[CANCER RESEARCH 56, 4516–4521, October 1, 1996]

Estradiol Activation of Human Colon Carcinoma-derived Caco-2 Cell Growth[1]

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ABSTRACT

This is the first report on estrogen-dependent growth of human-derived colon carcinoma cells. Under selected conditions, growth of subconfluent Caco-2 cells is triggered by estradiol. Cell growth is estradiol concentration dependent, with maximal effect occurring at about 0.4 nM. Growth is prevented by two different antiestrogens: the partial agonist, OH-Tamoxifen, and the pure antagonist, ICI 182,780. The growth effect is specific for estradiol since other hormonal steroids tested do not affect cell growth. The amount of estradiol receptor in subconfluent Caco-2 cells, detected by blot with monoclonal antibodies directed against the receptor as well as estradiol binding assays, is similar to that of the classical estradiol-responsive, human mammary cancer-derived MCF-7 cells.

Estradiol treatment of subconfluent Caco-2 cells rapidly and reversibly stimulates four important intermediates in a signal transduction pathway that is known to trigger cell proliferation: two members of the large family of c-src-related tyrosine kinases, c-src and c-yes; and two serine/threonine kinases, the mitogen-activated protein (MAP) kinases, erk-1 and erk-2. Tyrosine kinases activated by estradiol are up-stream MAP kinases and Caco-2 cell proliferation. In fact, genistein, a specific tyrosine kinase inhibitor, abolishes the estradiol stimulatory effect on both erk-2 activity and cell proliferation. Our findings show that in subconfluent Caco-2 cells, the estradiol-receptor complex activates the c-src, c-yes/MAP kinase pathway and activates growth. This could have important implications for the understanding of human intestinal carcinogenesis.

INTRODUCTION

Previous findings suggest that steroids and their receptors might have a role in proliferation of intestinal tumor cells (1–3). In the present study, we have further investigated this possibility.

Caco-2 is a human colon cancer line unique in its property to spontaneously differentiate when cells reach confluency and their growth stops. In contrast, at subconfluence they are undifferentiated and actively grow (4). We have initially investigated whether growth of subconfluent Caco-2 cells is estradiol responsive; thereafter, we have analyzed the mechanism for this steroid. Our findings show a receptor-mediated proliferative activity of estradiol. In these cells, the estradiol-receptor complex activates a classical signal transduction pathway that is known to be a target of different peptide growth factors. In fact, we observed rapid and reversible stimulation of two members of the large c-src-related kinase family (5), c-src and c-yes. Much evidence suggests a central role for the activation of these two tyrosine kinases in intestinal cell growth and carcinogenesis; the src activity is elevated in malignant and premalignant colonic epithelia (6, 7) as well as in human colon carcinoma HT-29 cells (6), a cell line similar to Caco-2 cells (4). The tyrosine kinase activity and protein levels of c-yes are elevated in colonic cell lines and human primary colonic tumors (8) and in premalignant colonic lesions (9). We observed that estradiol stimulates MAP[3] kinase activities in Caco-2 cells with kinetics similar to those of c-src and c-yes activation. MAP kinases are serine/threonine kinases and are intermediates of a phosphorylation cascade activated by receptor- and nonreceptor-tyrosine kinases like c-src. Once activated, MAP kinases trigger cell proliferation through phosphorylation of transcriptional factors and induction of proto-oncogenes (10–12). The relevance of the observed stimulation of c-yes and c-src to the activation by estradiol of MAP kinases and cell proliferation is indicated by the inhibition of hormonal effects on MAP kinases and growth by genistein, a specific inhibitor of protein tyrosine kinases including c-src (13). Interestingly, the effects of estradiol on the proliferative signaling pathway of Caco-2 cells are similar to those recently observed in estradiol-responsive human mammary cancer-derived MCF-7 cells when treated with the same steroid (14). Therefore, these effects could have an important role in human mammary, as well as intestinal, cell growth and carcinogenesis.

