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Characterization of Nerve Growth Factor Precursor Protein Expression by Human Prostate Stromal Cells: A Role in Selective Neurotrophin Stimulation of Prostate Epithelial Cell Growth

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BACKGROUND. Nerve growth factor (NGF) immunoreactive proteins derived from human prostatic stromal cells (hPS) have been implicated in the paracrine regulation of prostate epithelial cell growth. However, mature NGFβ does not appear to be expressed by these cells. In order to determine whether NGF precursors are expressed by these cells, we investigated the potential processing and expression of precursor forms of NGF by human prostatic stromal cells, and examined the effects of NGF precursor moieties along with the other members of the neurotrophin family of gene products on soft agar colony formation of prostate epithelial cells.

METHODS. Specific antibodies to the peptide domains defined as N4 and L38, and the NGFβ moiety of prepro-NGF, were used in immunoblot assays to characterize the molecular weight forms of precursor NGF secreted by human prostatic stromal cells. The potential processing of NGF precursors with two enzymes, NGFγ and trypsin, was performed by incubation with stromal cell secretory protein containing precursor NGF. The selective effects of the N4, L38, and NGFβ peptide domains of precursor NGF, along with the remaining members of the neurotrophin family, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), were examined for their ability to stimulate growth of prostate tumor epithelial cells in an assay of soft agar colony formation.

RESULTS. Immunoblot analysis of stromal cell secretory protein identified NGF precursors of 35 kDa and 27 kDa, along with the partially processed 22-kDa form of pro-NGF, whereas mature NGFβ was not observed. Treatment of precursor NGF with NGFγ and trypsin did not produce the large intermediate forms of pro-NGF, although these two enzymes did appear to cleave the N-terminal peptide from NGFβ. Of the N4, L38, and NGFβ peptide domains of precursor NGF, only NGFβ significantly stimulated the anchorage-independent growth of TSU-pr1 prostate epithelial cells in soft agar. The other members of the neurotrophin family of gene products had no effect on the anchorage-independent growth of prostate tumor cells.

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CONCLUSIONS. Human prostate stromal cells secrete the 35-kDa and 27-kDa precursor forms of NGF arising from alternate start sites, and the partially processed 22-kDa form of pro-NGF. Whereas the N4, L38, and NGFβ peptide domains present within pro-NGF were previously shown to induce phosphorylation of the high-affinity NGF receptor, tropomyosin receptor kinase (Trk), only the NGFβ moiety was able to stimulate anchorage-independent growth of prostate tumor cells. Likewise, the other neurotrophin family members did not stimulate anchorage-independent growth of prostate tumor cells. Hence, it would appear that NGF may be the predominant neurotrophic growth factor for prostate growth, albeit via precursor forms of NGF, and that its effect appears to be selectively mediated via the NGFβ moiety of these NGF precursors. *Prostate 41:39–48, 1999.* © 1999 Wiley-Liss, Inc.

KEY WORDS: **NGF precursor; prostate growth; stromal cells**

INTRODUCTION

Nerve growth factor (NGF) immunoreactive proteins secreted by prostate stromal cells are capable of stimulating the proliferation of prostate carcinoma cells [1]. Whereas recombinant 13-kDa NGFβ also stimulates proliferation of prostate tumor cells in vitro [2], the mature 13-kDa form of endogenous NGFβ does not appear to be secreted by prostate stromal cells, raising the possibility that the stromal cell-derived NGF immunoreactive proteins could be precursors of NGF. NGF and related neurotrophin family members are all synthesized as precursor proteins which contain dibasic amino-acid proteolytic cleavage sites which may generate several intermediate precursor forms as well as the mature neurotrophin proteins [3–5]. Expression of NGF and other neurotrophins in vitro, as well as by various cell types transfected with neurotrophin genes, demonstrated the production of precursors which could be cleaved to mature forms by proteolysis with specific enzymes [6,7]. In particular, NGFγ has been observed to be capable of processing NGF precursors to various molecular weight forms as well as to mature 13-kDa NGFβ [8]. Cleavage of the NGF precursor at dibasic amino-acid proteolytic cleavage sites yields peptides corresponding to the sequences −71 to −43 (denoted N4) and to −40 to −3 (denoted L38). Antisera against synthetic peptides corresponding to the N4 and L38 domains identified NGF precursors in the mouse submaxillary gland, rat thyroid, and parathyroid tissue [4], rat hippocampus [5,9] and brain cortex [9], and retinal ganglion cells [10], as well as rodent germ cells [11] and guinea pig prostate [12]. Hence, in this study we undertook the characterization of the NGF immunoreactive protein secreted by prostate stromal cells with antibodies that specifically recognize components of the NGF precursor protein.

The ability of tumor cells to form colonies in a semisolid medium, such as soft agar or methyl cellulose, strongly correlates with the ability of these cells to form tumors in vivo in immune-deficient nude mice [13]. Exogenous NGFβ was previously shown to promote colony formation in soft agar by the androgen-responsive prostate tumor cell line, LNCaP [14]. NGF is capable of stimulating a proliferative response via the high-affinity Trk receptor, and alternatively can mediate programmed cell death via the low-affinity $p75^{NTR}$ receptor [2]. Interestingly, the Trk receptor is present in normal and malignant prostate epithelia [15], as well as in tumor cell lines [16], whereas the $p75^{NTR}$ receptor is progressively lost from the malignant prostate [15] and is not expressed in prostate tumor cell lines derived from metastases [17]. Hence, the biological activity of NGF must be exclusively mediated via the Trk receptor in these prostate tumor cell lines. Moreover, chemically synthesized peptides corresponding to the N4 and L38 domains of the NGF precursor also exhibit biological activity in that they stimulate phosphorylation of the Trk receptor, as well as F-actin redistribution, an early event in neurite formation [18]. This raises the possibility that an NGF precursor protein containing the N4, L38, and NGFβ moieties could exhibit multiple biological activities on a tumor cell expressing the Trk receptor. Hence, we subsequently examined the ability of these peptide domains encompassed within the NGF precursor, as well as other neurotrophins (BDNF, NT-3, and NT-4/5), to promote colony formation by the androgen-refractory cell line TSU-pr1.

