Role of glycosylation in transport and enzymic activity of neutral endopeptidase-24.11

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Neutral endopeptidase (NEP, EC 3.4.24.11) is a major ectoenzyme of the brush-border membrane. The ectodomain of NEP contains five putative N-glycosylation sites. In order to determine the role of the addition of sugar moieties on the activity and intracellular transport of NEP, we have used site-directed mutagenesis to remove all or some of the five potential sites of sugar addition in membrane-bound and secreted forms of the enzyme. Expression of NEP glycosylation mutants in COS-1 cells showed that all five sites are used for sugar addition. Immunoblotting of NEP in COS-1 cell extracts or culture media indicated that total expression of normal membrane-bound NEP was not affected by mutations at glycosylation sites, whereas this expression level appeared to be strictly dependent on the number of glycosylation sites retained on the soluble form. The transport to the cell surface was also reduced by decreased glycosylation, but again the phenomenon appeared more drastic in the case of the soluble form than for the membrane-bound enzyme. Enzyme activity was decreased by deglycosylation. However, the presence of either of two crucial sites (sites 1 and 5; numbered from the N-terminus of the protein) was sufficient to recover close-to-normal enzymic activities. Transport to the cell surface and enzyme activity of NEP are thus both dependent on sugar residues, probably through different conformational constraints. These constraints seem to be local for enzyme activity but more global for transport to the cell surface.

INTRODUCTION

Neutral endopeptidase (EC 3.4.24.11; NEP) is a major ectoenzyme of the brush-border membrane of kidney proximal tubules and small intestine. It is also present in many other tissues, and is apparently involved in the degradation of small regulatory peptides [1–7].

The ectodomain of NEP contains oligosaccharide side chains [8]; their role and importance is still unknown. On other glycoproteins, postulated roles for sugar moieties include increased stability of proteins and participation in the folding of polypeptide chains [8–13]. It has also been suggested that sugar residues are important for transport of the protein to the cell surface. However, this latter role may be the consequence of a participation of sugar moieties in protein folding, since it is known that retention of proteins in the rough endoplasmic reticulum (ER) is generally the result of protein misfolding [14–16].

Ectoenzymes of the brush-border membrane, such as NEP, are an important class of glycoproteins for which the role of sugar moieties has never been carefully examined. We thus used oligonucleotide-directed mutagenesis to alter each of the consensus N-glycosylation sites in NEP. The recombinant proteins were transiently expressed in COS-1 cells by transfection of an expression vector. Transport of the glycosylation mutants to the cell surface was compared with that of the wild-type enzyme. Their enzymic activity was also determined. These studies were performed on the normal membrane-bound enzyme as well as on a secreted form obtained by replacement of the cytosolic and transmembrane domain by a cleavable signal peptide as described previously [17].

Our results suggest that all five consensus N-glycosylation sites of rabbit NEP are used. The presence of the sugar moieties appears to increase the stability of the protein and to facilitate its transport to the cell surface, as well as its enzymic activity. The requirement for sugar moieties is more stringent for the soluble than for the membrane-bound form of the enzyme. Interestingly, the presence of a glycosylation site at either one of two crucial positions is sufficient to restore normal enzymic activity, while transport to the cell surface occurred at the same rate for all mutants where one single glycosylation site was present at any of the five positions.

MATERIALS AND METHODS

DNA manipulations and vector constructions

All DNA manipulations were performed according to standard procedures [18,19]. DNA sequencing was performed on either M13 single-stranded DNA or denatured double-stranded plasmid DNA using T7 DNA polymerase according to the manufacturer's instructions (Pharmacia T^7 sequencing kit). The five AAC or AAT asparagine codons involved were thus changed to CAG glutamine codons by oligonucleotide-directed *in vitro* mutagenesis [20,21] using a commercially available kit (Amersham). Single-stranded DNAs obtained from M13 vectors containing cloned fragments of NEP were used as templates for mutagenesis. Mutated fragments were recovered from double-stranded replicative forms of M13 vectors and substituted for corresponding non-mutated segments in the appropriate expression vectors. Vectors for expression of membrane-bound or secreted forms of NEP proteins have already been described [17,22].

Abbreviations used: NEP, neutral endopeptidase (EC 3.4.24.11); ER, endoplasmic reticulum; endo-F, endo-β-*N*-acetylglucosaminidase F; endo-H, endo-β-*N*-acetylglucosaminidase H.

