

A cyclic peptide derived from α -fetoprotein inhibits the proliferative effects of the epidermal growth factor and estradiol in MCF7 cells

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Abstract. A cyclic peptide derived from the active domain of α -fetoprotein (AFP) significantly inhibited the proliferation of MCF7 cells stimulated with the epidermal growth factor (EGF) or estradiol (E_2). The action of these three agents on cell growth was independent of the presence of calf serum in the culture medium. Our results demonstrated that the cyclic peptide interfered markedly with the regulation of MAPK by activated c-erbB2. The cyclic peptide showed no effect on the E_2 -stimulated release of matrix metalloproteinases 2 and 9 nor on the shedding of heparin-binding EGF into the culture medium. We propose that the AFP-derived cyclic peptide represents a valuable novel antiproliferative agent for treating breast cancer.

Introduction

Estrogens have a major role in the growth and development of the mammary gland where they interact with other hormones, growth factors and cytokines in the precise regulation of proliferation and differentiation. Mammary cancer, the leading cancer in women worldwide, is known to be under hormonal control (1). Estradiol (E_2) stimulates the growth of certain breast tumors, activating relatively slow genomic processes, elicited by an association to nuclear estrogen receptors (ERs) and subsequent transactivation of target genes (2). Other cellular responses to E_2 involve the association of the hormone to cognate receptors located at the cell membrane

(mER) that promote the so-called 'non-genomic' E_2 effects (3). Depending on the cell type and context, the association of E_2 to the mER triggers several molecules, including calcium channels (4), cAMP (5), phospholipase C (6), inositol phosphate (7) and a variety of kinases (8). In addition, the association of E_2 to mER transactivate members of the family of epidermal growth factor receptors (EGFRs), leading, among others, to downstream signaling by extracellular factor regulated kinases (ERK) (9-12).

Recent studies suggest that the EGFRs could be a nodal point of convergence for many membrane cytokine receptors with the capacity to activate ERK. E_2 is known to activate signaling molecules, including IGF-IR, EGFR and MAPK in MCF7 breast cancer cells (13). Rapid ER α membrane translocation and interaction with membrane IGFR have been shown as prerequisite steps for E_2 -induced MAPK activation in these cells (14).

Hormonal therapies are important in treating ER-positive breast cancer patients and currently two pharmacological approaches are used to diminish the proliferative effects of E_2 . One is the inhibition of E_2 action using anti-estrogens that interact with the ER, the other is the inhibition of estrogen synthesis by aromatase inhibitors (15). However, not every ER-positive breast cancer is sensitive to these treatments and, even worse, certain treated tumors eventually acquire a resistance to these therapeutical drugs. The development of antihormone resistance has been related with the sustained operation of extranuclear ER α pathways (8). Therefore, compounds that repress or annihilate E_2 -dependent activation of these pathways in growing cancer cells could become valuable therapeutic agents.

Studies by Mesfin *et al* demonstrated that a cyclic peptide (cP) containing a minimal active sequence from α -fetoprotein (AFP) deter the growth of estrogen-dependent human mammary tumor cells xenografted into severely immunodeficient (SCID) mice (16). Furthermore, these authors demonstrated that the cP inhibits the uterotrophic action of E_2 and tamoxifen in immature mice and the proliferation of estrogen-dependent human tumor cells in culture (17,18). The cyclic peptide showed stability in solution and is fully active in delaying mammary tumor growth when administered orally to rats (19).

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Recently, we demonstrated that E₂-stimulated proliferation of mammary tumor cells was inhibited by the presence of the cyclic peptide in the culture medium (20). The peptide was effective slowing-down the proliferation of estrogen-dependent tumor cells, though not that of estrogen-independent MDA-MB231 cells. The cP did not increase the MCF7 cell death rate, nor altered cell E₂ binding capacity, nor modified cell aromatase activity (20).

The aim of this study was to compare the effect of the AFP-derived cyclic peptide on EGF- and E₂-stimulated proliferation of MCF7 cells. Results indicate that the cP interferes in MAPK regulation by c-erbB2. Neither the E₂-stimulated release of MMP2 and MMP9 nor the shedding of heparin-binding EGF (HB-EGF) into the culture medium were affected by the cyclic peptide.

Materials and methods

Materials. The AFP-derived nonapeptide cyclo (EKTOV NOGN) (where O is hydroxyproline) and the control-scrambled peptide were obtained by solid-phase peptide synthesis using N-(9-fluorenyl) methoxycarbonyl, as already described (18).

