Interaction of mammalian neprilysin with binding protein and calnexin in *Schizosaccharomyces pombe*

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Neutral endopeptidase (neprilysin or NEP, EC 3.4.24.11) is a zinc metallo-endopeptidase expressed in many eukaryotic cell types and displaying several important physiological roles. In the brain (and central nervous system), this enzyme is involved in the molecular mechanism of pain by its action in the degradation of enkephalin molecules. In the kidney, NEP is implicated in the degradation of regulatory factors involved in the control of arterial pressure, including atrial natriuretic peptide and bradykinin. In this study we assessed the potential of the fission yeast *Schizosaccharomyces pombe* to overproduce rabbit NEP and secreted NEP (sNEP, a soluble derivative of this integral membrane protein). Both recombinant NEP and sNEP were produced at high levels (5 mg/l) in this system. Enzymic studies revealed that these recombinant proteins were fully active and exhibit kinetic parameters similar to those of the *bona fide* enzyme.

Immunofluorescence microscopy and enzymic assays demonstrated that recombinant NEP is correctly targeted to the cell membrane. Furthermore, co-immunoprecipitation studies showed that folding intermediates of NEP and sNEP, produced in *S. pombe*, interact in the endoplasmic reticulum (ER) with binding protein (BiP) and calnexin (Cnx1p). The amount of sNEP coprecipitated with both BiP and Cnx1p augmented when cells were subjected to various stresses causing the accumulation of unfolded proteins in the ER. The interactions of NEP with BiP and Cnx1p were, however, more refractive to the same stresses.

Key words: chaperone, endoplasmic reticulum, fission yeast, heterologous expression, protein secretion.

INTRODUCTION

Neutral endopeptidase (neprilysin or NEP, EC 3.4.24.11) was first identified as the most abundant zinc metallo-endopeptidase of the brush-border membrane [1]. Subsequently, it has been shown that NEP is also expressed in many other mammalian cell types, including the microvilli of the intestine [2], cells of the lung [3], lymphocytes [3], neutrophils [4], reticular cells of the lymph [5], cells of the adrenal gland [6] and cells of the central nervous system [7,8]. NEP is a class-II integral membrane glycoprotein consisting of a short N-terminal cytosolic domain with a length of 27 residues, a single-pass transmembrane domain of 22 hydrophobic amino acids forming a helical secondary structure and a 700-residue-long ectodomain containing five glycosylated Asn residues and six disulphide bonds [9]. The catalytic centre of this enzyme is located in its large extracellular domain [10]. Functionally, NEP has been shown to be implicated in the degradation of small bioactive peptides at the cell surface. The best substrates for this protease appear to be small peptides rather than proteins [6]. Hydrolysis of substrates by NEP occurs at the N-terminal side of hydrophobic residues.

In the mammalian brain, NEP has been reported to inactivate enkephalins. This is supported by the observation that specific inhibition of NEP by thiorphan prevents degradation of enkephalins *in vitro* and induces analgesia *in vivo*, an effect that can be reversed by the opioid-receptor antagonist naloxone [11]. Another clinically interesting aspect of NEP is its capacity to cleave and inactivate the natriuretic and vasodilatory hormone atrial natriuretic peptide, a peptide having an important role in the control of arterial pressure (for a review, see [12]). Furthermore, it has been shown that NEP inhibitors potentiate

endogenous levels of atrial natriuretic peptide (for reviews, see [13,14]).

The identification of novel inhibitors of NEP is evidently of great interest for their eventual application in clinical treatments. In this respect, structural studies on this protein constitute a major step in this direction as they are a prerequisite for the rational design of drugs. Due to the unavailability of a three-dimensional model for NEP, the structural studies carried out to date on this enzyme have been mostly limited to the comparison with the crystallized bacterial zinc metalloprotease thermolysin. This approach is validated by the fact that thermolysin displays certain structural similarities with NEP, most particularly at the level of its catalytic site [15]. To overcome the problems inherent to the purification and crystallization of a membrane-bound protein, a soluble secreted form of NEP (designated secreted NEP, or sNEP) has been engineered and has been overproduced in different expression systems including Sf-9 [16], MDCK [17], COS-1 [18] and LLC-PK1 [19].

