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Activation of pro-BDNF by the pericellular serine protease plasmin

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Abstract **Brain-derived neurotrophic factor (BDNF) is secreted as either a mature furin-processed form or an unprocessed pro-form. Here, we characterise the extracellular processing of pro-BDNF by the serine protease plasmin. Using recombinant BDNF, maintained in the pro-form by site-directed mutagenesis or inhibition of furin, we demonstrate that plasmin (but not related proteases) is a specific and efficient activator of pro-BDNF. The proteolytic cleavage site is identified as Arg^{125}-Val, within the consensus furin-cleavage motif (RVRR), generating an active form that stimulated neurite outgrowth on TrkB-transfected PC12 cells. Furthermore, we demonstrate that this processing can also occur in the pericellular environment by the action of cell-associated plasminogen activators.**

Keywords: BDNF; Furin; Neurotrophin; Plasmin; Proteolytic processing

1. Introduction

The neurotrophins are a family of five structurally related proteins (BDNF, nerve growth factor (NGF), NT-3, NT-4/5, and NT-6) with established roles in the development, maintenance and repair of the nervous system [1], and increasingly recognised to have similar roles in the cardiovascular system [2,3]. They signal through a dual-receptor system, involving Trk receptors (A, B and C), selective for individual neurotrophins, and the pan-neurotrophin receptor $p75^{NTR}$, thereby mediating apoptotic as well as trophic signals [4].

Neurotrophins are translated as 30–35 kDa preproproteins consisting of a preprodomain, a prodomain and a C-terminal mature neurotrophin domain. The proproteins undergo intracellular cleavage by furin-like proprotein convertases C-terminally of the consensus sequence R–X–K/R–R, to produce mature proteins that are subsequently secreted [5,6]. However, pro-neurotrophins have been detected both in cell culture [6,7] and in vivo [8], and shown to signal preferentially through $p75^{NTR}$ to induce apoptosis [7,9]. The identification of extracellular proteolytic processing of the pro-neurotrophins NGF and BDNF [7], has added a further level of complexity to this system, potentially representing a mechanism for the regulation of neurotrophin activity.

These findings have redirected focus into investigating pro-neurotrophins as functional ligands, and the proteases that regulate their extracellular processing. Many extracellular proteases are involved in controlling cell behaviour [10]. The plasminogen activation system plays a key role by generating the broad-specificity serine protease plasmin, which has a wide range of potential protein substrates [11]. In the pericellular environment, the generation of plasmin is tightly regulated by the interaction of the urokinase plasminogen activator (uPA) with its cellular uPA receptor (uPAR) [11–13]. Plasmin has trypsin-like primary specificity, cleaving after Lys/Arg residues, but its extended substrate specificity is incompatible with cleavage at the consensus furin-cleavage site [14].

Here, we characterise the proteolytic processing of pro-BDNF by plasmin, using BDNF maintained in the pro-form, either by mutagenesis of the furin-cleavage site or by inhibition of furin. We find that plasmin efficiently processes pro-BDNF in a purified system, identify the unique plasmin-cleavage site in pro-BDNF, and demonstrate that the plasmin-processed form can activate TrkB. Furthermore, we show that processing of pro-BDNF by plasmin also occurs in the pericellular environment by the action of cell-associated plasminogen activators.

2. Materials and methods

2.1. Cell culture

Drosophila Schneider 2 cells (S2) were grown in Schneider's Drosophila medium (Invitrogen) with L-glutamine, 10% heat-inactivated FBS at 27 °C in air. The prostate cancer cell line PC3 was maintained in RPMI 1640 with L-glutamine (Invitrogen), 10% FCS at 37 °C/5% CO_2. PC12 cells stably transfected with TrkB were a gift from Dr. David Dawbarn (University of Bristol, UK) and were maintained in RPMI 1640, 10% horse serum, 5% FCS at 37 °C/5% CO_2.

2.2. Generation of BDNF expression plasmids

Human pre-proBDNF cDNA was subcloned into the insect expression vector pMT-V5/His (Invitrogen) at the SpeI–NotI restriction sites of the polylinker region. The native stop codon was mutated to allow expression of the epitope tag. A construct for the expression of furin-cleavage-resistant pro-BDNF was generated from the resulting plasmid pMT-BDNF-V5/His using primers designed to mutate the RVRR furin consensus site (residues 125–128) to RVAA by QuikChange Site-Directed Mutagenesis (Stratagene). A further pro-BDNF mutant construct with RVAA mutated to AVAA was generated from the original mutant using the same method.