Tissue culture materials were obtained from NalgeNunc (Rochester, NY, USA), PVDF membranes and Precision Plus Protein Standards were purchased from BioRad Laboratories (Hercules, CA, USA). Recombinant human (rh) EGF (Cat GF001) was obtained from Chemicon International, Inc (Temecula, CA, USA). Protease inhibitor cocktail III and E₂ were purchased from Calbiochem, EMD Biosciences (La Jolla, CA, USA). Most of the other substances used in this study were from Sigma-Aldrich (St. Louis, MO, USA).

Antibodies: mouse anti-c-erbB2 monoclonal antibody (sc-08); goat anti HB-EGF (sc-1414); rabbit anti-ERK polyclonal antibody (sc-154) and FITC-conjugated goat anti-mouse IgG (sc2010) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-MAP kinase (M2360-02A, specifically recognizing ERKs p44, p42, phosphorylated in Thr202,Tyr204) was purchased from United States Biological (Swampscott, MA, USA). Mouse anti-EGFR monoclonal antibody (GR13) was obtained from Calbiochem, EMD Biosciences and Alexa Fluor 488 conjugated donkey anti-rabbit IgG (A21206) was purchased from Molecular Probes-Invitrogen Corp. (Carlsbad, CA, USA).

Cell lines. E₂-sensitive MCF7 epithelial cells established from human metastatic breast cancer tissue (HTB 22; ATCC, USA), were cultured in MEM-Eagle media containing 10% calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Calf serum was used instead of fetal bovine serum to avoid the presence of traces of AFP.

In the proliferation studies with the medium containing calf serum, the cells were transferred 24 h after seeding to DMEM F12 containing ITS (insulin, transferrin, selenium), 5% charcoal/dextran-twice-treated calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Whereas in the proliferation studies under serum-free conditions, the cells were transferred to DMEM F12 containing ITS (insulin, transferrin, selenium), 3% hydroxyethylated starch (HAES),

0.5% human serum albumin, 50 U/ml penicillin and 50 µg/ml streptomycin. The E₂-insensitive cell line MDA-MB231 (HTB-26; ATCC, USA), used for control studies, was cultured as described previously (20). In each of the experiments, the cells were incubated at 37°C in a humidified incubator, under a 5% CO₂ atmosphere.

Proliferation studies. MCF7 (12,000 cells/cm²) and MDA-MB231 (6,000 cells/cm²), were seeded and incubated for 24 h to allow attachment, non-adherent cells and media were removed. The remaining cells were washed and further incubated for various periods with the respective culture medium containing either 2 nM E₂, or 5 ng rh EGF, in the absence or presence of 2 µg/ml of cyclic peptide, with a change of the culture medium every 48 h. Parallel control experiments containing no E₂ or cyclic peptide, or 2 µg/ml of a control, scrambled peptide were performed. At the completion of the incubation period, cells were washed with phosphate-buffered saline (PBS), detached (0.25% trypsin in 0.2 mM EDTA), re-suspended in PBS, counted and assessed for viability (trypan blue). Each experiment was performed three times.

Immunofluorescence studies. Cells exposed to different experimental conditions were grown on a sterile coverglass and then fixed (methanol, 20 min at -20°C), rinsed (PBS containing 2% BSA) and incubated with the primary antibodies (1 h at RT). After extensive washes (PBS containing 2% BSA), cells were incubated with the appropriate secondary antibody and nuclei counterstained with Hoechst 33240. After immunolabeling, cells were washed, mounted and viewed with a Zeiss Axiophot epifluorescence microscope fitted with a color CCD camera. In each experiment, the images were obtained under fixed settings of illumination, exposure times and camera gain.

Western blot analysis. For total cell protein extraction, cells were sonicated in 62.5 mM Tris, pH 6.8 containing 2% SDS, 50 mM DTT, 10% glycerol and 0.01% bromophenol blue. For electrophoresis, 10-20 µg protein samples were incubated for 5 min at 95°C and loaded in 10% polyacrylamide gels. Electrophoresis was carried out at RT at 100 V using BioRad's Miniprotean chambers. Bands were electro-transferred onto PVDF membranes, immunodetection was performed by using the appropriate primary antibodies and peroxidase-labelled secondary antibodies and visualizing by enhanced chemiluminescence (Renaissance Western blot chemiluminescence kit from Perkin Elmer, Boston, MA, USA). Phosphorylated ERK1/2 proteins were detected with the specific anti-phospho antibody and the rabbit antibody-antigen complexes were tagged as indicated above. Relative levels of total ERK1/2 protein in each sample were determined by stripping the phospho-specific ERK1/2 antibodies from the membrane and reprobing with antibodies to non-phosphorylated ERK1/2. The immunoblot bands were analyzed with NIH Image J software.

