NEP antibody followed by labelling with FITC-conjugated anti-mouse IgG (green). For a better definition of the cell orientation, nuclei were stained with propidium iodide (red). (B) The cells were labelled with a monoclonal anti-cadherin antibody revealed with FITC conjugated anti-mouse IgG (green) and propidium iodide for visualisation of the nuclei (red). (C) For a better definition of the apical versus basolateral membrane, the cells were labelled with guinea pig polyclonal anti-NEP antibody (green) and co-stained for the lateral protein E-cadherin (red).
extracellular conformational epitope on rabbit NEP [24]. The monoclonal antibody against the lateral-membrane E-cadherin was a generous gift of Dr. I.R. Nabi (University of Montreal). Rhodamine-conjugated anti-guinea pig antibody was purchased from Jackson Labs. FITC-conjugated anti-guinea pig antibody came from Chemicon. Anti-mouse FITC- or rhodamine-conjugated antibodies were from Dakopatts a/s. Indirect immunofluorescence staining was performed on cells grown to confluence in Costar Transwell cell culture chambers essentially as described [25]. Filters were visualised in a dual channel laser scanning confocal microscope (Scanning confocal microscope Bio-Rad MRC 600 Ar-Kr laser).

2.4. Cell-surface immunoprecipitation and pulse-chase studies

MDCK cells expressing various recombinant forms of NEP were metabolically labelled with Trans[^35S] label (ICN biomedicals) and cell-surface immunoprecipitation was performed exactly as described previously [15,22]. Treatment with endo-β-N-acetyl-glucosaminidase H (Endo H: New England Biolabs) was performed as described by the manufacturer.

2.5. Sucrose gradients

Sucrose gradient analysis of Triton X-100-insoluble residue was performed as previously described [11]. Fractions of 1 ml were harvested from the top of the gradient. NEP was then immunoprecipitated from each fraction using the polyclonal antibody against NEP before being subjected to SDS-PAGE. Radiolabelled proteins were visualised by fluorography. For non-radioactive proteins Western blotting was performed with a monoclonal anti-(rabbit NEP) antibody that recognises all the different forms of the NEP.

3. Results

3.1. Apical expression of WT-NEP, GPI-NEP and DA-NEP in MDCK cells at steady state

Confocal immunofluorescence microscopy of MDCK cells grown on filters reveals a clear apical localisation for WT-NEP, GPI-NEP and DA-NEP (Fig. 1A,C). E-cadherin is visible exclusively on the lateral membrane of MDCK cells (Fig. 1B,C). Negative controls were done by omitting the primary antibody against either NEP or E-cadherin and showed no labelling of these proteins for any of the three stable cell lines (data not shown), thus confirming the specificity of the antibodies used. Moreover, as labelling was performed after permeabilisation of the cells, a lack of intracellular staining indicates that at the steady state, the vast majority of these three forms of NEP are present at the apical surface of MDCK cells.

3.2. WT-NEP, GPI-NEP and DA-NEP transit rapidly from the Golgi apparatus to the cell surface

To determine the kinetics of the intracellular transport of the various forms of NEP from their site of synthesis in the rough ER to the cell surface, cells were pulsed for 15 min in medium containing [^35S]Met and chased as indicated in Fig. 2. NEP immunoreactive material was immunoprecipitated, and treated with Endo H. NEP-related molecules were then analysed by SDS-PAGE. Fig. 2 shows that for WT-NEP and DA-NEP, Endo H resistance is observed between 20 and 40 min after the beginning of the chase. At that time it is thus likely that most of these NEP-related molecules have passed through the Golgi apparatus where Endo H resistance is ac-

![Fig. 2. Kinetics of passage through the Golgi apparatus of WT-NEP, GPI-NEP and DA-NEP. MDCK cells expressing either WT-NEP, GPI-NEP or DA-NEP were labelled with[^35S]methionine for 15 min before being chased for the indicated times. Cells were then solubilised in 1-O-α-octyl-β-D-glucopyranoside and NEP was immunoprecipitated from the lysates, before being subjected to Endo H digestion. Proteins were resolved by SDS-PAGE and visualised by fluorography.](image-url)
quired. Fig. 2 also shows that for GPI-NEP, Endo H resistance first appears after 40 min of chase. However, an important proportion of the protein is still sensitive to Endo H after 180 min of chase, suggesting that the protein is trapped somewhere between its site of synthesis in the ER and the cis-Golgi where complex glycosylation occurs.

