Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases

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Abstract In order to define the enzymes responsible for the maturation of the precursors of brain-derived neurotrophic factor (proBDNF) and neurotrophin-3 (proNT3), we have analysed their biosynthesis and intracellular processing by the proprotein convertases furin, PC1, PC2, PACE4, PC5 and its isoform PC5/6-B. In these studies, we utilized a vaccinia virus expression system in either BSC40 or the furin activity-deficient LoVo cells. Results demonstrated that in both cells furin and, to a lesser extent, PACE4 and PC5/6-B effectively process proBDNF and proNT3. Furthermore, we have determined that human proNT3 is sulfated, suggesting that processing of proNT3 occurs following the arrival of the precursor to the Trans Golgi Network.

Key words: BDNF; NT3; Convertase; Furin; PACE4; PC5/6-B

1. 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 coworkers discovered nerve growth factor (NGF), the first member of the family, over forty years ago (for review see [1]). Since the cDNA structure coding for the precursor of NGF (proNGF) was reported [2], four other members of the family have been identified and their cDNAs cloned: brain-derived neurotrophic factor (BDNF) [3], neurotrophin-3 (NT3) [4,5], neurotrophin-4/5 (NT-4/5) [6] and neurotrophin-6 (NT-6) [7]. Sequence data predict that all neurotrophins are generated from 31–35 kDa precursors that contain at their N-termini, hydrophobic signal peptides followed by proregions containing sequences of contiguous basic amino acids. Intracellular cleavage of the proneurotrophins to produce active growth factors occurs following pairs of basic amino acids of the type I precursor motif [8] Arg-X-(Lys/Arg)-Arg↓, where X = Ser, Val or Arg for proNGF/proNT-4/5, proBDNF/proNT-6 and proNT3, respectively.

Recently, a family of mammalian processing enzymes (called convertases) evolutionary related to the serine proteinases of the bacterial subtilisin and yeast kexin types were characterized and shown to be responsible for the intracellular processing of many precursors at both single and pairs of basic residues (for recent reviews, see [8–10]). So far, seven members of this subtilisin/kexin-like convertase family are known and are named furin, PC1 (also called PC3), PC2, PACE4, PC4, PC5 (also called PC6) and the very recently discovered PC7 [11]. Of the known convertases, only furin [10], PC5/6-B [12], a C-terminally extended isoform of PC5 [13] and possibly PC7 [11] contain a C-terminal transmembrane domain. Since all members of the convertase family can cleave precursors at the consensus type I cleavage site Arg-X-(Lys/Arg)-Arg↓ [14], it became apparent that aside from cleavage specificity the cognate convertase(s) must also be correctly colocalized and possibly coregulated with their substrate within either the Trans Golgi Network (TGN) or in secretory granules [9,15,16]. Accordingly, studies on the colocalization of the neurotrophin mRNAs with those of the convertases and their coregulation revealed that more than one convertase can colocalize with the neurotrophins [17]. Indeed, biochemical analysis of the processing of proNGF by the five possible candidate convertases revealed that furin, PACE4 and PC5/6-B are the best proNGF processing enzymes in both constitutively secreting and regulated cells [18].

In this work, we analysed the processing of proNT-3 and proBDNF by the convertases furin, PC1, PC2, PACE4 and PC5 and its isoform PC5/6-B. The very recent discovery of PC7 [11] precluded its analysis in this report. For this purpose, we coexpressed each candidate convertase with the precursor substrates in either the kidney epithelial cell line BSC40 or the furin activity-deficient colon carcinoma cell line LoVo [19]. Pulse and/or pulse-chase data elucidated the biosynthetic pathway of proNT3 and proBDNF and their processing into NT3 and BDNF.

2. Materials and methods

2.1. Vaccinia virus (VV), VV infections and biosynthetic labelings

Purified recombinant VVs using the full-length mouse (m) PC1, mPC2, mPC5, mPC5/6-B and human (h) furin and PACE4 have been previously described [20]. The VV:hBDNF [21] and VV:hNT3 [10] using the full-length coding regions were obtained essentially as described [18,20]. The full-length cDNAs of hBDNF and hNT3 were generously provided by Regeneron. Cellular expression, biosynthetic labeling with [^{35}S](Met + Cys), endoglycosidase H and *N*-glycanase digestions were performed as described [18,20].