2.3. Protein expression in S2 cells

S2 cells were transfected using Cellfectin (Invitrogen), according to the manufacturers recommendations and selected for stable expression. Expression was induced with 0.5 mM $CuSO_4$ in serum-free

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Abbreviations: BDNF, brain-derived neurotrophic factor; wtBDNF, wild-type BDNF; mtBDNF, mutated BDNF; NGF, nerve growth factor; uPA, urokinase plasminogen activator; uPAR, uPA receptor; Dec-RVKR-CMK, decanoyl-Arg-Val-Lys-Arg-chloromethylketone

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medium (Invitrogen), routinely yielding 0.5 mg/l BDNF. Cells expressing wild-type BDNF (wtBDNF) were treated with 7.5 μM decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Dec-RVKR-CMK) (R&D Systems) to inhibit furin activity. Cell lysates and media were collected by centrifugation 48 h after induction and protein expression analysed by SDS–PAGE followed by Western blotting using an anti-V5 monoclonal antibody (Invitrogen). Pro-BDNF was purified to homogeneity using Ni-NTA resin (Qiagen, Crawley, UK) equilibrated in 50 mM $Na_2H_2PO_4$ pH 8.0, 300 mM NaCl, 10 mM imidazole. Conditioned medium was applied to the column, washed and eluted with a linear gradient of 10–250 mM imidazole. Fractions containing pro-BDNF were pooled, dialysed against equilibration buffer and further purified using the same procedure. The pooled pro-BDNF was dialysed against PBS, and stored at a final concentration of 25 μg/ml as determined by BCA assay (Pierce).

2.4. Plasmin processing of pro-BDNF

RVAA-mtBDNF, purified by Ni-NTA affinity chromatography (Qiagen), was incubated with 5 nM plasmin (American Diagnostica, Stamford, USA) at 37 °C. Aliquots were removed at timed intervals and the reaction halted by addition of 5× SDS–PAGE sample buffer containing DTT and analysed by 12.5% SDS–PAGE followed by Western blotting (anti-V5). S2 conditioned medium containing RVAA-mtBDNF, AVAA-mtBDNF or wtBDNF expressed in the presence Dec-RVKR-CMK, was incubated with varying concentrations of plasmin (0–100 nM) for 0–30 min at 37 °C. Plasmin processing was analysed by Western blotting. For mass spectrometry analysis, SDS–PAGE gels were Coomassie-stained, bands excised and subject to in-gel tryptic digestion. Tryptic peptides were analysed on a Reflex III MALDI-ToF instrument (Bruker UK Ltd., Coventry).

2.5. Cellular activation assays

PC3 cells were plated in 96-well plates at 5×10^4 cells/well. After overnight incubation the medium was replaced and cells were treated with mutated BDNF (mtBDNF) in the presence or absence of 5 nM Lys-plasminogen (Enzyme Research Laboratories, Swansea, UK) for 10 min at 37 °C. Each condition was replicated in the absence of cells as a control. Medium was collected and subject to Western blot analysis (anti-V5).

2.6. Analysis of neurotrophin activity

TrkB-PC12 cells were plated in 96-well plates at 5000 cells/well. Varying concentrations of purified BDNF preparations were added and incubated for 48 h. Human recombinant BDNF (R&D Systems, Abingdon, UK) served as a positive control. BDNF activity was assessed by its ability to stimulate neurite outgrowth, using phase-contrast microscopy, and was quantified as the percentage of cells with neurites greater than one cell-body in length. Data were analysed by Student's *t*-test.

3. Results

3.1. BDNF protein expression

To investigate the extracellular processing of pro-BDNF, it was necessary to produce a furin-cleavage resistant form of BDNF that would allow its secretion in an unprocessed pro-form. Arg^{127} and Arg^{128} at positions P2 and P1, respectively (Schechter and Berger [15] nomenclature), of the consensus furin-cleavage site were mutated to alanine (RVRR to RVAA). Both wild-type and the furin-cleavage resistant mutant BDNF (RVAA-mtBDNF) were successfully expressed using an inducible vector in Drosophila S2 cells. The mutation of the furin-cleavage site resulted in the expression of completely intact proprotein (Fig. 1A), compared to the 50:50 ratio of mature to pro-BDNF observed in S2 cells expressing wtBDNF (Fig. 1B). Treatment of these cells with the membrane-permeable furin inhibitor Dec-RVKR-CMK at the time of induction also led to the accumulation of unprocessed pro-BDNF in the conditioned medium (Fig. 1C).

