

Epidermal growth factor receptor–related peptide inhibits growth of PC-3 prostate cancer cells

Dorota J. Marciniak, Arun K. Rishi, Fazlul H. Sarkar, and Adhip P.N. Majumdar

Departments of Internal Medicine and Pathology, John D. Dingell Veterans Affairs Medical Center, Karmanos Cancer Center, Wayne State University School of Medicine, Detroit, Michigan

Abstract

Interference with the activation of growth factor receptors, specifically epidermal growth factor receptor (EGFR), represents a promising strategy for the development of novel and selective anticancer therapies. We reported that EGFR-related peptide (ERRP), a recently isolated negative regulator of EGFR, could be a potential therapeutic agent for colorectal cancer. To determine whether ERRP could potentially be a therapeutic agent for prostate carcinoma, we examined the effect of recombinant ERRP on the growth of the prostate cancer cell line PC-3 *in vitro*. Events of the EGFR signal transduction pathways were also examined. ERRP caused a marked inhibition of cell growth in a dose- and time-dependent manner and also induced apoptosis. The latter was evidenced by increased number of apoptotic cells, activation of caspase-3, and cleavage of poly(ADP-ribose)polymerase. The transforming growth factor- α -induced stimulation of cell growth and activation of EGFR was also inhibited by ERRP. These changes were accompanied by a concomitant attenuation of activation of Akt and mitogen-activated protein kinases as well as basal and transforming growth factor- α -induced activation of nuclear factor- κ B. Inhibition of EGFR activation by ERRP could be partly attributed to increased sequestration of EGFR ligands. In summary, our data show that ERRP inhibits the growth of prostate cancer cells by attenuating EGFR signaling processes. ERRP could potentially be an effective therapeutic agent for prostate cancer. [Mol Cancer Ther 2004;3(12):1615–21]

Introduction

Prostate adenocarcinoma is the most common cancer and the second leading cancer-related cause of deaths among

men in industrialized countries (1, 2). Whereas the early-stage prostate-confined disease is curable by surgery, locally advanced or metastatic tumors remain essentially incurable. Although androgen ablation is initially found to be successful, tumors overcome androgen blockade and develop a hormone-unresponsive phenotype that become resistant to therapy. Progression of therapy refractory prostate cancer has, in part, been explained by the hypersensitivity of tumor cells to residual circulating androgens, growth factors, and other circulating regulators (3). Therefore, there is a need for development of new therapeutic strategies for treatment of prostate cancer.

Interference with the activation of growth factor receptors, specifically epidermal growth factor (EGF) receptor (EGFR), represents a promising strategy for the development of novel and selective anticancer therapies. Prostate carcinoma has been shown to express several growth factors and their receptors, including EGFR (4). It is known that several growth factors, especially EGF, plays a critical role in supporting the tumor growth, and the prostate tissue becomes more susceptible to the growth-promoting action of EGF family of peptides during androgen withdrawal (5, 6). EGFR and its ligand, transforming growth factor- α (TGF- α), a structural and functional analogue of EGF, are overexpressed in preneoplastic and neoplastic prostate tissue (7–10). Moreover, cell lines derived from different prostatic cancers and their metastases overexpress TGF- α and its receptor, the EGFR (8, 11). It is now well established that many different growth factors are involved in cross-talk between androgens that stimulate prostatic stromal cells to secrete growth factors, which in turn stimulate prostate epithelial cells and control the normal development and homeostasis of this gland (12–14). Cell culture studies have shown that proliferation of prostate epithelial cells is not induced by androgens alone but stimulated by several growth factors, such as EGF, insulin-like growth factors I and II, platelet-derived growth factor, and keratinocyte growth factor (5, 15, 16).

Progression from normal prostate epithelium to androgen-responsive tumor, and finally to hormone-refractory carcinoma, is a multistep process involving changes in proto-oncogenes and tumor suppressor genes. Many of the major new therapeutic approaches for many epithelial cancers, including prostate cancer, are directed against growth factor signaling pathways involving the EGFR family of receptors and the downstream components that transduce signals to the cell nucleus (17, 18). Several approaches, such as monoclonal antibodies to EGFR and pharmacologic inhibitors of EGFR tyrosine kinase, have been used, but with limited success, primarily because of toxicity or lack of specificity. Therefore, identification of endogenous factors that may inhibit EGFR activation and its signaling pathways is of paramount therapeutic