Gelatin zymography. MMP activities secreted into the culture medium by MCF7 cells were analyzed by gel electrophoresis. The cells were incubated for 48 h in a medium with 5%

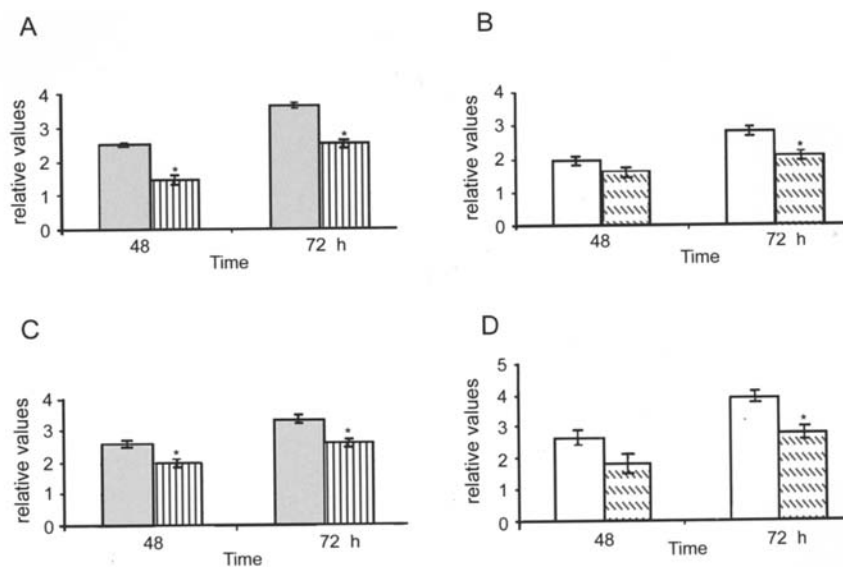


Figure 1. Effect of the cP on mitogen-stimulated proliferation of MCF7 cells in media with or without serum. MCF7 cells were incubated for indicated times in a medium with 5% CDCS containing 2 nM E₂ (grey columns), 2 nM E₂ plus 2 μg/ml cP (vertically hatched columns) (A), 5 ng/ml EGF (open columns) or 5 ng/ml EGF plus 2 μg/ml cP (hatched columns) (B). MCF7 cells were incubated for indicated times in a medium with HAES in the presence of 2 nM E₂ (grey columns), with 2 nM E₂ plus 2 μg/ml cP (vertically hatched columns) (C); 5 ng/ml EGF (open columns) or 5 ng/ml EGF plus cP (hatched columns) (D). Proliferation is expressed as a relative cell number in relation to control cell numbers (24 h after seeding = 11,700 cells/cm²). Values are mean ± SD of 3 experiments in triplicate. *P<0.05 compared with results without cP.

charcoal-treated calf-serum and then incubated for different periods of time with 2 nM E₂ in the presence or absence of 2 μg/ml cP. The cell proliferation medium was removed, mixed with two volumes of SDS sample buffer (0.125 M Tris-HCl, pH 6.8 containing 10% SDS, 8% sucrose and 0.05% bromophenol blue), incubated for 30 min at 25°C and applied on 7.5% polyacrylamide co-polymerized with 0.1% gelatin. The electrophoresis was performed as indicated previously. After the run, the gels were washed three times, 20 min each, with 2.5% Triton X-100 at room temperature and then incubated for 10 h at 37°C in 50 mM tris pH 7.4 containing 5 mM CaCl₂ and 0.5 mM NaN₃. The gel was stained with 0.5% Coomassie blue and destained in acetic acid/methanol solution.

Statistical analyses. Student's t-test was used to evaluate differences between samples and the respective controls. P<0.05 was considered significant. Data were analyzed with Statistica for Windows Software, release 6, Statsoft Inc., USA.

Results

Effect of the cyclic peptide (cP) on mitogen-stimulated proliferation of MCF7 cells. It has been suggested that the E₂-dependent growth of cancer cells might involve the participation of the EGF signaling pathway, therefore, we analyzed whether the cyclic peptide modified the EGF-dependent proliferation of MCF7 cells. For comparison, the cells were treated with 2 nM E₂, in the presence or absence of 2 μg/ml cP.

E₂-stimulated MCF7 cell proliferation in a serum containing culture medium, which was significantly inhibited in the presence of 2 μg/ml of the cyclic peptide by 46 and 29% after 48 and 72 h of treatment, respectively (Fig. 1A).