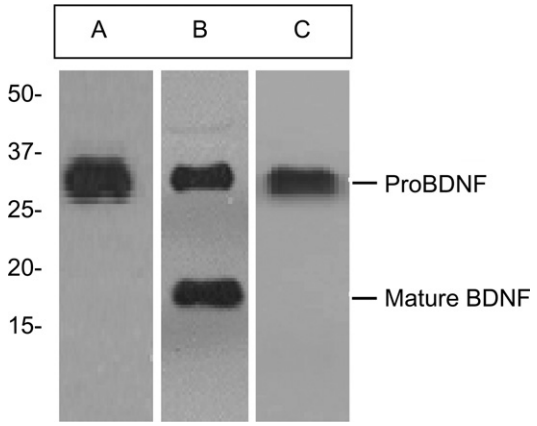

Fig. 1. Expression of pro-BDNF. Various forms of BDNF were expressed in S2 cells, and conditioned medium analysed by Western blotting for the C-terminal V5 epitope. (A) RVAA-mtBDNF, (B) wtBDNF, (C) wtBDNF expressed in the presence of the furin inhibitor Dec-RVKR-CMK. Mature BDNF has a mobility corresponding to a molecular weight of 18 kDa, consistent with furin-processed V5/His tagged protein (predicted molecular weight 17 564 Da).

3.2. Pro-BDNF processing by the serine protease plasmin

Purified furin-resistant RVAA-mtBDNF was incubated with plasmin in solution. The pro-form was rapidly processed to an 18 kDa fragment at a low concentration of plasmin (Fig. 2). This C-terminal fragment is equivalent in size to the furin-processed, mature form of wtBDNF (Fig. 1B). The plasmin cleavage appears to be unique, as no other C-terminal fragments were detected. Wild-type pro-BDNF expressed in the presence of the furin inhibitor was also efficiently processed by plasmin (data not shown). A range of other trypsin-like serine proteases, including thrombin, hepsin and matriptase, did not process pro-BDNF under similar conditions (data not shown).

3.3. Identification of the plasmin cleavage site

To identify the plasmin cleavage site, RVAA-mtBDNF was incubated with plasmin and the resultant fragments purified by anion exchange chromatography, run on SDS–PAGE and the 18 kDa band excised. However, we were unable to determine the identity of this fragment, either by N-terminal sequencing or by MALDI-ToF mass spectrometry. Therefore, the fragment was subjected to tryptic digestion prior to mass spectrometry. A peptide with mass 923.47 was identified, consistent with the residues 126–134 of RVAA-mtBDNF (VAAHSDPAR, theoretical mass 923.469). The presence of this peptide indicates that the plasmin cleavage site is not C-terminal to the Arg^{128} furin cleavage. Potential plasmin-cleavage sites N-terminal of the furin consensus sequence occur at Lys^{76}, Arg^{78}, Lys^{85}, Arg^{93} and Lys^{114}, however cleavage at these sites would generate fragments significantly larger than mature wtBDNF and no tryptic peptides N-terminal of the 126–134 sequence were detected. These observations indicated that the plasmin cleavage may occur at Arg^{125} within the furin consensus sequence. To test this a second mutant, AVAA-mt BDNF, was constructed with this residue substituted. This mutant was not processed to the 18 kDa fragment by plasmin, under conditions in which RVAA-mtBDNF was efficiently processed (Fig. 2B). These experiments demonstrate that the preferential plasmin-cleavage site in pro-BDNF lies at Arg^{125}-Val^{126}.