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Requests for reprints: Adhip P.N. Majumdar, Research Service, Room B 4238, John D. Dingell Veterans Affairs Medical Center, 4646 John R, Detroit, MI 48201. Phone: 313-576-1000, ext. 4460; Fax: 313-576-1112. E-mail: a.majumdar@wayne.edu

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importance. Recently, we reported the isolation and characterization of a negative regulator of EGFR, called EGFR-related peptide (ERRP; accession no. AF187818), that possesses a significant homology to the extracellular ligand-binding domain of EGFR (19). Results from our earlier studies suggest that ERRP could be a potential therapeutic agent for epithelial cancers (20). We have observed that overexpression of ERRP in colon cancer cell lines (HCT-116 and Caco-2) or exposure of them to recombinant ERRP not only inhibits proliferation in matrix-dependent and matrix-independent systems but also attenuates EGFR activation (19, 20). Moreover, recombinant ERRP causes regression of colon cancer xenograft tumors in some severe combined immunodeficient mice and arrests growth in others (20). To determine whether ERRP could also be effective against other epithelial cancers, specifically prostate cancer, the present investigation examines the effect of recombinant ERRP on the growth of prostate cancer cell line PC-3 *in vitro*. To further determine whether ERRP-induced inhibition of growth of prostate cancer cells could be attributed to EGFR signaling, changes in EGFR activation, and the downstream signaling events of the receptor, particularly activation of Akt and mitogen-activated protein kinase, were also examined.

Materials and Methods

Cell Lines

PC-3, a human prostate cancer cell line derived from bone metastasis, was purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (Invitrogen) at 37°C in the atmosphere of 95% air and 5% CO₂. The culture medium was changed every 2 to 3 days.

Generation of Recombinant ERRP

ERRP fusion protein was generated using the *Drosophila* expression system (Invitrogen) as described previously (20). The stable Schneider 2 *Drosophila* cell lines containing pMT/ERRP-V5-His plasmid were induced for 24 hours with 0.5 mmol/L CuSO₄ to express V5-His-tagged ERRP fusion protein and subsequently lysed in lysis buffer (50 mmol/L Tris, 100 mmol/L NaCl, 2.5 mmol/L EDTA, 1% Triton X-100, 1% NP40, 2.5 mmol/L Na₃VO₄, 25 µg/mL aprotinin, 25 µg/mL leupeptin, 25 µg/mL pepstatin A, 1 mmol/L phenylmethylsulfonyl fluoride). ERRP was then purified from Schneider 2 cell lysate by two sequential immunoaffinity columns: anti-ERRP antibodies followed by a second column of anti-polyhistidine antibodies as described previously (20, 21).

Growth Inhibition Assay

Inhibition of cell growth in response to recombinant ERRP was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (20). Briefly, cells were dispersed by trypsin-EDTA treatment and 2.5 × 10⁴ cells/mL were resuspended in RPMI 1640 containing 10% of FBS and seeded into 96-well culture plates with six replicates. After 24 hours

of plating, the medium was replaced with one that contained 2.5% of FBS, and the incubation was continued in the absence (control) or presence of recombinant ERRP as described in the figure legends. In some experiments, growth was induced by TGF-α (10 nmol/L) in the absence or presence of recombinant ERRP (5 µg/mL). In all experiments, the reaction was terminated by adding of 20 µL of 5 mg/mL stock of MTT to each well. The reaction was allowed to proceed for 3 to 4 hours at 37°C. The culture medium was then removed. The formazan crystals were then dissolved by adding 0.1 mL DMSO. The intensity of the color developed, which is the reflection of number of live cells, was measured at a wavelength of 570 nm. All values were compared with the corresponding controls. All assays were done with six replicates.

Assessment of Apoptosis

PC-3 cells (~1 × 10⁵/well) were plated in DMEM-10% FBS. After 24 hours of plating, the medium was changed to contain 2.5% FBS to minimize the contribution of serum-derived growth factors and subsequently incubated for 24 hours in the absence (control; vehicle added) or presence of ERRP (5 µg/mL). At the end of the incubation period, the cells were lysed, and the rate of apoptosis was determined using the Cell Death Detection ELISA^{PLUS} kit from Roche Diagnostics GmbH (Penzberg, Germany), which measures the cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes).