In the present study, we investigated the fission yeast *Schizosaccharomyces pombe* as a potential host for the overproduction of NEP and sNEP. Like *Saccharomyces cerevisiae*, *S. pombe* offers the great advantages of easy molecular-genetic manipulation along with rapid and inexpensive culture. More interestingly, we speculated that *S. pombe* could be a useful organism for the overproduction of active NEP and sNEP because this fission yeast displays many striking similarities with mammalian cells, including protein glycosylation and secretion (for reviews, see [20,21]). Consequently, *S. pombe* lends itself as an attractive surrogate organism for the study of protein folding and secretion, except for the paucity of endogenous proteins that could serve to monitor these processes. In this respect, for the

Abbreviations used: BiP, binding protein; ER, endoplasmic reticulum; NEP, neutral endopeptidase; sNEP, secreted NEP; Cnx1p, calnexin; MM, minimal medium; DTT, dithiothreitol; IPB, immunoprecipitation buffer; endo F, peptide N-glycosidase F.

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following reasons NEP stands out as an ideal protein to examine the mechanisms of folding and secretion: (i) it is a glycosylated polypeptide containing several disulphide bridges that can be expressed as an integral membrane protein or in a secreted form (sNEP), thus the existence of different folding mechanisms can be explored; (ii) the enzymic activity of NEP can be determined in simple and rapid assays [22], and thereby detection and correct folding can be assessed quantitatively; (iii) both polyclonal [23] and monoclonal [22] anti-NEP antibodies are available; and (iv) a plethora of glycosylation and disulphide-bond mutants, including some that are secretion-impaired, are readily available for the study of the various aspects of the protein folding and secretion mechanisms. As a first step in this direction, we have explored the possible interaction between NEP and sNEP in the endoplasmic reticulum (ER) with binding protein (BiP) and calnexin (Cnx1p).

EXPERIMENTAL

Strains and media

Escherichia coli ES1301 *mutS* and JM109 bacterial strains (Promega) were used for the mutagenesis steps (see the Plasmid constructions section). The *S. pombe* strain used was SP556 (*h⁺ ade6-M216 ura4-D18 leu1-32*), and was kindly provided by Dr. Paul Nurse (Imperial Cancer Research Fund, London, U.K.). *S. pombe* minimal medium (MM) was used for the culture of transformed strains, and was prepared as described [24]. *S. pombe* transformations were performed by the lithium acetate procedure [24]. Liquid cultures were carried out in shake flasks.

Plasmid constructions

Standard DNA manipulations were carried out as described previously [25]. The cloning of the cDNA encoding rabbit NEP and the engineering of sNEP have been reported previously [10,23]. To facilitate the subcloning of the rabbit NEP and sNEP coding sequences into various expression vectors, an expression cassette was constructed by creating, using site-directed mutagenesis, the restriction sites *NdeI* (CA↓TATG; where the arrow indicates the actual cleavage site.) at the 5' end of the coding sequence (for NEP, 5'-TT TAG CATATG GGA AGA TCA GAA AGT C-3'; and for sNEP, 5'-CCG GAA GCT CAT ATG CCG AGA TTG TGC GG-3', where underlined nucleotides represent restriction sites) and *BamHI* (G↓GATCC) at the 3' end of the coding sequence (5'-CTT CTT CCCTAGGTC ACC AAA CCC GGC-3'). To avoid the requirements of partial restriction for the subcloning, an endogenous *NdeI* (CA↓TATG) site was also removed by mutagenesis (5'-CT TGT ACA GCG TAT GTG GAT TTT ATG-3'). Site-directed mutagenesis was performed with the Altered Sites II *in vitro* Mutagenesis Systems, using the mutagenesis vector pALTER-1 (both from Promega), according to the manufacturer's recommendations. DNA sequences were finally verified by sequencing of the double-stranded DNA with the T7 sequencing kit (Pharmacia). The NEP and sNEP expression cassettes were then cloned in the *S. pombe* vector pREP2 carrying the inducible *nmt1* promoter. To induce heterologous expression of the proteins, cells were washed twice and resuspended in fresh growth media without thiamin [26–28].

SDS/PAGE and immunoblot analyses

Protein samples for gel electrophoresis were dissolved in 3 × Laemmli sample buffer [29] and boiled for 5 min. Separation of proteins was performed on SDS/PAGE (7.5% gel) and

proteins were transferred on to nitrocellulose membrane (0.45 mm, Schleicher & Schuell) overnight. Incubation with anti-NEP monoclonal antibody [22] at a 1:200 dilution was performed and immune complexes were visualized by chemiluminescence with the ECL kit (Amersham). Quantification of immunoblots was done by soft-LASER densitometry using an LKB 2222-02 UltroScan XL instrument (Pharmacia) and comparing with a set of samples of known amounts of purified rabbit kidney NEP, which were run simultaneously on a gel, as described previously [30].