2.2. Western blot analysis

Concentrated conditioned media from LoVo cells expressing BDNF and one of the convertases were applied to a 13–22% T, 2.7% C gradient SDS-PAGE, electrophoresed and transferred to Trans-Blot Supported

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT3, neurotrophin-3; VV, vaccinia virus.

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Nitrocellulose Membrane (Bio-Rad). Non-specific binding sites were blocked by using 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Blots were probed with antibodies raised against a conserved synthetic hBDNF decapeptide (amino acids 40–49; a gift from Dr. Eric Shooter) overnight at 4°C. After four washes (10 min each) in TBS-T, the primary antibody was visualised using horse radish peroxidase-coupled donkey antirabbit antibodies (Jackson ImmunoResearch Laboratories), followed by peroxidase-catalysed chemiluminescence (enhanced chemiluminescence; Amersham) according to the manufacturer's instructions.

3. Results

3.1. Processing of proNT3

The candidate processing enzymes of proNT3 [10] were analysed by their ability to process proNT3 following their coexpression in BSC40 cells using the vaccinia virus expression system. Fortunately, our NGF antibody readily cross-reacts with NT3 [22] and immunoprecipitates both proNT3 and NT3. As shown in Fig. 1, pulse-labeling the constitutive BSC40 cells for 30 min with [^{35}S](Met + Cys) followed by a chase of 120 min demonstrated the formation of two forms of proNT3 of apparent masses of 33.5 kDa (major form) and 35 kDa, both of which are secretable and convertase-sensitive. When proNT3 and furin were coexpressed, both proNT3 forms diminished in intensity with a parallel increase in the ratio of mature 14 kDa NT3 to proNT3. It is interesting to note that, similar to proNGF, the NT3 precursor contains a single *Asn*-glycosylation site in the prosegment, 8 amino acids N-terminal to its presumed processing site within the sequence AsnArgThrSer**ArgArgLysArg**↓TyrAlaGlu. From the relative intensities of the secreted and the remaining precursor forms estimated by scanning the autoradiogram, we propose that the decreasing order of proNT3 processing by the convertases is similar to that for proNGF [18], viz. furin being the best candidate, followed by PACE4; PC5/6-B with PC1 and PC5 being also somewhat effective but PC2 is inactive. This is especially apparent from the remaining amount of proNT3 in the medium. Notice that the proNT3 levels are about the same in the PC2 and wild-type experiments suggesting that PC2 does not cleave proNT3. In contrast, PC5 and PC1 partially cleave proNT3 as evidenced by the reduced amounts of proNT3 in the medium. In the cases of furin, PACE4 and PC5/6B, we could not detect proNT3 in the medium (Fig. 1).

In order to further substantiate the precursor–product relationship of proNT3/NT3, we pulsed BSC40 cells expressing either proNT3 alone or in combination with furin for 120 min (P^{120}) with $Na_2[^{35}SO_4]$ or for 15 min (P^{15}) with [^{35}S](Met + Cys) and then chased for 30 ($P^{15}C^{30}$), 60 ($P^{15}C^{60}$) or 120 ($P^{15}C^{120}$) min (Fig. 2). Analysis of the immunoprecipitated radiolabeled proteins revealed the presence of a 33.5 kDa proNT3 form at each time point and the gradual appearance of a 35 kDa form of proNT3. Concomitant with the increase in the levels of the (33.5 + 35) kDa forms of proNT3, we observed an increase in the levels of a 14 kDa form of NT3 both in the cells and media. It appears that both the 33.5 and 35 kDa NT3 precursors are sulfated whereas mature NT3 (14 kDa) is not. We also detected a 23 kDa immunoreactive sulfated protein in medium and cell extract which might be an N-terminally truncated form of proNT3, possibly resulting from cleavage of human proNT3 at the predicted sequence **Arg**GlnGln**Arg-Arg**TyrAsnSer [10].