Activation of EGFR and Downstream Signaling Molecules

These studies were done using six-well plates. Aliquots (~10⁵ cells/well) of PC-3 cells in RPMI-10% FBS were plated, and after 24 hours, they were serum starved for 48 hours to minimize the contributions of growth factors, particularly EGF family of peptides, and to synchronize the cells. The cells were then preincubated with or without ERRP (5 µg/mL) for 5 minutes and then incubated for another 5 minutes in the absence or presence of 10 nmol/L TGF-α (Oncogene, San Diego, CA) as stated in the figure legends. The reaction was terminated by adding lysis buffer. The lysate was collected and clarified (10,000 × g; 10 minutes), and the supernatant was used to determine the activation of EGFR, Akt, and extracellular signal-regulated kinase (ERK) by measuring the levels of phosphorylated forms of these molecules by Western blot with appropriate antibodies as described below.

Western Immunoblot Analysis

Western blot analysis was done essentially according to our standard protocol (22, 23). Briefly, the cells were solubilized in lysis buffer as stated above. Following clarification at 10,000 × g for 15 minutes, the supernatant was used for Western blot analysis. In all analyses, protein concentration, determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA), was standardized among the samples. Aliquots of cell lysates containing 50 µg of protein were separated by SDS-PAGE. Following electrophoresis, protein was transferred electrophoretically onto supported nitrocellulose membranes (Osmonics, Gloucester, MA). Membranes were incubated for 1 hour at room temperature

with blocking buffer, TBS-T [20 mmol/L Tris (pH 7.6), 100 nmol/L NaCl, 0.1% Tween 20], and 5% nonfat dry milk with gentle agitation. After washing the membranes with TBS-T, they were incubated overnight at 4°C in TBS-T buffer containing 5% milk and with one of the following antibodies: phospho-EGFR (Tyr¹¹⁷³; Upstate, Lake Placid, NY), phosphorylated ERKs (p44/p42; Thr²⁰²/Tyr²⁰⁴; Cell Signaling, Beverly, MA), phosphorylated Akt (Ser⁴⁷³; Cell Signaling), poly(ADP-ribose)polymerase (Santa Cruz Biotechnology, Santa Cruz, CA), or caspase-3 (Santa Cruz Biotechnology). The membranes were washed thrice with TBS-T and subsequently incubated with appropriate secondary antibodies [IgG from Upstate, Cell Signaling, or Chemicon (Temecula, CA)] in TBS-T containing 5% milk for 2 hours at room temperature with gentle agitation. The membranes were washed again with TBS-T, and the protein bands were visualized by enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). The membranes containing the electrophoresed proteins were exposed to X-Omat film, and the signals were quantitated by densitometry using ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics, Sunnyvale, CA). Membranes were stripped (2× for 15 minutes at 55°C) in stripping buffer containing 100 mmol/L 2-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.7). The membranes were then reprobed for total (nonphosphorylated) EGFR (Upstate), ERKs (Cell Signaling), or Akt as well as for α -tubulin (Oncogene) using corresponding antibodies. α -Tubulin was used as an internal control. All Western blots were done at least thrice for each experiment.

Immunoprecipitation and Western Blot Analysis

This methodology was used to examine sequestration of the EGFR ligands, TGF- α , and heparin-binding EGF (HB-EGF) to ERRP. Briefly, following incubation of PC-3 cells in the absence (control) or presence of recombinant ERRP (5 μ g/mL) for 24 hours, they were homogenized in homogenizing buffer [HEPES (pH 7.4), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, 10 μ g/mL leupeptin, 1 μ g/mL aprotinin, and 1 mmol/L 1,10-phenanthroline] and centrifuged at 1,000 \times g for 15 minutes. The supernatant was then centrifuged at 30,000 \times g for 30 minutes to obtain a crude membrane fraction (30,000 \times g pellet). The crude membranes were solubilized in lysis buffer as stated above, and following protein determination, the solubilized membranes from the control and ERRP-treated cells containing the same amount of protein (1 mg) were diluted with an equal volume of TTA buffer [50 mmol/L Tris (pH 7.6), 0.15 mol/L NaCl, 0.5% Tween 20, 0.1% bovine serum albumin]. The samples were mixed with Sepharose G beads (20 μ L) and anti-ERRP antibodies, which were generated in our laboratory as described previously (20). The samples were incubated overnight at 4°C and subsequently washed several times with TT buffer [50 mmol/L Tris (pH 7.6), 0.15 mol/L NaCl, 0.5% Tween 20]. The immunoprecipitates were resuspended in loading buffer and subjected to Western blot analysis with anti-TGF- α or HB-EGF as described above.

