

Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases

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In order to define the enzymes responsible for the maturation of the precursor of nerve growth factor (proNGF), its biosynthesis and intracellular processing by the pro-protein convertases furin, PC1, PC2, PACE4, PC5 and the PC5 isoform PC5/6-B were analysed using the vaccinia virus expression system in cells containing a regulated and/or a constitutive secretory pathway. Results demonstrate that in both cell types furin, and to a lesser extent PACE4 and PC5/6-B, are the best candidate proNGF convertases. Furthermore, two processed NGF forms of 16.5 and 13.5 kDa were evident in constitutively secreting cell lines such as LoVo and BSC40 cells, whereas only the 13.5 kDa form was observed in AtT20 cells, which contain secretory granules. Both forms display the same N-terminal sequence as mature NGF, and were also produced following site-directed mutagenesis of the C-terminal Arg-Arg sequence of NGF into Ala-

Ala, suggesting that the difference between them is not at the C-terminus. Co-expression of proNGF with furin and either chromogranin B or secretogranin II (but not chromogranin A) in BSC40 cells eliminated the 16.5 kDa form. Data also show that N-glycosylation of the pro-segment of proNGF and trimming of the oligosaccharide chains are necessary for the exit of this precursor from the endoplasmic reticulum and its eventual processing and secretion. Sulphate labelling experiments demonstrated that proNGF is processed into mature NGF following the arrival of the precursor in the *trans*-Golgi network. This comparative study shows that the three candidate mammalian subtilisin/kexin-like convertases identified process proNGF into NGF and that the nature of the final processed products is dependent on the intracellular environment.

INTRODUCTION

Neurotrophins are a family of chemically related proteins that promote the survival, growth and maintenance of neurons in the central and peripheral nervous systems. Levi-Montalcini and co-workers discovered nerve growth factor (NGF), the first member of the family, over 40 years ago (for a review see [1]). Since the complete primary structure of NGF [2] and the cloning of its cDNA [3] were reported, four other members of the family have been identified: brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6 (for reviews see [4,5]). The monomers of each of the neurotrophins share a number of chemical characteristics, including similar molecular sizes (13.2–15.9 kDa, and exceptionally 21 kDa for NT-6), primary sequence identities that approach or exceed 50%, isoelectric points in the range 9–10, and six conserved half-cystines in the same relative positions that give rise to three intrachain disulphide bonds. Sequence data predict that all neurotrophins are generated from 31–35 kDa precursors that contain at their N-termini hydrophobic signal peptides followed by pro-regions containing sequences of contiguous basic amino acids. Intracellular cleavage of the pro-neurotrophins to produce active growth factors takes place following pairs of basic amino acids of the type I precursor motif [6,7] Arg-Xaa-(Lys/Arg)-Arg↓, where Xaa is Ser, Val and Arg for proNGF/proNT-4/5, proBDNF/proNT-6 and proNT-

3 respectively. This general motif is also found in the recently described glial-derived neurotrophic factor, in which Xaa is Val [8]. As yet, the processing enzymes responsible for generating each mature neurotrophin within cells have not been unequivocally identified.

Over the past few years a family of mammalian processing enzymes (called convertases) that are evolutionarily related to the serine proteinases of the bacterial subtilisin- and yeast kexin-type families have been molecularly characterized and shown to be responsible for the intracellular processing of many precursors at both single and pairs of basic residues (for reviews see [9–12]). So far, six members of this subtilisin/kexin-like convertase family are known and are named furin, PC1 (also called PC3), PC2, PACE4, PC4 and PC5 (also called PC6). Of these, only PACE4, PC4 and PC5 exhibit multiple isoforms, most likely resulting from the generation of tissue-specific mRNAs by alternate splicing (for reviews see [7,9]). Of the known convertases only furin [12] and PC5/6-B [13], a C-terminally extended isoform of PC5 [14], contain a transmembrane domain within their C-terminal sequences.

Furin is ubiquitously expressed [10,12,15], whereas PACE4 and to a lesser extent PC5 are distributed in some endocrine and non-endocrine cells [10,14,16,17]. PC1 and PC2 are predominantly expressed in neural and endocrine cells [10,16,18], whereas PC4 is exclusively expressed in testicular germ cells

Abbreviations used: BDNF, brain-derived neurotrophic factor; Endo H, endoglycosidase H; NB-DNJ, *N*-butyldeoxynojirimycin; NGF, nerve growth factor; NT-3 (etc.), neurotrophin-3 (etc.); POMC, pro-opiomelanocortin; proNGF, precursor of NGF; TGN, *trans*-Golgi network; VV, vaccinia virus; the prefixes m and h denote mouse and human respectively.

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[¶] The contributions to this study by these authors were of equal scientific value.

