Mitogenic Effect of Nerve Growth Factor (NGF) in LNCaP Prostate Adenocarcinoma Cells: Role of the High- and Low-Affinity NGF Receptors

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We have investigated the effect of nerve growth factor (NGF) in the androgen-dependent, prostate adenocarcinoma LNCaP cell line. Exposure of LNCaP cells to NGF resulted in a significant increase of cell proliferation. The effect was concentration dependent and equally present in serum- or charcoal-stripped serum-supplemented and serum-deprived conditions. The mitogenic action of NGF was accompanied by an enhanced expression of prostate-specific antigen (PSA) and resulted additive to the proliferative effect of dihydrotestosterone. The proliferative effect of NGF appeared to be mediated by the high-affinity NGF receptor, p140^{trka}. Only p140^{trka}, but not the low-affinity NGF receptor, p75^{LNGFR}, was expressed in LNCaP cells; both the proliferative response and the phosphorylation of p140^{trka} upon NGF treatment were prevented by the tyrosine kinase inhibitor K252a. LNCaP cells transiently transfected with the cDNA encoding for p75^{LNGFR} appeared more sensitive to NGF, as demonstrated by the increased number of p75^{LNGFR}-transfected LNCaP cells exposed for 72 h to NGF compared with wild LNCaP cultures. However, p75^{LNGFR}-transfected LNCaP cells rapidly underwent apoptotic death when deprived of NGF. Our study demonstrates the physiological relevance of NGF in the regulation of prostate cell proliferation and the relative contribution of the high- and low-affinity NGF receptors in this control. (Molecular Endocrinology 14: 124–136, 2000)

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INTRODUCTION

Paracrine and autocrine mechanisms play a major role in the control of normal and aberrant growth of the prostate (1, 2). In this respect, growth factors and their receptors seem to be primarily involved (3). Thus, insulin-like growth factor-I (IGF-I), epidermal growth factor, and transforming growth factor-α exert their biological effects on prostate epithelium being produced by stromal and/or epithelial cells (4–7). Peptides of the fibroblast growth factor (FGF) family are also considered essential factors for prostate proliferation (8, 9), and changes in the expression of FGF and/or FGF receptors positively correlate with the malignant progression and metastatic potential of several neoplasias (10, 11), including prostate tumors (12, 13).

Nerve growth factor (NGF) has been identified in human prostate (14, 15) where it localizes to the stromal compartment (16). The modulatory action of a NGF-like protein released by prostatic stromal cells on the proliferation of human prostatic epithelial cells (17) clearly indicates a paracrine function for NGF in the control of prostatic growth.

NGF exerts its action through stimulation of a high-affinity receptor, identified as a member of the tyrosine kinase receptor family, termed p140^{trka}, and a low-affinity high-capacity transmembrane receptor, named p75^{LNGFR}, whose transduction signal has not been completely elucidated yet (all reviewed in Ref. 18). p75^{LNGFR} is expressed in prostate epithelium *in vivo* (16) and in primary cultures of normal prostate epithelial cells (19), but it is absent from prostate stroma *in vivo* (16) and from a human prostatic stromal

cell line (19). More interestingly, in benign prostate hyperplasia and in prostatic adenocarcinoma, $p75^{LNGFR}$ expression is reduced or absent (19, 20), and it is completely missing in prostate metastatic cell lines (19), suggesting an inverse association of $p75^{LNGFR}$ expression and the neoplastic progression of human prostate.

The human prostatic adenocarcinoma LNCaP cells, obtained from a supraclavicular lymph node metastasis (21), represent one of the metastatic prostate tumor cell lines that do not express $p75^{LNGFR}$ (19). LNCaP cells are largely used for the study of prostatic cancer biology as they maintain several features of human prostatic carcinoma including expression of high affinity androgen receptors and production of prostate-specific antigen (PSA), a marker of cellular differentiation in prostatic epithelial cells (21). By using the LNCaP cell line, we have tried to characterize the biological response of NGF in prostatic adenocarcinoma by focusing our attention on the relative contributions of high- and low-affinity NGF receptors in this action.

RESULTS

NGF Affects LNCaP Cell Proliferation

Exposure of LNCaP cells to NGF resulted in a significant increase of cell proliferation. Interestingly, the entity of the stimulatory effect produced by NGF was comparable to that induced by dihydrotestosterone (DHT; 10 nM; Table 1) and was equally present in cells cultured in the presence or in the absence of serum (Table 1) or in charcoal-stripped serum (CSS)-containing culture media (not shown). As expected, stimulation of LNCaP proliferation by DHT was attenuated in the presence of serum (Table 1). Because of the androgen dependence of LNCaP cells for their cellular growth and of the similar response observed in different culture conditions, CSS-supplemented medium was used in all subsequent experiments. A first significant stimulatory effect on LNCaP cell proliferation by NGF was observed at a concentration of 3 ng/ml, as assessed by either cell counting and [^{3}H]thymidine incorporation (Fig. 1, *upper panel*), and was maximal in a range of concentrations of 10–25 ng/ml. This proliferative effect of NGF exhibited also time dependency, as it was already present after a single treatment with NGF (48 h), was more pronounced after a repeated stimulation (7 days), and maximal after a much more prolonged exposure to the growth factor (2 week-treatment; Fig. 1, *lower panel*). Under these conditions no apparent change of LNCaP cell morphology and phenotype was evident by phase contrast microscopy observation (data not shown). The stimulatory action of NGF on LNCaP cell growth was not unique as other growth factors including basic FGF and IGF-I were also able to stimulate LNCaP cell proliferation (Table 2). Although, as mentioned above, treatment with NGF and DHT modified LNCaP cell proliferation to a similar extent, the mechanisms involved in their action seem to be completely independent as shown by the additive effect on LNCaP cell proliferation observed after repeated, combined addition of increasing concentrations of DHT and NGF (Fig. 2).

Table 1. Effect of NGF and DHT on the Proliferation of LNCaP Cells Grown in Serum-Supplemented and Serum-Deprived Conditions

	Cells/Well ($\times 10^3$)	
	+ Serum	− Serum
Control	153 ± 11.0	110 ± 2.0
NGF	217 ± 16.0[a]	163 ± 7.9[a]
DHT	205 ± 5.0[a]	181 ± 6.6[a]

LNCaP cells were either deprived of serum or maintained in serum and treated with NGF (25 ng/ml) and DHT (10 nM) for 48 h before counting with the hemocytometer. Values are mean ± SE of three independent studies performed in triplicate.
[a] $P < 0.05$ *vs.* respective control.

Table 2. Effect of Different Growth Factors on the Proliferation of LNCaP Cells

	Cells/Well ($\times 10^3$)
Control	189 ± 1.3
NGF	385 ± 4.6[a]
bFGF	424 ± 6.6[a]
IGF-I	307 ± 3.1[a]

LNCaP cells were cultured in CSS and treated with NGF (25 ng/ml) and basic FGF or IGF-I (both at 10 ng/ml) for 7 days. Growth factors were added every other day. Values are mean ± SE of three to four independent studies each run in triplicate.
[a] $P < 0.05$ by Student's *t* test.