Endoglycosidase digestion

Approx. 100–200-ng amounts of recombinant NEP and sNEP from cell extracts or supernatants, and purified rabbit NEP, were boiled for 5 min in denaturation buffer [50 mM sodium acetate (pH 5.5)/25 mM EDTA/1% β-mercaptoethanol/0.02% SDS/0.5mM PMSF] and incubated with 1 unit of peptide N-glycosidase F (endo F; Boehringer Mannheim) overnight at 37 °C, according to the manufacturer's recommendations. Digestion products were loaded on an SDS/polyacrylamide gel and subjected to immunoblot analysis as described above.

Enzyme assays

From a culture with an attenuation of 1, recombinant *S. pombe* NEP or intracellular sNEP were solubilized in breakage buffer [100 mM Tris/HCl/1 mM dithiothreitol (DTT)/20% glycerol/1 mM NaF/1 mM NaHSO₃/2 mM PMSF]. Samples from the secreted form of sNEP were from the yeast culture supernatants (MM). The secreted proteins were concentrated and the culture medium was exchanged with 50 mM Mes (pH 6.5) buffer using Centriprep-30 tubes (Amicon). Enzymic activity was measured essentially as described previously [22] except that incubations were carried at 37 °C in 50 mM Mes/NaOH (pH 6.5), instead of 50 mM Tris/HCl (pH 7.4), at room temperature. Catalysis was monitored by using 50 nM of the tritiated substrate [tyrosyl-(3,5-³H)(D-Ala²)-Leu-enkephalin (50 Ci/mmol; Commissariat à l'Énergie Atomique, Gif-sur-Yvette, France), and metabolite separation from substrate was performed by chromatography on Sep-Pak C₁₈ cartridges (Waters). The IC₅₀ values were calculated for the NEP-specific inhibitor thiorphan, the K_m values were determined by isotopic dilution and the k_{cat} values were established by densitometric dosage of the proteins after Western blotting, as described above and by Dion et al. [30].

Immunoprecipitations

Co-immunoprecipitation assays were carried out essentially as described previously [31]. Briefly, exponential-growth-phase cultures of *S. pombe* cells expressing NEP or sNEP were prepared starting from a culture inoculum with an attenuation of 0.05 for an overnight preculture. When the cultures reached an A₅₉₅ of 0.4, they were subdivided and cells were subjected to different treatments for 2 h: control conditions (30 °C); heat shock (39 °C); reducing conditions, to inhibit the formation of disulphide bridges (+10 mM DTT); inhibition of N-glycosylation (+10 µg/ml tunicamycin); and inhibition of glucosidases (+1 mM 1-deoxynojirinomycin). Samples containing 10⁸ cells were taken for each immunoprecipitation and washed in ice-cold PBS containing 10 mM Na₃. Cells were resuspended in 150 µl of immunoprecipitation buffer [IPB; 50 mM Hepes (pH 7)/50 mM NaCl/1% Nonidet P-40/1 mM CaCl₂/10 mM iodoacetamide/1 mM PMSF/1 µg/ml of each aprotinin, pepstatin and leupeptin], and mechanically broken with glass beads, in 10 cycles of 30-s vortex/30-s pause, on ice. For each immuno-

precipitation, the equivalent of 10^8 cells in 500 μ l was used. Serum incubations were performed with a 1:200 dilution of polyclonal guinea-pig anti-NEP [23], polyclonal rabbit anti-Cnx1p [31] or polyclonal anti-BiP [32] for 1 h at 4 °C. To each tube, a one-tenth volume of a 10% solution of Protein A-Sepharose in IPB was added and incubation was continued for an additional 1 h at 4 °C. Subsequently, immune complexes were washed five times with IPB. Following this, 25 μ l of 3 $\times$ Laemmli sample buffer were added and samples were boiled for 4 min. Samples were fractionated by SDS/PAGE (12.5% gels) and immunoprecipitated proteins detected by Western blotting.