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Transfection of COS-1 cells

COS-1 cells were seeded the day before transfection at a density of 10^6 cells/100 mm-diameter Petri dish. Plasmid DNAs were introduced into COS-1 cells [23,24] by the calcium phosphate co-precipitation procedure [25] modified to include a 1 min shock with 15% glycerol. Cell-associated proteins were recovered by octyl glucoside solubilization of cells. Secreted proteins were recovered in serum-free spent culture medium as previously described [17,22]. One-tenth fractions of cellular proteins or of proteins contained in medium were then analysed by immunoblotting.

Immunoblotting

Proteins were resolved by SDS/PAGE [26] and electrotransferred on to nitrocellulose filters [27]. Detection of NEP was done with a guinea-pig polyclonal anti-NEP antiserum as described in [17]. When necessary, signals on the filters were quantified by laser-densitometric analysis using a Personal laser densitometer (Molecular Dynamics). Endoglycosidase digestions of proteins were performed by an already published procedure [17].

Determination of enzyme activity

Enzyme activity was determined by monitoring the cleavage of D-Ala$_2$[*tyrosyl*-3,5-^{3}H][leucine]enkephalin (50 Ci/mmol; CEA, Gif-Sur-Yvette, France) [28]. Identical amounts of NEP were used in all enzymic assays. Quantification of NEP was done by comparing the relative intensities on immunoblots using laser densitometry. K_m and $V_{max.}$ were determined by the isotopic-dilution method with unlabelled substrate. Calculations of these values and margin of error were facilitated by using the ENZFITTER microcomputer program (Elsevier-Biosoft, Cambridge, U.K.).

RESULTS

There are five consensus N-glycosylation sites [Asn-Xaa-Ser/Thr (Xaa being any amino acid except proline)] [29] in the primary structure of rabbit NEP [30] (Figure 1). These sites are also present in human and rat enzymes [31–34], the latter containing one additional site. In order to demonstrate the role of these glycan moieties, asparagine residues in consensus glycosylation sites were replaced with glutamine by site-directed mutagenesis.

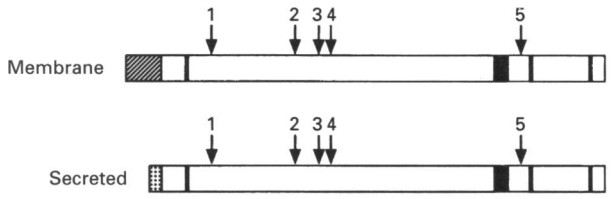

Figure 1 Structure of membrane-bound and secreted forms of NEP

Open boxes (□) represent the extracellular C-terminal domain of NEP. Hatched regions (▨) are the transmembrane and intracytoplasmic region present on the membrane form of the protein, whereas the dotted region (▩) is the pro-opiomelanocortin signal peptide fused by genetic engineering to the extracellular domain to generate the secreted form. Black (■) regions in the extracellular domain indicate the positions of amino acids involved in enzyme activity, as described in the text. The position of all glycosylation sites is indicated by numbered arrows.

Expression of glycosylation site mutants of NEP in COS-1 cells

The wild-type and glycosylation mutants were expressed in COS-1 cells and the NEP-related proteins analysed by immunoblotting. Each transfection experiment was repeated at least three times for the different plasmid DNAs. The analysis of some mutants is presented in Figure 2(a). The expression of wild-type NEP in this system normally results in the production of a single band revealed by immunoblotting [17] (see also Figure 3b); however, the membrane-bound enzyme lacking two glycosylation sites (lane f) was resolved into two different forms. Furthermore, the wild-type enzyme is normally resistant to endo-β-*N*-acetylglucosaminidase H (endo H)-treatment [17] (see also Figure 3b); in contrast, the lower form of the partially deglycosylated enzyme is sensitive to endo H treatment, whereas the upper one appears to be resistant (Figure 2a). Moreover, the band resulting from endo H digestion migrated with the same mobility as the endo-β-*N*-acetylglucosaminidase F (endo F)treated enzyme, (results not shown).

Resistance to endo H is acquired on transport of the protein to the Golgi apparatus. The endo H-sensitive form of NEP observed in partially deglycosylated recombinant proteins is therefore believed to represent NEP molecules present in some intracellular compartment located before the Golgi apparatus in the pathway of membrane-bound protein transport to the cell surface. Moreover, as the transit time from the Golgi apparatus to the plasma membrane is very short, the majority of proteins with endo H-resistant sugars are most probably located on the cell surface. For each mutant, the percentage of NEP molecules reaching the cell surface can therefore be estimated by comparing the relative intensities of endo H-sensitive and -resistant bands. Figure 2(a) shows that progressive removal of glycosylation sites increased the percentage of the endo H-sensitive moieties, thereby suggesting retention of the protein in the ER. It is also noteworthy that the electrophoretic mobility of NEP increased with the number of mutated glycosylation sites, suggesting the use of all five potential glycosylation sites on the protein (Figure 2a; results not shown).