Effect of NGF on the Expression of PSA

Continuous exposure of LNCaP cells to NGF markedly enhanced the expression of PSA, a typical marker of cellular differentiation in prostatic epithelial cells. Increased immunostaining for PSA was observed after exposure of LNCaP cells to NGF as assessed by the percentage of immunopositive cells (Fig. 3). Treatment with DHT produced a similar stimulatory effect on the production of PSA (Fig. 3). Western blot analysis revealed a single band with a molecular mass of approximately 30 kDa that was clearly enhanced after treatment of LNCaP cells with either NGF or DHT (Fig. 3).

The High-Affinity NGF Receptor $p140^{trka}$ Mediates the Stimulatory Action of NGF on LNCaP Cell Growth

To analyze in more detail the effect of NGF on LNCaP cells, the expression of high- and low-affinity receptor

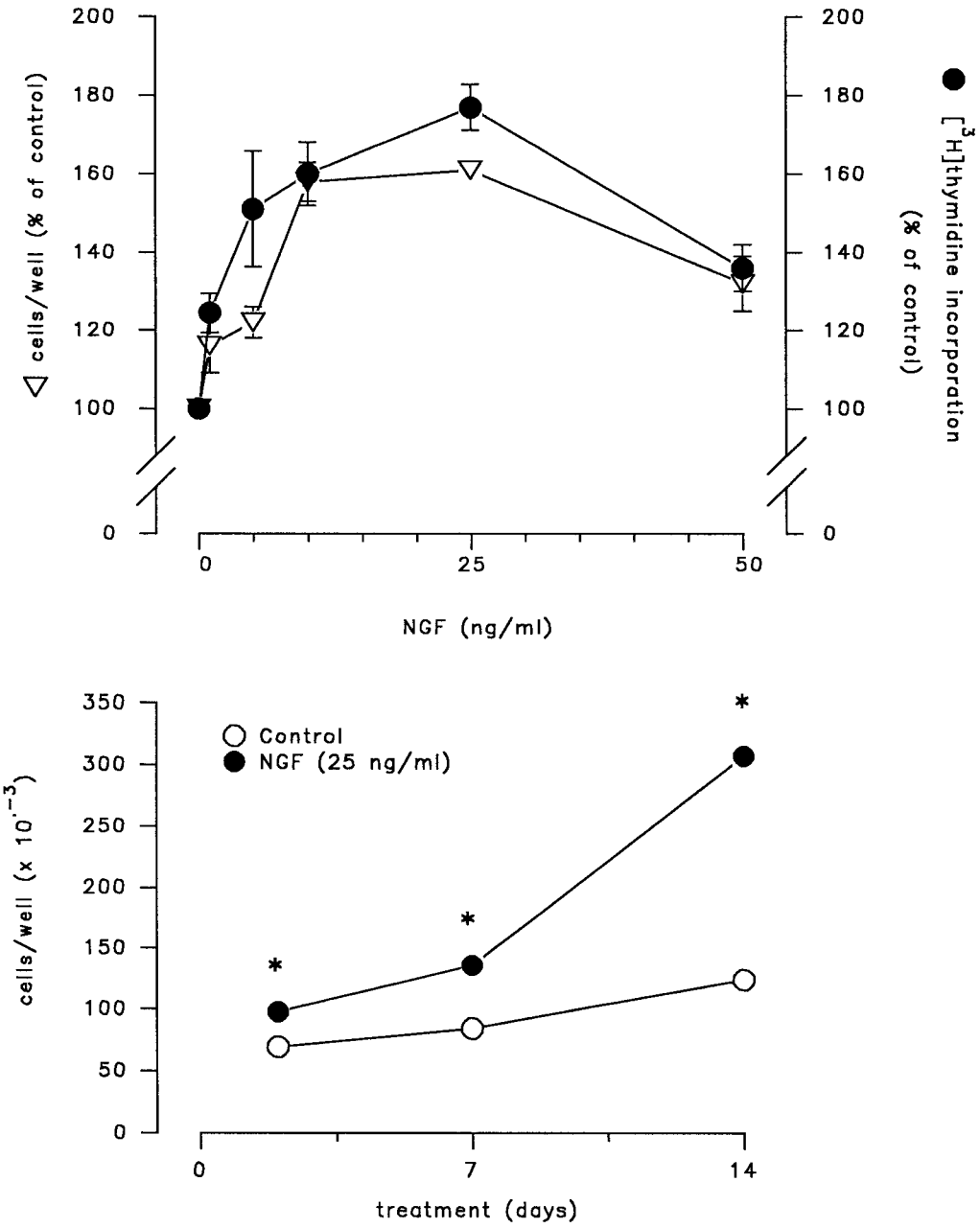

Fig. 1. Concentration-Response and Time Course Curves of NGF Effect on LNCaP Cell Proliferation

LNCaP cells maintained in CSS were exposed to increasing concentrations of NGF for 8 days (*upper panel*). Cells were then either harvested and counted at the hemocytometer or incubated with 1 μCi/ml [^{3}H]methylthymidine during the last 6 h of incubation and evaluated at a scintillation counter after precipitation of the acid-insoluble cellular fraction. The *lower panel* shows the effect of different exposure times to 25 ng/ml NGF on LNCaP cell growth as evaluated by cell counting. All data are mean ± SE from three to five independent studies performed in triplicate. *, $P < 0.01$ *vs.* respective control.

proteins for NGF was examined. LNCaP cells expressed the high-affinity NGF receptor p140^{trka} (Fig. 4), but not the low-affinity p75^{LNGFR} (not shown) as revealed by immunocytochemistry. In addition, the presence of p140^{trka} in LNCaP cells was validated by cytofluorimetric analysis that revealed more than 97% LNCaP cells positive for p140^{trka}. Expression of p140^{trka} in LNCaP cells and its phosphorylation upon NGF treatment were further confirmed by Western blot analysis (Fig. 5). On a functional basis, the involvement of p140^{trka} in NGF action was suggested by complete prevention of the stimulatory effect on LNCaP cell growth by treatment with the tyrosine kinase inhibitor K252a (Fig. 5) that, at the concentration used (1 nM), exhibits relative specificity for *trka* (22). In addition, in the presence of K252a, the enhanced tyrosine phosphorylation induced by receptor activation by NGF was completely prevented (Fig. 5).