Brefeldin A treatment and 35 S-labelling *in vivo* of proteins

S. pombe cells were grown overnight at 30 °C in MM supplemented with adenine and leucine, until the culture reached an A_{600} of 1. From this culture, 2×10^8 cells (1 ml of culture at an A_{600} of 1 = 2.5×10^7 cells) were spun down and resuspended in 1 ml of the same medium. To block intracellular trafficking from the ER to the Golgi complex, 0.4 mM brefeldin A (Sigma) was added for 30 min, and then cells were labelled for 3 h in the presence of 300 μ Ci of Translabel 35 S-labelling mix ($[^{35}\text{S}]\text{Cys}$ and $[^{35}\text{S}]\text{Met}$; ICN). Following this, cells were placed on ice and NaN_3 was added to a final concentration of 10 mM, spun down and washed with 1 ml of ice-cold PBS containing 10 mM NaN_3 . Lysates were prepared by breaking the cells with glass beads, and immunoprecipitation experiments were performed as described above with the following modifications: immune complexes were washed twice with IPB and twice with RIPA buffer [31], and radiolabelled proteins were immunoprecipitated using guinea pig anti-NEP antibodies at a 1:50 dilution.

Immunofluorescence

From an overnight exponential pre-culture, a 25-ml culture with an A_{595} of 0.2 was started and incubated for 4 h (until reaching an A_{595} of about 0.5). Fresh 17.5% formaldehyde solution and 10 $\times$ PBS stock solution were added to the culture to a final concentration of 4% formaldehyde and 1 $\times$ PBS. Cells were shaken twice for 30 min each time (100 rev./min), with one buffer change. Cells were then pelleted and resuspended in 500 μ l of 10 mM NaN_3 /1 $\times$ PBS, and 20 μ l of this suspension was placed in wells of poly-L-lysine-coated toxoplasmosis slides (Belloco). Excess fluid was removed and slides were allowed to air dry for 15 min. Polyclonal guinea pig anti-NEP antibodies at a 1:100 dilution in 1% BSA/1% non-fat dried milk/10 mM NaN_3 /1 $\times$ PBS were added dropwise into each well, and incubation proceeded overnight, at 4 °C. After eight washes with 1% BSA/10 mM NaN_3 /PBS, the same incubation conditions were performed for an additional 5 h in the dark with the secondary FITC-coupled antibodies (Chemicon International). Following eight additional washes with 1% BSA/10 mM NaN_3 /PBS, slides were allowed to air dry slightly (5 min), and mounting solution (1 mg/ml *p*-phenylenediamine/0.1 $\times$ PBS/glycerol) was added between wells. Finally, slides were sealed with nail polish. Microscopy was done with a Leica optical microscope at 1000 $\times$ magnification. Controls were performed on untransformed cells under the same incubation conditions. Photographs were taken on 400 ASA T-MAX 35-mm film (Eastman Kodak).

RESULTS

Heterologous production of NEP and sNEP in *S. pombe*

In order to produce NEP and sNEP in *S. pombe*, their respective coding sequences were inserted in the pREP2 shuttle vector (see the Experimental section). Expression of inserted genes in this

Figure 1 Glycosylation analysis of the *S. pombe*-produced NEP and sNEP

Endoglycosidase digestions (with endo F) were performed, as described in the Experimental section: on extracts of *S. pombe* (*Sp*) cells expressing wild-type NEP (160 ng; lanes 5 and 6); extracts of *S. pombe* cells expressing the secreted form (sNEP, 200 ng; lanes 1 and 2); or supernatant of *S. pombe* cells [*Sp* (sup.)] expressing sNEP (80 ng; lanes 3 and 4). Protein samples were fractionated subsequently by SDS/PAGE and analysed by immunoblotting with anti-NEP antibodies. As a control, endo F restriction was carried out on purified rabbit (Rab.) NEP (200 ng; lanes 7 and 8).

Table 1 Kinetic parameters of recombinant NEP and sNEP

K_m and k_{cat} ($\pm$ S.E.) were determined with the D-Ala²-enkephalin substrate on proteins from total cell extracts (for wild-type NEP) or concentrated supernatant (for sNEP). The IC_{50} values ($\pm$ S.E.) were determined for the NEP-specific inhibitor thiorphan. The concentrations of the sNEP and NEP proteins produced in *S. pombe* were determined by quantitative Western-blot analysis as described by Dion et al. [30]. Catalytic reactions were performed at 37 °C in 100 μ l of Mes buffer, using 13 ng of sNEP and NEP from *S. pombe* and 14 ng of sNEP from insect cells.