Expression of deglycosylated soluble forms of NEP in COS-1 cells

Deglycosylated mutants of the secreted form of NEP (sec-NEP) were also expressed in COS-1 cells. Surprisingly, in contrast with the membrane-bound form, the total amount of sec-NEP (cell extract plus spent medium) considerably decreased with the progressive removal of glycosylation sites (Figure 2b). Removal of four of the five glycosylation sites can result in an almost tenfold decrease of total sec-NEP produced.

The amount of NEP recovered in cell extracts was next compared with that secreted in the medium. The percentage of sec-NEP remaining in an intracellular compartment was estimated by comparing the amount of cell-associated protein with the amount released into the medium. As shown in Figure 2(b), an increase in intracellular retention was again observed and was even more drastic than the one observed with the membrane-bound form. For example, the percentage of cell-associated material was as high as 80% when four of the sites were removed (Figure 2b).

Expression and activities of singly glycosylated NEP mutants

We then performed a systematic study in which each potential site was added back separately to a completely deglycosylated

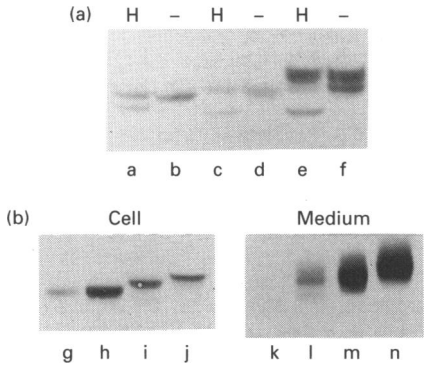

Figure 2 Immunoblot analysis of deglycosylated forms of NEP

Proteins from COS-1 cells transfected with the different NEP expression plasmids were recovered and analysed by immunoblotting as described in the Materials and methods section. (**a**) Proteins obtained following transfection of plasmids encoding membrane-bound forms of the protein lacking different numbers of glycosylation sites; proteins were either treated with endo H (H) or left untreated (—). Lanes a and b, mutant lacking four sites (sites 2–5); lanes c and d, mutant lacking three sites (sites 2, 3 and 5); lanes e and f, mutant lacking two sites (sites 2 and 5). (**b**) Protein retained in the cell compared with protein recovered into the medium when cells were transfected with expression vectors encoding soluble forms of NEP. Lanes g and k, mutant lacking four sites (sites 2–5); lanes h and l, mutant lacking two sites (sites 1 and 5); lanes i and m, mutant lacking one site (site 1); lanes j and n, wild-type secreted form of NEP.

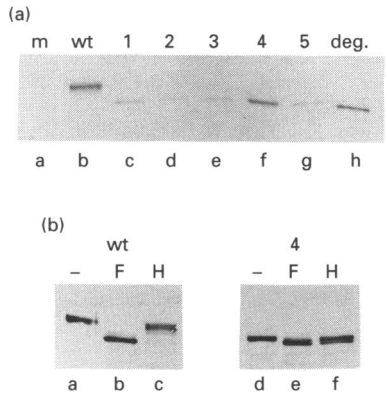

Figure 3 Immunoblotting analysis of the mutants of the membrane-bound form of the protein harbouring only one glycosylation site

(**a**) Proteins were recovered from transfected COS cells and analysed by immunoblotting as described in the Materials and methods section. Lane a, mock-transfected cells; lane b, wild-type protein (five sites present); lane c, site 1 present; lane d, site 2 present; lane e, site 3 present; lane f, site 4 present; lane g, site 5 present; lane h, totally deglycosylated form of NEP (no residual site). (**b**) Proteins recovered from transfected cells were either left untreated (lanes a and d), treated with endo F (F) (lanes b and e) or treated with endo H (H) (lanes c and f). Lanes a–c, wild-type (w.t.) NEP; lanes d–f, mutant harbouring only the fourth glycosylation site.

membrane-bound form of the enzyme. The various mutants were expressed at a level sufficient for their further analysis, although some variations were observed in their expression levels (Figure 3a, lanes c–g). All the mutants also appeared to be glycosylated, since their electrophoretic mobility was slightly decreased compared with the completely deglycosylated form (lane h). Furthermore, they were all sensitive to endo F digestion (a typical result is presented in Figure 3b). This further confirmed that all five potential glycosylation sites are used on NEP. The five singly glycosylated mutants were only partially resistant to endo H (Figure 3b), indicating again a partial retention of the protein in the ER. However, this retention always represented less than 50% of the total protein and appeared to be similar for each mutant (results not shown).

