Expression of an enzymically active glycosylphosphatidylinositol-anchored form of neutral endopeptidase (EC 3.4.24.11) in Cos-1 cells

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Neutral endopeptidase (EC 3.4.24.11, NEP) is a type-II integral membrane protein found in a wide variety of cell types. We previously produced a secreted form of the enzyme by deletion of the cytoplasmic and transmembrane domains and in-frame fusion of the cleavable signal peptide of pro-opiomelanocortin [Lemay, Waksman, Roques, Crine and Boileau (1989) *J. Biol. Chem.* **264**, 15620–15623]. Here we have used this secreted form of NEP and fused to it the glycosylphosphatidylinositol (GPI)-anchor attachment signal of decay-accelerating factor to produce a GPI-anchored form. Expression of this chimeric form in Cos-1 cells resulted in cell-surface activity. This activity could be released from the cell surface by phosphatidylinositol-specific phospho-

lipase C and radiolabelling studies showed that the protein could incorporate [³H]ethanolamine, indicating that the enzyme was GPI-anchored. The *K_m* value, using [D-Ala²,Leu⁵]enkephalin as substrate, of GPI-anchored NEP ($62 \pm 5 \mu\text{M}$) was comparable with that of wild-type NEP ($70 \pm 4 \mu\text{M}$), as were the sensitivities to the inhibitors phosphoramidon and thiorphan. However, pulse-chase studies showed that the biosynthesis and cell-surface delivery of GPI-anchored NEP was delayed compared with that of the wild-type transmembrane form of NEP. These results suggest a lower rate of biosynthesis and/or cellular transport for GPI-anchored NEP compared with its transmembrane counterpart.

INTRODUCTION

An increasing number of proteins are now known to be anchored to the plasma membrane via a covalently attached C-terminal glycosylphosphatidylinositol (GPI) moiety [1]. Some of the most widely studied GPI-anchored proteins include the variant surface glycoprotein of *Trypanosoma brucei*, decay-accelerating factor (DAF) and more recently, some brush-border hydrolases, e.g. membrane dipeptidase (for reviews see [1–4] respectively). A signal sequence for GPI-anchor addition has been identified and is becoming increasingly well characterized [5–9]. It lies at the C-terminus of proteins and is composed of a stretch of hydrophobic amino acids preceded, 10–12 amino acids to the N-terminal side, by a cleavage/attachment site. Upon recognition of the GPI-signal during protein synthesis the GPI-anchor is believed to be added *en bloc* to the polypeptide chain. Studies on a number of proteins have shown that as the GPI-anchor is added the remainder of the GPI-signal is cleaved in the endoplasmic reticulum (for review see [10]). The function of the GPI-anchor over the more conventional transmembrane anchor is, as yet, unresolved. Many functions have been proposed for GPI-anchors. For example, the GPI-anchor of the folate receptor has recently been suggested to play a role in folate uptake [2]. However, there is increasing evidence for GPI-anchor involvement in the apical targeting of GPI-anchored proteins in polarized epithelia (for reviews see [1,11,12]).

Neutral endopeptidase (EC 3.4.24.11; neprilysin; NEP) is an ectoenzyme that can hydrolyse a wide variety of biologically active peptides (for review see [13]). It has recently been cloned and shown to be identical with the leucocyte differentiation antigen CD10 or CALLA (see [14] for review). NEP is widely

distributed throughout many sites in the mammalian body [13,15] and has many proposed functions depending on its location. For example, in the central nervous system it is ideally located to hydrolyse neuropeptides at the synapse [16]. At other sites its function is far less clear. It has been suggested to play a protective role in the kidney and choroid plexus [13,17], to play a role in neutrophil migration into sites of infection and inflammation [18], and recently evidence has emerged to suggest a role in bone formation and resorption, and Ca²⁺ homeostasis [19,20]. Structurally NEP exists as a type-II integral membrane protein (see [14] for review). The enzyme consists of a short N-terminal cytoplasmic domain of 27 amino acids, a transmembrane domain of 22 hydrophobic residues and a large ectodomain of 700 residues containing the catalytic site. We have recently produced a secreted form of the enzyme by deletion of the cytoplasmic and transmembrane domains and in-frame fusion of the pro-opiomelanocortin-cleavable signal peptide [21]. The polarized secretion of this truncated form of NEP in Madin–Darby canine kidney cells enabled us to propose that the signal responsible for apical targeting is contained in the ectodomain [22].