Nuclear Factor- κ B Transcriptional Activity

PC-3 cells were seeded (1×10^5 /well in six-well plates in two duplicates) in RPMI 1640 medium containing 10% of FBS. After 24 hours when the cells were properly attached, medium was replaced with fresh medium devoid of serum and antibiotic. The cells were then transfected with a combination of 0.8 μ g of the plasmid pNF- κ B-TATA-Luc (Stratagene, La Jolla, CA) and 0.2 μ g of internal control pRSVZ plasmid as described previously (23). Three hours after transfection, the cells were incubated with RPMI containing 2.5% FBS. After 24 hours, they were treated with TGF- α (10 nmol/L) or ERRP (5 μ g/mL) or a combination of both for an additional 48 hours and subsequently assayed for luciferase and β -galactosidase activities as described previously (23). Transfection efficiency was normalized according to β -galactosidase activity. The data are expressed as means \pm SE.

Statistical Analysis

Where applicable, results were statistically evaluated with Student's *t* test for unpaired values with *P* < 0.05 as the level of significance.

Results

Earlier, we reported that exposure of colon cancer cell lines to recombinant ERRP resulted in attenuation of tyrosine phosphorylation of EGFR and inhibition of cell growth. This, together with the observation that ERRP also inhibits colon cancer xenograft tumors in severe combined immunodeficient mice, prompted us to suggest that ERRP could be an effective therapeutic agent for colorectal cancer. To determine whether ERRP would also be effective against prostate cancer, we studied the effect of recombinant ERRP on cell growth and apoptosis of PC-3 prostate cancer cell line. We observed that recombinant ERRP inhibited cell growth and stimulated apoptosis of PC-3 cells in a dose-dependent manner, revealing ~75% inhibition of proliferation and 1,500% stimulation of apoptosis with a dose of 2 μ g/mL ERRP with no further significant changes occurring at higher doses of ERRP (Fig. 1A). To further determine the events of apoptosis signaling, we examined activation of caspase-3 as well as cleavage of its substrate poly(ADP-ribose)polymerase. Exposure of PC-3 cells to recombinant ERRP (5 μ g/mL) for 24 hours caused a reduction in the levels of procaspase-3, when compared with the controls, and was accompanied by an increase in the levels of the 85-kDa protein, the cleaved product of 112-kDa poly(ADP-ribose)polymerase (Fig. 1C). No apparent change in α -tubulin levels between controls and ERRP-treated cells was observed (Fig. 1C).

Results of the time course changes in cell growth of PC-3 cells revealed that in the absence of ERRP (controls) cell growth increased gradually over the 96-hour incubation period reaching a value of ~200% of the initial control, whereas in the presence of ERRP (5 μ g/mL) cell growth declined to ~10% of the initial value at 72 hours and

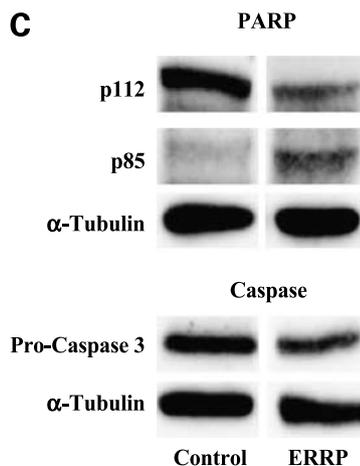
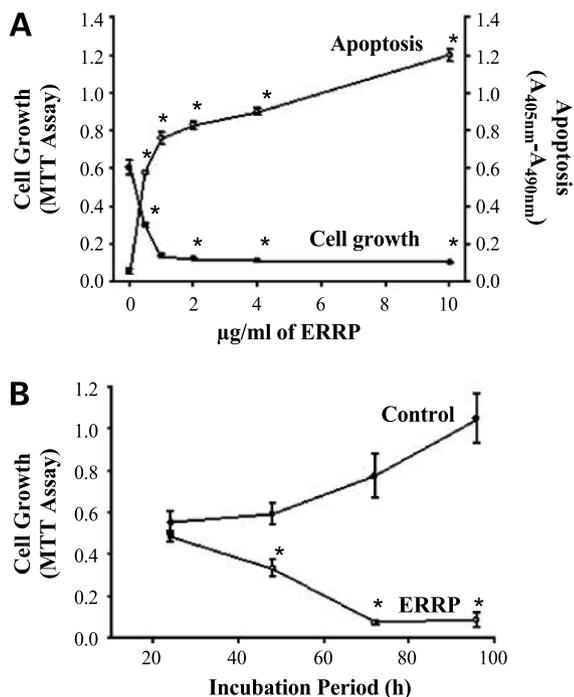