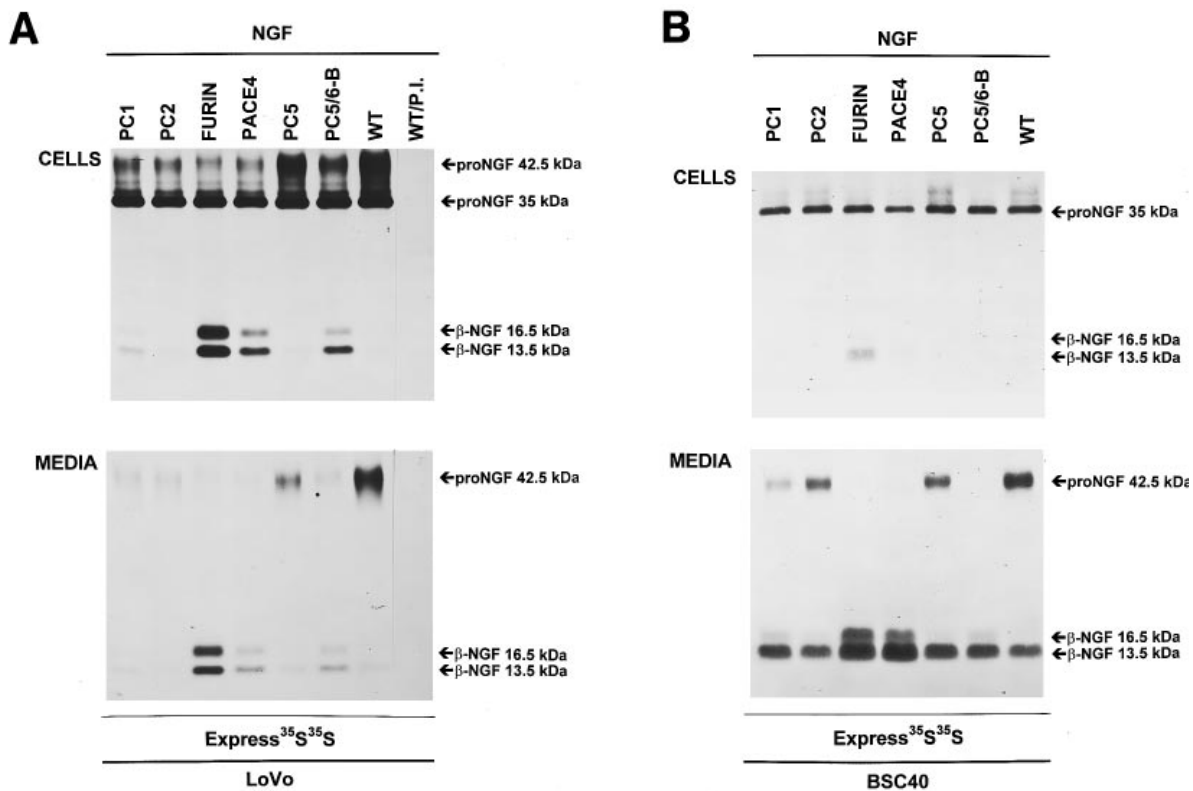


Figure 1 Processing of proNGF in LoVo and BSC40 cells

Autoradiograms of a 15% total/1.3% cross-linker slab SDS/PAGE gel of (A) LoVo and (B) BSC40 cells co-infected with VV:mNGF and either wild type (VV:WT) or the VV recombinants of the convertases (VV:mPC1, VV:mPC2, VV:hFurin, VV:hPACE4, VV:mPC5 and VV:mPC5/6-B). The rabbit antibody used in these immunoprecipitations is directed against mouse β -NGF, and as a control we used a preimmune (P.I.) rabbit serum for the NGF + WT experiment. Following infection and overnight incubation, the cells were pulsed with ^{35}S -labelled Met + Cys for 30 min followed by a chase of 2 h. The Figure also depicts the molecular masses estimated from the migration positions of immature proNGF (35 kDa), secreted proNGF (42.5 kDa) and β -NGF (16.5 and 13.5 kDa). The specificity of the immunoprecipitation was further verified in a separate experiment in which inclusion of 50 μg of pure β -NGF completely abolished the immunoprecipitation of labelled proteins (results not shown).

[10,19]. Furin is localized in the *trans*-Golgi network (TGN) and cycles to the cell surface [20]. The neural and endocrine convertases PC1 and PC2 are localized within the TGN and in dense-core secretory granules [21]. Little is known about the intracellular localization of PACE4, PC5 or PC5/6-B. Furin processes precursors generated in constitutively secreting cells, and usually cleaves substrates at the consensus type I cleavage site Arg-Xaa-(Lys/Arg)-Arg↓ [6,7,12,22]. In contrast, PC1 and PC2 have a broader selectivity and require only solvent-accessible single or paired basic residues [7,23].

Neurotrophins are produced both by constitutively secreting cells (e.g. fibroblasts and glial cells) and in cells containing dense-core secretory granules (e.g. granular tubule cells in mouse mandibular glands, neurons and mast cells). Therefore it is of interest to define which of the known subtilisin/kexin-like mammalian convertases could be candidate processing enzymes for neurotrophin precursors. Following the first demonstration that proNGF could be processed intracellularly in both constitutively and regulated cells [24], Bresnahan et al. [6] showed that yeast kexin and human furin are capable of processing mouse proNGF to NGF. Since then, no comparative analyses of the ability of the other convertases to process proNGF have been carried out.

In the present study we tested whether the prohormone convertases can process the precursor of NGF. We have used the

vaccinia virus (VV) expression system to co-express proNGF and convertases in mammalian cell lines and analysed processing of the precursor by metabolic labelling and gel electrophoresis. Results indicate that some members of the convertase family effectively generate mature forms of NGF in both regulated and/or constitutively secreting cells. Our data confirm that furin is an effective pro-neurotrophin convertase, but also demonstrate that other convertases such as PACE4 and PC5/6-B could participate in this process. The results show that the processing intermediates of proNGF and the secretory products generated in constitutively secreting cells differ from those produced in regulated cells; some of these differences are eliminated when the NGF precursor is processed in the presence of either chromogranin B or secretogranin II, but not chromogranin A. Taken together, these results suggest that the processing of NGF could vary depending upon the cell type which produces it.

EXPERIMENTAL

VV recombinants

Purified recombinant VVs using the full-length mouse (m)PC1, mPC2 [25] and human (h)Furin have been described previously [26]. VV:hPACE4 [27], VV:mPC5 [14], VV:mPC5/6-B [13] and VV:mNGF [3], using the full-length coding regions, were obtained essentially as described for mPC1 and mPC2 [26,28].

The full-length cDNA of mNGF was generously provided by Regeneron Inc. VV recombinants of the human chromogranin A ([29]; kindly provided by Dr. G. Hendy, Calcium Laboratory, McGill University), mouse chromogranin B short (0.8 kb) [30] and long (2.3 kb) [31] forms and rat secretogranin II [32] were also prepared. Site-directed mutagenesis of pro-mNGF to mutate [Arg³⁰⁵,Arg³⁰⁶]proNGF [3] into [Ala³⁰⁵,Ala³⁰⁶]proNGF was performed using an M13 mutagenesis kit (Bio-Rad) and the mutagenic oligonucleotide AGGAAGGCTACAGCAGCTGGCTGACTT, where the bold, italic nucleotides represent the variant ones, and which further incorporated a *PvuII* site (CAGCTG) permitting easier selection of the mutant. VV:mPOMC (where POMC is pro-opiomelanocortin) was a gift from Dr. G. Thomas (Vollum Institute, Portland, OR, U.S.A.).

VV infection, biosynthetic labelling and microsequencing

All VV infections were performed as described previously [25,26]. Following VV infection and incubation for 17 h, the cells from 25 cm² dishes were pulse- or pulse-chase-labelled at 37 °C for the specified time with Express³⁵S³⁵S containing L-[³⁵S]-methionine + L-[³⁵S]-cysteine (0.2 mCi), L-[³⁵S]-methionine (10 cm² dishes; 60 µCi) or Na₂³⁵SO₄ (0.5 mCi) (DuPont-New England Nuclear) in methionine/cysteine-free, methionine-free or methionine/cysteine/SO₄-free RPMI-1640 (Gibco) medium respectively. In the tunicamycin (Sigma) experiments, this drug was present at 5 µg/ml during both the 60 min preincubation and the pulse-chase analysis periods. In experiments performed with *N*-butyldeoxynojirimycin (NB-DNJ) (Sigma), 0.2 mg/ml (2 mM) of the α₁-glucosidase inhibitor was added during both the preincubation and labelling periods. Cells were collected and kept on ice for 30 min in the presence of 0.35 ml of RIPA lysis buffer (150 mM NaCl, 1 % Nonidet P-40, 50 mM Tris/HCl, pH 7.5, 0.1 % SDS, 0.5 % sodium deoxycholate) to which 10 mM methionine was added. The cell lysates or media were incubated overnight at 4 °C with 4 µl of anti-NGF serum (dilution 1:250) followed by incubation with Protein A-agarose (Calbiochem) for 2 h. Slab-gel SDS/PAGE was performed on 15 % total/1.3 % cross-linker polyacrylamide gels in 0.1 % SDS. The gels were fixed and treated with ENTENSIFY (DuPont-New England Nuclear) according to the manufacturer's instructions. For microsequencing, the [³H]Val- and [³H]Phe-labelled as well as the [³⁵S]Met-labelled proteins were eluted from an SDS/PAGE gel and subjected to microsequencing analysis on an Applied Biosystem gas-phase sequencer model 470A, as previously described [25,26].

Endoglycosidase H (Endo H), N-Glycanase and aryl sulphatase treatments

Following metabolic labelling of BSC40 cells with [³H]Phe or of AtT20 cells with Na₂³⁵SO₄ for 2 h and immunoprecipitation procedures, the antigen-antibody complexes were subjected to SDS/PAGE analyses. The [³H]Phe-labelled 42.5, 35 and 13.5 + 16.5 kDa proteins and the ³⁵SO₄-labelled 39 and 34 kDa proteins were then eluted from the gel and divided into three equal portions. Each reaction was performed in a volume of 100 µl at 37 °C for 17 h. One portion was treated with Endo H incubation buffer (100 mM sodium citrate, pH 5.5) to which Endo H (10 m-units) was added. The second portion was digested with 1.5 units of peptide:*N*-glycosidase F (N-Glycanase; Oxford GlycoSystems) in a buffer containing 20 mM sodium phosphate, pH 7.5, and 50 mM EDTA. The third sample served as an untreated control and did not contain enzyme. For the aryl sulphatase treatment, the labelled samples obtained from AtT20 cells were digested with 0.5 unit of aryl sulphatase (Sigma) in a

buffer containing 0.2 M sodium acetate, pH 5. All reaction products were then analysed on SDS/PAGE as described above.