Analysis of the state of glycosylation of proNT3 (Fig. 3) revealed that following a pulse of 2 h, the (33.5 + 35) kDa intracellular forms are both *N*-glycanase-sensitive and the 33.5 kDa form is partially Endo H-sensitive whereas the mature 14 kDa NT3 is not digested by these enzymes. In the media, only small amounts of precursor are detected; however, the secreted 33.5 kDa mature form of proNT3 was found to be *N*-glycanase-sensitive but Endo H-resistant. This suggests that proNT3 is glycosylated at its predicted single Asn-glycosylation site present at a distance of 8 amino acids preceding the major processing site whereas, as expected, NT3 is not N-glycosylated [10].

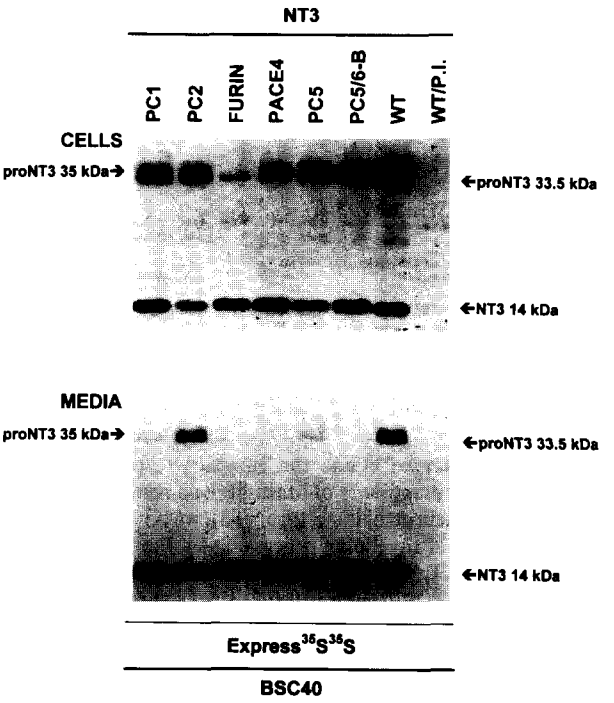

Fig. 1. Processing of proNT3 by the convertases. BSC40 cells were infected with either [VV:hNT3 + VV:WT], [VV:hNT3 + VV:mPC5/6-B], [VV:hNT3 + VV:mPC5], [VV:hNT3 + VV:hPACE4], [VV:hNT3 + VV:hfurin], [VV:hNT3 + VV:mPC2] or [VV:hNT3 + VV:mPC1]. Following overnight incubations the cells were pulse-labeled with [^{35}S](Met + Cys) for 30 min. followed by a chase of 2 h. The media and cell extracts were then immunoprecipitated with a β-NGF antibody which cross-reacts with NT3. Notice the presence of two forms of proNT3 (major 33.5 kDa and minor 35 kDa) both of which can be secreted and converted to the mature NT3 (14 kDa). A control immunoprecipitation of the [VV:hNT3 + VV:WT] infection with a preimmune rabbit serum (WT/P.I.) is also shown.

3.2. Processing of proBDNF

In order to further evaluate the candidate convertases of proneurotrophins, we analysed the capability of some of the convertases to process human proBDNF [21]. This neurotrophin is well known to be expressed in a number of neuronal and non-neuronal tissues, including the hippocampus, Schwann cells and fibroblasts [4,23,24]. Similar to proNGF and proNT3 the predicted protein structure of human proBDNF [21] suggests that it contains a single *Asn*-glycosylation site 8 amino acids N-terminal to its presumed major cleavage site in the sequence AsnMetSerMet**ArgVal**ArgArg↓HisSer.

One of the major hurdles with analysing the processing of proBDNF is its relatively poor cross-reactivity with NGF antibodies as compared to NT3 and to the lack of BDNF-specific

immunoprecipitating antibodies. Therefore, we opted for the analysis of proBDNF by western blots using a specific BDNF-peptide antibody. As shown in Fig. 4, analysis of the media of the furin activity-deficient LoVo cells expressing proBDNF alone or in combination with either furin, PACE4, PC1, PC2 or PC5 demonstrated that furin can promote the conversion of proBDNF into BDNF (14.5 kDa). The other convertases which are effective include PACE4, PC1 and PC5. As with proNGF [18] and proNT3, PC2 does not process proBDNF. Using these methods, we did not detect any proBDNF secreted into the medium.