Table 1 Enzymic activity of deglycosylated membrane-bound forms of NEP

Glycosylation sites present	$V_{max.}$ (pmol/min) Expt. 1	Expt. 2
Wild-type protein	171±16	277±1
Site no. 1 present	149±24	205±1
Site no. 2 present	28±1	24±1
Site no. 3 present	24±3	41±2
Site no. 4 present	31±1	15±2
Site no. 5 present	139±9	327±1
No site present (deglycosylated enzyme)	26±7	31±20

Enzymic activity of the mutants was then determined by measuring $V_{max.}$ values for the same amount of enzyme. The same experiment was performed with proteins recovered from separate transfection experiments; the results obtained in two separate experiments are presented with margin of errors provided by calculations with the ENZFITTER program (Table 1). The activity of the totally deglycosylated NEP was significantly reduced (10–15% of unmutated NEP). However, addition of a single glycosylation site at either position 1 or 5 was sufficient to restore the activity close to its normal value. Addition at either one of the other three sites had no significant effect on the catalytic activity of the deglycosylated NEP (Table 1). In contrast with $V_{max.}$, K_m values did not appear to be significantly altered by the position of glycosylation (results not shown).

DISCUSSION

Until recently, studies concerning the role of sugar moieties on polypeptide chains took advantage of viral proteins as models. The availability of cloned cDNA encoding cellular transmembrane proteins and the advent of site-directed mutagenesis has allowed extension of these studies to other protein models. In the present study we examined the role of sugar-residue addition to the NEP polypeptide chain.

Most studies suggest that sugar moieties do not play a significant role in the biological activity of polypeptide chains (reviewed in [10]). In the present study we prevented glycosylation of NEP by replacing asparagine residues with glutamine residues. This is quite a conservative change physicochemically and evolutionarily [35]. As expected, completely deglycosylated NEP retained enzymic activity. However, a significant decrease in the $V_{max.}$ value was observed. Interestingly, restoration of either glycosylation site 1 or 5 was sufficient to recover nearly full enzymic activity. This observation is similar to a previous report showing that erythropoietin activity is dependent upon the presence of glycan moieties at one specific N-glycosylation site out of three [36]. It is noteworthy that NEP glycosylation sites 1 and 5, which are important for catalytic activity, are located closest to amino acids previously shown to be involved in this activity (see Figure 1) [22,37–40]. Site 1 (asparagine-145) is close to arginine-102, and site 5 (asparagine-628) is close to histidine-583 and -587, glutamic acid-584 and -646 and arginine-747. One explanation for these results could be that the sugar moieties

participate in local folding or stabilization of the polypeptide chain.

A role of glycans in the folding of NEP is also consistent with the intracellular trapping observed for the partially glycosylated forms of the enzyme. This retention is most probably at the level of the ER, since these proteins are sensitive to endo H treatment. It has been known for some time that such retention of proteins is generally the result of misfolding of polypeptide chains (see, for instance, [14–16,41,42]). It did not appear, however, that any one site was more important for intracellular transport than another, but that there was rather a cumulative effect of sugar addition at the five sites.

The difference observed in the intracellular transport of the membrane-bound and secreted forms of NEP mutants is unexplained, but clearly illustrates that the stability as well as the transport competency of different polypeptide chains is affected to different extents by the presence of sugar residues [43]. The stability of the secreted forms of the NEP mutants appeared to be drastically affected by the removal of glycosylation sites, since total accumulation of these proteins, as measured by immunoblotting, was greatly reduced. The expression vectors encoding these different proteins were identical except for the mutations, and thus we must conclude that the reductions in the amounts of proteins are due to post-translational events. Our observations are consistent with numerous reports indicating that, in some proteins, the addition of glycan is important for their transport competency and/or stability (see, for instance [11–13,36,41,43–46]), while in others the sugar residues appeared dispensable (see, e.g., [47–49]). It is noteworthy, however, that the intracellular transport of two polypeptide chains that differed only by the presence of a membrane anchor showed such differences in their requirements for glycosylation. There is at least one other example of very similar proteins that showed different requirements for sugar moieties: a single amino acid substitution in the vesicular-stomatitis-virus protein G appears sufficient to change its glycan requirement for transport [50].

Transport to the cell surface and the enzymic activity of NEP are thus both dependent on sugar residues, probably through conformational constraints. These constraints seem to be local for enzyme activity (sites 1 or 5), but more global for transport since all sites can have an effect.

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