Here we have constructed a chimeric form of NEP using the GPI-signal sequence of DAF attached to the C-terminus of secreted NEP (sec-NEP). Expression of this chimera in Cos-1 cells resulted in an enzymically active GPI-anchored form of NEP (GPI-NEP) compared with the transmembrane form found *in vivo*. However, by changing the mode of anchorage and reversing the orientation of the ectodomain of NEP the biosynthesis and cell-surface delivery of this novel form was delayed. The significance of this is discussed. It is hoped that GPI-NEP can prove useful in targeting studies in different polarized cell lines.

Abbreviations used: DAF, decay-accelerating factor; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; NEP, neutral endopeptidase; octyl glucoside, *n*-octyl β-D-glucopyranoside; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI-NEP, GPI-anchored form of NEP; sec-NEP, secreted NEP; PBS-BSA, PBS containing 0.1% BSA; PBS-CM, PBS containing 1 mM CaCl₂ and 1 mM MgCl₂; endo H, endoglycosidase H.

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Figure 1 The deduced C-terminal extension of sec-NEP produced by addition of the DAF GPI-anchor signal

The last four amino acids of sec-NEP are boxed. The hydrophobic sequence within the DAF GPI-anchor signal is underlined and the putative GPI-attachment site (serine) is indicated by an asterisk [3].

EXPERIMENTAL

Materials

The monoclonal antibody used against NEP was 2B12 [23]. The guinea-pig polyclonal antibody raised against rabbit NEP has been described previously [21]. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-(mouse IgG) antibody was from Dimension Laboratories, Ontario, Canada.

All oligonucleotides were synthesized using a Pharmacia Gene Assembler. The GPI-anchor signal of DAF was constructed by ligating two pairs of annealed oligonucleotides. 5'-GGGACCA-CCAGCGGCACAACACGACTTCTCAGCGGACACACG-TGCTTTACC-3' was annealed to 5'-AAGCACGTGTGTC-CGCTGAGAAGTCGTGTTGTGCCGCTGGTGGTCCC-3' and 5'-CTGACCGGACTGCTTGGCACTCTGGTGACAAT-GGGCCTACTCACA-3' was annealed to 5'-TGTGAGTAG-GCCATTGTCAACAGAGTGCCAAGCAGTCCGGTCA-GGGTA-3'. These two DNA fragments were ligated together to produce a 96 bp blunt DNA fragment, which when expressed would code for 32 of the 37 amino acids of the DAF GPI-anchor signal.

Construction of expression vectors and transfection in Cos-1 cells

The production of sec-NEP has been described previously [21]. An *EcoRI* DNA fragment of NEP was subcloned into m13mp19. This fragment contained the coding region for the C-terminus of NEP. Site-directed mutagenesis on single-stranded m13 was performed as described by Taylor et al. [24] and a *ScaI* site introduced at the site of the stop codon of NEP (TGATCT→AGTACT). After re-subcloning of the mutated DNA into sec-NEP (sec-NEPM1) the 96 bp DNA fragment encoding the DAF GPI-anchor signal was ligated into a partial *ScaI* digest of sec-NEPM1. Restriction and sequence analyses showed that this fragment had entered into a second *ScaI* site in sec-NEPM1. The DAF fragment in this second *ScaI* site was amplified by the PCR and the PCR product ligated into a *ScaI* partial digest of sec-NEPM1. Sequence analysis confirmed the insertion of the PCR product at the C-terminus of sec-NEP producing the deduced amino acid sequence shown in Figure 1.

