Neutral endopeptidase (NEP, EC 3.4.24.11) is a type II membrane protein that possesses a large extracellular domain anchored to the plasma membrane by a N-terminal transmembrane spanning domain (for review see [1]). Various molecular forms of NEP have been produced that differ in the type of anchoring mechanism(s) used to attach the protein to the membrane (reviewed in [2]). Briefly, a soluble form of NEP (sNEP) was produced by replacing the transmembrane region and short cytoplasmic domain with a cleavable signal peptide [3]. A glycosyl-phosphatidylinositol (GPI)-anchored form of NEP (GPI-NEP) was created by the addition of a GPI-anchor signal attachment sequence to the soluble form [4].

NEP is found on a variety of mammalian cell types. The protein is abundantly expressed at the apical surface of epithelial cells, in particular those of the choroid plexus, lining of the small intestine and renal cortex. In an attempt to understand the targeting and sorting mechanisms in polarised epithelia we have studied different molecular forms of NEP expressed in various epithelial cell lines (for review see [5]). When NEP was expressed in the pig kidney proximal tubule cell line, LLC-PK1, it was shown to be directly targeted to the apical surface [6]. However, when sNEP was expressed in LLC-PK1 cells an unaltered secreted distribution of the protein was observed. Here we report on the effect of the addition of a GPI-anchor on the targeting of the NEP ectodomain (sNEP) in LLC-PK1 cells.

The GPI-NEP encoding region of the vector pSVGPINEP [4] was subcloned into the pRcCMV vector (Invitrogen, CA) downstream of the cytomegalovirus promoter and enhancer elements to give pRcCMVGPINEP. This vector was then transfected into LLC-PK1 cells using the CaPO4 precipitation method and selection undertaken using the neomycin analog, G418, in accordance with the protocol previously described for NEP [6].

Evidence for the presence of the GPI-anchor in GPI-NEP was obtained by labelling cells with [3]H ethanolamine: a component of the core structure of the GPI-anchor. GPI-NEP was shown, by fluorography, to incorporate [3]H ethanolamine. Control samples, from LLC-PK1 cells expressing NEP, did not incorporate [3]H ethanolamine. Conformation that GPI-NEP was indeed anchored via a GPI-anchor came from studies using phosphatidylinositol-specific phospholipase C (PI-PLC). PI-PLC specifically cleaves GPI-anchored proteins and can liberate them from cellular membranes [7]. Membranes, from LLC-PK1 cells expressing GPI-NEP, were subjected to PI-PLC and NEP immunoreactive material was released. This was not the case when using membranes from LLC-PK1 cells expressing wild-type NEP.

In order to assess the polarity of GPI-NEP, cells were grown on filters and proteins from both the apical and basolateral surfaces were tagged with a biotinylating reagent using previously documented techniques. Biotinylated proteins were then precipitated with streptavidin-agarose and GPI-NEP was specifically revealed by immunoblotting. As shown in figure 1, GPI-NEP is almost exclusively present at the apical surface. A control, following biotinylation of either the apical or basolateral surface, one tenth of the solubilised material was directly subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Figure 1 (control) shows that equal quantities of GPI-NEP were present in monolayers labelled from either the apical or basolateral surface. These results are consistent with the earlier report by Lisanti and coworkers [8] in which it was shown that all endogenous PI-PLC sensitive proteins were localised at the apical domain of LLC-PK1 cells. Thus, GPI-anchoring can restore the polarised expression of the ectodomain of NEP in LLC-PK1 cells.

An interesting question that arises is whether NEP and GPI-NEP use the same transport vesicles to reach the apical surface. It has been suggested that GPI-anchored proteins cluster in so called 'glycolipid rafts' and that these rafts are then targeted to the apical surface thus delivering the GPI-anchored proteins [9]. It is currently unknown whether apically targeted transmembrane proteins use the same transport vesicles as their GPI-anchored counterparts. Thus, we are currently using both NEP and GPI-NEP to investigate the pathways membrane proteins take to reach the apical surface in epithelial cells.

Abbreviations used: GPI, glycosyl-phosphatidylinositol; NEP, neutral endopeptidase; PI-PLC, phosphatidylinositol-specific phospholipase C; sNEP, soluble form of NEP.

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