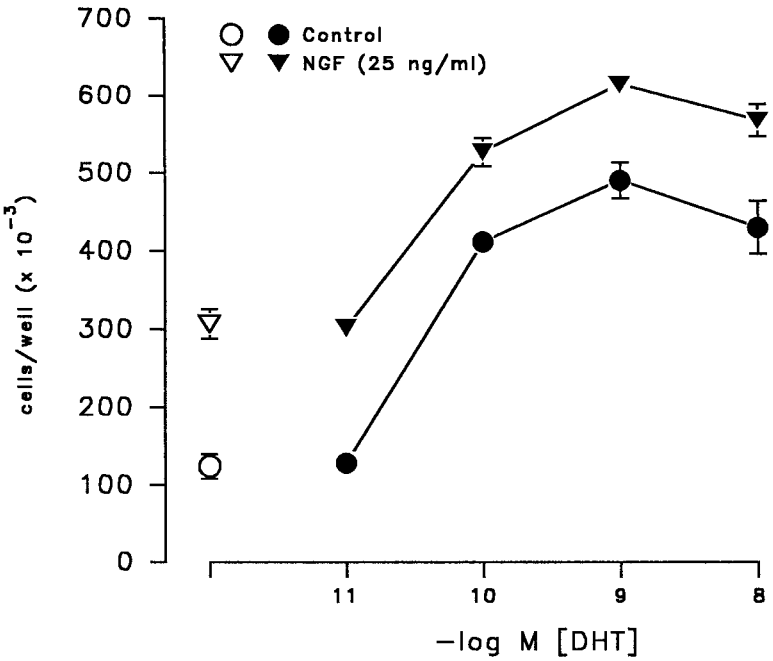

Fig. 2. Additive Effect of DHT and NGF in Stimulating LNCaP Cell Proliferation

CSS-cultured LNCaP cells were repeatedly treated with NGF (25 ng/ml) and increasing concentrations of DHT (10 nM) for 15 days before detachment with trypsin and counting at the hemocytometer. Data are mean ± SE of four independent studies.

Evaluation of Cellular Growth and Viability in P75^{LGFNR}-Transfected LNCaP Cells

To control for p75^{LNGFR} membrane expression in p75^{LNGFR}-transfected LNCaP cells, cytofluorometric analysis was performed by indirect immunofluorescence with a monoclonal antibody recognizing p75^{LNGFR}. The efficacy and specificity of the anti-p75^{LNGFR} antibody were evaluated in parallel cultures of differentiated SK-N-BE human neuroblastoma cells expressing p75^{LNGFR} (not shown). Figure 6 depicts the shift in immunofluorescence intensity in p75^{LNGFR}-transfected (solid histogram) *vs.* vector-transfected LNCaP cells. Immuno-4 processing (Coulter Electronics, Hialeah, FL) was applied to calculate the percentage of positive cells (40.6 ± 2.5%). Expression of p75^{LNGFR} in p75^{LNGFR}-transfected cells was confirmed by immunocytochemistry that revealed a subpopulation of cells positively stained by the anti-p75^{LNGFR} antibody (Fig. 6, *right photomicrograph*). To assess the proliferation rate of LNCaP cells expressing p75^{LNGFR}, cells were grown in 24-well multiwell plates, and incubated with NGF from day 1 after transfection for 72 h. NGF (25 ng/ml) was added daily. This treatment produced an increase in cell proliferation that was 70–75% over control (Fig. 7) and exhibited time dependency as cells deprived of the growth factor 1 day before harvesting arrested their proliferation rate (Fig. 7). The 24-h wash-out period was chosen to be sure that p75^{LNGFR} was absolutely free of ligand due to the specific kinetics of binding of NGF to this receptor, which exhibits very fast association and dissociation rates (18). The stimulatory effect on cell growth induced by NGF (25 ng/ml) was more pronounced (~35% over that induced in untransfected cells) in p75^{LNGFR}-transfected LNCaP cells (Fig. 7). However, this potentiation was evident only at this maximally effective concentration of NGF. In fact, treatment of p75^{LNGFR}-transfected LNCaP cells with submaximal concentrations of NGF (between 1 and 10 ng/ml) was unable to produce a further increase in cell proliferation when compared with untransfected cultures (not shown). Interestingly, complete removal of NGF from the transfected cell culture during the last 24 h of incubation resulted in a final cellular density that was below that observed in untreated p75^{LNGFR}-transfected LNCaP cells (Fig. 7). No significant change of growth rate could be detected in p75^{LNGFR}-transfected LNCaP cells when not exposed to NGF (not shown).

Apoptotic death in p75^{LNGFR}-transfected LNCaP cells was first evaluated by assessing fluorescein isothiocyanate (FITC)-conjugated annexin V binding to phosphatidylserine externalized on the cell surface. This specific method to evaluate apoptotic death was chosen to make sure to detect early apoptotic events. Immunofluorescence analysis at the flow cytometer revealed the appearance of a distinct cell population, corresponding to about 30% of the total cell population examined, that was immunopositive for annexin V only in p75^{LNGFR}-transfected LNCaP cells deprived of NGF for 24 h after a 48 h treatment with the growth factor (Fig. 8, *upper panel*). Conversely, minimal levels of annexin V binding (< 4%) were detectable in NGF-treated p75^{LNGFR}-transfected LNCaP cells and either NGF-treated or NGF-deprived untransfected LNCaP