MATERIALS AND METHODS

Cell Culture

The TSU-pr1 human prostate carcinoma cell line [19] was maintained in RPMI-1640 medium (Mediatech Cellgro™, Mediatech, Washington, DC) supplemented with 5% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO) and 1% antibiotic-antimycotic. Human prostate stromal cells were derived from tissue obtained by transurethral prostatic resection of an adult male at Georgetown University Medical Center, as previously described [1]. These human prostate stromal cells were maintained in RPMI-

1640 medium supplemented with 10% FBS, 10^{-7} M testosterone, and 1% antibiotic-antimycotic. NIH 3T3 cells which overexpress Trk, TrkB, or TrkC were a gift from Dr. David Kaplan (McGill University, Montreal, Quebec, Canada), and were maintained in RPMI-1640 medium supplemented with 5% FBS and 1% antibiotic-antimycotic. Cells were maintained at 37°C in 5% CO_2/95% air, and the growth media were replaced every third day.

Reagents

Human recombinant NGF was a gift from Genentech, Inc. (South San Francisco, CA). Human recombinant BDNF, NT-3, and NT-4/5, as well as polyclonal rabbit antisera against each of these neurotrophins, were gifts from Dr. Craig Dionne (Cephalon, Inc., West Chester, PA). Polyclonal rabbit antisera directed against the peptides encoded by bases −40 to −3 (anti-L38) and bases −71 to −43 (anti-N4) of the NGF precursor gene product were a gift from Dr. Eleni Dicou (INSERM, France). NGFγ was a gift from Dr. Philippe Brachet (INSERM, France). The NGFβ antibody used for immunoblot analysis was obtained from Collaborative Research, Inc. (Bedford, MA). All other reagents were obtained from Sigma Chemical Co. unless otherwise noted.

Human prostate stromal cell secretory proteins (hPS) were prepared as described previously [1]. Human prostate stromal cells were cultured to 80–90% confluence in prostate growth medium, washed three times, and cultured in RPMI-1640 supplemented with 10^{-7} M testosterone and 1% antibiotic-antimycotic solution for 24 hr at 37°C in 5% CO_2/95% air. This procedure was successively alternated with a 24-hr incubation of cells in prostate growth medium. Conditioned serum-free medium was concentrated/dialyzed with a hollow-fiber filter cartridge of M_r 10,000 exclusion limit (Cole-Parmer Instruments Co., Chicago, IL), using ice-cold deionized water for dialysis. The concentrated dialyzed medium was lyophilized and stored at −20°C until use, at which time secretory proteins were reconstituted in deionized water.

Immunoblotting With Anti-NGFβ and Anti-NGF Propeptide Antisera

Stromal cell-secreted proteins (hPS) and human recombinant NGFβ were boiled for 5 min in electrophoresis sample buffer (6% sodium dodecyl sulfate (SDS), 10% glycerol, 0.8 M dithiothreitol, 0.25% bromophenol blue), resolved by SDS-PAGE by the method of Laemmli [20] on 12% polyacrylamide gels, and transferred to nitrocellulose filter (0.2 μm, Hoefer, San Francisco, CA) by the method of Burnette [21], and the blots were incubated in 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS) (137 mM NaCl, 20 mM Tris-HCl, pH 8.0) at room temperature for 1 hr. Blots were incubated overnight at 4°C with anti-NGFβ antiserum diluted 1:2,000, or anti-pro-NGF peptide antisera diluted 1:200 in TBS containing 1% Tween-20 (TBST). Blots were washed with TBST, incubated for 1 hr at room temperature with horseradish peroxidase-conjugated goat anti-rabbit antiserum (Boehringer-Mannheim, Indianapolis, IN) diluted 1:20,000 in TBST, and washed with TBST, and immunoreactive proteins were visualized using ECL chemiluminescence reagent (Amersham Life Science, Arlington Heights, IL).

Activity of NGFγ and Trypsin

The activity of NGFγ and trypsin was determined using the method described by Thomas and Bradshaw [22]. The substrate Nα-benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA) was prepared at 100 mM in DMSO at 25°C. Trypsin was dissolved in 0.001 N HCl to 100 μg/μl. One μl NGFγ (0.71 μg/μl) or trypsin (0.1 μg/μl, 1 μg/μl, 10 μg/μl, or 100 μg/μl) was added to 96 μl of 0.1 M Tris-HCl, pH 8.0, 1 μl 0.001 N HCl, and 1 μl 2.5 mM Na_2-EDTA in microcentrifuge tubes, and as a control, in tubes containing the same proportion of reagents, but without enzymes. Samples were incubated at 37°C for 1 hr, after which samples were read at 414 nM in 96-well microtiter plates in an EIA reader (Bio-Rad Model 2550, Bio-Rad, Richmond, CA).