Cos-1 cells in 100-mm-diam. Petri dishes were transfected using the calcium phosphate co-precipitation method described previously [25]. Cells were harvested, 44 h after transfection, as follows. After removing the culture media the cells were rinsed twice with PBS (130 mM NaCl, 10 mM KCl, 10 mM sodium phosphate buffer, pH 7.4) and then incubated for 5 min at 37 °C with PBS containing 4 mM EGTA. Detached cells were centrifuged (1000 *g*, 4 min), resuspended in PBS and stored on ice until needed. Cells were used within 1 h of collection. Some transfections were performed with pSVENK19 which encodes wild-type rabbit NEP and has been described before [26].

Table 1 Distribution of NEP types expressed in Cos-1 cells

See the Experimental section for details. Cell-surface activity is that determined on whole cells. Intracellular activity was calculated by subtracting the cell-surface activity from that obtained when the cells were solubilized by incubation with 1% (w/v) octyl glucoside for 1 h. The results are expressed as the mean of two determinations. n.d., not detectable.

Distribution	Activity (% of total)		
	GPI-NEP	NEP	sec-NEP
Cell surface	51	75	n.d.
Intracellular	45	25	2
Cell culture medium	4	n.d.	98

Figure 2 Release of GPI-NEP from transfected Cos-1 cells with PI-PLC

See the Experimental section for details. The results are expressed as the mean of three determinations and the error bars are $\pm$ S.E.M. ○, GPI-NEP; ●, NEP.

Enzyme assays

NEP was assayed using [*tyrosyl*-3,5-³H] [D-Ala²,Leu⁵]enkephalin (50 Ci/mmol; Commissariat à l'Énergie Atomique, Gif-sur-Yvette, France) as substrate as described previously [27]. *K_m* values were determined using isotopic dilution with [D-Ala²,Leu⁵]enkephalin and calculations performed with the program ENZFITTER.

Phosphatidylinositol-specific phospholipase C (PI-PLC) release of proteins from Cos-1 cells

Cos-1 cells were incubated with various concentrations of *Bacillus cereus* PI-PLC (Boehringer-Mannheim, Canada) for 30 min at 37 °C. Released proteins were separated from the cells by centrifugation (12000 *g*, 5 min at 4 °C) and the supernatants assayed for NEP activity.

Immunofluorescent staining

Cos-1 cells were grown on glass coverslips in six-well plates (Falcon). Coverslips were transferred to clean six-well plates 44 h after transfection and immunofluorescent staining performed

Figure 3 Immunofluorescent staining of GPI-NEP expressed on Cos-1 cells

See the Experimental section for details. (a), (b) and (c) are micrographs viewed using phase-contrast optics of (d), (e) and (f) respectively; (d) mock transfection; (e) cells stained for GPI-NEP using 2B12; (f) cells treated with PI-PLC (1 unit/ml for 30 min at 37 °C) and then stained for GPI-NEP using 2B12. Note the decrease in staining intensity due to the release of GPI-NEP by treatment with PI-PLC. Exposure time on Kodak TMAX400 film was 15 s. Bar, 20 μ m.

essentially as described previously [28]. Non-specific binding sites were blocked using 0.1 % BSA diluted in PBS (PBS-BSA). The primary antibody used to detect NEP was 2B12 diluted in PBS-BSA (final concentration 5 μ g/ml). Rabbit anti-(mouse FITC-conjugated IgG) second antibody was used to detect the primary antibody.

[³H]Ethanolamine labelling

For labelling with [³H]ethanolamine, cells were incubated, 44 h post-transfection, at 37 °C for 1 h in serum-deficient Dulbecco's minimal essential medium. Fresh medium containing [³H]ethanolamine (33 μ Ci/ml, 35 Ci/mmol; Amersham) was added and the incubation continued for a further 4 h. Cells were

then washed twice with PBS and scraped off the substratum into PBS. Cells were solubilized by incubation with 1 % *n*-octyl β -D-glucopyranoside (octyl glucoside) for 1 h at 0 °C. The solution was then centrifuged (12000 *g*, 10 min at 4 °C) and the supernatant collected. Immunoprecipitation of labelled NEP/GPI-NEP was performed as described previously [22]. Immunoprecipitated proteins were then subjected to SDS/PAGE [29] and fluorography as described before [30].