RESULTS

Candidate convertases for the processing of proNGF in constitutive and regulated cells

We first evaluated which of the mammalian convertases of the subtilisin/kexin family are candidate processing enzymes of proNGF, and also tested whether the enzyme specificity varies as a function of cell type. Using the VV expression system we co-expressed mouse proNGF with individual convertases both in constitutively secreting (LoVo and BSC40) cells and in regulated (AtT20 and GH4C1) cells. The six enzymes tested were furin, PACE4, PC5 (plus its differentially spliced form PC5/6-B), PC1 and PC2.

Processing of proNGF in constitutive cells

As shown in Figure 1, in the absence of co-expressed convertases a high- and a low-molecular-mass form of proNGF were evident in LoVo cell extracts following electrophoresis. The lower-molecular-mass form (35 kDa) represents the NGF precursor predicted from its cDNA and primary structure. The higher-molecular-mass form (42.5 kDa) is a glycosylated intermediate that is processed into NGF (as detailed below). Small amounts of processed 13.5 and 16.5 kDa forms of NGF could barely be

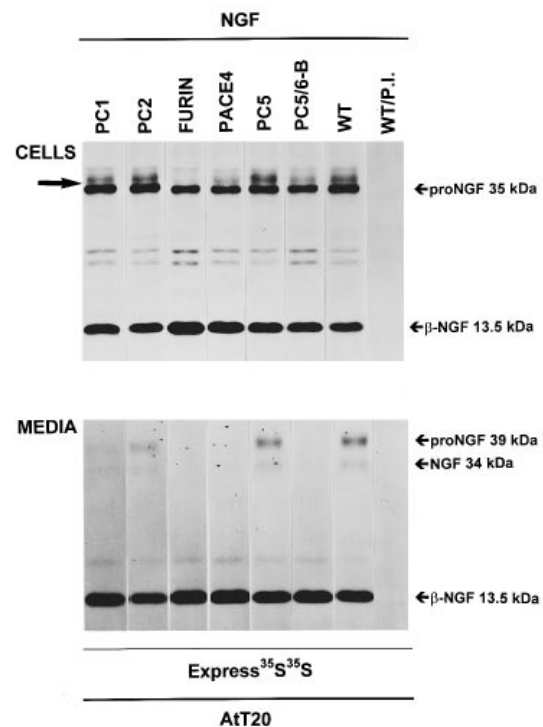


Figure 2 Processing of proNGF in AtT20 cells

Autoradiograms of a 15% total/1.3% cross-linker slab SDS/PAGE gel of AtT20 cells co-infected with VV:mNGF and either wild-type (VV:WT) or the VV recombinants of the convertases (VV:mPC1, VV:mPC2, VV:hfurin, VV:hPACE4, VV:mPC5 and VV:mPC5/6-B). Following infection and overnight incubation, the cells were pulsed with ³⁵S-labelled Met + Cys for 30 min followed by a chase of 2 h. The migration positions of molecular mass standards, of immature proNGF (35 kDa) and secretable proNGF (39 kDa; left-hand arrow) and of mature β-NGF (13.5 kDa) are indicated.

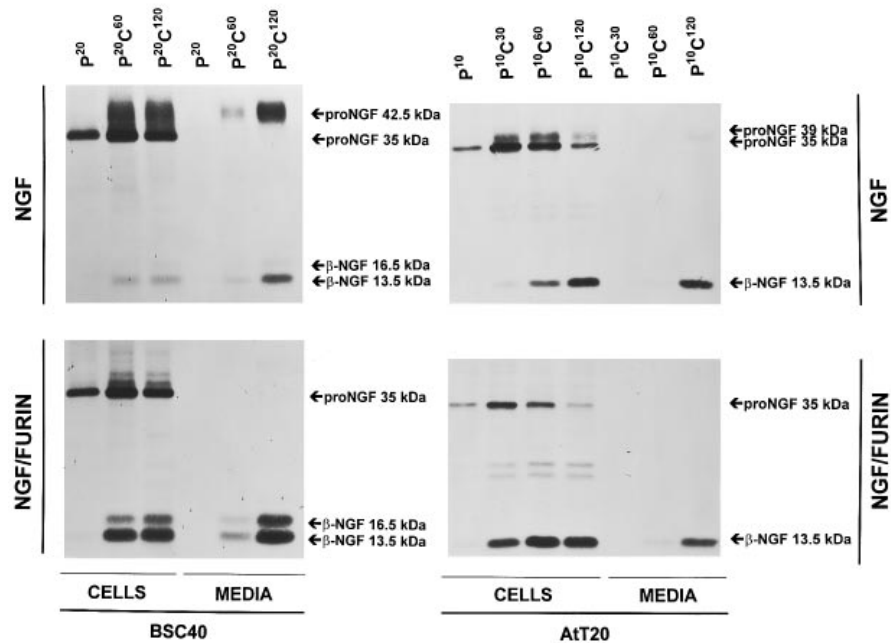


Figure 3 Pulse-chase analysis of proNGF processing in BSC40 and AtT20 cells in the absence or presence of furin

BSC40 or AtT20 cells were co-infected with either VV:mNGF + VV:WT or VV:mNGF + VV:hfurin. Following overnight incubation, the cells were pulse-labelled with ^{35}S -labelled Met + Cys for either 20 min (P^{20} ; BSC40 cells) or 10 min (P^{10} ; AtT20 cells) followed by a chase of 30, 60 or 120 min (C^{30} , C^{60} and C^{120} respectively). The media and cell extracts were then immunoprecipitated with an anti- β -NGF antibody and the immunoprecipitates analysed on a 15% total/1.3% cross-linker slab SDS/PAGE gel. The molecular masses of immature proNGF (35 kDa), secreted proNGF (42.5 kDa) and mature β -NGF (16.5 and 13.5 kDa) are indicated. Similar precursor-product results were obtained in AtT20 cells, except that the 42.5 kDa form migrated as a 39 kDa molecule and no 16.5 kDa form was detected. The apparent increase in the level of the 35 kDa form in the first period of chase is probably due to the continued incorporation of radioactivity which entered the cells during the pulse period.

seen in the medium of LoVo cells (Figure 1A, lower panel). In contrast, BSC40 cells expressing only proNGF secreted appreciable amounts of NGF into the conditioned medium (Figure 1B, lower panel). Comparison of Figures 1(A) and 1(B) suggests that LoVo cells (which lack endogenous furin activity [33]) are less capable than BSC40 cells of endogenous processing of proNGF.

Northern blot analyses (results not shown) demonstrated that, of the other known mammalian subtilisin/kexin-like convertases, LoVo cells also express PACE4 [10], suggesting that this enzyme could be responsible for the endogenous processing of proNGF. Supporting this idea, the data reveal that in both LoVo (Figure 1A) and BSC40 (Figure 1B) cells furin, PACE4 and PC5/6-B are the three best candidate proNGF convertases. This conclusion is based on two lines of evidence: (i) the decrease in the level of the 42.5 kDa proNGF intermediate, and (ii) the corresponding increase in the levels of two forms of NGF of apparent molecular mass 16.5 kDa (β -NGF 16.5 kDa) and 13.5 kDa (β -NGF 13.5 kDa). The relative amount of these forms varies between 15 and 40%, as determined by densitometry.