Figure 1. Effect of increasing concentrations of recombinant ERRP on (A) growth and apoptosis of PC-3 prostate cancer cells. Cells were incubated in the absence (controls) and increasing concentrations of recombinant ERRP for 48 hours. Cellular growth was determined by MTT assay. Apoptosis was determined using the Cell Death Detection ELISA^{PLUS} kit from Roche Diagnostics and by measuring (C) the levels of 112-kDa full-length poly(ADP-ribose)polymerase and its 85-kDa cleaved product and procaspase-3. (B) shows time course changes in cell growth in response to recombinant ERRP (5 µg/mL). Points, mean of five to six observations; bars, SE. *, $P < 0.01$, compared with the corresponding controls.

remained at that level during the rest of the experimental period (Fig. 1B). Although the dose-response study has shown a 75% inhibition of cell growth at a dose of 2 µg/mL ERRP, all subsequent experiments, including the time course study, were done with an ERRP dose of 5 µg/mL. Because recombinant ERRP was generated in batches and biological activity varied among the batches, we chose a higher dose of ERRP to ensure that the growth inhibitory effect was observed.

Numerous studies have shown that TGF- α , one of the primary ligands of EGFR, stimulates growth of normal and neoplastic cells (5, 6, 24). To determine whether ERRP would inhibit TGF- α -induced growth of PC-3 cells, 48-hour serum-starved cells were incubated with recombinant ERRP (5 µg/mL) in the absence or presence of 10 nmol/L TGF- α for 48 hours. As expected, TGF- α caused ~50% stimulation of growth of PC-3 cells over the controls (Fig. 2A). This stimulation was abrogated by ERRP. In fact, in the presence of ERRP, there was ~50% reduction in cell growth when compared with the controls (Fig. 2A). Recombinant ERRP by itself decreased proliferation of PC-3 cells by ~70% when compared with the controls (Fig. 2A).

To determine whether the ERRP action would be reversible, PC-3 cells were incubated for 48 hours in the absence (control) or presence of recombinant ERRP (5 µg/mL), whereafter the incubation medium was replaced with that contained either fresh recombinant ERRP (5 µg/mL) or an equivalent volume of vehicle (control). The incubation was continued for another 48 hours. The presence of ERRP throughout the 96-hour incubation period resulted in ~60% reduction in cell growth (Fig. 2B). However, when ERRP was removed from the medium after 48 hours and

incubation continued for another 48 hours in the absence of ERRP, there was a 20% to 25% increase in cell growth compared with that noted in cells maintained in the continuous presence of ERRP (Fig. 2B).

Earlier, we suggested that ERRP inhibits cell proliferation by attenuating EGFR activation (20). To further determine the role of EGFR signaling, we examined the effect of recombinant ERRP on TGF- α -induced activation of EGFR as well as its downstream signaling processes, particularly the activation of Akt and mitogen-activated protein kinases (ERKs; p44/p42). The reason for analyzing Akt and mitogen-activated protein kinases was that whereas activation of mitogen-activated protein kinases is associated with stimulation of cell proliferation, induction of Akt has been linked to increased cell survival (25–27). In 48-hour serum-starved PC-3 cells, exposure to TGF- α for 5 minutes caused a marked stimulation of phosphorylation of EGFR (Tyr¹¹⁷³) and Akt (Thr³⁰⁸/Ser⁴⁷³) as well as ERK1/2 (p44/p42; Thr²⁰² and Tyr²⁰⁴). These increases were abrogated in the presence of ERRP (Fig. 3A). Under the present experimental conditions, the levels of total (non-phosphorylated forms) EGFR or ERKs were not affected by either TGF- α or ERRP (Fig. 3A).

Akt-induced stimulation of cell survival partly involves activation of nuclear factor- κ B (NF- κ B; refs. 25, 26). To determine, whether ERRP would affect the function of NF- κ B, we examined the changes in basal and TGF- α -induced activation of NF- κ B promoter in PC-3 cells in the absence (control) or presence of recombinant ERRP (5 µg/mL). This was determined following transient transfection of NF- κ B promoter luciferase reporter construct in PC-3 cells. Relative luciferase activity, as a measure of NF- κ B