4. Discussion

Developmental growth of the nervous system, neuronal survival and repair of damaged neurons requires the production and release of one or more neurotrophin [25]. Synergy between two or more neurotrophins may be required for some neurons as well [26]. The neurotrophin precursors are subject to posttranslational modifications, including signal peptidase cleavage, N-glycosylation, in some cases sulfation, and limited proteolysis at specific pairs of basic residues. The regulation of the synthesis and processing machinery of each neurotrophin would, therefore, have to be finely tuned in order to allow for their coordinate release and actions.

The results presented in this work on proBDNF, proNT3 and in a previous study on proNGF [18], demonstrated that among the known mammalian serine proteinases of the subtilisin/kexin family furin, PACE4 and PC5/6-B represent the best convertases of the neurotrophins. Therefore, the sum of the present knowledge suggest that more than one convertase can process the neurotrophins, but that furin is the most efficient in processing these precursors. Whether other convertases not yet defined could also participate in this process is not yet known, especially with the recent discovery of the novel convertase PC7 which is widely expressed, including in areas known to synthesize the neurotrophins [28].

It must be stressed that the work presented in this study and an earlier one [18] was performed under conditions where both the substrate and the convertase were expressed at high levels. In vivo, the enzyme to substrate ratios may differ widely from one type of cell to the other, which may influence the nature of the convertase responsible for the processing of each neurotrophin precursor.

Our pulse-chase analysis data (Figs. 3,4) demonstrated that proNT3 (but not mature NT3) is sulfated, suggesting that processing of proNT3 occurs following the arrival of this precursor at the TGN. A similar conclusion was also reached for proNGF [18]. These results are in agreement with others which suggested that the convertases do not become fully active until they reach the late compartments of the Golgi apparatus [8–10,14,27].

In conclusion, the present data provided the first study on the processing of the neurotrophin precursors proBDNF and proNT3 and together with a previous analysis of proNGF processing [18], suggested a unifying hypothesis whereby all the three neurotrophins could be processed by the same convertase(s). However, since the tissue-distribution of the neurotrophins varies, it will be important in the future to colocalize the cognate convertase(s) with each neurotrophin under basal and activated cell states (e.g. during epileptic seizures) in order to

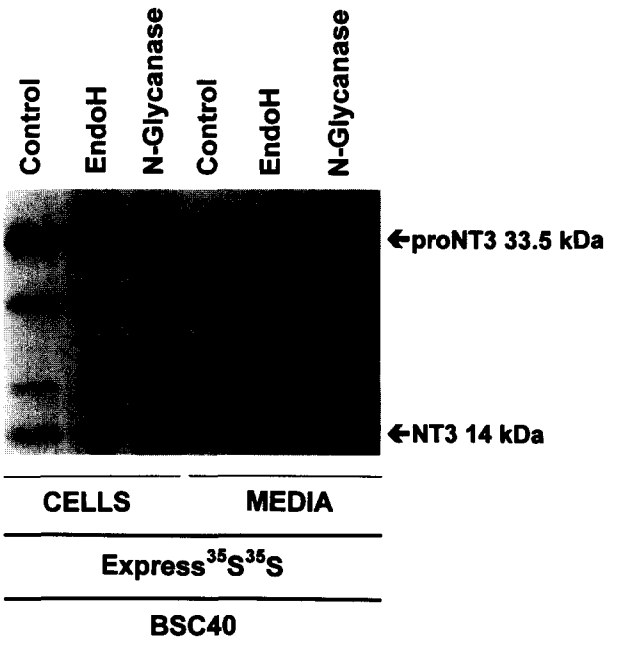

Fig. 3. EndoH and *N*-glycanase digestions of proNT3 and NT3. BSC40 cells were infected with [VV:hNT3] and following an overnight incubation the cells were pulse-labeled with [^{35}S](Met + Cys) for 2 h and the cell extracts were immunoprecipitated with a β-NGF antibody. The cell and media immunoprecipitates were then divided into three equal fractions: a control sample, one which was digested with EndoH and another with *N*-glycanase prior to electrophoresis on a 15% T, 1.3% C slab SDS-PAGE gel.