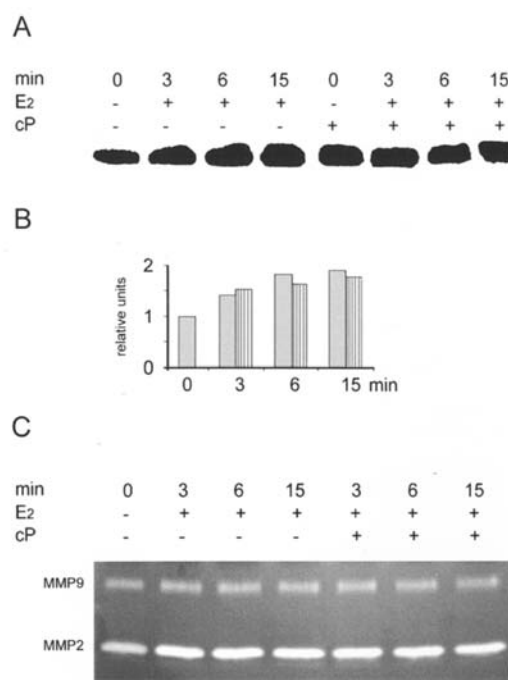


Figure 2. HB-EGF shedding upon E₂ stimulation. (A) Western blot of HB-EGF after short-time stimulation of MCF7 cells with E₂ in the absence or presence of cP. (B) Histogram of HB-EGF shedding after E₂ stimulation in the absence (grey bars) and presence of cP (hatched bars). (C) Analysis of MMP2 and MMP9 secretion into the culture medium. The cells were incubated with 2 nM E₂ in the absence or presence of 2 μg/ml cP. The MMP2 and -9 gelatinase activities in the culture medium were analyzed by zymography.

Studies on MCF7 cell growth in serum-containing medium with different amounts of recombinant human EGF showed that 5 ng/ml EGF was a dose sufficient to obtain maximal proliferation. The cells grew slower under EGF than

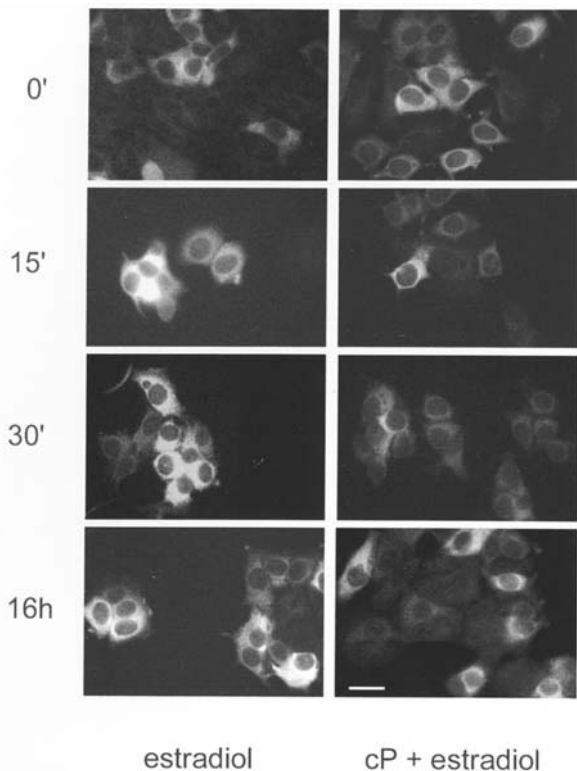


Figure 3. Immunofluorescence for c-erbB2 in MCF7 cells stimulated with E_2 . Representative images of c-erbB2 in MCF7 cells grown for 48 h in a medium containing 5% CDGS and then stimulated for indicated times with 2 nM E_2 alone (left) or E_2 and 2 $\mu\text{g/ml}$ cP (right), or vehicle alone (0 min). The bar represents 50 μm .

under E_2 (Fig. 1B). The cyclic peptide at 2 $\mu\text{g/ml}$ inhibited effectively the EGF-stimulated cell proliferation, allowing a significant inhibition of 18 and 25% after 48 and 72 h, respectively.

The cyclic peptide exhibited significant antiproliferative effects on other mammary tumor cells such as ZR-75 and various ER α -positive primarily-cultured canine mammary tumor cells (unpublished data), however the compound had no effect on the growth of E_2 -independent tumor cells, such as MDA-MB231 (20) nor on the proliferation of normal cells, such as HUVEC (not shown).