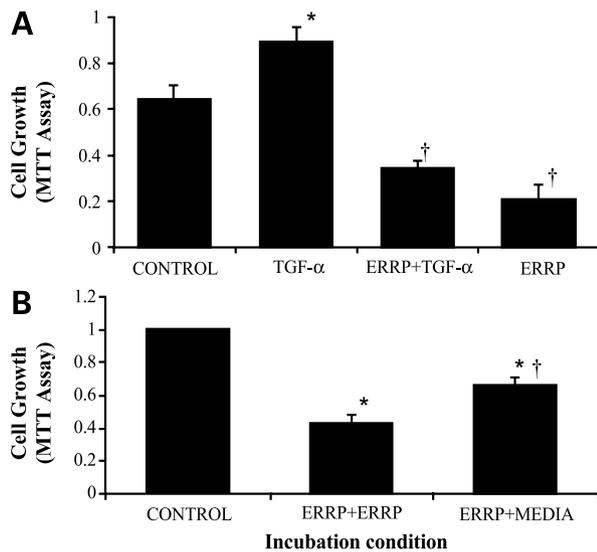


Figure 2. **A**, effect of recombinant ERRP on TGF- α -induced growth of PC-3 prostate cancer cells. Cells were incubated with 10 nmol/L TGF- α in the absence or presence of recombinant ERRP (5 μ g/mL) for 48 hours. Control incubations contained neither 10 nmol/L TGF- α nor recombinant ERRP. **B**, proliferation of PC-3 cells in the presence and after removal of recombinant ERRP. Cells were incubated for 48 hours in absence (CONTROL) or presence of recombinant ERRP (5 μ g/mL). After 48 hours, the medium was replaced to contain fresh recombinant ERRP (5 μ g/mL) or vehicle, and the incubation was continued for another 48 hours. Negative controls were incubated for 96 hours in the absence of recombinant ERRP. Columns, mean of six observations; bars, SE. *, $P < 0.001$, compared with the control; †, $P < 0.05$, compared with the values from cells that were incubated continuously for 96 hours.

promoter function, was increased by $\sim 70\%$ at 48 hours following exposure to TGF- α (10 nmol/L) compared with the controls (Fig. 3B). This increase was abrogated by ERRP (Fig. 3B). ERRP also inhibited basal NF- κ B promoter activity by $\sim 40\%$ (Fig. 3B).

To determine whether and to what extent ERRP will affect the constitutive EGFR in PC-3 cells, the levels of total (nonphosphorylated) and active (tyrosine-phosphorylated) forms of EGFR were measured in PC-3 cells over a period of 96 hours following exposure to either ERRP (5 μ g/mL) or vehicle (controls). In the absence of ERRP, the basal levels of both tyrosine-phosphorylated and nonphosphorylated (total) forms of EGFR increased gradually over the 96-hour incubation period, whereas in the presence of ERRP these increases were attenuated (Fig. 4A and B).

Although the precise mechanisms by which ERRP attenuates EGFR signaling pathways and in turn inhibit the growth of PC cells remain to be fully delineated, we hypothesize that ERRP, with a significant homology to the ligand-binding domain of EGFR, binds EGFR ligand(s) rendering them unavailable for binding to and activation of EGFR. To test our hypothesis, we measured the amount of EGFR ligands TGF- α and HB-EGF bound to ERRP in membranes of PC-3 cells following incubation of the cells in the absence (control) and presence of exogenous ERRP (5 μ g/mL) for 24 hours. We observed that the amount of TGF- α and HB-

EGF bound to ERRP was $\sim 30\%$ higher in the membranes from the cells that were incubated with recombinant ERRP when compared with the corresponding vehicle-treated controls (Fig. 4C).

Discussion

There is increasing evidence that the malignant behavior of some of the tumors is sustained by deregulated activation of several growth factor receptors (28–30). Interference with the activation of growth factor receptor and/or with intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of novel and selective anticancer therapies (18). A large body of experimental evidence has been provided for a key role for EGFR activation in a variety of human epithelial cancers (31–33). EGFR-driven intracellular signaling controls not only cancer cell proliferation but also several processes that are important for tumor progression, including apoptosis, invasion, angiogenesis, and metastasis (34).