Proteolytic Cleavage of Prostatic Proteins by Trypsin and NGFγ

HPS protein (25 μg) and human recombinant NGFβ (50 ng) were incubated with trypsin (0.01 μg/ml, 0.1 μg/ml, or 1.0 μg/ml) or NGFγ (0.014 μg/μl) at 37°C in 0.1 M Tris-HCl (pH 8.0), 0.3 M NaCl, and 0.025 mM Na_2-EDTA for 1 hr. Samples were mixed with one-fifth volume of 6 × reducing sample buffer, boiled for 5 min, and electrophoresed on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, and after blocking with 2% BSA/TBS the membrane was immunoblotted with rabbit anti-NGFβ as described above, using horseradish peroxidase-conjugated goat anti-rabbit antiserum as secondary antibody, and ECL chemiluminescence reagent (Amersham Life Science) as developing agent.

Cell Proliferation Assays

NIH 3T3 cells which overexpressed either Trk, TrkB, or TrkC were plated in growth medium in 24-

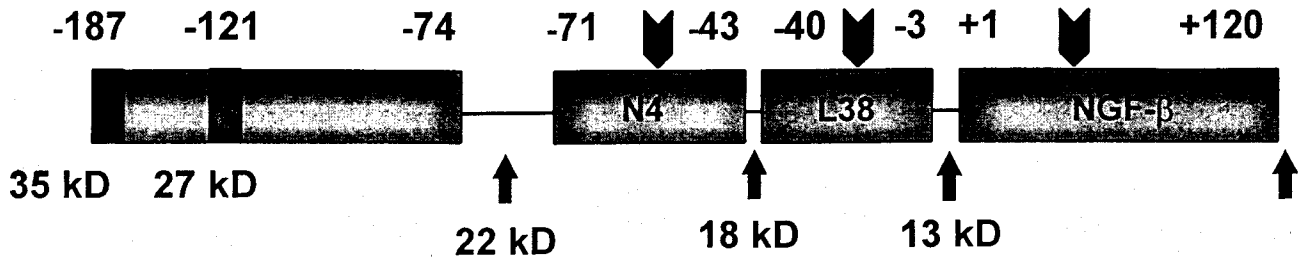

Fig. 1. Structure of NGF precursor. NGF can be translated as long (35-kDa) and short (27-kDa) precursors (prepro-NGF), which potentially can be proteolytically cleaved at dibasic sites (denoted by underside arrows) to generate proforms of 22-kDa, 18-kDa, and mature 13-kDa NGFβ. The locations of three putative glycosylation sites (denoted by arrowheads) are also shown (adapted from Ullrich et al. [3], Dicou [5], and Dicou et al. [18]).

well cluster culture plates at a [redacted] × 10^4 cells/well and incubated at 37°C in [redacted]5% air for at least 12 hr. Cells were rinsed [redacted]n RPMI-1640 and serum-deprived in RPM[redacted] 6 hr, after which the medium was repla[redacted]either RPMI-1640 with the appropriate co[redacted]nt, or RPMI-1640 containing either NGFβ, BDNF, NT-3, or NT-4/5, and incubated at 37°C in 5% CO_2/95% air for 18 hr, after which 1 μCi of [^{3}H]-thymidine (ICN Radiochemicals, Costa Mesa, CA) (20 μCi/ml in RPMI-1640) was added to each well. After 6 hr incubation at 37°C in 5% CO_2/95% air, cells were washed three times with RPMI-1640, and fixed with ice-cold 5% trichloroacetic acid (TCA) for 20 min, followed by three 5-min 5% TCA fixations. Cells were air-dried and extracted with 0.5 M NaOH, and the extracts were neutralized by adding an equal volume of 0.5 M HCl. Equal fractions of each sample were mixed with Eco-Lite scintillation fluid (ICN Radiochemicals, Costa Mesa, CA), and the amount of incorporated [^{3}H]-thymidine was measured by liquid scintillation spectrometry using a Beckman (Fullerton, CA) scintillation counter. Relative proliferation was expressed as the amount of [^{3}H]-thymidine incorporated (cpm) in treated cells vs. controls.

Effects of Neurotrophins and NGF Propeptides on Anchorage-Independent Growth of Prostate Tumor Cells

Cells were plated in 60-mm dishes at a density of 8,800 cells/dish in agar (0.36% in RPMI-1640/5% FBS) over a layer of agar (0.6% in RPMI-1640/5% FBS). NGFβ was diluted in RPMI-1640/5% FBS to 0.1, 1, and 10 ng/ml. BDNF, NT-3, and NT-4/5 were diluted in RPMI-1640/5% FBS to 10, 50, and 100 ng/ml. Chemically synthesized peptides L38 and N4 were diluted in RPMI-1640/5% FBS to 10^{-7} M, 10^{-9} M, and 10^{-11} M. Neurotrophin or peptide solutions were overlaid onto the dishes in triplicate, with dishes overlaid with RPMI-1640/5% FBS without neurotrophins or peptides plus the appropriate solvent included as controls. The dishes were incubated for 8 days at 37°C in a humid chamber, and colonies were counted using an Omnicon 3600 Image Analysis System.

Statistical Analysis

Results of cell proliferation assays and anchorage-independent growth assays were analyzed by the Bonferroni or Dunnett's multiple comparisons tests. Statistical analysis was performed using the Instat program for DOS (GraphPad Software, Inc., San Diego, CA).