Parallel studies were run in a medium without serum. Previously, we tested several serum-free culture formulations that allowed cell proliferation for at least 96 h, selecting as appropriate DMEM F12 containing 3% HAES, 10 $\mu\text{g/ml}$ insulin, 5.5 $\mu\text{g/ml}$ transferrin and 0.5% human serum albumin. Results shown in Fig. 1C allowed discarding serum effects on the cP inhibitory action, since analogous inhibition of 40 and 22% after 48 and 72 h of treatment respectively, were observed in a medium without serum. The inhibitory effect of the cP was structure-dependent as the addition 2 $\mu\text{g/ml}$ of a scrambled peptide did not affect the estrogen-dependent growth of these cells (not shown).

As shown in Fig. 1D, the stimulatory effect of 5 ng/ml rh EGF on cell proliferation was effective in a serum-free medium: 2 $\mu\text{g/ml}$ cyclic peptide significantly inhibited EGF-stimulated cell growth by 36 and 29% after 48 and 72 h, respectively.

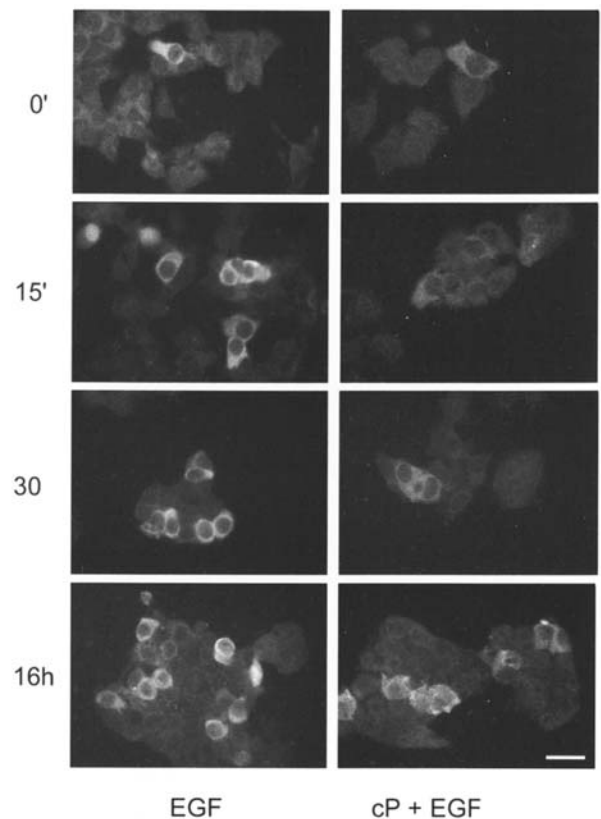


Figure 4. Immunofluorescence for c-erbB2 in MCF7 cells stimulated with EGF. Representative images of c-erbB2 in MCF7 cells grown for 48 h in a medium containing 5% CDGS and then stimulated for indicated times with 5 ng/ml rh EGF alone (left) or rh EGF and 2 $\mu\text{g/ml}$ cP (right), or vehicle alone (0 min). The bar represents 50 μm .

The cP inhibitory action is not related to HB-EGF releasing by cells. E_2 treatment of MCF7 cells activates the rapid shedding of HB-EGF, enhancing the availability of the growth factor (Fig. 2A). We tested whether the presence of the cyclic peptide altered this E_2 effect on cells. Western blots for HB-EGF showed that after E_2 stimulation of cells either in the absence or presence of the cyclic peptide, the level of this growth factor in cells increased rapidly (Fig. 2B). In agreement with this observation, we determined that the cyclic peptide had no effect on the E_2 -stimulated release of MMP2 and MMP9 into the culture medium (Fig. 2C).

The inhibitory effect of cP is related to c-erbB2 reactivity. The expression of the c-erbB2 protein by MCF7 cells after short-term treatment with E_2 , in the absence or presence of the cyclic peptide was analyzed by using indirect immunofluorescence. Fig. 3 shows that 2nM E_2 increased c-erbB2 immunoreactivity in the cells within 15 min of treatment. In contrast, c-erbB2 immunofluorescence was markedly reduced in the presence of the cyclic peptide, remaining low even after 16 h of treatment (Fig. 3). The treatment of cells with the scrambled peptide did not affect c-erbB2 immunoreactivity. Analogous results were observed in cells incubated in a serum-free medium (not shown). Similar results were observed upon 5 ng/ml rh EGF treatment of MCF7 cells; this growth factor induced an early increase in c-erbB2