In these experiments we could not determine the exact level of activity of each enzyme in the virus-infected cells, since doing so would require an active-site titrant which for the convertases is not yet available. As the next best approach we estimated the relative levels of convertase mRNAs by Northern blot analyses. Using the VV expression system, which utilizes the same promoters for each convertase and for proNGF, similar mRNA expression levels for each convertase were measured ([25,28]; results not shown).

By the criteria we used, the results of Figure 1 suggest that

furin is the most efficient of the three convertases. PC1 and PC5 can also process proNGF, albeit much less effectively than furin. PC2, which usually requires a regulated cell environment for maximal activity [21,25,34], does not appear to significantly enhance the processing of proNGF. Under similar expression conditions in BSC40 cells, PC2 does process POMC into β -endorphin [25]. Finally, the 42.5 kDa form of proNGF is present both in the cell extracts (especially apparent in LoVo cells) and in the media, whereas the 35 kDa precursor is not secreted and represents the major intracellular form in both LoVo and BSC40 cells (Figure 1).

Processing of proNGF in cells containing secretory granules

To analyse the processing of proNGF in cells containing dense-core secretory granules, we repeated the experiments shown in Figure 1 in corticotroph AtT20 cells (Figure 2) and somatomammotroph GH4C1 cells. Densitometry revealed that 5, 8 and 7% of proNGF (39 kDa) remained in cells expressing furin, PACE4 and PC5/6-B respectively, in contrast to cells containing the other enzymes or the wild-type control, where 12% of the radioactivity appeared to be associated with the unprocessed precursor. Processed NGF product (13.5 kDa) accounted for 61, 57 and 58% of the total, respectively, in the presence of the same three enzymes, whereas control levels were about 48%. ProNGF (39 kDa) could not be detected in medium conditioned by cells expressing exogenous furin, PACE4 or PC5/6-B, as compared with levels of radioactivity varying between 9 and 15% of the total in cells containing the wild-type virus or recombinant PC1, PC5 or PC2 together with proNGF. Therefore, in agreement

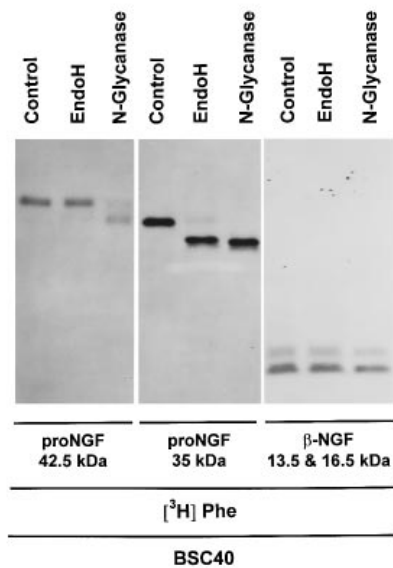


Figure 4 Endo H and N-Glycanase digestion of proNGF and NGF

BSC40 cells were infected with VV:mNGF, and following an overnight incubation they were pulse-labelled with [3 H]Phe for 2 h and the cell extracts were immunoprecipitated with an anti- β -NGF antibody. The 42.5, 35 and 16.5 + 13.5 kDa proteins were purified from a 15% total/1.3% cross-linker slab SDS/PAGE gel. The eluted proteins were divided into three equal portions: a control sample and samples digested with either Endo H or N-Glycanase prior to re-electrophoresis under the same conditions. Overexposure of the gel confirmed that the 13.5 and 16.5 kDa NGFs are insensitive to digestion by either Endo H or N-Glycanase (results not shown).

with the results obtained with the constitutively secreting LoVo and BSC40 cells (Figure 1), furin, PACE4 and PC5/6-B are also the most effective proNGF convertases in the regulated AtT20 cells.

Comparison of Figures 1 and 2 reveals two major differences between AtT20 cells and LoVo/BSC40 cells. Whereas LoVo/BSC40 cells generate both 16.5 and 13.5 kDa forms of NGF, cell extracts and media conditioned by AtT20 cells contain only the 13.5 kDa form of the protein, with no detectable 16.5 kDa form (Figure 2). Since the 16.5 kDa molecule may be a post-translationally modified form of NGF (see below), this result suggests that the intracellular milieu of the regulated cells we tested favours the production of the 13.5 kDa form, a result confirmed in GH4C1 cells, another regulated secretory cell (not shown). Secondly, the molecular mass of mature proNGF in AtT20 cells is lower (39 kDa; Figure 2) than that of the form detected in constitutive cells (42.5 kDa) (Figure 1). This difference presumably relates to a different post-translational modification of the 35 kDa precursor (compare Figures 1 and 2, and see below). In addition, media conditioned by AtT20 cells contain minor amounts of a processing intermediate with an apparent molecular mass of 34 kDa (Figure 2). Finally, the degree of processing of proNGF in the control samples was much higher in AtT20 cells (Figure 2) than in LoVo or BSC40 cells (Figure 1).

Precursor-product relationship

In order to define the processing pathway of proNGF we pulsed BSC40 and AtT20 cells expressing proNGF either alone (Figure 3, top panels) or in combination with furin (Figure 3, bottom panels) with 35 S-labelled Met + Cys for either 20 min (BSC40 cells) or 10 min (AtT20 cells), followed by a chase in unlabelled

medium for up to 120 min. After the short pulse period in cells expressing NGF alone, only the 35 kDa proNGF form was detectable in cell extracts, and no processed NGF was evident in the medium. The 35 kDa proNGF form was not secreted from either cell type (Figures 1–3), suggesting that it represents an immature intermediate within the endoplasmic reticulum which is post-translationally modified to give rise to the mature precursor form seen in both cell types. This hypothesis is further reinforced by the sensitivity of the 35 kDa proNGF to digestion by both Endo H and N-Glycanase (Figure 4, middle panel). Progressively upon chase, in both cells and media, we observed (Figure 3) in BSC40 cells an increase in the level of the 42.5 kDa proNGF and 16.5/13.5 kDa forms of NGF, and in AtT20 cells an increase in the 39 kDa precursor and 13.5 kDa processed NGF. The 42.5 kDa form (Figure 4, left panel) is likely to be the mature form of proNGF, since it was sensitive to digestion with N-Glycanase but resistant to treatment with Endo H (Figure 4). We also observed that, in contrast to the 35 kDa form, from which carbohydrate chains were completely removed by N-Glycanase (Figure 4, middle panel), under the same incubation conditions the digestion of the 42.5 kDa form by this endoglycosidase was only partial (Figure 4, left panel). In addition, the N-Glycanase-processed form of the 42.5 kDa proNGF migrated with an apparent molecular mass higher than that obtained following the same treatment of the 35 kDa form. This result suggests that one or more of the oligosaccharide chains on the 42.5 kDa form are resistant to N-Glycanase or that another, as yet undefined, post-translational modification is present on the 42.5 kDa but not the 35 kDa form of proNGF. From the predicted protein structure of mouse proNGF [3,24], three N-glycosylation sites are proposed, two in the pro-segment (positions 51 and 96) and one in the NGF molecule (position 148). Interestingly both the 16.5 and 13.5 kDa forms of NGF (Figure 4, right panel) were resistant to digestion with N-Glycanase or Endo H, confirming that neither form of NGF is N-glycosylated and demonstrating that Asn¹⁴⁸ is not appreciably glycosylated. However, in other experiments we observed in the medium faint protein bands (apparent molecular masses of 22 and 18 kDa) which seemed to be insensitive to Endo H. When digested with N-Glycanase, however, their molecular masses were decreased to that of unglycosylated NGF.