To determine the kinetics of appearance of WT-NEP, GPI-NEP and DA-NEP at the cell surface, MDCK cells were pulsed for 15 min in medium containing \(^{35}\text{S}\)Met and chased at 37°C as indicated in Fig. 3 before being subjected to cell-surface immunoprecipitation. For both WT-NEP and DA-NEP, the protein was found to appear at the cell surface between 20 and 40 min after the beginning of the chase. By comparing the time of transport through the Golgi apparatus with that of cell-surface delivery, it can be seen that transport from the Golgi to the cell surface of these two forms of NEP occurs very rapidly in MDCK cells. The same experiment was performed on MDCK cells expressing GPI-NEP. Fig. 3 shows that this protein appears at the cell surface only between 40 and 60 min after the beginning of the chase. Thus, although GPI-NEP seems to be retained in the ER longer than WT-NEP and DA-NEP, it appears at the cell surface almost immediately after its mature form is detected. As can be observed on the bottom part of Fig. 3, the apparition of cell-surface material is simultaneous with a diminution of the intracellular immunoreactive proteins for the three chimeras.

In the previously reported secNEP construct, the original transmembrane domain of WT-NEP was replaced by the pro-opiomelanocortin signal peptide. The resulting chimaeric protein retains full enzymatic activity, and is apically secreted in MDCK cells [23,26]. The maturation and cell-surface delivery of both secNEP and GPI-NEP were found to be slower than for WT-NEP. Thus it seems that the presence of the N-terminus transmembrane anchor of NEP would be necessary for either the proper folding of the protein or its eventual association with a chaperone protein in the ER. Moreover, as was already discussed by Howell et al. [15], the addition of the GPI anchor reverses the topology of the ectodomain. This too may result in a longer time for the correct
folding of the protein and hence for its exit from the ER.

3.3. DA-NEP and GPI-NEP associate with low density fractions at steady state whereas WT-NEP is recovered with the soluble proteins

To study the detergent partition of the different forms of NEP at the cell surface, MDCK cells expressing either WT-NEP, GPI-NEP or DA-NEP were grown to confluence before being lysed in Triton X-100 and subsequently fractionated on a sucrose gradient as described in Section 2. An aliquot of each fraction was then subjected to SDS-PAGE and Western blotting using NEP-specific monoclonal antibody 18B5 [27]. As seen in Fig. 4, WT-NEP is recovered in the higher density fractions of the gradient together with Triton X-100-soluble proteins. In contrast, GPI-NEP and DA-NEP, which both contain a GPI anchor, partition in the light fractions where the GPI-anchored proteins that associate with low-density lipids are usually recovered [11,18,28–30]. The behaviour of DA-NEP indicates that the GPI anchor is the dominant determinant for the partition of NEP into low-density fractions after Triton X-100 solubilisation at low temperature.

At that stage we cannot exclude the possibility that the behaviour of these chimaeric forms of NEP is an artefact of the extraction procedure due to some peculiar physico-chemical properties of the chimaeric proteins. To rule out this possibility, we decided to study the partition of newly synthesised NEP molecules at different stages of their intracellular transport. Indeed, GPI addition occurs in the rough ER within minutes after translocation of the newly synthesised molecule [31–33] whereas detergent insolubility is acquired only when the protein reaches the glycolipid rafts in the Golgi complex [11,10].

3.4. Newly synthesised DA-NEP is soluble in Triton X-100 when still in the rough ER

Cells expressing either WT-NEP or DA-NEP were pulsed for 15 min in medium containing [35S]Met and immediately lysed in Triton X-100 at 4°C. Cell lysates were centrifuged on sucrose gradients and
NEP-related molecules were immunoprecipitated from each fraction. Immunoprecipitates were then analysed by SDS-PAGE and fluorography. After the 15 min pulse, WT-NEP and DA-NEP were found to contain Endo H sensitive oligosaccharide side chains (see Fig. 2). Fig. 5 shows that at that stage, WT-NEP and DA-NEP are both recovered from bottom of the gradient, colocalising with the Triton X-100-soluble proteins.

3.5. The insolubility of DA-NEP in Triton X-100 appears in the Golgi apparatus

It was previously observed that by cultivating MDCK cells at reduced temperature (20°C) the transport of proteins is blocked in the Golgi, whereas their glycosylation program was at least partially completed [34].

Cells were pulsed for 15 min in medium containing [35S]Met at 37°C, and chased for 90 min at 20°C. Cell-surface immunoprecipitation in these conditions shows that WT-NEP and DA-NEP were still localised inside the cell (Fig. 6A). Separation of Triton X-100 lysates on sucrose gradients shows that WT-NEP colocalises with Triton X-100-soluble proteins in the bottom fractions of the sucrose gradient whereas DA-NEP is found in the light fractions (Fig. 6B). This pulse-chase experiment was repeated in the same conditions except that the chase incubation was done at 37°C. In this case WT-NEP colocalises with Triton-soluble proteins whereas DA-NEP migrates to the light fractions of the gradient, with Triton-insoluble proteins (Fig. 6C). Under those conditions proteins have reached the cell surface and exhibit the same behaviour as non-labelled molecules previously recovered at the steady state.