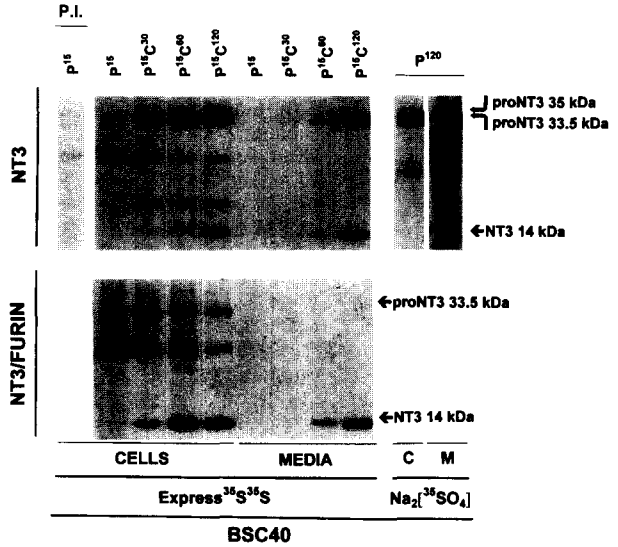

Fig. 2. Pulse and pulse-chase analysis of the processing of proNT3 in BSC40 cells in the absence or presence of furin. BSC40 cells were infected with either [VV:hNT3 + VV:WT] or [VV:hNT3 + VV:hfurin]. Following overnight incubations the cells were pulse-labeled with [^{35}S](Met + Cys) for 15 min (P^{15}) followed by a chase of 30 ($P^{15}C^{30}$), 60 ($P^{15}C^{60}$) or 120 ($P^{15}C^{120}$) min or with $Na_2[^{35}SO_4]$ for 2 h. The media and cell extracts were then immunoprecipitated with a β-NGF antibody. Notice that proNT3 and a processing intermediate (23 kDa) is sulfated but that NT3 (14 kDa) is not. A control immunoprecipitation of the 15-min pulse with a preimmune rabbit serum (P.I.) shows the immunoprecipitation of four faintly labeled intracellular non-specific proteins which are not secreted and which are also found in the immunoprecipitates using the β-NGF antibody.

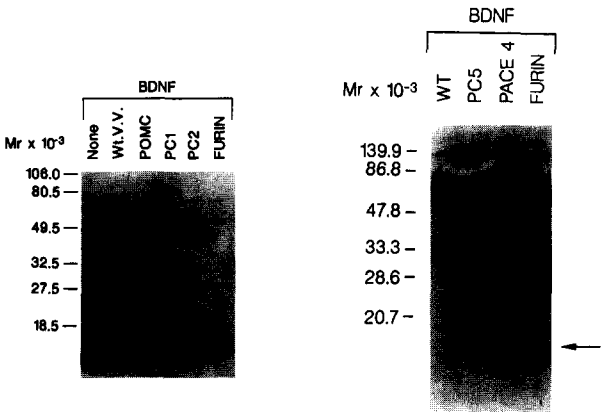

Fig. 4. Processing of proBDNF by the convertases. In two separate experiments, LoVo cells were infected with either (A): [VV: hBDNF + VV:WT], [VV: hBDNF + VV:mPOMC], [VV: hBDNF + VV:mPC1], [VV: hBDNF + VV:mPC2] or [VV: hBDNF + VV:hfurin] or (B): [VV:hBDNF + VV:WT], [VV: hBDNF + VV:mPC5], [VV: hBDNF + VV:hPACE4] or [VV: hBDNF + VV:hfurin]. Following overnight incubations the cells were pulse-labeled with [^{35}S](Met + Cys) for 2 h and the media were electrophoresed on a 13–22% T, 2.7% C gradient SDS-PAGE and the gels were blotted onto a nitrocellulose filter. Immunoblots were then performed with a specific BDNF antibody. The arrow indicates the migration position of mature (14.5 kDa) BDNF.

study their possible coregulation [17]. For example, in the dentate gyrus of the hippocampus which expresses significant amounts of mRNAs coding for furin and PC1 [16] as well as PC7 [11] but not for PACE4 or PC5/6-B [28], the cognate processing enzymes may be reduced to the former two with the possible participation of PC1 in the processing of proBDNF (Fig. 4) [17]. Future studies of the regulatory mechanism(s) of neurotrophin and convertase coexpression and cohabitation in neuronal and non-neuronal cells should be quite rewarding.

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