Identification of proNGF and its processed forms

In order to unambiguously characterize the proteins immunoprecipitated in the above experiments, we microsequenced the NGF-containing 35, 42.5, 16.5 and 13.5 kDa forms. As shown in Figure 5, both the 35 kDa (Figure 5A) and the 42.5 kDa (Figure 5B) forms contain in their N-terminal sequence Val⁸ and Val¹⁴, demonstrating that their sequence starts at the expected position following the predicted signal peptidase cleavage site [24]. The signals apparent at Val⁹ and Val¹⁵ (Figure 5B) probably represent excessive carry-overs in the presence of limiting amounts of radioactivity. Alternatively, they may reflect some miscleavage of preproNGF at the less favoured Gly-Val-Gln↓Ala-Glu-Pro-Tyr sequence rather than at the preferred Gly-Val-Gln-Ala↓Glu-Pro-Tyr sequence [24]. The size difference between these two forms is due to post-translational modifications such as N-glycosylation, trimming (Figure 4) and sulphation (see Figure 10 below). The 16.5 and 13.5 kDa forms of NGF revealed the same N-terminal sequence as that of native β -NGF, demonstrating the presence of Met⁹ (Figures 5C and 5D), Phe⁷ and Phe¹² (Figures 5E and 5F), as well as Val at positions 6, 14, 18 and 20 (results not shown). These data demonstrate that the 16.5 and 13.5 kDa forms result from the cleavage of proNGF at the sequence Asn-Arg-Thr-His-

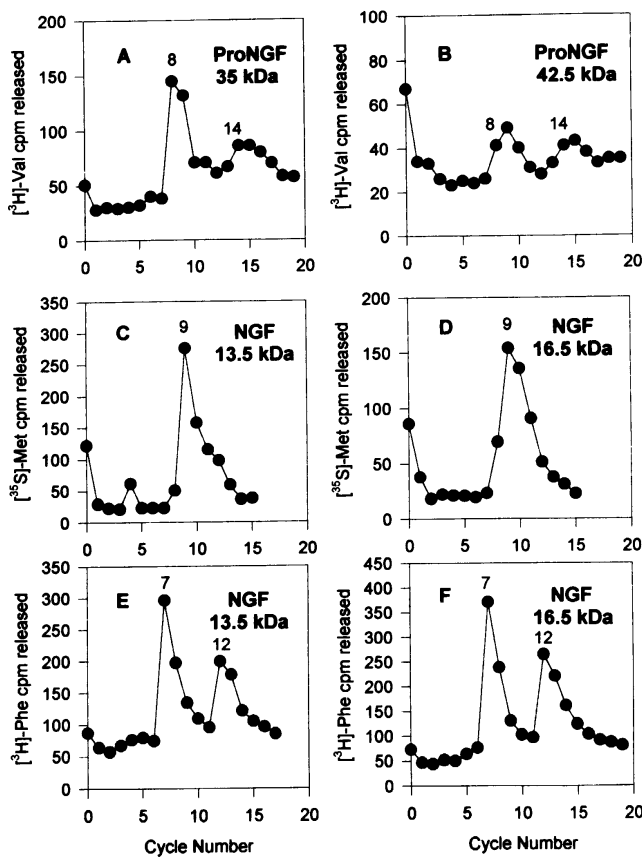


Figure 5 Microsequence analysis of NGF-containing products

Shown are the results of microsequencing of [^3H]Val-labelled 35 kDa proNGF (A) and 42.5 kDa proNGF (B); of [^{35}S]Met-labelled 13.5 kDa NGF (C) and 16.5 kDa NGF (D); and of [^3H]Phe-labelled 13.5 kDa NGF (E) and 16.5 kDa NGF (F). The deduced sequence positions are indicated. For the 42.5 kDa sample (B), which had limited counts, we observed a large degree of carry-over from cycle to cycle.

Arg-Ser-Lys-Arg¹⁰³↓Ser-Ser-Thr [3,4]. Note the presence of an Asn glycosylation site eight amino acids N-terminal to the cleavage site. The difference between the 16.5 and 13.5 kDa forms must therefore reside elsewhere than at the N-terminus.

In order to assess whether the processing of the C-terminal Arg-Arg³⁰⁶↓Gly sequence [3] is responsible for the difference between the 16.5 and 13.5 kDa forms, we mutated these two arginine residues into alanines. As shown in Figure 6, upon co-expression of either [Arg³⁰⁵,Arg³⁰⁶]proNGF or [Ala³⁰⁵,Ala³⁰⁶]proNGF with furin in BSC40 cells both the 16.5 and 13.5 kDa forms were still apparent in the cell extract and in the medium. In contrast, only the 13.5 kDa form was detected in AtT20 cells for both the wild-type (Figures 2, 3 and 6) and mutant (results not shown) NGF. Therefore the difference between the two mature NGF forms seen in constitutive cells is not due to either N-terminal or C-terminal extensions, but is probably the result of some unidentified post-translational modification within the NGF protein that occurs in BSC40 and LoVo cells but not in AtT20 cells or GH4C1 cells.

Secretogranins and the processing of proNGF

Cells containing secretory granules express a number of proteins which could be implicated in granule biogenesis, some of which may influence the TGN environment in which the processing

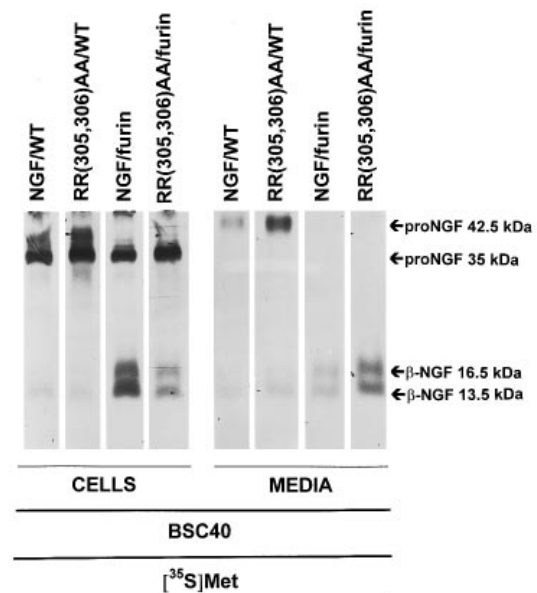


Figure 6 Biosynthetic analysis of the [Ala³⁰⁵,Ala³⁰⁶]proNGF mutant

Autoradiograms of a 15% total/1.3% cross-linker slab SDS/PAGE gel of BSC40 cells co-infected with VV:mNGF/VV:WT, VV:[Ala³⁰⁵,Ala³⁰⁶]mNGF [RR(305,306)AA]/VV:WT, VV:mNGF/VV:hfurin or VV:[Ala³⁰⁵,Ala³⁰⁶]mNGF [RR(305,306)AA]/VV:hfurin (see the text for details). The cells were pulsed with [^{35}S]Met for 3 h. The migration positions of immature proNGF (35 kDa), secretory proNGF (42.5 kDa) and mature β -NGF (16.5 and 13.5 kDa) are indicated.

reaction begins. The secretogranins represent a family of acidic neuroendocrine-specific secretory proteins of unresolved function and are widespread constituents of the secretory granules in neuroendocrine cells [35]. We therefore sought to test the influence of chromogranin A, chromogranin B and secretogranin II on the processing of proNGF in constitutive cells lacking these proteins. As shown in Figure 7, the furin-mediated processing of proNGF in BSC40 cells, generating the 16.5 and 13.5 kDa NGF products, was not significantly affected by the co-expression of a short form generated by alternative splicing (0.8 kb mRNA) of chromogranin B [30], and the level of the 16.5 kDa form was only partially reduced in the presence of exogenous chromogranin A [29]. In contrast, full-length chromogranin B (2.3 kb mRNA) [31] or secretogranin II [32] completely eliminated the formation of the 16.5 kDa NGF product. This result suggests that the absence of the 16.5 kDa form from regulated cells such as AtT20 (Figure 2) or GH4C1 (results not shown) cells may well be due to the effect of endogenous secretogranins on the processing reaction.