3.6. GPI-NEP behaves like DA-NEP

In order to verify that the behaviour observed for DA-NEP was a direct consequence of the presence of the GPI anchor, we performed the same type of experiments with the MDCK cell-line stably expressing GPI-NEP. As a consequence of the delayed cell-surface delivery for this form of NEP, we had to treat the immunoprecipitated material with Endo H to determine the form of the protein that was present at each time-point throughout the kinetic experiment. At the end of a 15 min pulse, with [35S]Met, immediately followed by lysis of the cells in Triton X-100 at 4°C, fully Endo H-sensitive GPI-NEP was recovered in the high density fractions of the gradient (data not shown), as was previously observed for both WT-NEP and DA-NEP (see Fig. 5).

After an additional 90 min chase at 37°C, part of GPI-NEP is still Endo H sensitive (see Fig. 2), suggesting these molecules have not yet reached the cis-Golgi. These proteins however are fully soluble in Triton X-100 and consequently localise at the bottom of the gradient. Moreover, when the proteins acquire Endo H resistance, they shift from the high-density to the low-density fractions of the sucrose gradients (from lanes 10–12 to lanes 4–5 in Fig. 7). Thus the association of GPI-NEP with the low-density material occurs shortly after the acquisition of complex carbohydrate side chains in the Golgi apparatus, as indicated by the presence of high molecular weight bands in lanes 10–12 (Fig. 7).

4. Discussion

Glycosphingolipids (GSL) are synthesised in the Golgi complex and must be delivered preferentially to the apical membrane of epithelial cells to achieve their polarised cell-surface distribution [13]. It has been proposed that they associate in the Golgi apparatus and form microdomains called glycolipid rafts [10,35]. These glycolipid rafts have been claimed to be indistinguishable from the Triton X-100-insoluble structures recovered in the lighter fraction of sucrose density gradients after cell fractionation [1,13]. GPI-anchored proteins have been proposed to associate
with these microdomains via their GPI moiety [10]. These clusters would then bud off from the TGN membrane to serve as transport vesicles to the apical membrane [1,10,35]. It is still unclear whether the same targeting mechanisms apply for both GPI- and peptide-anchored apical proteins.

Several observations support this theory. Arreaza and Brown [18] have shown that GPI-anchored placental alkaline phosphatase (PLAP) associates with Triton-insoluble glycolipid rafts in MDCK cells whereas transmembrane forms of PLAP do not appear to associate with GSL-enriched microdomains. More recently, Graichen et al. have shown that gp80 (clusterin), an apically secreted glycoprotein, is not included in detergent-insoluble complexes in MDCK cells [29]. Furthermore, in FRT cells, which target GPI-anchored proteins preferentially to the basolateral surface, gp80 was shown to be secreted apically. Finally, GPI-anchored proteins but not transmembrane apical proteins have been shown to be mis-sorted in FRT cells [17] and in concanavalin A-resistant MDCK cells [28]. Taken together, these results suggest the existence of distinct intracellular routes for GPI-anchored proteins, transmembrane proteins, and secreted glycoproteins.

However, the structural determinants responsible for sorting proteins into different transport vesicles are still unknown. To better understand the mechanisms leading to the choice of one particular vesicle, we decided to study the transport of different forms of NEP to the cell surface of MDCK cells.

The results presented in this paper show that GPI-NEP and DA-NEP both behave as previously studied GPI-anchored proteins [11,28] with regard to their solubility in Triton X-100 and migration in sucrose gradient after equilibrium sedimentation. As DA-NEP contains both a transmembrane and a GPI anchor, these results suggest that the GPI anchor constitutes the predominant element to determine solubility in Triton X-100 and association with low density material in sucrose gradients. In our study, DA-NEP and WT-NEP differ only by the presence of the GPI anchor. The strategy developed in this paper thus allowed to rule out any effect due the removal of the transmembrane anchor of the reporter protein.

Many argue that the separate phases observed after Triton X-100 extraction might not exist in membranes until detergent exposure. In that case, the differences observed in their behaviour after solubilisation in Triton X-100 could simply reflect differences in their physicochemical properties once they have entered the TGN where they can or not associate with other membrane proteins and lipids that cluster in this compartment [28]. However, a recent paper by Schroeder et al. [36] provides substantial evidence that detergent extraction does not induce the artifactual formation of ordered domains. In view of these recent results, it is most likely that the partitioning of both GPI-NEP and DA-NEP in Triton X-100-insoluble fractions reflects their physiological association in specialised domains of the Golgi membrane. These domains could bud off into apical transport vesicles that would be different from the ones used by WT-NEP. Isolation of the TGN-derived vesicles containing either DA-NEP or WT-NEP, and further analysis of the factors that regulate the transport of these two proteins would make it possible to confirm this hypothesis beyond any doubt. In this respect, monoclonal antibodies that recognise the cytosolic region present in both NEP and DA-NEP [37] would constitute very useful tools for the isolation of these vesicles.

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