Protein	Source	K_m (μ M)	k_{cat} (min^{-1})	IC_{50} (nM)
NEP*	<i>S. pombe</i>	50 ± 5	460 ± 2.5	29 ± 0.01
sNEP*	<i>S. pombe</i>	47 ± 1	240 ± 1.5	34 ± 0.02
sNEP*	Insect cells	32 ± 4	275 ± 2.5	34 ± 0.04
sNEP†	Insect cells	22	508	4.7
NEP†	Rabbit kidney	87	573	2.1

* This study.

† From [16].

vector is driven by the regulatable promoter *nmt1*, which is repressed by the addition of thiamin to the culture medium [26–28]. *S. pombe* strains bearing these plasmid constructs were cultured in the exponential growth phase. To assess the production of NEP and sNEP, cell extracts, and supernatants in the case of sNEP clones, were analysed by Western blotting (Figure 1). After 16 h of cell culture, about 5 mg of NEP and sNEP per litre of culture at an A_{595} of 1 were produced with the pREP2-based plasmids. Enzymic activity was detected in crude extracts from NEP- and sNEP-producing cells (see Table 1); however, no significant activity was detected in sNEP-containing supernatants. Because of the sensitivity of sNEP to low pH and the lack of apparent proteolytic degradation (results not shown), we inferred that the lack of enzymic activity was due to culture conditions. Consequently, we assessed the effect of different buffers in various culture media and the highest enzymic activity was observed in MM containing 50 mM Hepes (pH 7.0).

Figure 2 Targeting of recombinant NEP in *S. pombe*

Immunofluorescence microscopy was performed on intact, non-permeabilized cells as described in the Experimental section, with polyclonal guinea pig anti-NEP antibodies [45]. (A) Cells transformed with the vector not expressing NEP, or (B) transformed with a plasmid expressing wild-type NEP. Secondary antibody incubations were carried out with an anti-guinea pig antibody coupled to the FITC-conjugated fluorescent marker.

Targeting of recombinant NEP to the *S. pombe* cell surface

The above-described experiments determined that the *S. pombe*-produced intracellular NEP and sNEP were functional, and that sNEP was secreted into the culture medium in an active conformation. Indirect immunofluorescence microscopy was carried out to examine whether NEP was targeted correctly to the *S. pombe* cell membrane. As it is possible to see in Figure 2, NEP accumulated at the surface of *S. pombe* cells carrying the NEP expression plasmid (Figure 2B); in contrast, no detectable immunofluorescence was observed on control cells (Figure 2A). Furthermore, repeatedly washed, intact, cells expressing NEP displayed significant specific enzymic activity (see Table 1), whereas no activity was detected in control cells (results not shown). These results suggest both the correct targeting of NEP to the cell surface and the proper orientation and folding of the protein produced in *S. pombe*.

Glycosylation of recombinant NEP and sNEP

To characterize the oligosaccharides attached to the various forms of NEP produced in *S. pombe*, the proteins of interest were treated with endo F and the digestion products analysed by Western blotting (Figure 1, lanes 1–6). For comparison, NEP from rabbit kidney was subjected to the same treatment and analysis (Figure 1, lanes 7 and 8). Rabbit NEP migrated with an apparent molecular mass of 95 kDa, whereas recombinant NEP and sNEP migrated as 97- and 93-kDa polypeptides, respectively. Both NEP and sNEP intracellular preparations contained an additional band with slightly higher electrophoretic mobility, corresponding to the ER core-glycosylated forms of these proteins (see below and Figure 3B). Upon deglycosylation with endo F, both recombinant NEP and the native protein purified from rabbit kidney migrated as single bands with an apparent molecular mass of 82 kDa. Likewise, deglycosylated sNEP migrated with an apparent mobility of 79 kDa, as observed previously for the protein produced in other expression systems [23]. Identical results were obtained upon treatment with endo-glycosidase H (results not shown). These results confirm that glycosylation of proteins in *S. pombe* is similar to that occurring in cells of higher eukaryotes [20].