MATERIALS AND METHODS

Genistein was from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal 327 mouse anti-src monoclonal antibodies, antimouse IgG, and antirat IgG goat antibodies were from Oncogene Science, Inc. (Manahasset, NY). Anti-erk-1 and anti-erk-2 rabbit polyclonal antibodies as well as the control peptide (SC-154P) were purchased from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA). Acrylamide, BIS, *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulfate, SDS, Tween 20, and protein assay kit were from Bio-Rad (Richmond, CA). Prestained molecular weight markers for protein electrophoresis and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham Corp. (Buckinghamshire, England). BA-85 nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Gelatin and Norit A were from Serva (Heidelberg, Germany). Antimouse and antirat IgG AP conjugate antibodies were from Promega Corp. (Madison, WI). Protein G-Sepharose, estradiol, BSA (fraction V), sodium orthovanadate, enolase, MBP, HEPES, PIPES, Tris, glycine, EDTA, Triton X-100, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, antipain, transferrin, sodium selenite, 17β-estradiol, 17α-estradiol, estrone (Δ1,3,5(10)-estratrien-3-ol-17-one), triamcinolone acetonide, and dihydrotestosterone (5α-androstan-17β-ol-3-one) were from Sigma Chemical Co. (St. Louis, MO). R5020 (17α-21-dimethyl-19-norpregn-4,9-diene-3,20-dione) was produced by Roussel-UCLAF (Romainville, France). OH-Tamoxifen was from ICI (Macclesfield, United Kingdom. Reagents from cell culture media including FCS were from Life Technologies, Inc. (Gaithersburg, MD). All of the other reagents were of analytical grade.

Cell Culture. Caco-2 cells were routinely grown in 10% CO_2 in an air atmosphere in DMEM supplemented with phenol red, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 10% FCS. Before beginning the experiments, subconfluent cells were maintained for 5 days in phenol red-free DMEM with 10% FCS. Serum was pretreated twice with dextran-coated charcoal using a method described previously (15). The cells were then kept for 18 h in the same medium lacking FCS, to which was added 25 μg/ml transferrin, 25 ng/ml insulin, and 20 nM sodium selenite; then the cells were treated with estradiol. Cells were passaged every 7 days with trypsin and EDTA, and the medium was changed every 2–3 days. Steroids used in this study were dissolved in 100% ethanol and added at 1:1000 dilution to the medium, except for estrone, which was solubilized in 50% dioxan. Cells were counted in a hemocytometer in quadruplicate.

Received 4/11/96; accepted 7/30/96.

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[1] This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro, from Consiglio Nazionale delle Ricerche, Progetti Speciali Ingegneria Genetica (950062599) and Applicazioni Cliniche della Ricerca Oncologica (9401089), and from Ministero dell'Università e della Ricerca Scientifica (Fondi 40 e 60%).

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[3] The abbreviations used are: MAP, mitogen-activated protein; BIS, *N,N′*-methylene-bis-acrylamide; AP, alkaline phosphatase; MBP, myelin basic protein.

Scatchard Plot Analysis of Hormone Binding to Estradiol Receptor. Cytosols of Caco-2 cells were incubated with different concentrations of [^{3}H]estradiol (from 0.01 to 6 nM) in the absence and in presence of an excess of radioinert hormone, and the estradiol bound to the receptor was assessed by the dextran-coated charcoal method (15).

Immunoprecipitation of erk-1 and erk-2 Kinases. Caco-2 cells treated or not with 10 nM 17β-estradiol in the absence or in presence of 1 μM ICI 182,780 were washed three times with ice-cold PBS (pH 7.4) and scraped. Cells were then added to 2 ml of ice-cold lysis buffer (50 mM Tris-HCl, 4 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of antipain, leupeptin, and pepstatin, 1% aprotinin, 1 mM sodium orthovanadate, and 1% Triton X-100, pH 7.4). Lysate was left at 4°C under shaking and centrifuged at 15,000 × g for 30 min; protein concentration of the clear supernatant was assayed. Cell lysates were diluted to a protein concentration of 1 mg/ml, incubated with 1 μg/ml of rabbit polyclonal anti-erk-1 or anti-erk-2 antibodies for 90 min at 4°C, and then added to 40 μl of a 50% suspension of protein G-Sepharose and incubated for an additional 30 min. Control parallel samples were incubated with either rabbit immunoglobulins or anti-erk-2 antibodies in the presence of the control peptide (SC-154 P) to verify the specificity of erk-1 or erk-2 immunoprecipitation, respectively. The immunoprecipitates were washed with 1 ml of lysis buffer four times and used for the MAP kinase assay.