RESULTS

Immunoblots Against NGFβ and NGF Propeptide Moieties

In order to determine whether the NGF immunoreactive proteins secreted by human prostate stromal cells represent NGF precursors, antisera directed against the propeptide regions of pro-NGF were used to immunoblot hPS-secreted proteins. A schematic diagram of prepro-NGF, illustrating potential proteolytic cleavage sites and glycosylation sites, is shown in Figure 1. Anti-NGFβ antiserum recognized a protein with approximate M_r of 35 kDa (Fig. 2, lane A1). The diffuse appearance of this 35-kDa band may be due to disruption of the conformational epitope following electrophoresis and electrotransfer. As expected, the identical antiserum was observed to react strongly with human recombinant NGFβ (Fig. 2, lane A2). An antibody to the L38 domain of pro-NGF (Fig. 1) recognized prominent hPS proteins with approximate M_r of 35 kDa, 27 kDa, and 22 kDa (Fig. 2, lane B1), consistent with incompletely processed forms of the NGF precursor (Fig. 1), and, as expected, this antibody did not react with human recombinant NGFβ (Fig. 2, lane B2). An antibody to the N4 domain of pro-NGF (Fig. 1)

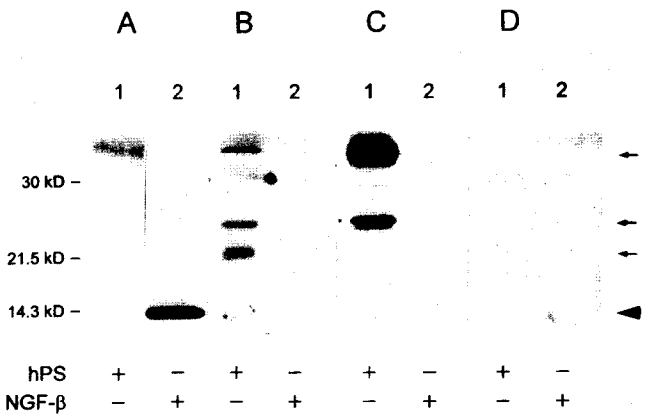

Fig. 2. Immunoblot with anti-NGFβ and anti-NGF propeptide antisera. Secretory protein from human prostatic stromal cells (25 μg hPS, **lane 1**) and purified human recombinant NGFβ (50 ng, **lane 2**) were resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose filter, and blocked in 2% BSA in TBS. Blots were probed with antisera against NGFβ (**lanes A**), L38 NGF propeptide (**lanes B**), N4 NGF propeptide (**lanes C**), and preimmune serum (**lanes D**), as described in Materials and Methods. Arrows indicate positions of NGF precursor proteins. Arrowhead indicates position of NGFβ.

recognized prominent hPS proteins with approximate M_r of 35 kDa and 27 kDa (Fig. 2, lane C1). As expected, this antibody did not react with human recombinant NGFβ (lane C2). Neither the putative NGF precursors in hPS (Fig. 2, lane D1) nor human recombinant NGFβ (Fig. 2, lane D2) were recognized by the preimmune L38 serum.

Effects of NGFγ and Trypsin on Proteolytic Cleavage of Prostatic Proteins

Since mature NGFβ was not observed in hPS, we further investigate the potential processing of these NGF precursors by proteolytic cleavage. The functional activity of trypsin and NGFγ was demonstrated by their ability to release the p-nitroaniline group from the substrate DL-BAPNA, which was observed as an increase in optical density at 414 nm (Table I). Treatment of hPS with increasing concentrations of trypsin resulted in a decrease in intensity and slight decrease in M_r of the 35-kDa NGF precursor (arrow on left) as compared to control (Fig. 3, lanes 1–4). However, no corresponding increase in the 13-kDa form of mature NGFβ was observed. Trypsin treatment of human recombinant NGFβ also resulted in a partial reduction of 13-kDa NGFβ immunoreactivity and a small decrease in M_r as compared to controls (Fig. 3, lanes 5–8), suggesting that either there is some loss of mature NGFβ, or of the epitope recognized by the antibody. Treatment of hPS proteins with NGFγ (Fig. 4) did not

TABLE I. Activity of Trypsin and NGFγ*

Enzyme	Concentration (mg/ml)	$Absorbance_{414}$
Without trypsin	0.0	0.092
With trypsin	0.01	0.114
With trypsin	0.1	0.125
With trypsin	1.0	0.466
With trypsin	10.0	0.489
With NGFγ	7.1	0.341

*NGFγ and trypsin were incubated with the substrate Nα-benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA) at 37°C for 1 hr, after which samples were read at 414 nm in 96-well microtiter plates in an EIA reader (Bio-Rad model 2550).

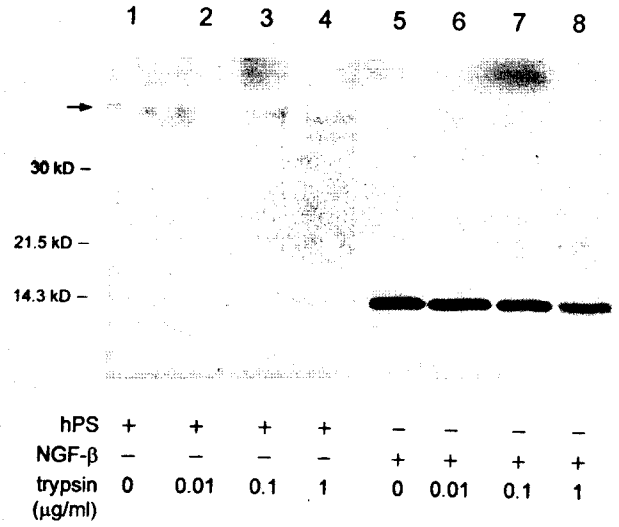

Fig. 3. Proteolytic cleavage of prostatic proteins by trypsin. hPS proteins (**lanes 1–4**) and human recombinant NGFβ (**lanes 5–8**) were incubated at 37°C in the presence (+, lanes 2–4, 6–8) or absence (–, lanes 1 and 5) of trypsin. Samples were resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose filter, and blocked in 2% BSA in TBS. Blots were probed with antisera against NGFβ, as described in Materials and Methods. Arrow indicates position of NGF precursor. Locations of molecular weight standards are shown at left.