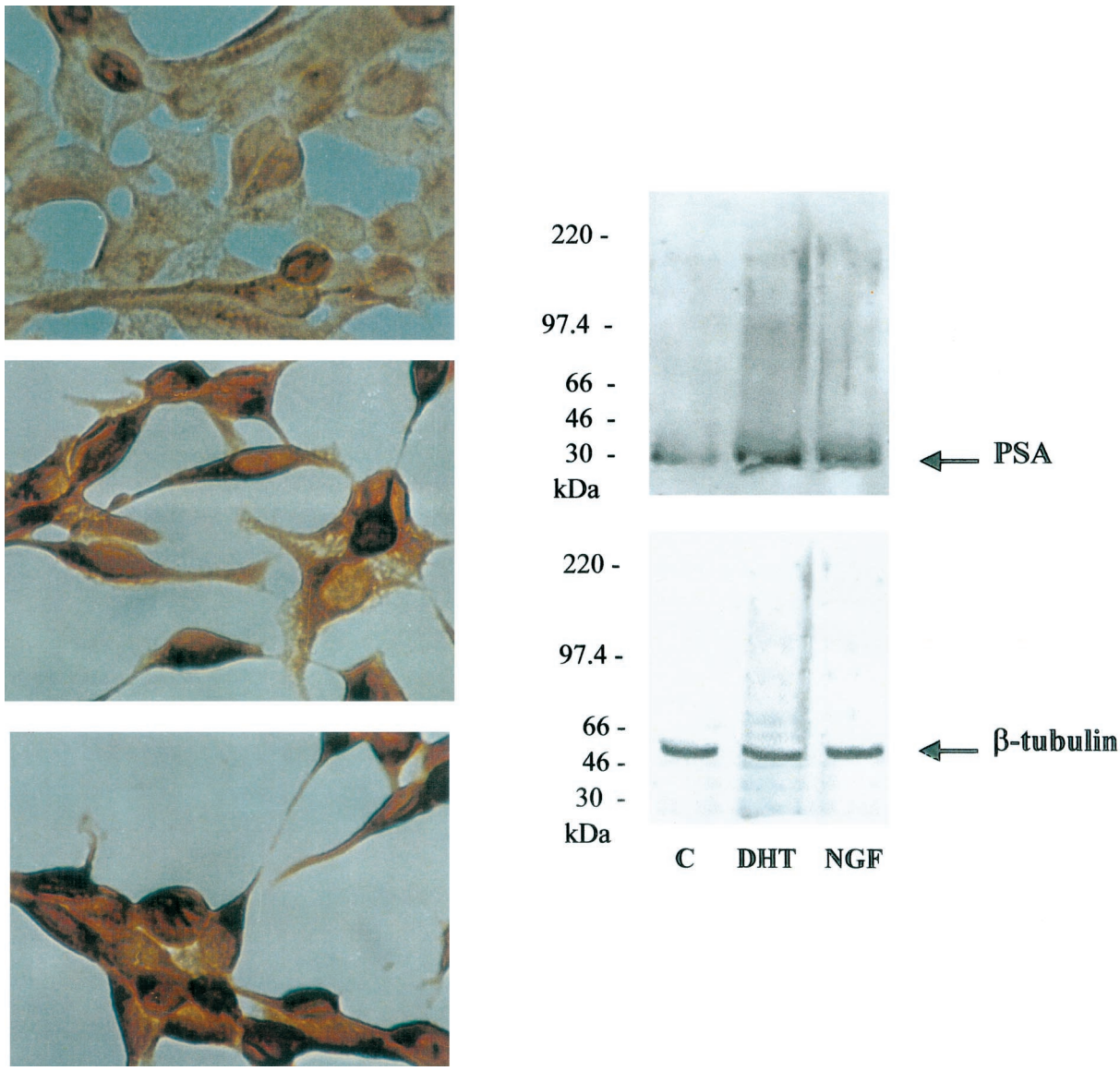

Fig. 3. NGF Treatment Increases the Expression of PSA in LNCaP Cells

LNCaP cells exposed for 7 days to either vehicle, NGF (25 ng/ml), or DHT (10 nM) were fixed with 4% paraformaldehyde for immunocytochemistry or lysed and processed for protein extraction and Western blot analysis. An antihuman anti-PSA antibody raised in rabbits was used. On the *left*, positive PSA immunostaining in NGF- (*middle*) and DHT-treated (*lower panel)* cultures, compared with control (*upper panel*), was visualized with diaminobenzidine. Protein bands (on the *right*) were visualized by chemiluminescence. The same membrane was probed with a polyclonal anti-β-tubulin antibody raised in goat (1:200) to control for protein loading.

cells (Fig. 8*, upper panel*). Similar results were obtained by evaluating apoptosis by assessment of the presence of a hypodiploid cell population in propidium iodide-labeled cells (Fig. 8*, lower panel*).

DISCUSSION

The mechanisms underlying prostate tumoral growth are still poorly understood. The interaction between stromal and epithelial compartments within the gland and the involvement of paracrine and autocrine events regulated by growth factors and their receptors seem to play a major role in this regard (3). Since the early work demonstrating the presence of NGF-like proteins in the prostate gland of the guinea pig (14), a large body of evidence has indicated a prominent role for NGF in the regulation of prostate growth. In particular, the discovery that an NGF-like protein secreted by prostate stromal cells stimulated growth of a prostatic epithelial tumor cell line (17) allowed a more precise understanding of the effects mediated by NGF at the

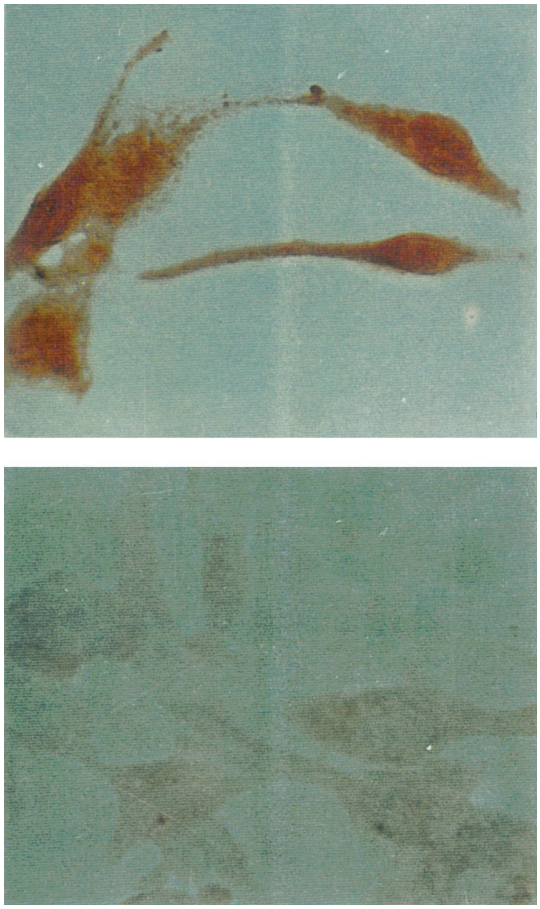

Fig. 4. LNCaP Cells Show Positive Immunostaining for the High-Affinity NGF Receptor p140^{trka}

LNCaP cells were fixed with 4% paraformaldehyde, exposed to Triton-X100 for 5 min at 4 C, and analyzed by immunocytochemistry after labeling with a goat antihuman p140^{trka} (1 μg/ml) followed by a biotinylated anti-rabbit IgG (1:200). A culture in which incubation with the primary antibody was omitted is shown for comparison (*lower panel*).

prostate. Our data obtained in the prostatic adenocarcinoma LNCaP cells indicate that NGF exerts a mitogenic action in human prostate epithelial cells. Although better known for the differentiating activity exerted in nerve cells (23), interaction of NGF, as well as of other neurotrophins, with its specific receptors is also able to elicit proliferation phenomena (24–26). These have been observed in several cellular systems including undifferentiated hematopoietic cells (27), normal human keratinocytes (28), a pancreatic carcinoid (29), and a thyroid epithelial (30) cell line.

The stimulatory action induced by NGF on LNCaP cell growth was concentration- and time-dependent; the entity of the effect produced was comparable to that induced by basic FGF, but greater than that of IGF-I probably because of the autocrine regulation of LNCaP cell proliferation by this growth factor (31). In addition, the stimulatory action on LNCaP cell growth was not influenced by culture conditions as it was equally effective in the presence or absence of serum in the culture media or when only steroids were removed from the incubation buffer by the use of CSS. In line with this, stimulation of cell proliferation by NGF has been reported in androgen-independent prostate carcinoma cell lines (32, 33). Furthermore, cotreatment of LNCaP cells with NGF and DHT produced an additive effect, suggesting that the two agents acted in parallel, through activation of completely independent intracellular mechanisms.

Similarly to what was observed after treatment with DHT, a long-term exposure of LNCaP cells to NGF was also able to induce increased expression of the cellular differentiation marker, PSA. Androgens have been reported to stimulate LNCaP cell proliferation at low concentrations and to promote the production of PSA at high concentrations (34). In the present study, the concentration-response curve of DHT on LNCaP cell growth was shifted to the right compared with that previously reported (34), a phenomenon that can be ascribed to the batch of LNCaP cells used or to the different culture conditions in which experiments were carried out. In our hands, however, 10 nM DHT, a concentration that was fully effective in inducing increased LNCaP cell proliferation, was also able to enhance PSA expression. Accordingly, NGF, at maximally effective concentrations in promoting LNCaP cell growth, was also able to enhance PSA production, suggesting that, in prostate adenocarcinoma cells, NGF can maintain, at least in part, its differentiating properties while inducing enhanced proliferation. Such a combined action of NGF has already been reported in other cellular systems (35). The effect of NGF on LNCaP cells, however, was not accompanied by any major change of cellular phenotype or cytostructural modification. Moreover, although other growth factors able to modulate LNCaP cell growth are known to control proliferation and differentiation events in this cell line inversely (36), the regulation of these phenomena appears extremely complex as it involves a series of interactions between the androgen-regulatory system and the growth factor-regulatory system that are likely to take place at multiple intracellular levels in prostatic cells.