Further analyses of the post-translational modifications of proNGF and NGF

In an effort to define more completely the post-translational modifications of proNGF and NGF, we analysed the production of these proteins in the presence of a number of drugs that affect post-translational processing.

The importance of N-glycosylation

In order to probe the significance of N-glycosylation of the prosegment of proNGF, we pulse-labelled BSC40 cells expressing proNGF in the absence or presence of co-expressed furin and the

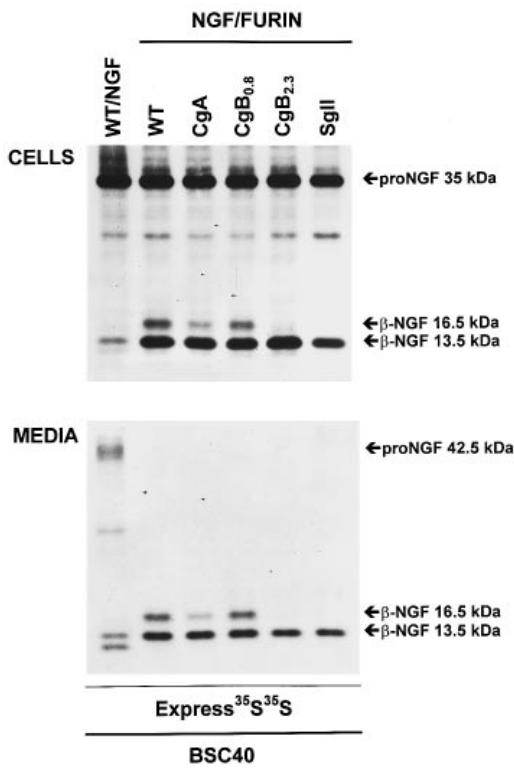


Figure 7 Secretogranins and the processing of proNGF in constitutive cells

Autoradiograms of a 15% total/1.3% cross-linker slab SDS/PAGE gel of BSC40 cells co-infected with VV:mNGF [1 plaque-forming unit (pfu)]/VV:WT (6 pfu), VV:mNGF (1 pfu)/VV:hfurin (2 pfu)/VV:WT (4 pfu) or VV:mNGF (1 pfu)/VV:hfurin (2 pfu)/VV:secretogranin (4 pfu). The secretogranins include human chromogranin A (CgA), mouse chromogranin B short (CgB_{0.8}) and long (CgB_{2.3}) forms and bovine secretogranin II (SgII). The cells were pulsed with ³⁵S-labelled Met + Cys for 2 h. The migration positions of immature proNGF (35 kDa), secreted proNGF (42.5 kDa) and mature β-NGF (16.5 and 13.5 kDa) are indicated. The band migrating below 13.5 kDa represents a non-specific protein which is usually seen in the absence of NGF and which is not displaceable by excess unlabelled NGF (results not shown).

N-glycosylation inhibitor tunicamycin for 30 min, followed by a chase of 120 min. As shown in Figure 8, tunicamycin treatment in the presence or absence of furin decreased the molecular mass of the cellular 35 kDa form of proNGF to about 30 kDa and caused the virtual disappearance from the cells and media of the 42.5 kDa precursor and the 16.5/13.5 kDa forms of NGF. The residual 35 kDa form which is detectable probably arises from incomplete inhibition by tunicamycin which is often observed at the chosen lower (and less toxic) tunicamycin concentration of 5 μg/ml. This result implies that N-glycosylation of the prosegment of proNGF is essential for its efficient exit from the endoplasmic reticulum to the Golgi apparatus and its subsequent processing and secretion. The fate of unglycosylated proNGF within the endoplasmic reticulum is not known, but evidently it is destroyed by resident proteolytic enzymes, as observed for unglycosylated proPC1 and proPC2 [26].

If N-glycosylation is important for the exit of proNGF from the endoplasmic reticulum and its processing into NGF, then trimming of its N-glycosyl moieties may also be necessary to allow its maturation. To test this hypothesis, we pulse-labelled BSC40 cells expressing either proNGF or proNGF plus furin for 120 min with ³⁵S-labelled Met + Cys in the absence or presence of the α₁-glucosidase-I inhibitor NB-DNJ [36]. The enzyme α₁-glucosidase-I hydrolyses the terminal Glcα1-2Glc linkage in N-

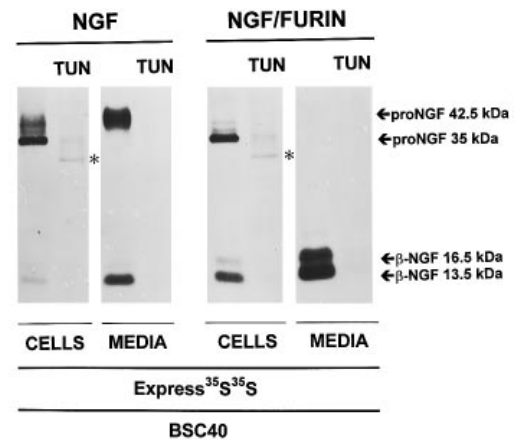


Figure 8 Importance of N-glycosylation of proNGF for its exit from the endoplasmic reticulum

BSC40 cells were infected with either VV:mNGF + VV:WT or VV:mNGF + VV:hfurin. Following overnight incubation the cells were pulse-labelled with ³⁵S-labelled Met + Cys for 30 min followed by a chase of 2 h in the absence or presence of tunicamycin (TUN) at 5 μg/ml. The immunoprecipitates of media and cell extracts were resolved on a 15% total/1.3% cross-linker slab SDS/PAGE gel. Note the disappearance of proNGF and NGF from the media of cells treated with tunicamycin. The apparent mass of the unglycosylated proNGF was estimated to be about 30 kDa (*). Similar results were obtained with PACE4 or PC5/6-B co-expressed with proNGF (not shown).