Pulse-chase studies

Cos-1 cells, 44 h post-transfection, were incubated for 1 h in cysteine- and methionine-free RPMI (Gibco). The cells were then incubated with Tran³⁵S-label (33 μ Ci/ml, 1125 Ci/mmol; ICN

Biomedicals) for 30 min and then chased for various times in media containing cysteine and methionine (both at 0.67 mM). Cells were then solubilized, NEP/GPI-NEP immunoprecipitated and subjected to SDS/PAGE and fluorography as described above.

To determine the time taken for cell-surface delivery of GPI-NEP and NEP, cell proteins were labelled with Tran³⁵S-label (100 μ Ci/ml, 1125 Ci/mmol) for 15 min. After various chase times subsequent steps were performed at 4 °C. Cell monolayers were washed four times with PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ (PBS-CM) for 5 min. Cell-surface proteins were biotinylated (0.5 mg/ml NHS-SS-Biotin; Pierce, Rockford, IL, U.S.A.) twice for 20 min and washed three times with PBS-CM containing 50 mM NH₄Cl to quench biotinylation. Cells were then solubilized, NEP/GPI-NEP immunoprecipitated and biotinylated proteins were isolated by incubation with immobilized streptavidin (Pierce). Eluted proteins were subjected to SDS/PAGE and fluorography as described above.

Other methods

In some experiments immunoprecipitated proteins were digested with 10 munits of endoglycosidase H (endo H) for 20 h as described previously [31]. The effects of inhibitors were determined at a substrate concentration of 50 nM in the presence of various concentrations of the inhibitors.

RESULTS

Distribution of NEP activity expressed in Cos-1 cells

Cos-1 cells were transfected with the various forms of NEP. Table 1 shows the NEP activities (as a percentage of the total) in various cell preparations. As can be seen on assays of whole cells 51 % of the total GPI-NEP activity was detectable on the cell surface with only 4 % released into the medium. The remainder was intracellular. In comparison, 75 % of the total activity of wild-type NEP was expressed on the cell surface. These results indicate that GPI-NEP is primarily located on the cell surface. As a comparison, Cos-1 cells transfected with sec-NEP possessed no detectable cell-surface NEP activity, the majority of the activity (98 %) was secreted into the cell culture medium.

Evidence for a GPI-anchored form of NEP

One of the criteria used to distinguish GPI-anchored proteins from transmembrane proteins is their susceptibility to PI-PLC [32,33]. After incubation of transfected cells with various amounts of PI-PLC, released NEP activity was determined. As can be seen in Figure 2 when cells were transfected with wild-type NEP no PI-PLC released activity was detectable. However, cells transfected with GPI-NEP released NEP activity in a PI-PLC-concentration-dependent manner. After a 30 min incubation with PI-PLC (1 unit/ml) an activity of 0.85 pmol/1 $\times 10^6$ cells per min corresponded to a release of 70 % of the total cell-surface activity.

Transfected cells grown on glass coverslips were subjected to immunofluorescent staining. Control cells which had undergone a mock transfection (omitting DNA) lacked any cell-surface immunostaining (Figure 3d). Figure 3(e) shows cell-surface immunostaining of GPI-NEP. This cell-surface immunofluorescent staining was markedly reduced in intensity by the treatment of the cells with PI-PLC before fixation (Figure 3f). This is in line with the previous observation whereby 70 % of the total cell-surface activity could be released with PI-PLC (see above).

Confirmation that GPI-NEP was GPI-anchored was obtained by the incorporation of [³H]ethanolamine. After fluorography two bands were observed with apparent M_r s of 92 000 and 94 000 (Figure 4, lane 1). These values compare well with those of sec-NEP and wild-type NEP, M_r s 90 000 and 94 000 respectively [21]. Wild-type NEP did not incorporate any [³H]ethanolamine (Figure 4, lane 2).