NGF is known to exert its action through two distinct receptors: a high-affinity tyrosine kinase receptor, p140^{trka}, and a low-affinity binding protein, p75^{LNGFR} (reviewed in Ref. 18). Although p75^{LNGFR} and p140^{trka} are coexpressed in many NGF-responsive cell types, independent expression of individual receptors has also been observed. The ratio of the two receptors seems to be crucial for the functional response elicited by NGF, and coexpression of p75^{LNGFR} and p140^{trka} is known to contribute to the formation of a high-affinity binding site (18).

Prostatic LNCaP cells expressed p140^{trka}, but not p75^{LNGFR}, as detected by immunocytochemistry, cytofluorimetric, and Western blot analysis. The lack of p75^{LNGFR} in LNCaP cells was not surprising as a progressive loss of the low-affinity NGF receptor from normal prostate to adenocarcinoma and metastatic

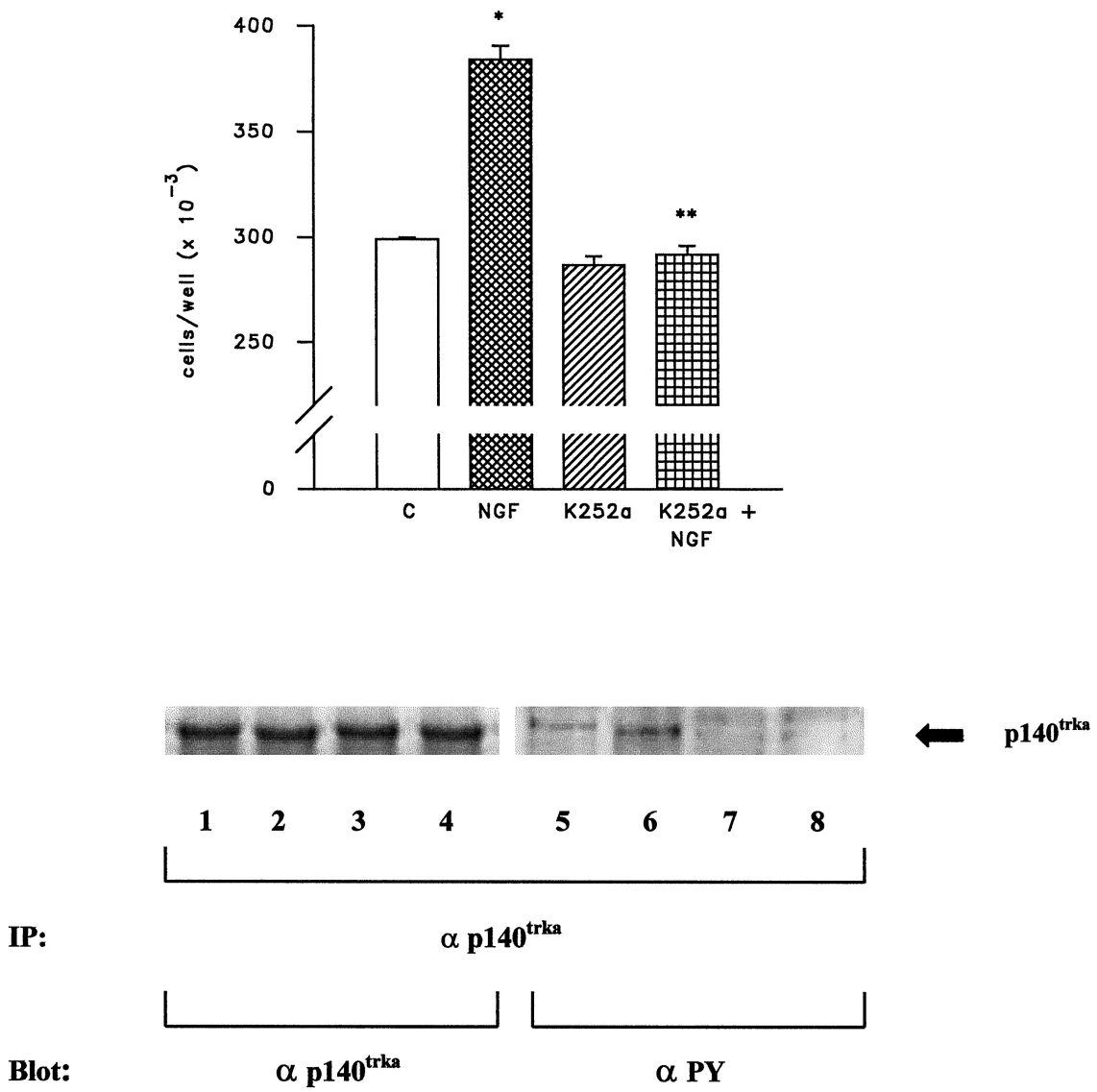

Fig. 5. Inhibitory Action of K252a on NGF-Stimulated LNCaP Cell Proliferation and Tyrosine Phosphorylation of p140^{trka}

A, LNCaP cells were exposed for 48 h to K252a (1 nM) either alone or in combination with NGF (25 ng/ml). Cells were counted with the aid of a hemocytometer. Data are mean ± SE of four independent studies performed in triplicate. *, $P < 0.05$ *vs.* control; **, $P < 0.05$ *vs.* NGF-treated. **B,** Expression and tyrosine phosphorylation of p140^{trka} in basal conditions (lanes 1 and 5) and after treatment with NGF (lanes 2 and 6), K252 (lanes 3 and 7), and NGF plus K252 (lanes 4 and 8). Cell lysates were immunoprecipitated with the polyclonal anti-p140^{trka} antibody. Membranes were immunoblotted with either the polyclonal anti-p140^{trka} antibody (lanes 1–4) or a monoclonal antiphosphotyrosine antibody (αPY; lanes 5–8).

prostate tissue has already been described (19). Accordingly, the mitogenic effect triggered by NGF in LNCaP cells appeared to be mediated exclusively by activation of the p140^{trka} as demonstrated by the blockade of the stimulatory action of NGF by the tyrosine kinase inhibitor K252a, similarly to that already observed in BON pancreatic carcinoid cells (29) and human keratinocytes (28). In strong support of a specific involvement of p140^{trka} in NGF-induced cell proliferation, treatment with K252a was able to completely prevent the phosphorylation at tyrosine residues of p140^{trka} observed upon NGF stimulation. However, the involvement of p140^{trka} in the regulation of cellular growth is much more complex, as activation of the high-affinity NGF receptor is also known to mediate growth arrest and differentiation not only in neuronal cells such as neuroblastoma (37) and PC12 cells (38), but also in NIH-3T3 fibroblasts, where NGF can elicit growth arrest by induction of a cyclin-dependent kinase inhibitor (39), in various tumors of neuroendocrine origin (reviewed in Ref. 40) and in human small cell lung carcinoma cell lines (41).