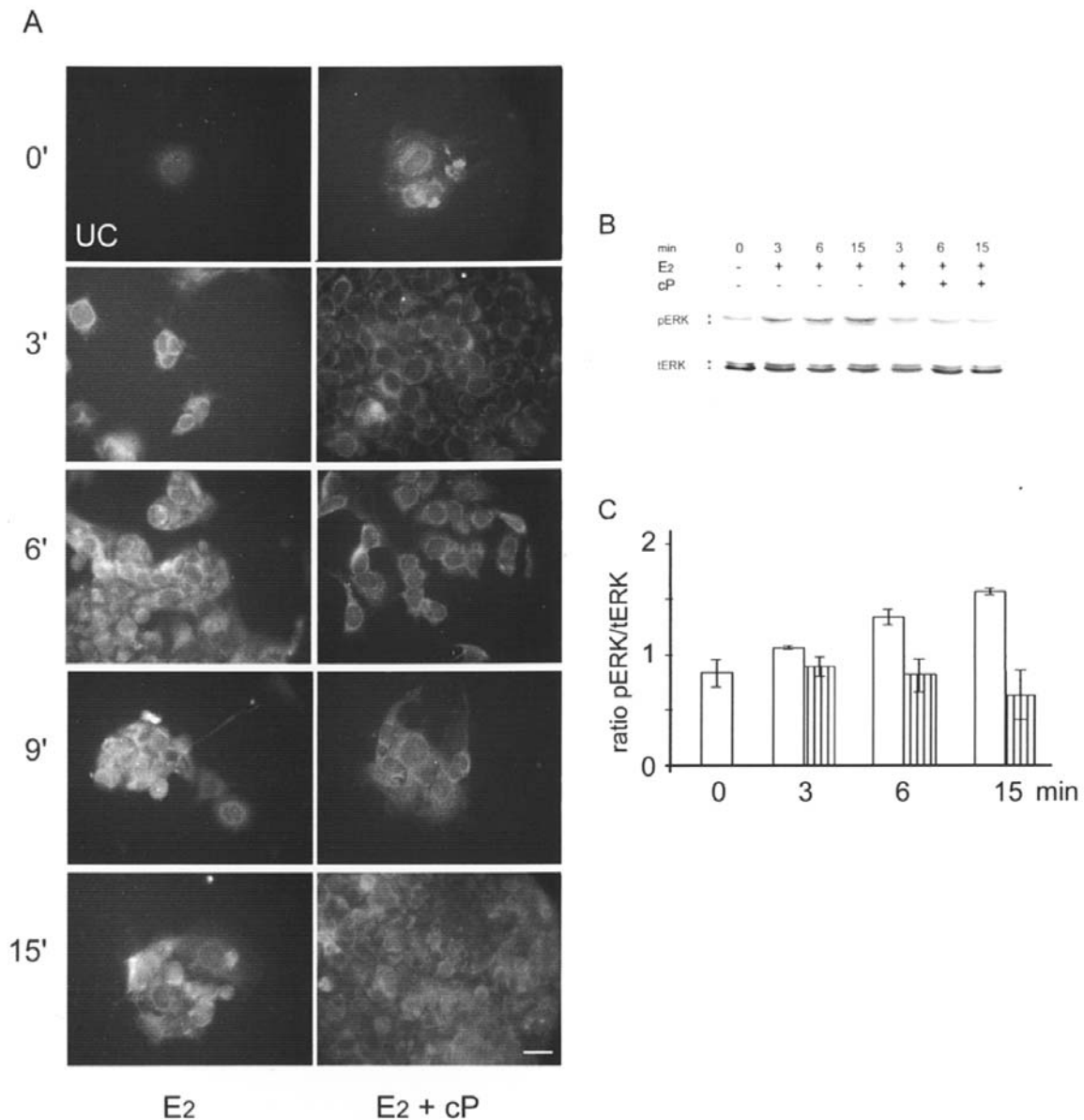


Figure 5. Immunological studies of MAPK in MCF7 cells stimulated with E₂. (A) Immunofluorescence for pErk1/2 in cells grown for 48 h in a medium containing 5% CDSS and stimulated for indicated times with 2 nM E₂ (left) or E₂ plus 2 μg/ml cP (right) or after the addition of vehicle alone (0 min). UC, Unspecific control. The bar represents 50 μm. (B) MCF7 cells were treated with 2 nM E₂ in the absence or presence of 2 μg/ml cP for the indicated periods of time. Cell lysates were collected and ERK1/2 phosphorylation was assessed in 10 μg protein samples by Western blot analysis. The histogram shows the ratios between phosphorylated and total ERK1/2 in cells stimulated for the indicated times with E₂ in the absence (open bars) or presence of cP (hatched bars); mean ± range from 2 independent experiments.

immunoreactivity, which was clearly inhibited by the addition of 2 μg/ml cyclic peptide to the culture medium (Fig. 4).