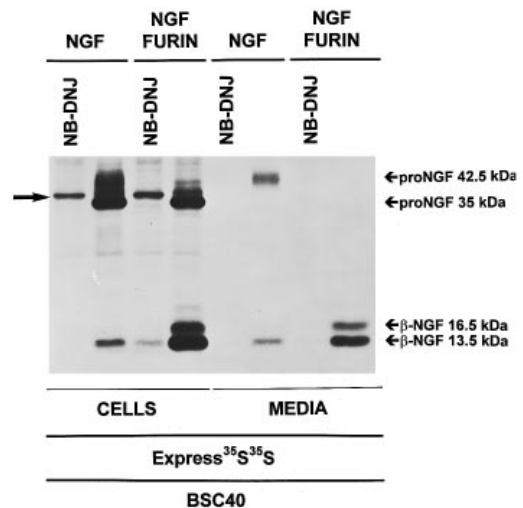


Figure 9 Importance of carbohydrate chain trimming for the exit of proNGF from the endoplasmic reticulum

BSC40 cells were infected with either VV:mNGF + VV:WT or VV:mNGF + VV:hfurin. Following overnight incubation the cells were pulse-labelled with ³⁵S-labelled Met + Cys for 2 h in the absence or presence of NB-DNJ at 200 μg/ml. The immunoprecipitates of the media and cell extracts were then resolved on a 15% total/1.3% cross-linker slab SDS/PAGE gel. Note the increase in the molecular mass of the intracellular 35 kDa form to about 37 kDa in the presence of NB-DNJ (left-hand arrow) and the lack of secretion of both proNGF and NGF from cells treated with this α₁-endoglycosidase-I inhibitor.

glycosylated proteins. NB-DNJ, an iminosugar derivative, inhibits α₁-glucosidase-I by mimicking the pyranosyl moiety of glucose; in so doing it prevents the normal processing of the first glucose residue of N-linked glycoproteins and hence effectively shuts down the trimming process. Figure 9 demonstrates that

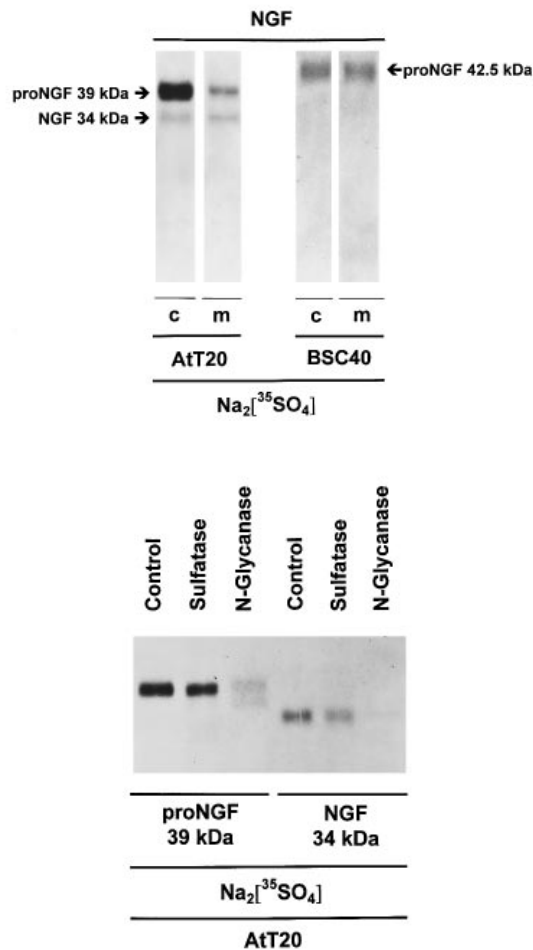


Figure 10 The pro-segment of ProNGF is sulphated

Top: AtT20 cells and BSC40 cells were infected with VV:mNGF. Following overnight incubation the cells were pulse-labelled with $\text{Na}_2^{35}\text{SO}_4$ for 2 h. The media and cell extracts were then immunoprecipitated with an anti- β -NGF antibody. A major sulphated proNGF (42.5 and 39 kDa in BSC40 and AtT20 cells respectively) was present, along with a minor 34 kDa intermediate form which appeared in AtT20 cells. Note the lack of sulphated mature NGF. Bottom: the purified 39 and 34 kDa forms of proNGF obtained from AtT20 cells were divided into three equal aliquots, which were kept as such (control) or treated with either aryl sulphatase or N-Glycanase; the samples were then re-electrophoresed under the same SDS/PAGE conditions used for the other experiments. Only N-Glycanase treatment resulted in a diminution of the labelling intensity of the 34 kDa form and, in addition, a lowering of the molecular mass of the 39 kDa form.

NB-DNJ treatment increased the molecular mass of the 35 kDa precursor slightly (to 37 kDa) and lowered the intensity of labelling; also, no 42.5 kDa form was detected. This suggests that the Endo H-sensitive 35 kDa form of proNGF already contains partially trimmed N-glycosylated moieties. Secondly, the lack of secretion of either proNGF or NGF suggests that treatment with NB-DNJ prevents proNGF from reaching the Golgi stacks and being processed into NGF. Therefore we conclude that not only are the N-glycosyl moieties on the pro-segment of proNGF required for its efficient exit from the endoplasmic reticulum and its processing, but so is the necessity to trim such sugars in order to allow proNGF to reach the Golgi apparatus and be processed by furin-like enzymes.

ProNGF is sulphated on N-oligosaccharide chains

In an attempt to further confirm that the 42.5 kDa form of proNGF is the convertase-sensitive form, we wanted to define the location in the Golgi apparatus of the conversion of proNGF into NGF. For this purpose we monitored the labelling of proNGF with $\text{Na}_2^{35}\text{SO}_4$, since the sulphotransferases are known to be localized within the TGN [37,38]. As shown in Figure 10 (top), pulse-labelling of AtT20 and BSC40 cells for 120 min with $\text{Na}_2^{35}\text{SO}_4$ resulted in the sulphation of proNGF but not of mature NGF. Furthermore, as expected, the 42.5 kDa form of proNGF in BSC40 cells and the 39 kDa form in AtT20 cells were sulphated, but the 35 kDa form was not, suggesting that the latter was not localized within the TGN. We did, however, detect lower levels of a sulphated processing intermediate of 34 kDa in AtT20 cells and media which was precipitated by our anti-NGF antibody. In view of the specificity of the convertases for cleavage after Arg-Xaa-Xaa-Arg [6,7,22,23], the 34 kDa intermediate probably represents proNGF cleaved at the Ala-Leu-Arg-Arg³²-Ala-Arg³⁴-Ser-Ala sequence [3]. We believe that sulphation of the 39 and 34 kDa forms of proNGF occurs primarily on an N-glycosyl moiety, since their labelling is greatly diminished following treatment with N-Glycanase but not with aryl sulphatase (Figure 10, bottom). We therefore conclude that proNGF is glycosulphated but is not sulphated on Tyr residues.

DISCUSSION

Developmental growth of the nervous system, neuronal survival and repair of damaged neurons requires the production and release of one or more neurotrophins, which may act in synergy on some neurons [39]. Following translation of the neurotrophin mRNAs, their precursors are subject to post-translational modifications including signal peptidase cleavage, N-glycosylation, in some cases sulphation, and finally limited proteolysis at specific pairs of basic residues. The regulation of the synthesis and processing machinery of each neurotrophin would therefore need to be finely tuned in order to allow for their co-ordinate release and actions.

As a prelude to defining the fine tuning of the regulatory machinery of neurotrophin synthesis, in the present work we have concentrated on the analysis of the candidate proNGF processing enzymes. Results indicate that, of the five possible subtilisin/kexin-like convertases which are expressed in constitutively secreting and/or regulated cells [9,10], the candidate processing enzymes of proNGF are furin, PACE4 and PC5/6-B. This conclusion was reached for both constitutive and regulated cells. Although not shown, an identical conclusion was reached from similar studies undertaken on proBDNF and proNT-3.

Our results are in agreement with those of Edwards et al. [24], who demonstrated that both constitutive and regulated cells have the ability to process and secrete biologically active NGF. In cells with a regulated pathway, such as AtT20 cells, these authors showed that NGF is stored intracellularly and can be released by appropriate secretagogues. Bresnahan et al. [6] also reported that yeast kexin and human furin efficiently processed proNGF to NGF in constitutively secreting BSC40 cells. Both studies used the VV expression system, since it was reported that transfection techniques did not yield stable transformants expressing significant levels of NGF [24]. However, neither study systematically examined other potential convertases; nor did they contrast the processing products of proNGF in constitutive versus regulated cells. In this paper we have presented a detailed comparative analysis of the processing of proNGF by the convertases in both cell types, and in addition we have provided

evidence for the influence of the secretogranins on the processing reaction.