Overexpression of TGF- α and/or EGFR has been detected in many solid tumors, including non-small cell lung cancer

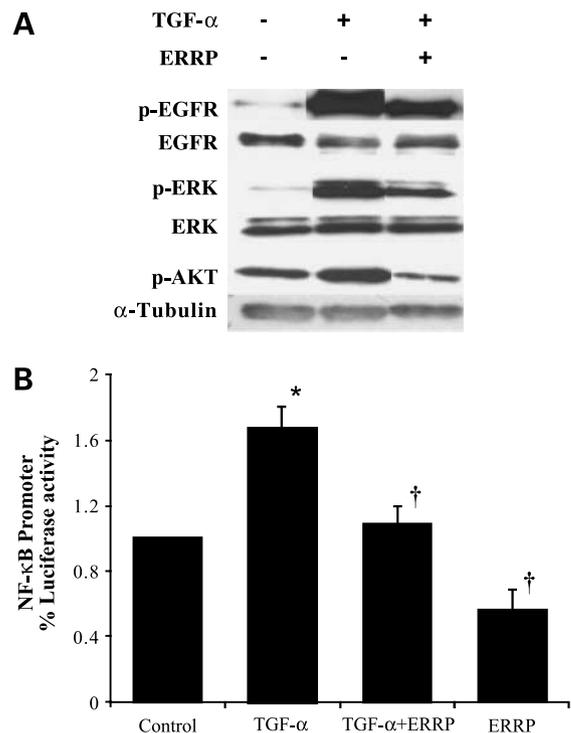


Figure 3. **A**, Western blot of the effect of ERRP (5 μ g/mL) on TGF- α -induced phosphorylation of EGFR, ERKs, and Akt in PC-3 cells. Cells were incubated for 5 minutes at 37°C in the absence or presence of ERRP and subsequently incubated with or without 10 nmol/L TGF- α . Membranes were stripped and reprobbed for total (nonphosphorylated) EGFR and ERKs, as well as for α -tubulin (internal control). **B**, effect of ERRP (5 μ g/mL) on TGF- α -induced NF- κ B promoter activity in PC-3 cells. Cells were transiently transfected with NF- κ B promoter luciferase reporter construct and subsequently incubated with 10 nmol/L TGF- α in the absence or presence of recombinant ERRP (5 μ g/mL). *, $P < 0.001$, compared to controls that contained neither TGF- α nor ERRP.

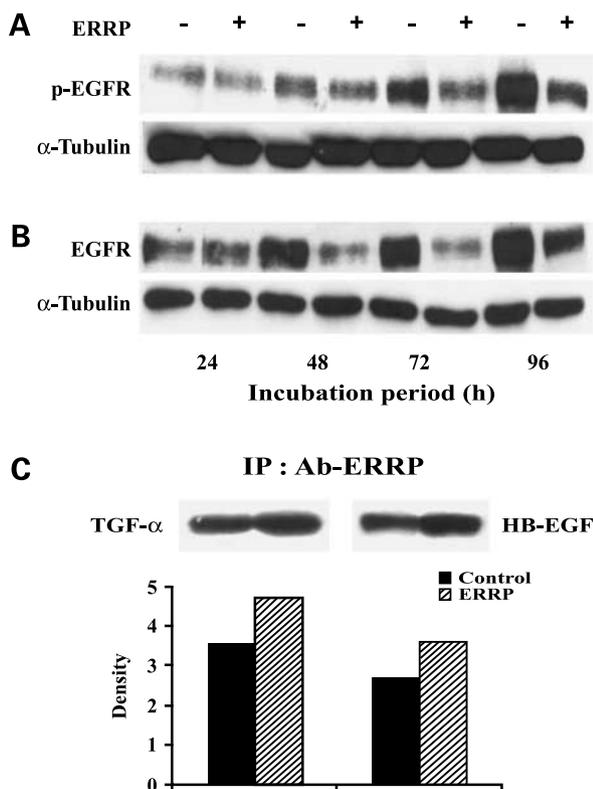


Figure 4. Western blot of time course changes in the levels of (A) phosphorylated and (B) total (nonphosphorylated) forms of EGFR in PC-3 cells in response to recombinant ERRP (5 μ g/mL). Controls were incubated with an equivalent volume of vehicle. In all experiments, the membranes were stripped and reprobed for α -tubulin. C, Western blot of the relative amount of the precursor form(s) of TGF- α (18–20 kDa) and HB-EGF (22–24 kDa) bound to ERRP in PC-3 cells that were incubated in the absence (Control) or presence (5 μ g/mL) of recombinant ERRP for 24 hours.