appear to process the NGF precursors into additional forms of smaller precursors, but did appear to decrease the intensity of the precursor proteins (small arrows, Fig. 4, lane 2) relative to untreated hPS proteins (Fig. 4, lane 1). Incubation of NGFβ with NGFγ resulted in a small decrease in M_r as compared to controls, as denoted by the large arrowhead (Fig. 4, lanes 3, 4).

Effects of Neurotrophins and NGF Propeptides on Anchorage-Independent Growth of Prostate Tumor Cells

The androgen-refractory cell line, TSU-pr1, was used to examine the effects of neurotrophins on colony

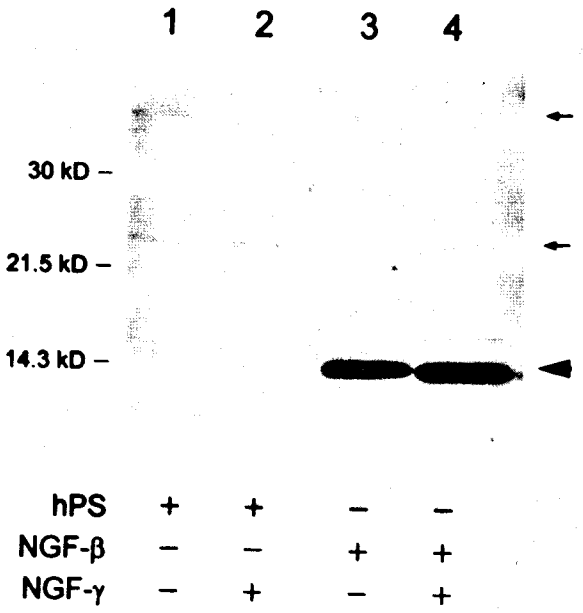

Fig. 4. Proteolytic cleavage of prostatic proteins by NGFγ. hPS proteins (**lanes 1** and **2**) and human recombinant NGFβ (**lanes 3** and **4**) were incubated at 37°C in the presence (+, lanes 2 and 4) or absence (–, lanes 1 and 3) of the NGFγ subunit. Samples were resolved by SDS-PAGE under reducing conditions, transferred to nitrocellulose filter, and blocked in 2% BSA in TBS. Blots were probed with antisera against NGFβ, as described in Materials and Methods. Arrows indicate positions of putative NGF precursors. Arrowhead indicates position of NGFβ.

formation in soft agar. The activity of the neurotrophins was confirmed by the stimulation of [^{3}H]thymidine incorporation (Fig. 5A,C,E) and soft agar colony formation (Fig. 5B,D,F) by 3T3 cells transfected with the appropriate Trk receptor. All of the neurotrophins exhibited bioactivity in assays of [^{3}H]thymidine incorporation into 3T3 cells transfected with the corresponding receptor (Fig. 5A,C,E), whereas bioactivity of NT-4/5 and NT-3 (Fig. 5D,F), but not NGF and BDNF (Fig. 5B,D), was observed in assays of soft agar colony formation.

In the TSU-pr1 prostate tumor cells, treatment with NGFβ induced a statistically significant increase in the number of colonies formed in soft agar, and this increase appeared to be concentration-dependent (Fig. 6A). Conversely, BDNF, NT-3, and NT-4/5 had no significant effect on the number of colonies formed by TSU-pr1 cells relative to controls (Fig. 6B). Similarly, neither the L38 peptide (Fig. 7) nor the N4 peptide (Fig. 7) exhibited significant effects on soft agar colony formation of TSU-pr1 cells.

DISCUSSION

The nucleotide sequence of the human NGF cDNA encodes two potential methionine initiation start sites which predict NGF precursor proteins (prepro-NGF) of approximately 35 kDa and 27 kDa [3]. Both the large and small forms of prepro-NGF contain four dibasic amino acid processing sites. The first and second proteolytic cleavage sites are situated in the proregion of the precursor protein, whereas the third and fourth proteolytic cleavage sites flank the NGFβ moiety [3]. Three N-linked putative glycosylation sites within the coding region of prepro-NGF provides for additional modification during posttranslational processing of the precursor protein [3]. Hence, many of the differences in the size of NGF immunoreactive proteins that have been reported [4,5,23] may reflect differences in tissue-specific and species-specific processing of the NGF precursor protein [24]. In this context, immunoblot analysis of hPS with anti-NGFβ antibody identified an immunoreactive band of approximately 35 kDa molecular weight, whereas the 13-kDa mature form of NGFβ was not observed. This anti-NGFβ immunoreactive protein in hPS is similar in size to the larger NGF precursor protein of 35 kDa [4]. The NGFβ antibody appeared to be specific to the extent that it recognized the mature 13-kDa form of purified NGFβ. Hence, these results suggest that the 35-kDa NGFβ immunoreactive protein in hPS is the larger precursor form of the NGF gene product. In order to investigate NGF precursors in hPS, two additional antibodies raised against biosynthetic peptides corresponding to –40 to –3 (L38 peptide) and –71 to –43 (N4 peptide) of the NGF precursor protein [7,28] were utilized for Western blot analysis of hPS. Immunoblot analysis of hPS with anti-N4 antibody identified two immunoreactive bands with molecular weights of approximately 35 kDa and 27 kDa, whereas, as expected, the anti-N4 antibody was not immunoreactive with the 13-kDa mature form of purified NGFβ, or in hPS. Furthermore, immunoblot analysis of hPS with anti-L38 antibody identified three immunoreactive bands with molecular weights of approximately 35 kDa, 27 kDa, and 22 kDa, respectively, whereas, as expected, the anti-L38 antibody was not immunoreactive with the 13-kDa mature form of purified NGFβ, or in hPS. In addition, the anti-L38 antibody appeared specific to the extent that preimmune L38 antibody did not recognize any of the precursor forms of NGF, or purified NGFβ. In contrast with the anti-L38 antibody, the anti-N4 antibody was not immunoreactive with the 22-kDa protein. Several possibilities exist for the lack of anti-N4 antibody immunoreactivity with the 22-kDa protein. For instance, a modified folding configuration of the 22-kDa protein may have masked the antibody recognition site in the N4 epitope of the 22-kDa protein and therefore prevented detection by the anti-N4 antibody. Alternatively, the anti-N4 antibody may recognize a conformational epitope that may have been dis-