Characterization of GPI-anchored NEP

The effect of the addition of a GPI-anchor on NEP kinetics was then investigated. Table 2 shows that there were no marked differences between the K_m values of GPI-NEP and wild-type NEP or with the previously reported K_m for sec-NEP [21]. Similarly, no marked differences were obtained in the sensitivity of GPI-NEP to phosphoramidon or thiorphan compared with wild-type NEP or sec-NEP.

Biosynthesis of GPI-anchored NEP and cell-surface delivery

The biosynthesis of GPI-NEP was compared with that of wild-type NEP. GPI-NEP was synthesized initially as an endo H-sensitive form that gradually acquired endo H resistance (Figure 5a). After 40–60 min approx. half of the GPI-NEP was endo H

Figure 4 Incorporation of [³H]ethanolamine into GPI-NEP

See the Experimental section for details. Lane 1, GPI-NEP; lane 2, wild-type NEP. Exposure time was 3 weeks on Kodak X-OMAT film.

Table 2 A comparison of NEP type with various kinetic parameters

See the Experimental section for details. K_m determinations are shown as the mean $\pm$ S.E.M. of three determinations.

NEP type	K_m (μ M)	IC_{50} (nM)	
		Phosphoramidon	Thiorphan
Wild-type NEP	70 $\pm$ 4	4.6	1
GPI-NEP	62 $\pm$ 5	2.8	1
Sec-NEP	49†	6†	4.7*

† and *, data from [21] and [34] respectively.

Figure 5 Biosynthesis of GPI-NEP and wild-type NEP in Cos-1 cells

Cos-1 cells transfected with either GPI-NEP or wild-type NEP were labelled with 33 μ Ci/ml Tran³⁵S-Label for 30 min. After various periods of time cells were solubilized and NEP or GPI-NEP immunoprecipitated, half was subjected to endo H digestion, proteins were resolved by SDS/PAGE and then visualized by fluorography. Exposure time was 24 h on Kodak X-OMAT film. (a) GPI-NEP. (b) wild-type NEP. Both the mature forms of NEP and GPI-NEP migrate with an apparent M_r of 94 000, whereas their respective immature (endo H-sensitive) forms both migrate with an apparent M_r of 88 000.

Figure 6 A comparison of cell-surface delivery of GPI-NEP with wild-type NEP

Cos-1 cells transfected with either GPI-NEP or wild-type NEP were labelled with 100 μ Ci/ml of Tran³⁵S-Label for 15 min and cell-surface NEP/GPI-NEP isolated as described in the Experimental section. Exposure time was 4 days on Kodak X-OMAT film. Shown here is a fluorograph of cell-surface GPI-NEP (a) and wild-type NEP (b) after various chase times.

resistant. In comparison, wild-type NEP was also synthesized initially as an endo H-sensitive form (Figure 5b). However, in less than 20 min more than half of the NEP synthesized was endo H resistant. As the acquisition of endo H resistance is believed to take place in the mid-Golgi, these results would suggest that the time taken to reach the mid-Golgi is 2–3-fold less for GPI-NEP than wild-type NEP.

The time taken for both proteins to reach the cell surface was then determined. GPI-NEP could be detected at the cell surface after 60 min (Figure 6a), whereas NEP could be detected at the cell surface after only 40 min (Figure 6b) under the conditions

used. These results are consistent with the delayed maturation of GPI-NEP compared with wild-type NEP.