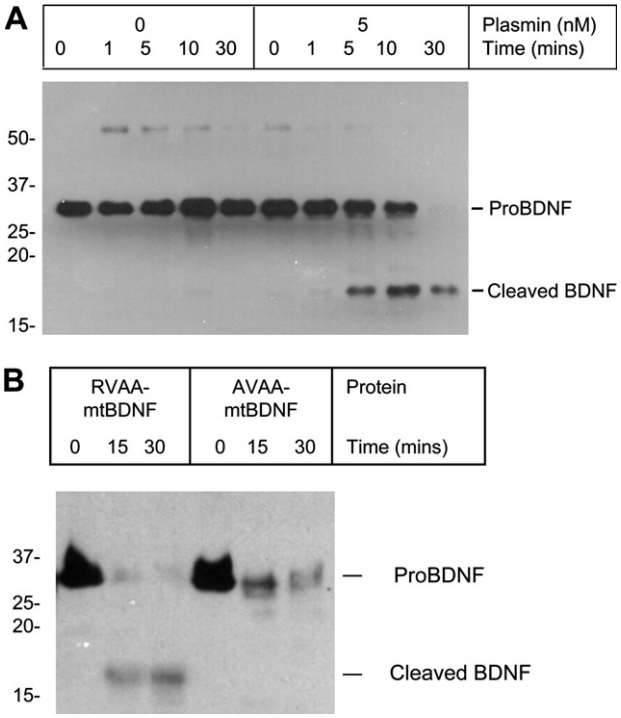

Fig. 2. Plasmin cleavage of pro-BDNF. (A) Purified RVAA-mtBDNF was incubated in the presence or absence of plasmin (5 nM). (B) Conditioned medium from RVAA-mtBDNF and AVAA-mtBDNF were incubated with plasmin (20 nM). Aliquots were removed at the indicated timed intervals and analysed by Western blotting (anti-V5). The reduction in signal intensity observed in the presence of plasmin with increasing incubation time is due to proteolytic cleavage of the C-terminal epitope-tag.

3.4. Biological activity of plasmin cleaved pro-BDNF

To test whether plasmin cleaved pro-BDNF has biological activity equivalent to mature wtBDNF its ability to stimulate neurite outgrowth in TrkB-expressing PC12 cells was determined. Cells were treated with mature wtBDNF, RVAA-mtBDNF or plasmin-cleaved RVAA-mtBDNF, and neurite outgrowth quantified after 48 h. These experiments show that the plasmin-processed form of BDNF stimulates neurite outgrowth to a similar extent to mature wtBDNF, in contrast to unprocessed pro-BDNF which had no significant activity (Fig. 3).

3.5. Cell surface activation of pro-BDNF

Having observed plasmin-mediated processing of pro-BDNF in purified systems, we tested whether this could also occur pericellularly. PC3 cells (which express both uPA and uPAR) rapidly processed RVAA-mtBDNF to the active 18 kDa form in the presence of plasminogen (Fig. 4). Processing was not observed in the absence of plasminogen, or in conditioned medium, either in the presence or absence of plasminogen. These data show that the activation of a low concentration of plasminogen by cell-associated plasminogen activators produces sufficient active plasmin to rapidly and efficiently process pro-BDNF in the pericellular environment.

4. Discussion

Recent evidence suggests that a significant proportion of neurotrophins are secreted as unprocessed pro-forms, that

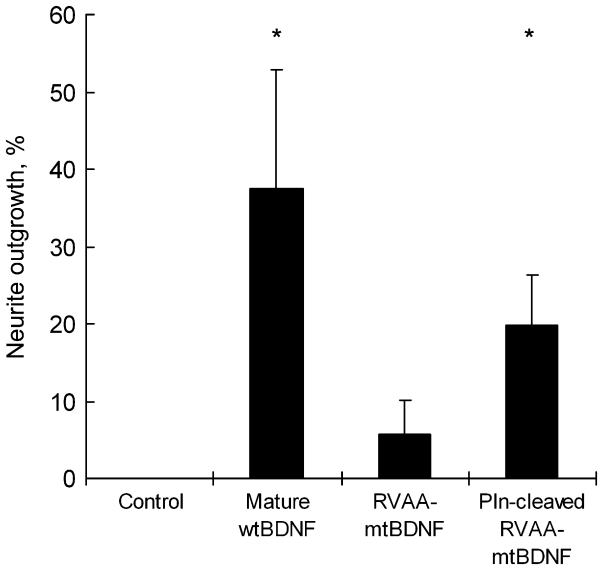

Fig. 3. BDNF-mediated neurite outgrowth. TrkB-PC12 cells were treated with various forms of BDNF for 48 h as indicated. Neurite positive cells are expressed as a percentage of the total cell population. Plasmin-cleaved RVAA-mtBDNF and mature wtBDNF were significantly different to both control and RVAA-mtBDNF ($^{*}P < 0.05$, $n = 4$). The difference between mature wtBDNF and plasmin-cleaved RVAA-mtBDNF was not statistically significant.