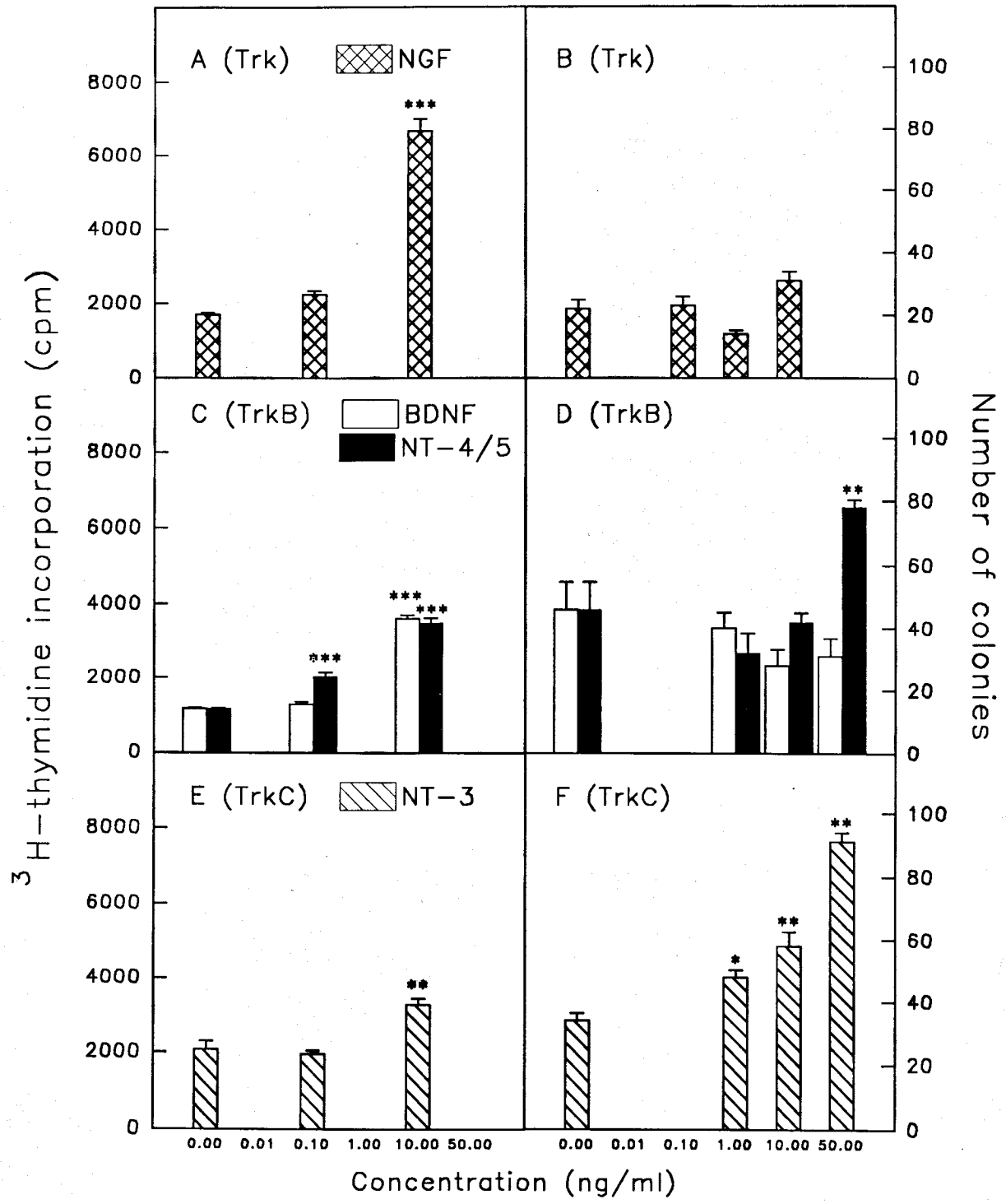

Fig. 5. Effects of neurotrophins on thymidine incorporation and anchorage-independent growth by Trk-expressing cells. 3T3 cells expressing Trk (**A, B**), TrkB (**C, D**) or TrkC (**E, F**), were plated at a density of 50,000 cells/35-mm dish on plastic (A, C, E) or plated at 8,800 cells/60-mm dish in agar (0.36% in RPMI-1640/5% FBS) (B, D, F) and overlaid with growth medium containing NGFβ (cross-hatched bars), BDNF (open bars), NT-3 (striped bars), or NT-4/5 (solid bars), with dishes overlaid with growth medium without neurotrophins plus the appropriate solvent included as controls. Cells on plastic were incubated for 24 hr at 37°C, and 1 μCi of [^{3}H]-thymidine was added to each well prior to fixation and extraction, as described in Materials and Methods. Cells in agar were incubated for 8 days at 37°C in a humid chamber, and colonies were counted using an Omnicon 3600 Image Analysis System. *$P < 0.05$. **$P < 0.01$. ***$P < 0.005$.