The activation of mRE₂ and EGFRs triggers intracellular signalling pathways leading to ERK phosphorylation and subsequent enhanced cell proliferation. In agreement with this, a rapid increase in immunoreactivity for phosphorylated ERK1/2 was observed after E₂ or EGF treatment of MCF7 cells (Figs. 5A and 6A). In contrast, in the presence of the cyclic peptide, the immunoreactivity for phosphorylated ERK1/2 remained low (Figs. 5A and 6A). These observations were corroborated by Western blotting, which showed a significant decrease of phosphorylated ERK1/2 in E₂- and EGF-stimulated cells in the presence of the cyclic peptide (Figs. 5B and C and 6B and C).

Discussion

The proliferation of MCF7 cells increased 20-35% over non-stimulated controls in the presence of either 2 nM E₂ or 5 ng/ml rh EGF (Fig. 1). Similar augments of cell growth were obtained using 5 ng/ml TGFα (not shown). A dose of 2 μg/ml cP effectively hindered the effect of mitogens on the proliferation.

The effects of these mitogens and of cP on the proliferation of MCF7 cells were also observed in serum-free medium, indicating that serum factors are not required for the activities of these molecules. The maintenance of cells in serum-free conditions proved that 10 μg/ml insulin present in the medium is sufficient for keeping the cells alive and

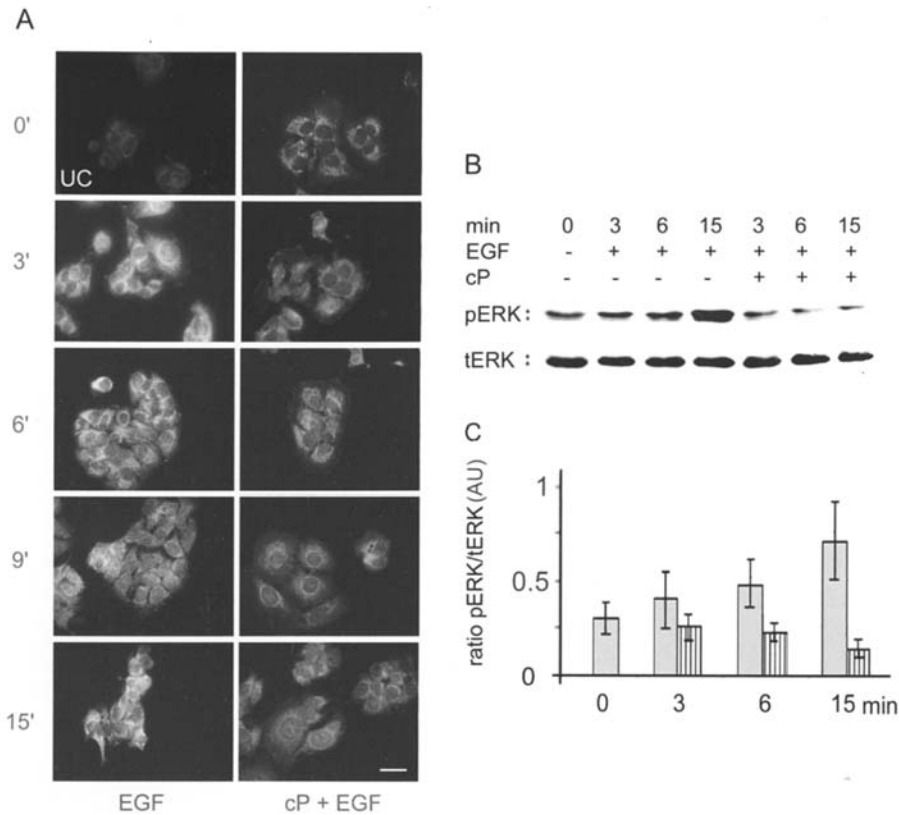


Figure 6. Immunological studies of MAPK in MCF7 cells stimulated with EGF. (A) Immunofluorescence for pERK1/2 in cells grown for 48 h in a medium containing 5% CDSS and stimulated for indicated times with 5 ng/ml EGF (left) or EGF plus 2 μ g/ml cP (right), or after the addition of vehicle alone (0 min). UC, Unspecific control. The bar represents 50 μ m. (B) MCF7 cells were treated with 5 ng/ml rh EGF in the absence or presence of 2 μ g/ml cP for the indicated period of time. Cell lysates were collected and ERK1/2 phosphorylation was assessed in 10 μ g protein samples by Western blot analysis. The histogram shows the ratios between phosphorylated and total ERK1/2 in cells stimulated for the indicated times with rhEGF in the absence (grey bars) or presence of cP (hatched bars); mean \pm range from 2 independent experiments.

proliferating, albeit at a reduced rate, for at least 96 h. The respective effects of E_2 , EGF, TGF α and of the cyclic peptide on the cell proliferation were observed, practically at the same magnitude, in MCF7 cells cultured in the presence or absence of calf serum, indicating that no other serum factors are required for the corresponding activities of these molecules.