Figure 3 Only the ER form of NEP co-immunoprecipitates with Cnx1p

(A) Immunoprecipitations (IPP) under mild conditions (see the Experimental section) were carried out on extracts of cells producing NEP with anti-NEP antibodies (α -NEP; lane 1), anti-Cnx1p antibodies (α -Cnx; lane 2), or pre-immune serum (lane 3). Immunoprecipitated material was fractionated on SDS/PAGE (10% gel) and precipitated NEP was detected by Western blotting with anti-NEP antibodies. Note that only the fastest-moving NEP band, corresponding to its ER form, coprecipitated with Cnx1p. (B) Cells were metabolically labelled with ^{35}S -translabel in the presence (BFA; lane 2) or the absence (control; lane 1) of brefeldin A to block transport from the ER to the Golgi apparatus. Subsequently, immunoprecipitations with anti-NEP antibodies were performed as described in the Experimental section. Immunoprecipitated material was fractionated on SDS/PAGE (10% gel) and transferred on to a nitrocellulose membrane and metabolically labelled proteins were visualized by exposure on a BioMax X-ray film. The positions of the mature (M) and core-glycosylated (CG) forms of NEP are indicated by arrows.

Catalytic properties of recombinant NEP

To further characterize recombinant NEP and sNEP produced in *S. pombe*, we assessed their kinetic parameters. The results of the Michaelis–Menten kinetic analysis and IC_{50} inhibition curves are summarized in Table 1. The enzymes produced in *S. pombe* displayed catalytic (k_{cat}) and Michaelis (K_m ; affinity) constants comparable with values published previously for the enzymes isolated from natural sources or produced in animal-cell expression systems. NEP produced in *S. pombe* had a catalytic constant of 460 min^{-1} whereas the k_{cat} of that purified from rabbit kidney was 573 min^{-1} . The k_{cat} of *S. pombe* sNEP was 240 min^{-1} , and thus comparable with that of the enzyme purified from the baculovirus expression system (508 min^{-1} ; [16]). Likewise, the K_m values of 50 and $47 \mu\text{M}$ for *S. pombe* NEP and sNEP respectively were also in agreement with those published by Fossiez et al. [16], who determined constants of 87 and $22 \mu\text{M}$ for NEP and sNEP, respectively. Finally, the thiorphan inhibition curve established further that the catalytic properties of the *S. pombe* recombinant NEP and sNEP were comparable, within an order of magnitude,

with the enzymes from rabbit sources or produced in insect cells, as published by Fossiez et al. [16]. Fluctuations in IC_{50} values may occur with different batches of thiorphan. Therefore, in this study we re-determined the catalytic constants and the thiorphan IC_{50} values for the insect-produced sNEP, as an internal control. As shown in Table 1, the *S. pombe* and the insect enzymes exhibited very similar IC_{50} values.

Interaction of recombinant NEP and sNEP with BiP and Cnx1p

Taken together, the results described above demonstrate that NEP and sNEP produced in *S. pombe* were fully active, strongly suggesting that these recombinant proteins were properly folded. As a first step in the elucidation of the mechanisms of NEP and sNEP folding in the ER, we investigated a possible interaction of these proteins with Cnx1p. To this end, extracts from NEP-producing *S. pombe* cells were immunoprecipitated with polyclonal guinea pig anti-NEP or rabbit anti-Cnx1p antibodies. Immunoprecipitated NEP was detected by Western-blot analysis with anti-NEP antibodies. As seen in Figure 3A (lane 1), anti-NEP antibodies immunoprecipitated two NEP species, a slow-moving one representing the mature form of NEP and a faster-moving one corresponding to the core-glycosylated form typical of the ER. That the faster-moving band corresponded to the ER-core-glycosylated form of NEP was demonstrated by blocking the ER to Golgi trafficking with brefeldin A followed by anti-NEP immunoprecipitation of metabolically labelled proteins (see Figure 3B, lane 2). Anti-Cnx1p precipitation revealed that Cnx1p interacts with the ER-core-glycosylated form of NEP. As the immunoprecipitations were carried out with total cell extracts of *S. pombe*, these results also strongly suggest that the interaction observed with Cnx1p is specific, as it occurs only with the ER form but not with the mature species. Furthermore, the use of pre-immune serum demonstrated that NEP was coprecipitated with Cnx1p rather than sedimenting due to unspecific aggregation. Treatments that induce misfolding of nascent polypeptides in the ER, such as exposure to heat shock or to the reducing agent DTT, have been shown to increase the expression and synthesis of chaperones [33,34]. It was of interest to see if these different conditions would increase the interactions of Cnx1p or BiP with its ligands. To this end, *S. pombe* cells expressing NEP or sNEP were exposed to heat shock, or cultured in the presence of DTT (to inhibit disulphide-bond formation), the core-glycosylation inhibitor tunicamycin or the glucosidase inhibitor 1-deoxynojirimycin. Total cell lysates were prepared and immunoprecipitations were carried out with anti-Cnx1p or -BiP antibodies, following which immune complexes were fractionated on SDS/PAGE. Immunoprecipitated NEP and sNEP were visualized by immunoblotting with anti-NEP antibodies. As we have observed previously for *S. pombe* acid phosphatase [31], heat shock (Figure 4B, lanes 2 and 7) and exposure to DTT (Figure 4B, lanes 3 and 8) increased the amount of sNEP associated with Cnx1p and BiP as compared with the control conditions (Figure 4B, lanes 1 and 6). The presence of tunicamycin in the culture medium (Figure 4B, lanes 4 and 9) did not disrupt the association of Cnx1p and BiP with sNEP, as we have observed previously with acid phosphatase [31]. It is also noteworthy that the inhibition of the glucosidases I and II (with 1-deoxynojirimycin, lanes 5 and 10) did not abolish the interaction of sNEP with BiP and Cnx1p. The association of NEP with BiP showed no major variations under the different growth conditions (Figure 4A, lanes 1–5). However, the amount of NEP coprecipitated with Cnx1p augmented when the cells were grown in the presence of DTT (1.7-fold; Figure 4A, lane 8) and tunicamycin (3.7-fold; Figure 4A, lane 9).