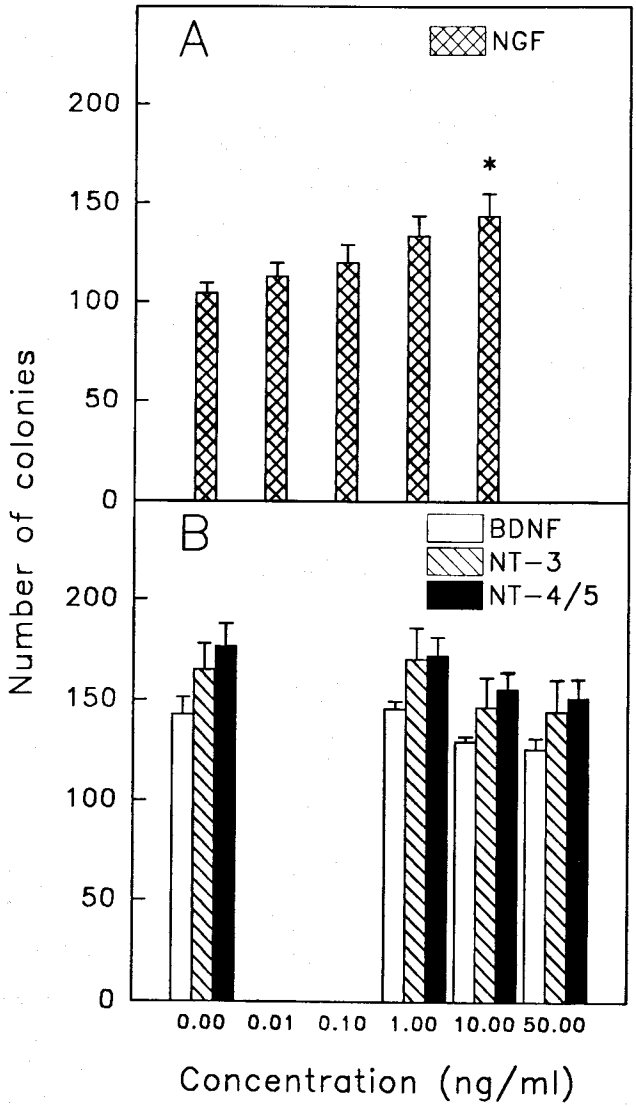

Fig. 6. Effects of neurotrophins on anchorage-independent growth of prostate tumor cells. TSU-pr1 cells were plated at a density of 8,800 cells/dish in agar (0.36% in RPMI-1640/5% FBS) and overlaid with growth medium containing (**A**) NGFβ (cross-hatched bars), (**B**) BDNF (open bars), NT-3 (hatched bars), or NT-4/5 (solid bars), with dishes overlaid with growth medium without neurotrophins plus the appropriate solvent included as controls. The dishes were incubated for 8 days at 37°C in a humid chamber, and colonies were counted using an Omnicon 3600 Image Analysis System. *$P < 0.05$.

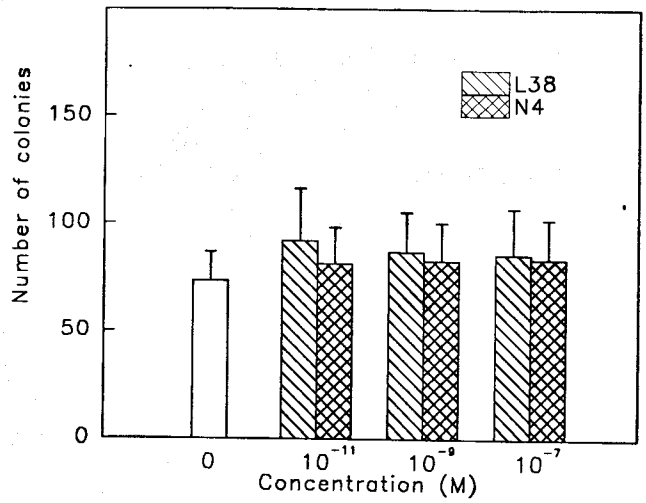

Fig. 7. Effects of NGF propeptides on anchorage-independent growth of prostate tumor cells. TSU-pr1 cells were plated at a density of 8,800 cells/dish in agar (0.36% in RPMI-1640/5% FBS) and overlaid with growth medium containing L38 NGF propeptide (hatched bars) or N4 NGF propeptide (cross-hatched bars), with dishes overlaid with growth medium without propeptides plus the appropriate solvent included as controls (open bar). Cells were incubated for 8 days at 37°C in a humid chamber, and colonies were counted using an Omnicon 3600 Image Analysis System.

rupted by electrophoresis and Western blot of the 22-kDa precursor. A similar effect of epitope masking and/or inability to recognize altered conformational epitopes may account for the inability of anti-NGFβ to recognize intermediate forms of pro-NGF. In addition, differences in relative abundance of NGF precursors between sample preparations have been described [7] that could also account for differences in propeptide antibody recognition of precursor proteins. On infrequent occasions, NGF immunoblots of hPS preparations have also identified a protein with approximate M_r of 18 kDa (data not shown), consistent with differences in the relative abundance of NGF precursors described previously [7]. In any event, it seems clear that human prostatic stromal cells express the 35-kDa and 27-kDa precursor forms of the prepro-NGF gene product, and that the processing of this gene product can produce the 22-kDa form of pro-NGF, but that processing is incomplete since the 13-kDa mature form of NGFβ was never observed.