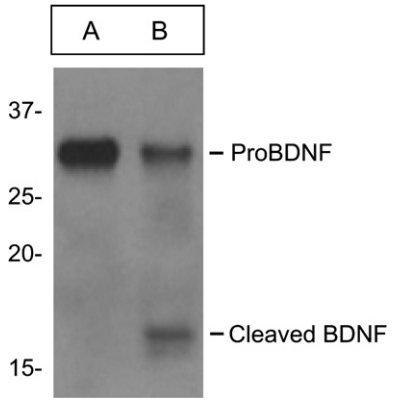

Fig. 4. Cell surface activation of pro-BDNF. PC3 cells maintained in SFM were incubated with RVAA-mtBDNF for 10 min either in the absence (A) or presence (B) of plasminogen. Conditioned medium was removed and BDNF processing analysed by Western blotting (anti-V5).

the processed and unprocessed forms have differing biological activities, and that the pro-forms are susceptible to processing by extracellular proteases. Here, we have produced intact forms of pro-BDNF, both by mutation of the consensus furin-cleavage site and by inhibition of furin activity. These pro-forms were efficiently and specifically processed by plasmin, requiring only a low concentration of protease and generating a single product similar in size to mature wtBDNF. The specific plasmin-cleavage site was identified and found it to be located within the consensus furin-cleavage sequence of BDNF, but occurring after Arg^{125} rather than Arg^{128} of the RVRR sequence. The P4-P1 sequence recognised by plasmin (MSMR) is consistent with its known substrate preference [14]. Interestingly, a SNP has been identified at Arg^{125} (Arg > Met) which would prevent plasmin processing.

Plasmin-processed BDNF was active against TrkB-expressing cells, stimulating neurite outgrowth, in contrast to the much lower activity observed with pro-BDNF. The plasmin-processed form characterised here is extended by three residues at the N-terminus compared to furin-processed BDNF. The crystal structure of mature BDNF shows that the six N-terminal residues are disordered [16]. Although the BDNF · TrkB complex has not been solved, the structure of NT-4/5 · TrkB suggests that receptor binding leads to an ordering of the neurotrophin N-terminus [17]. Consistent with this, mutations in this region reduce neurotrophin activity, although only to a minor degree [18].

We have also demonstrated that plasmin-processing of pro-BDNF occurs via cell-associated plasminogen activators in the pericellular environment, the primary site of plasminogen activation and plasmin activity in tissues [10–12]. A previous study failed to observe pericellular processing of furin-resistant pro-BDNF [19], however, Arg^{125} within the furin consensus motif had been mutated to Ala, inadvertently destroying the plasmin-cleavage site identified here. From the observed activity of plasmin-processed BDNF against TrkB and the known apoptotic effects of pro-BDNF [9], this pericellular processing would be expected to shift a pro-apoptotic response towards a pro-survival effect. The functional regulation of this system is likely to be complex as, for example, activation of $p75^{NTR}$, the preferential receptor for pro-neurotrophins, can lead to a reduction in plasmin generation [20], potentially suppressing pericellular activation of the pro-forms.

How pro-BDNF evades intracellular processing is unknown, however given that it has a distinct biological activity it is possible that this is a regulated process. It is notable that mutation of a single cleavage site abolishes processing of BDNF, whereas for NGF it is necessary to mutate three sites in order to prevent processing [21]. Consistent with this, expression of RKAA-mtNGF in S2 cells generates multiple partially processed forms (Gray and Ellis, unpublished data). A variety of proteins are produced as pro-forms that are subject to processing by the furin-like proprotein convertases including growth factors, receptors, cell adhesion molecules and matrix metalloproteases [22]. If any of these occur as extracellular proproteins requiring a proteolytic event to elaborate their activity, plasmin may also be responsible for mediating the activation of numerous, as yet unidentified proproteins.

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