Figure 4 Co-immunoprecipitation of NEP and sNEP with BiP and Cnx1p under different stress conditions

Co-immunoprecipitations were performed on extracts of cells expressing the wild-type NEP (**A**) or the secreted form (sNEP, **B**) produced in *S. pombe*. Cells were subjected to different stress conditions: lanes 1 and 6, control (30 °C); lanes 2 and 7, heat shock (39 °C); lanes 3 and 8, 10 mM DTT; lanes 4 and 9, 10 µg/ml tunicamycin; lanes 5 and 10, 1 mM 1-deoxynojirimycin. NEP or sNEP were co-immunoprecipitated with rabbit polyclonal anti-BiP antibodies (lanes 1–5) or rabbit anti-Cnx1p (CNX; lanes 6–10) antibodies. Immunoprecipitated material was fractionated by SDS/PAGE (10% gel) and precipitated NEP and sNEP were detected by Western blotting with anti-NEP antibodies. The fold increase of NEP or sNEP proteins coprecipitated with Cnx1p or BiP with respect to the control conditions (30 °C; lanes 1 and 6) were calculated by densitometric scanning of the films. The following values are the means of three experiments (units are -fold of the control). NEP coprecipitated with BiP: heat shock, 0.8 (lane 2); DTT, 1.0 (lane 3); tunicamycin, 1.2 (lane 4); and 1-deoxynojirimycin, 0.8 (lane 5). NEP coprecipitated with Cnx1p: heat shock, 0.9; DTT, 1.7; tunicamycin, 3.7; and 1-deoxynojirimycin, 0.6. sNEP coprecipitated with BiP: heat shock, 1.9; DTT, 1.7; tunicamycin, 2.9; and 1-deoxynojirimycin, 1.0. sNEP coprecipitated with Cnx1p: heat shock, 1.7; DTT, 2.3; tunicamycin, 4.1; and 1-deoxynojirimycin 0.9. The asterisk denotes the band in lane 10.

DISCUSSION

The first objective of this work was to assess whether *S. pombe* could serve as a host for the overproduction of active membrane-bound and secreted enkephalinase (NEP and sNEP, respectively). The characterization of the recombinant proteins demonstrated that indeed NEP and sNEP synthesized by the fission yeast were targeted correctly and fully active. As judged by the mobility on SDS/PAGE of the mature and endo F-digested NEP and sNEP, the *S. pombe*-produced proteins contained high-mannose oligosaccharides comparable in size with the complex oligosaccharide in mammalian cells (see Figure 1). These results bring further support to the notion that protein glycosylation in *S. pombe* resembles that occurring in higher-eukaryotic cells [20]. In contrast, *S. cerevisiae* presents the drawback that secreted proteins are generally hyperglycosylated and that their glycans are heterogeneous in size, which in turn may negatively affect the enzymic activity or the antigenic properties of the overproduced foreign proteins (reviewed in [35]). In this respect, it is important to stress that the kinetic parameters K_m , k_{cat} and IC_{50} observed for the *S. pombe* NEP and sNEP were comparable with those of the recombinant proteins produced in animal expression systems (see Table 1). Moreover, the *S. pombe*-synthesized proteins were immunologically indistinguishable from NEP and sNEP from animal cells. Thus overall, the fission yeast appears better suited than *S. cerevisiae* for the heterologous production of higher-eukaryotic proteins.