Furin is a likely candidate for processing of pro-neurotrophins since this enzyme is ubiquitously expressed, including in cells that generate neurotrophins [15,16], and it is produced early in embryonic development [40], before the appearance of the neurotrophins. However, our present data also revealed that production of mature NGF (Figure 1) occurs to a small extent in LoVo cells, which are devoid of furin activity [33], suggesting that other mammalian convertases, in addition to furin, can process neurotrophin precursors. PACE4, which is produced by LoVo cells [10], seems a likely additional candidate, as does PC5/6-B. The latter is almost double the size of the more widely expressed PC5 [14] and has an extended Cys-rich domain and a putative transmembrane sequence close to the C-terminus [13]. Presumably it derives from a single gene [41] by alternative splicing. PC5/6-B is abundantly expressed in the epithelial cells of the small intestine and in the adrenal cortex [9,10,13,14]. However, in order to substantiate the possible physiological involvement of PC5/6-B in pro-neurotrophin processing, it will be necessary to analyse the production of this protein in various tissues containing one or more members of the neurotrophin family.

In mouse salivary glands β -NGF is part of the 7 S NGF complex [42] along with two members of the kallikrein-like family of serine proteinases, termed the α - and γ -subunits. The stoichiometry is $\alpha_2\beta_2\gamma_2$ [43]. *In vitro*, the γ -subunit is able to cleave proNGF into the 118-amino-acid β -NGF only when stoichiometric (as opposed to catalytic) amounts of the two components are mixed together [44]. Therefore, in view of our results and those of Bresnahan et al. [6], which support the involvement of furin-like enzymes in the processing of proNGF, the importance of the γ -subunit in NGF processing within the submaxillary gland is questionable. Furthermore, many cells that synthesize NGF do not express the α - or γ -subunits [45]. Rather, it was suggested that the γ -subunit cleaves the C-terminus of mouse β -NGF at the sequence Arg-Lys-Ala-Pro-Arg¹¹⁸↓Arg-Gly-CO₂H and then remains associated as an enzyme-product complex, with the C-terminal Arg of β -NGF occupying the S1 subsite of the γ -subunit [46]. In NGF-expressing tissues other than the submaxillary gland the fate of the C-terminus of NGF is not known.

The results presented in this study demonstrate for the first time that the pro-segment of proNGF can be sulphated at its oligosaccharide chains. We do not know the function of such a post-translational modification, but we have exploited it to provide evidence that the processing of proNGF does not occur before it reaches the TGN (Figure 10). The other post-translational modification that we studied is the N-glycosylation of the pro-segment of proNGF. The data demonstrate that N-glycosylation and carbohydrate chain trimming are both important for the exit of this precursor from the endoplasmic reticulum and its ultimate processing into NGF (Figures 9 and 10). Blocking either N-glycosylation by tunicamycin treatment or the trimming of the nascent carbohydrate chain using NB-DNJ prevented the exit of proNGF to the Golgi apparatus and its subsequent secretion. This suggests that the carbohydrate chains of proNGF may be important for its correct folding within the endoplasmic reticulum. The importance of carbohydrates in the folding of proteins has been well documented [47]. In addition it was concluded that, although NGF contains the sequence Asn¹⁴⁸-Asn-Ser, representing a potential N-glycosylation site, the protein is primarily not glycosylated at this site, possibly because it is found within an α -helical sequence. From our results we predict that less than 5% of the total mature

NGF may be glycosylated at this site. Interestingly, Murphy et al. [48] reported that, in preparations of mature mouse submaxillary gland β -NGF isolated by standard methods, about 2% of the protein is N-glycosylated.

The data presented here show that the 16.5 and 13.5 kDa forms of NGF produced by constitutively secreting cells have the same N-terminal sequence (Figures 5C–5F), and hence that the des-octa-NGF isoform lacking the first eight amino acids isolated from mouse submaxillary gland extracts [45] is not generated by any of the convertases tested. Furthermore, the 16.5 and 13.5 kDa forms of NGF are not N-glycosylated (Figure 4), O-glycosylated (as indicated by their resistance to O-Glycanase; results not shown) or sulphated. The presence of the sequence Ala-Thr-Arg-Arg³⁰⁶-Gly at the C-terminus of proNGF suggested that processing at the Arg-Arg pair might be responsible for the two forms observed. However, site-directed mutagenesis of this pair into an Ala-Ala sequence eliminated this possibility (Figure 6). Therefore we still do not know what gives rise to the 3 kDa size difference between the 16.5 and 13.5 kDa forms of NGF. Nevertheless, we are intrigued by the observation that proNGF processing in constitutive cells leads to the formation of the 16.5 kDa intermediate (Figure 1), whereas only the 13.5 kDa form of NGF is evident in regulated cells (Figure 2). We reasoned that the absence of the 16.5 kDa product from regulated cells may be related to the presence of specific factors such as the acidic chromogranins A and B [49] and secretogranin II [50], which are expressed in these cell types but not in constitutive cells. Surprisingly, our data revealed that co-expression of either full-length chromogranin B or secretogranin II (Figure 7) together with proNGF and furin resulted in the disappearance of the 16.5 kDa product. In contrast, chromogranin A or a shorter variant form of chromogranin B had little or no effect on the processing of proNGF. A similar observation was also made in our laboratory on the processing of POMC by either PC1 or PC2, which in constitutive cells, in addition to generating β -lipotropin and β -endorphin respectively [25], gives rise to higher-molecular-mass immunoreactive β -endorphin-containing products (S. Benjannet and N. G. Seidah, unpublished work). In agreement with the notion that the intracellular environment can influence the processing reaction [28], our data further suggest that some secretogranins can exert an important influence on the processing of certain precursors such as proNGF. The mechanism by which these acidic proteins (pI close to 5.2) interact with the basic proNGF (pI 9.3) and/or its post-translational modification machinery is not yet defined. It will be informative in the future to identify the exact difference between the 16.5 and 13.5 kDa forms of NGF in order to identify which modification is inhibited by the presence of secretogranins. Since NGF is also synthesized in constitutive cells such as Schwann cells and fibroblasts, it will be interesting to investigate possible secretion from these cells of the two NGF forms observed in the present study and reported but not commented upon in previous studies using a similar VV expression system [6,24]. The function(s) of the 16.5 kDa form of NGF should also be scrutinized, and its binding to the NGF *TrkA* receptor [4,5] compared with that of the 13.5 kDa form.

In conclusion, the work presented in this paper provides a framework with which to begin to dissect the various steps involved in the biosynthesis of proNGF. Future studies of this complex phenomenon will afford many new insights into the mechanism of neurotrophin regulation and synthesis in neuronal and non-neuronal cells.

We acknowledge the excellent technical assistance of Aida Mamarbassi, Odette Th  berge and James Rochemont. We also thank Dr. K. Nakayama for kindly

providing the cDNA of mouse PC5/6-B, and Dr. G. D. Yancopoulos (Regeneron Inc.) for his gift of the neurotrophin cDNAs which were used to prepare the corresponding VV recombinants. We are grateful to Drs. H. Winkler and R. Fisher-Colbrie for the secretogranin II cDNA and to M. Mbikay for the chromogranin B cDNAs. This work was supported by Medical Research Council of Canada program grant nos. PG-11474 (N.G.S., C.L. and M.C.) and PG-11473 (R.A.M.), by Neuroscience Network grants (N.G.S., M.C. and R.A.M.) and fellowship (S.P.), and by J.A. De Sève Succession.

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