The fluorescence studies using the specific monoclonal antibody demonstrated early increases of c-erbB2 immunoreactivity in cells stimulated with E_2 or with rh EGF, again independently of the presence of serum. The stimulatory effects of E_2 and rh EGF (and of TGF α) on c-erbB2 IR were similar, supporting reported data from others and pointing to the sharing of the signal transduction pathway involving members from the EGF receptor family (10-13). The results demonstrated that the activation of EGFR2, highly expressed in MCF7 cells, increases following the treatment of cells with rh EGF or E_2 . The cyclic peptide hindered any increase of c-erbB2 immunoreactivity by the mitogens, suggesting that the compound might interfere with the dimerization of this receptor. It would seem that the ligand-induced increase in EGF-IR will lead to augmented tyrosine kinase-specific activity of c-erbB2.

The treatment of MCF7 cells with either rh EGF or E_2 caused a time-dependent augment of ERK1 phosphorylation and, to a lesser extent, of ERK2 (Figs. 5B and 6B). It has been shown that diverse ligand-activated receptors induce ERKs phosphorylation through members of the EGFR family

(21). The induction of ERK phosphorylation in MCF7 cells confirms the participation of the EGFR tyrosine kinase in E_2 and EGF signaling, as reported by several groups (8,10-15). Our experiments detected a clear reduction of ERK activation in the presence of cP. Therefore, we conclude that the cP prevents c-erbB2 transactivation of the rise in phosphorylated ERK1/2 observed after mitogenic stimuli. As intermediate effectors of the Ras/ERK pathway, phosphorylated ERKs participate in the control of cellular proliferation (12). The Ras/ERK pathway represents a major mitogenic pathway, though at present there is not enough information on the precise mechanisms by which ERKs influence the cell cycle (22). It has been proposed that the interplay between cyclins and p21KipC1 regulated by ERKs is of major significance in the regulation of the cycle (23). In this context, it is appropriate to refer to our former results, demonstrating that MCF7 cells exposed to cP exhibit increased nuclear p21KipC1 expression (20).

Many studies have shown that E_2 transactivates the EGFR via the shedding of the cell surface-bound HB-EGF. In MCF7 and other sensitive cells, the release of HB-EGF requires the active participation of MMP-2 and MMP-9 (11). We detected no effect of the cP on E_2 -stimulated shedding of HB-EGF by MCF7 cells and found no alterations in MMP2 and MMP9 secretion, as compared with cells stimulated with E_2 only (Fig. 2). Therefore, we localize the effect of cP on cell proliferation mainly at the level of c-erbB2 activity.

The precise molecular explanation of our findings remains to be determined, however a functional interplay between members of the EGF receptor family in mammary tumor cells seems to be in compliance with observations performed by other groups. Altogether, the results confirm the concept that the increment in the proliferation of mammary epithelial cells cultured in the presence of insulin depends upon EGF, TGF α or E₂. Many studies have shown that the stimulation of EGFR and IGF-IR is necessary for the sustained proliferation of mammary tumor cells (24) and plausible molecular explanations for these findings have been provided (21). Their main conclusion is that the EGFR pathway is necessary for the proliferation of mammary epithelial cells because of the control of MAPK activity. The involvement of this pathway in cell proliferation has supported the application of EGFR tyrosine kinase inhibitors for the treatment of diverse forms of cancers with the overexpression of EGFRs (25). Whether the cyclic peptide acts in a similar way to that of these inhibitors or not remains to be elucidated.

Breast cancer cells are often intrinsically resistant or become resistant to antiestrogens during treatment, therefore concrete requirement exists for additional effective drugs with minimal or no associated host toxicity in the treatment and prevention of breast neoplasias. The data presented in this study endorses the effectiveness of the AFP-derived cyclic peptide as an antiproliferative agent. This, together with previous data on its distinct mechanism of action, its low toxicity for the host and its effectiveness by oral administration (18-20), allows us to conclude that the cyclic peptide compound represents a valuable novel tool for fighting breast cancer.

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