p75^{LNGFR} has recently been included in the superfamily of death domain receptors, which comprises receptors for some cytokines and several surface antigens (reviewed in Ref. 42). The role of this p75^{LNGFR}

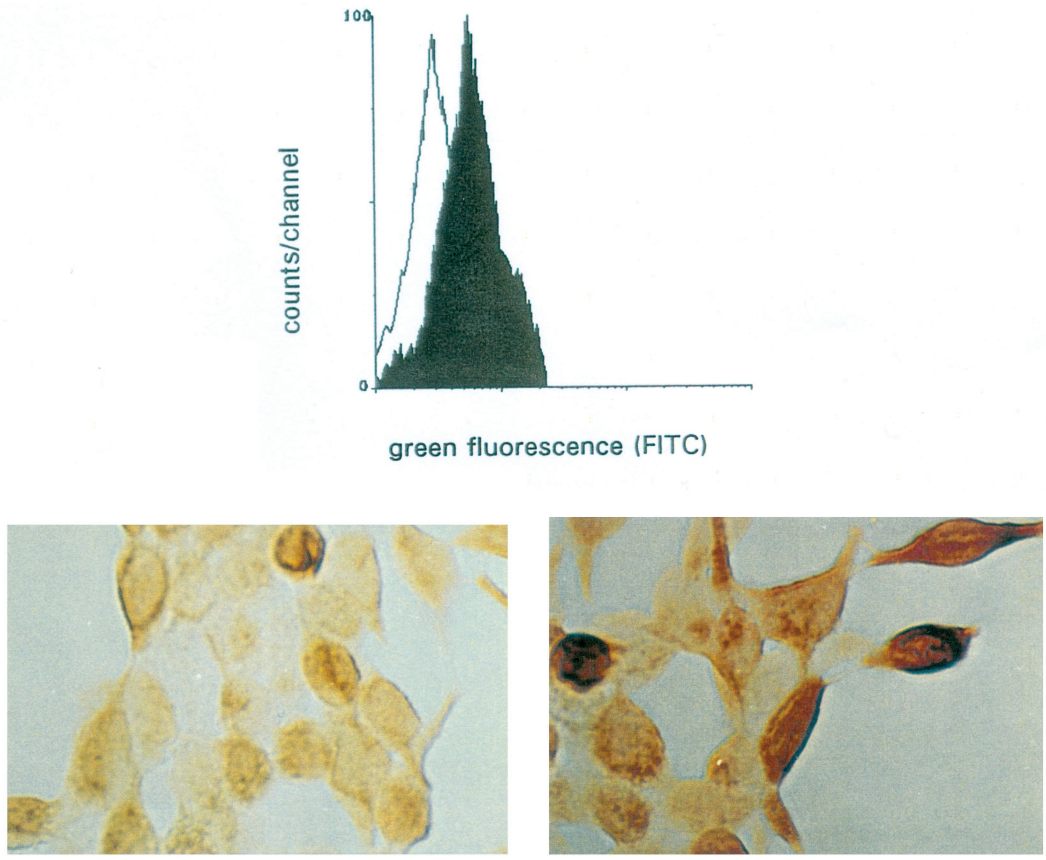

Fig. 6. Flow Cytometric and Immunocytochemical Analysis of $p75^{LNGFR}$ Expression in $p75^{LNGFR}$-Transfected LNCaP Cells

LNCaP cells transfected with the pCEP4 plasmid containing the human $p75^{LNGFR}$ cDNA (*top, solid histogram*) or the vector alone (*open histogram*) were detached from the dish and immediately incubated with mouse antihuman $p75^{LNGFR}$ (10 μg/ml) for 1 h followed by incubation with FITC-conjugated goat antimouse IgG (1:100) for 45 min. Cells were analyzed with a flow cytometer. At the *bottom*, photomicrographs showing three $p75^{LNGFR}$ immunopositive cells (*right)* in $p75^{LNGFR}$-transfected cultures. The lack of staining when primary antibody was omitted is shown for comparison (*left)*. Data shown are representative of several independent experiments.

in the regulation of cell death phenomena, however, is still controversial: intrinsic $p75^{LNGFR}$ activity, as opposed to ligand-dependent receptor effects, have in fact been described and they may alternatively initiate proapoptotic or antiapoptotic signals (43). In murine dorsal root ganglion neurons, $p75^{NGFR}$ seems to mediate cell survival at very early times of development whereas, at later stages, it triggers an intrinsic proapoptotic signal that is completely prevented by NGF (44). Similarly, in immortalized neural cells, NGF or an anti-NGF antibody abolish the death signal mediated by unbound $p75^{LNGFR}$ (45). On the other hand, however, NGF is also able to cause apoptotic death after binding to its low-affinity receptor (46).

In nonneuronal cells, the exact role of $p75^{LNGFR}$ has been much less investigated: the preferential involvement of $p140^{trka}$ in the proliferating response of tumoral, NGF-responsive cells (27–29) leads to the hypothesis that the low-affinity NGF receptor might play a counterbalancing role in the control of cellular growth. In LNCaP cells, however, expression of $p75^{LNGFR}$ determines cooperation with $p140^{trka}$, as suggested by the greater mitogenic effect observed in response to maximally effective concentrations of exogenously added NGF. The lack of potentiation at lower NGF concentrations, however, rules out possible changes in the affinity of NGF for $p140^{trka}$ but rather suggests potential interactions of $p140^{trka}$- and $p75^{LNGFR}$-mediated signaling at a postreceptor level. $p75^{LNGFR}$ seems to be endowed with intrinsic activity that is unmasked only after exposure to NGF, as revealed by the decreased cell number and the appearance of apoptotic cell death after withdrawal of the growth factor. The lack of apoptosis in response to NGF withdrawal in untransfected LNCaP cells excludes the possibility that LNCaP cells become dependent on NGF for their survival after a prolonged treatment with the growth factor. Partially in contrast with our results, Pflug and Djiakiew (32) reported a progressive reduced proliferation rate of TSU-prl prostatic epithelial cells expressing low, intermediate, or high levels of $p75^{LNGFR}$ in addition to the occurrence of apoptosis upon NGF removal (32). The loss of $p75^{LNGFR}$ expression in prostate adenocarcinoma