and cancers of the colon, breast, and prostate (4, 5, 31, 35, 36). This overexpression has been associated with resistance to cytotoxic drugs and is generally an indicator of poor prognosis (31, 37, 38). For these reasons, blockade of the TGF- α -EGFR autocrine pathway has been proposed as a target for anticancer therapy. Several approaches, including monoclonal antibodies to EGFR and pharmacologic inhibitor(s) of EGFR tyrosine kinase, have been used. Effectiveness of most of the therapeutic regimen has thus far been limited primarily because of toxicity and/or lack of specificity. Moreover, there are limitations in using antibodies that are recognized as foreign proteins and that eventually induce an immune response as anti-idiotypic antibodies are formed as a result of the repeated injections. Therefore, identification of endogenous factor(s) that may inhibit EGFR activation is of paramount importance. ERRP, a 53- to 55-kDa secretory protein that has been shown to inhibit EGFR signaling (19, 20), fits this requirement. Results of our earlier studies suggest that ERRP is a negative regulator of EGFR, which inhibits proliferation and transformation of colon cancer cells *in vitro* by attenuating EGFR activation (20). Moreover, *i.t.* or *s.c.*

(away from the tumor site) injections of purified recombinant ERRP caused regression of colon cancer xenograft tumors in severe combined immunodeficient mice (20, 39). These observations led us to suggest that ERRP could be an effective therapeutic agent for colorectal cancer (20, 39).

Our current observation that recombinant ERRP inhibits both basal and TGF- α -induced growth of PC-3 cells further suggests that ERRP could be a potential therapeutic agent for prostate cancer. Although no *in vivo* studies have been done to determine the effectiveness of ERRP in prostate cancer, the fact that the growth of PC-3 cells remained markedly attenuated throughout the 4-day incubation period in the presence of recombinant ERRP suggests that PC-3 cells, which are nonresponsive to androgen, are responsive to ERRP. This, together with the observation that removal of ERRP does not fully restore the growth of these cells, further suggests that the antiproliferative effect of ERRP on PC-3 cells is long lasting.

We have shown earlier that recombinant ERRP-induced inhibition of growth of colon cancer cells is associated with parallel reductions in both basal and TGF- α -induced EGFR activation, as evidenced by decreased levels of tyrosine-phosphorylated form of EGFR (20). Interestingly, the androgen-refractory prostate carcinomas tend to display activated EGFR signaling pathways (5, 6). Our current data suggest that ERRP is an effective inhibitor of EGFR signal transduction pathways in prostate cancer cells, particularly those that are androgen nonresponsive. We have observed that recombinant ERRP not only inhibits TGF- α -induced activation of EGFR but also attenuates phosphorylation of ERKs and Akt. Because induction of ERKs and Akt signaling pathways has often been shown to result in stimulation of cell growth and increase in survival of cells, respectively (25, 26), attenuation of these processes by recombinant ERRP in PC-3 cells suggests that ERRP not only inhibits cell growth but also stimulates apoptosis of prostate cancer cells. Indeed, we have observed that ERRP stimulates apoptosis of PC-3 cells. Further support for ERRP induction of apoptotic events in PC-3 cells comes from the observation that recombinant ERRP inhibits both basal and TGF- α -induced stimulation of NF- κ B promoter activity, a transcription factor whose induction is often associated with increased cell survival (25, 26).

Although the precise mechanisms by which ERRP attenuates EGFR activation and in turn inhibits cell growth remain to be fully delineated, we have reported that in colon cancer cells ERRP that possesses a substantial homology to the extracellular ligand-binding domain of EGFR binds/sequesters the EGFR ligand TGF- α and forms inactive heterodimers with EGFR resulting in inhibition of EGFR activation and its signaling (20). Additionally, we have observed that the age-related increase in EGFR activation in the gastric mucosa was accompanied by decreased levels of ERRP and the amount of precursor forms of TGF- α bound to ERRP (40). The latter was thought to raise the levels of unbound TGF- α rendering them available for binding to and activation of EGFR (40). Indeed, we reported that the amount of TGF- α bound to

EGFR was higher in the gastric mucosal membranes from young rats than those from aged rats (40). Our current observation of higher levels of the precursor forms of TGF- α and HB-EGF bound to ERRP in the membrane fraction of PC-3 cells from those treated with recombinant ERRP, over the controls, could thus be attributed to increased binding/sequestration of EGFR ligand(s) to exogenous ERRP rendering them unavailable for binding to and activation of EGFR signaling.

In summary, our current data show that ERRP inhibition of androgen-refractory prostate cancer cell growth is accompanied by attenuation of EGFR activation and its downstream signaling effectors such as Akt and mitogen-activated protein kinases. This inhibition involves reduction in cell growth and stimulation of apoptosis. The inhibition of EGFR activation by ERRP could be partly attributed to increased sequestration of EGFR ligands by ERRP.

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