Using shake flasks, *S. pombe* cells could be induced to secrete 5 mg of sNEP per litre of culture without compromising their

viability. Therefore, it can be expected that the production of sNEP in fermenters should reach significantly higher levels. It has been observed previously that both NEP and sNEP from animal cells are inactivated at low pH (P. Crine, unpublished work). *S. pombe* cultures in shake flasks reach pH 4 during an overnight incubation period. This resulted in the production of inactive sNEP when the cells were grown in MM. To produce fully active sNEP, Hepes was added to the medium in order to increase its buffering capacity and thereby prevent the acidification of the culture in shake flasks. Certainly, for large-scale production, fermenters provide the possibility of expressing active recombinant sNEP in a constantly controlled-pH medium, at reduced cost. Interestingly, *S. pombe* cells produced functional NEP when cultured in MM without the addition of Hepes, or other buffers. Hence, the membrane attachment of NEP and/or the surrounding environment (e.g. other membrane proteins) may stabilize the activity of this enzyme. Since NEP is active on the surface of *S. pombe* cells, it is conceivable to study mutants and design inhibitor screens using intact cells, without cell lysis or further purification of the enzyme. In addition, the fact that the membrane-bound proteins remain accessible to large molecules such as antibodies makes possible the simple and rapid screening of NEP-expressing cells expressing a foreign DNA, by using cytofluorimetric procedures (or FACS).

The second objective of this work was to assess whether NEP and sNEP could serve as model proteins to study, in *S. pombe*, the mechanisms involved in protein folding and secretion. The fact that both proteins were targeted correctly and active strongly indicated that the fission yeast mimics the natural folding, maturation and secretion processes in animal cells, validating therefore the use of *S. pombe* as a surrogate organism. Co-immunoprecipitation studies have revealed that Cnx1p or BiP associate only with the ER forms of NEP and sNEP, confirming thereby that, under our assay conditions, the interactions of Cnx1p with its ligands [31] were indeed specific to events taking place in the ER, and not the result of artifacts occurring during or after cell lysis. The amount of sNEP coprecipitating with Cnx1p and BiP substantially increased when the cells were subjected to stresses such as heat shock and inhibition of disulphide formation (presence of DTT). Mammalian Cnx1p has been shown to interact with glycoproteins with monoglucosylated oligosaccharide moieties ($\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$; [36–40]). Nevertheless, some reports have described the interaction of Cnx1p with ligands devoid of N-linked glycans, thus implying the interaction of folding intermediates with Cnx1p via peptide contacts [31,41–43]. In this respect, Cannon et al. [44] have shown that Cnx1p binds folding intermediates of unglycosylated proteins in large aggregates. As we have observed previously for acid phosphatase (Pho4p; [31]), inhibition of glucose trimming with 1-deoxynojirimycin (which inhibits glucosidases I and II), did not disrupt the binding of sNEP to Cnx1p. Furthermore, unglycosylated sNEP, resulting from the action of tunicamycin, did coprecipitate with Cnx1p, most probably as part of large protein aggregates formed in the ER when protein glycosylation is inhibited [31,44]. Thus altogether, these results bring further support to our proposal that, at least in *S. pombe*, untrimmed or unglycosylated proteins coprecipitate with Cnx1p due to its association with BiP in a functional complex [31]. Interestingly, although both NEP and sNEP coprecipitated with BiP and Cnx1p, the amount of protein interacting with the two chaperones under various stress conditions varied widely. When cells were subjected to heat shock or to DTT, the amount of sNEP recovered in the anti-BiP co-immunoprecipitation was larger than that for membrane-bound NEP. Therefore, in spite of NEP and sNEP being practically identical, the membrane topology

appears to prescribe a more modest role for BiP in the folding of NEP, and the putative involvement of other chaperones, yet to be determined. Consequently, it is tempting to speculate that, in general, proteins follow different folding mechanisms, using distinct sets of chaperones, not only due to their distinctive primary structure but also according to whether they are soluble or they have a particular membrane topology. The availability of the NEP/sNEP model opens the way to explore in detail this possibility.

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