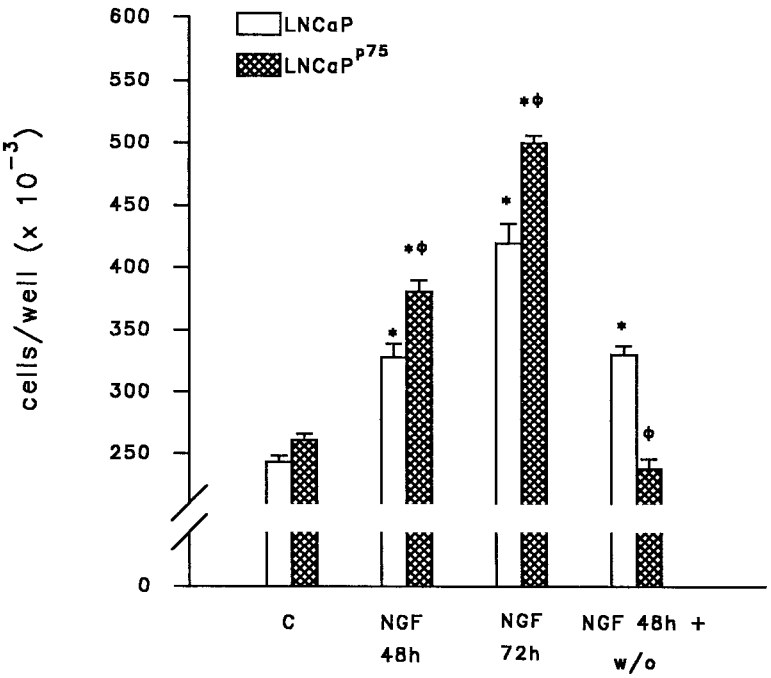

Fig. 7. Differential Response to NGF and NGF Withdrawal in Wild (LNCaP) and p75^{LNGFR}-Transfected (LNCaP^{p75}) LNCaP cells

LNCaP (*open bars*) and LNCaP^{p75} (*cross-hatched bars*) cells were treated with NGF (25 ng/ml, added daily) for 48 and 72 h or exposed to NGF for 48 h before deprivation for 24 additional h (wash-out period; NGF + w/o). Data are mean ± SE of one experiment representative of four, each run in triplicate.*, $P < 0.01$ *vs.* respective control; ϕ, $P < 0.05$ *vs.* corresponding LNCaP untrasfected cells.

cells may thus represent a self-regulatory mechanism through which proliferating cells control their growth processes. In line with this, expression of p75^{LNGFR} in human melanoma cells is positively correlated with their chemoinvasion potential (47). On the other hand, however, the ability of NGF deprivation to trigger apoptotic events in p75^{LNGFR}-transfected LNCaP cells suggests a pivotal role for this receptor in the complex regulation of proliferation phenomena by NGF.

MATERIALS AND METHODS

LNCaP Cell Culture

The androgen-sensitive human prostatic adenocarcinoma LNCaP cells (obtained from American Type Culture Collection, Manassas, VA), were maintained in RPMI-1640 medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% FCS, and penicillin (100 U/ml)/streptomycin (100 μg/ml) (all from Life Technologies, Inc.). In most studies, as specifically indicated, FCS was replaced with CSS, obtained by treatment with dextran-coated charcoal, to remove any steroid contaminant.

Cell Proliferation Studies

LNCaP cells plated at low density were cultured in FCS-, CSS-supplemented, or serum-deprived media as specified in detail. Human recombinant NGF (kindly provided from Genentech, Inc., South San Francisco, CA), the other growth factors and DHT (Sigma, St. Louis, MO) were added every other day. Cellular growth was assessed by total cell counts at the end of the treatment period and by the rate of [^{3}H]thymidine incorporation in a 6-h labeling period. For cell counting experiments, LNCaP cells, plated in 24-well multiwell plates, were detached using a 0.25% trypsin/0.2% EDTA solution (Life Technologies, Inc.) and counted with a hemocytometer. Cell viability was determined by the trypan blue (Sigma) exclusion test. For [^{3}H]thymidine incorporation studies, cells were incubated with 21 μCi/ml [^{3}H]methylthymidine (New England Nuclear, Milan, Italy; specific activity, 20 Ci/mmol) for 6 h before precipitation with 1 N $HClO_4$. Incorporated radioactivity was quantitated by liquid scintillation counting.

Immunocytochemistry

LNCaP cells were stained for the high-affinity NGF receptor p140^{trka} using a polyclonal anti-trk antibody raised in rabbits (1 μg/ml; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). A monoclonal antihuman p75^{LNGFR} (10 μg/ml; Roche Molecular Biochemicals, Mannheim, Germany) was used to detect p75^{LNGFR} immunostaining in LNCaP cells. Immunopositivity for PSA was assessed by using a polyclonal anti-PSA antibody raised in rabbits (4 μg/ml; DAKO Corp., Glostzup, Denmark). Preparation of cells for immunostaining was performed by fixing cells for 30 min with 4% paraformaldehyde. After permeabilization in 0.1% Triton X-100 (Sigma) for 10 min at room temperature (this step was omitted when looking for p75^{LNGFR} staining because the antibody used recognizes an extracellular epitope of the receptor), cells were repeatedly washed with phosphate buffer and exposed to the primary antibody at 4 C overnight followed by incubation with biotinylated antirabbit (or antimouse) IgG for 1 h. After reaction with avidin-biotin-horseradish peroxidase (Vectastain ABC-Elite kit, Vector Laboratories, Inc., Burlingame, CA), staining was