Antisera directed against mature NGFβ and propeptide regions of the NGF precursor recognized proteins secreted by human prostate stromal cells, with M_r corresponding to the long and short forms of NGF precursors, as well as partially processed cleavage products of the precursors. The lack of secretion of the mature form of NGFβ by prostatic stromal cells may reflect the lack of the appropriate factor(s) for complete processing to the mature form. Hence, we investigated whether these NGF precursor proteins could be cleaved in vitro to generate mature NGFβ. The dibasic cleavage sites in the NGF precursor are illustrated in Figure 1. NGFγ has been observed to be capable of processing NGF precursors to the various molecular weight forms as well as to mature 13-kDa NGFβ [8]. In addition, studies with human recombinant NGFβ showed that trypsin treatment results in a cleavage of the N-terminal peptide, and these prod-

ucts exhibit increased electrophoretic mobility [25,26]. Thus, trypsin and NGFγ were used to digest prostate stromal cell proteins to determine whether these enzymes could produce a mature form of neurotrophin. While both the NGFγ subunit and trypsin appeared to cause a small increase in the electrophoretic mobility of the NGF precursor, these proteases failed to cleave the precursor to a mature form, while resulting in an apparent decrease in precursor. This suggests a different folding of precursor, since it has been observed that NGFγ and trypsin degrade the mature NGFβ-moiety of in vitro NGF precursor translation products, suggested to be a consequence of improper folding [6–8,23]. While neither NGFγ nor trypsin appeared to cleave the stromal NGF precursor to NGFβ, it is possible that another enzyme could cleave the protein to the mature form. Although NGFγ is not expressed in humans, related kallikrein enzymes with similar activity are expressed [27]. Prostate-specific antigen (PSA) and human kallikrein 2 (hK2), which possess chymotrypsin-like or trypsin-like activity, respectively, are both expressed in prostate tissue [28,29]. In addition, coexpression of the NGF precursor with several proprotein convertases, particularly furin, was shown to result in the secretion of processed NGF in vitro [30], leading to the suggestion that furin is a candidate processing enzyme for NGF [30,31]. Thus, the NGF precursor secreted by prostate stromal cells may be processed by enzymes other than those used in this study. Alternatively, the incomplete processing of immature NGF may result from a binding protein that prevents cleavage of the NGF precursor. In this regard, high molecular weight NGF-immunoreactive proteins have been described in excess of 35 kDa [1] that could result from a complex of NGF precursors and binding proteins expressed by prostate stromal cells. In any event, the NGF precursor may not require proteolytic cleavage for functional expression of biological activity. Indeed, in vitro translated NGF precursors have been shown to have biological activity in stimulating the outgrowth of neurites from chick dorsal root ganglia [6]. Thus, even unprocessed NGF precursors could potentially stimulate prostate epithelial cell growth.

Immunoprecipitation of prostate stromal proteins with an NGF antibody was shown to decrease the ability of these proteins to stimulate proliferation of the TSU-pr1 prostate tumor cell line [1]. Subsequently, exogenous NGFβ was shown to promote soft agar colony formation of the androgen-responsive LNCaP prostate tumor cell line [14]. In addition, exogenous NGFβ was also shown to stimulate proliferation of the TSU-pr1 [2], DU-145, PC-3, and LNCaP cell lines [32] in vitro. We now report that exogenous NGFβ also stimulates soft agar colony formation of the TSU-pr1 prostate tumor cell line. Neither BDNF, NT-3, nor NT-4/5 showed significant effects on TSU-pr1 colony formation. This coincides with the observation that NGFβ, but not BDNF or NT-3, stimulated the phosphorylation of a Trk receptor in TSU-pr1 cells [15]. Hence, of all the neurotrophins examined, it appears that only NGFβ, but not BDNF, NT-3, or NT-4/5, stimulates proliferation of TSU-pr1 prostate tumor cells. These observations suggest that NGF may be the predominant neurotrophic growth factor for prostate growth. Since the NGF propeptides, L38 and N4, have been observed to induce early NGF responses, including Trk phosphorylation [18], and these propeptide domains occur within prepro-NGF, we investigated whether the L38 and N4 peptides could stimulate proliferation of TSU-pr1 cells. It is clear that these NGF propeptides, at biologically active concentrations [18], did not promote colony formation by TSU-pr1 cells. Thus, it is likely that stimulation of prostate epithelial cell proliferation is a late response which requires the presence of the NGFβ moiety. It is also possible that these NGF propeptides may have other functions in the prostate that have yet to be determined.

In conclusion, it seems clear that human prostate stromal cells express precursor forms of the NGF gene product, and that the NGFβ moiety of these NGF precursor proteins contains biological activity for the stimulation of anchorage-independent growth, consistent with the paracrine regulation of prostate tumor cell growth.

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