DISCUSSION

Proteins destined to become GPI-anchored possess a GPI-anchor attachment signal [3]. In recent years the exact nature of this signal has become more clearly defined [3,8,9]. In 1991, Moran and co-workers [7] showed that placement of the latter 33 of the 37 amino acids of the DAF GPI-anchor signal at the C-terminus of human growth hormone (normally a secreted protein) resulted in a GPI-anchored form of this protein. We used this and the secreted form of NEP, sec-NEP, to construct a form of NEP ending with part of the DAF GPI-anchoring signal. Expression of this construct in Cos-1 cells resulted in detectable cell-surface activity which was shown to be GPI-anchored. By changing the mode of anchorage of NEP from a transmembrane to a GPI-anchored protein this effectively reverses the orientation of the ectodomain from an N-terminal- to a C-terminal-anchored protein. To date, other proteins that have been converted from either a transmembrane form into a GPI-anchored form (e.g. herpes glycoprotein [35]) or from a GPI-anchored form into a transmembrane form (e.g. alkaline phosphatase [36]) have retained the original orientation of their ectodomain. The GPI-anchored form of NEP described here is one of the first GPI-anchored proteins that has been constructed from a type-II membrane protein.

It is now well-established that PI-PLC can release GPI-anchored proteins from the cell surface [32,33]. However, not all GPI-anchored proteins are released from the cell surface by PI-PLC. In fact, the amount released can depend on the source of the protein under investigation. For example, the percentage of the total aminopeptidase P activity released from pig kidney microvillar membranes was approx. 75%, whereas from human kidney microvillar membranes approx. 25% could be released [37]. Here, 70% of the total GPI-NEP activity was released from the cell surface of Cos-1 cells and the remaining enzyme could be visualized using indirect immunofluorescent staining. Incomplete release may be due to the inaccessibility of PI-PLC towards all of the GPI-anchored molecules, or may be due to cell specificity shown by a number of PI-PLCs (for review see [38]). However, as yet, the differences seen here await clarification.

Arg⁷⁴⁷ of NEP has been implicated in substrate and inhibitor binding [39]. As Ser⁷⁵⁴ is a likely candidate for the GPI-anchor attachment site (see Figure 1) the GPI-anchor may well be within close proximity (7 amino acids) of an important substrate-binding region in the GPI-anchored form of NEP. A comparison of various kinetic parameters of GPI-NEP, wild-type NEP and sec-NEP showed no marked differences. Both the K_m to [D-Ala², Leu⁵]enkephalin and the sensitivities to two inhibitors, phosphoramidon and thiorphan, were similar, suggesting that, although the protein is 'upside-down' and attached to the membrane by its C-terminus, the active site remains preserved. These observations would suggest that the C-terminus of the protein is not grossly altered by the addition of, and attachment to the membrane by, a GPI-anchor.

When the maturation and cell-surface delivery times of GPI-NEP were compared with those of wild-type NEP marked differences were seen between the two forms. GPI-NEP showed consistently slower maturation and cell-surface delivery. The addition of the GPI-anchor is reported to take place in the endoplasmic reticulum within 1 min of protein synthesis [40,41]. This would suggest that anchor addition alone is not responsible for the slower maturation and cell-surface delivery of the protein. However, it is becoming apparent that proteins must be correctly

folded before exiting the endoplasmic reticulum and that incorrectly folded proteins are retained [42]. Therefore, the slower maturation and cell-surface delivery may be due, in part, to protein folding events perhaps linked to the acquisition of the GPI-anchor. As previously discussed the addition of the GPI-anchor reverses the orientation of the ectodomain. This may result in a longer time being taken for correct folding of the protein and hence exit from the endoplasmic reticulum. In fact, close examination of the kinetics of cell-surface delivery suggest that exit from the endoplasmic reticulum may be the rate-limiting step in the transport of GPI-NEP. Indeed, both GPI-NEP and NEP appear at the surface as soon as their mature form is detected. This would suggest that the rate of transport from mid-Golgi to the cell surface is the same regardless of the type of membrane anchor.

Taken together, the results presented here support the idea that the ectodomain of NEP is a relatively compact stable structure, as it can tolerate membrane attachment via its C-terminus and remain enzymically viable. However, changing the mode of anchorage of NEP perturbs its biosynthesis and/or cellular transport. Work to determine the effect of GPI-anchoring on the endocytosis of NEP is in progress. Furthermore, GPI-NEP may well prove useful in targeting studies in different polarized cell lines.

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