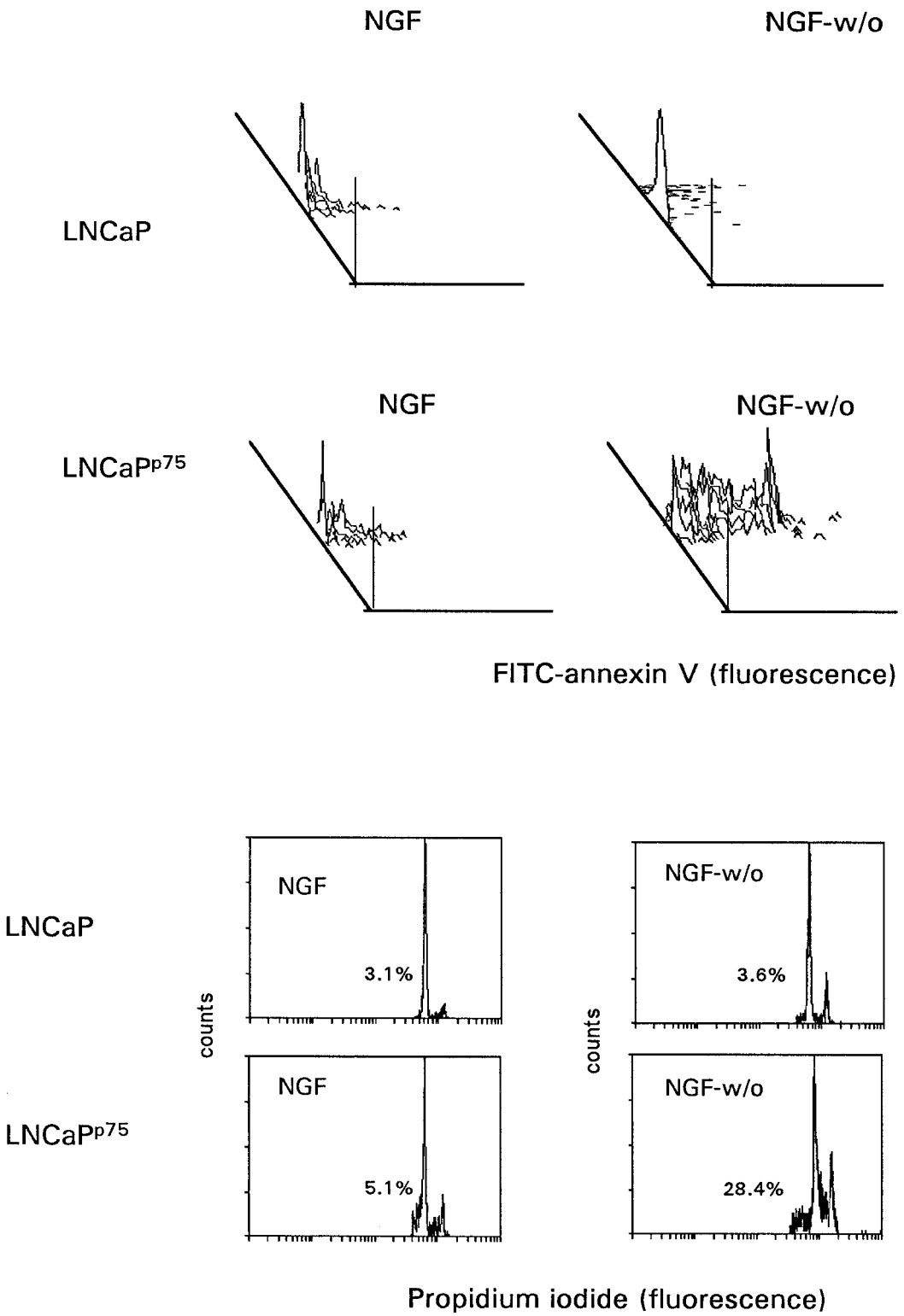

Fig. 8. Detection of Apoptotic Cell Death in p75^{LNGFR}-Transfected LNCaP Cells by Flow Cytometric Analysis

LNCaP and p75^{LNGFR}-transfected LNCaP cells were either continuously exposed to NGF (25 ng/ml) for 72 h with daily addition (NGF) or treated with NGF and then deprived of the growth factor during the last 24 h of incubation (NGF-w/o). Cells were then detached and incubated with annexin V-FITC (1 μg/ml) or propidium iodide before analysis by flow cytometry. The appearance of a clear annexin V-positive cell population was evident only in NGF-deprived p75^{LNGFR}-transfected LNCaP cells (*upper panel*). The intensity of fluorescence is plotted against cell distribution. Similarly, a distinct hypodiploid cell population, as detected by propidium iodide staining, appeared only in NGF-deprived p75^{LNGFR}-transfected LNCaP cells. Percent values refer to the entity of the apoptotic population (*lower panel*).

developed by exposure to 0.05% diaminobenzidine/0.01% H_2O_2 for 10 min.

Western Blot Analysis

LNCaP cells plated in 100-mm plates were harvested at 4 C with a lysis buffer containing phenylmethylsulfonylfluoride, aprotinin, pepstatin A, and leupeptin. After centrifugation at 15,000 × *g* at 4 C, the supernatant was processed for protein concentrations according to the method of Bradford (48). Samples were diluted in sample buffer and boiled for 5 min. Electrophoresis was performed in 10% SDS-PAGE (30 mA/h) using 20 μg of total protein per lane. After separation, proteins were transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Italia, Milan, Italy), for 45 min at room temperature using a transblot semidry transfer cell. After blocking, the membrane was incubated with anti-PSA antibody (6.4 μg/ml) for 2 h at room temperature, and then repeatedly washed and exposed to horseradish peroxidase-conjugated antirabbit IgG for 1 h at room temperature. Proteins were visualized using the enhancing chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Arlington Heights, IL). The same procedure was used for the Western blot analysis of p140^{trka} protein; to detect the phosphorylation status of the receptor, proteins were incubated overnight at 4 C with anti-p140^{trka}. Immunocomplexes were precipitated with antirabbit IgG-agarose beads for 1 h at 4 C before elution, electrophoresis separation, and blotting with anti-p140^{trka} antibody or antiphosphotyrosine antibody (kindly provided by Dr. Oreste Segatto, Rome, Italy).

Transfection of pCEP4-p75^{LNGFR} in LNCaP Cells

LNCaP cells were seeded in 100-mm dishes or 24-well multiwell plates (for cell counting studies) and transiently transfected with the pCEP4 plasmid (kindly provided by Prof. G. Della Valle, University of Bologna, Italy) containing the human p75^{LNGFR} cDNA. Cells were transfected using a liposome mixture complexed with DNA in a ratio 12:1, as described (49). Control cultures were transfected with the same vector lacking the DNA coding sequence for p75^{LNGFR}. Briefly, LNCaP cells, grown at 65–70% confluency, were deprived of serum and incubated for 4 h at 37 C with 7.2 μg/ml DNA complexed with liposomes. FCS was then added back to the cultures and cells were maintained for 72 h in the absence or presence of NGF, as specifically indicated, before harvesting for cell counting, assessment of p75^{LNGFR} expression, or detection of apoptotic death.

Assessment of the Expression of High- and Low-Affinity NGF Receptors in LNCaP Cells

To control for p140^{trka} expression, LNCaP cells were harvested from the dish with a rubber policeman and fixed in 70% ethanol at 4 C, overnight. In parallel, p75^{LNGFR}-transfected cells to be checked for p75^{LNGFR} expression were detached from the dish and used immediately. This protocol was chosen because of the known requirement of an intact membrane for the p75^{LNGFR}. Cells were repeatedly washed and incubated for 1 h at room temperature with either a mouse anti-p75 monoclonal antibody (10 μg/ml) or a rabbit anti-p140^{trka} polyclonal antibody (1 μg/ml). This step was followed by incubation with fluorescein isothiocyanate (FITC)-conjugated goat either antimouse (for p75^{LNGFR} detection) or antirabbit (for p140^{trka}) IgG, for 45 min at room temperature. Controls included omission of the primary antibody and substitution with nonimmune serum. Samples were analyzed using an Elite flow cytometer (Coulter Electronics). At least 10,000 forward and side scatter gated events were collected per specimen. Cells were excited at 488 nm, and the fluorescence was monitored at 525 nm. FITC fluorescence was collected using logarithmic amplification.

Evaluation of Apoptotic Death in p75^{LNGFR}-Transfected LNCaP Cells

Externalization of phosphatidylserine on the cell membrane, detectable by interaction with annexin V, which represents an early index of apoptotic death, was evaluated by incubating harvested cells with FITC-conjugated annexin V, at the concentration of 1 μg/ml for 15 min, according to manufacturer's instructions (CLONTECH Laboratories, Inc., Palo Alto, CA). Immunofluorescence for annexin V-FITC was analyzed with an Elite flow cytometer. At least 5,000 forward scatter gated events per specimen were collected. Annexin V-FITC fluorescence was collected using logarithmic amplification.

The appearance of a prediploid cell population, indicative of damaged and fragmented DNA, was evaluated cytofluorimetrically in LNCaP cells stained with the nuclear dye propidium iodide. After fixation with 70% ethanol, overnight at −20 C, cells were incubated with RNAse (100 μg/ml) for 2 h at 37 C and stained with propidium iodide (final concentration, 50 μg/ml). Analysis was carried out in a flow cytometer and restricted to cells with diploid and hypodiploid DNA content.

Statistical Analysis

Data were analyzed by Student's *t* test and, where appropriate, by one- and two-way ANOVA as specifically indicated.

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