MAP Kinase Assay. Cell lysates (2 ml) were immunoprecipitated with anti-erk antibodies as described above, and immunoprecipitates were assayed at 30°C for 20 min for MBP phosphorylation in a final volume of 50 μl of a mixture containing 50 mM HEPES (pH 8.0), 10 mM $MnCl_2$, 1 mM DTT, 1 mM benzamidine, 0.3 mg/ml MBP, and 50 μM [γ-^{32}P]ATP (10 μCi). Reactions were stopped with 2× SDS sample buffer, and samples were analyzed by SDS-polyacrylamide gel electrophoresis (13.5% acrylamide) and gel autoradiography.

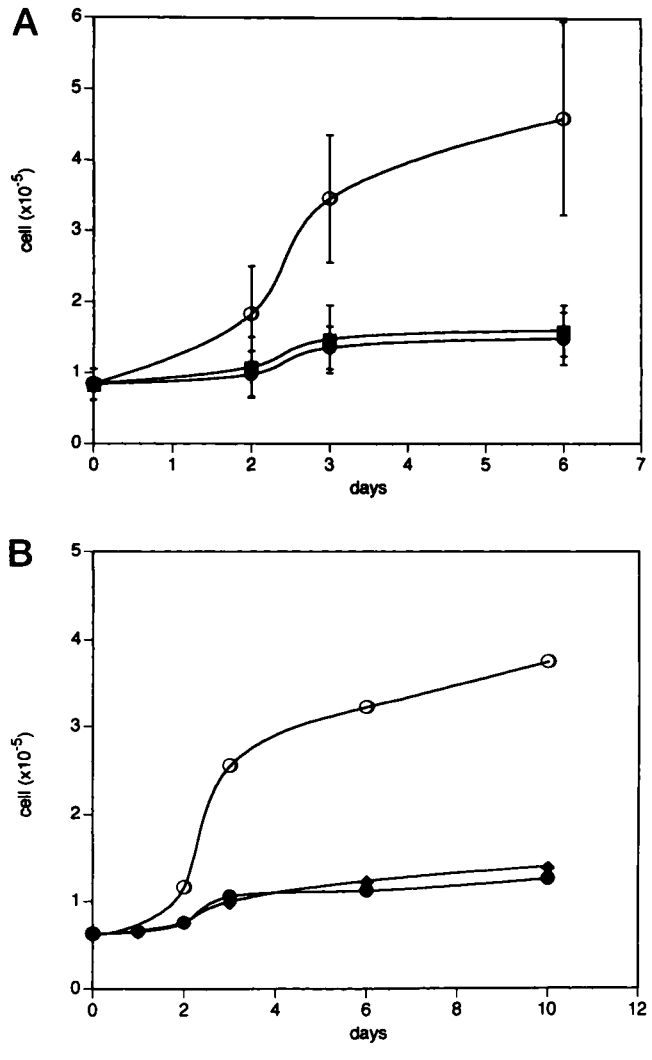

Fig. 1. Caco-2 cell growth rate dependence on 17β-estradiol. *A*, Caco-2 cells were grown in the absence (●) and in presence of either 10 nM 17β-estradiol (○) or 10 nM 17β-estradiol and 1 μM OH-Tamoxifen (■). The values represent mean values of cell number of each well averaged from three different experiments; *bars*, SD. *B*, Caco-2 cells were grown in the absence (●) and in presence of either 10 nM 17β-estradiol (○) or 10 nM 17β-estradiol and 1 μM ICI 182,780 (▲).

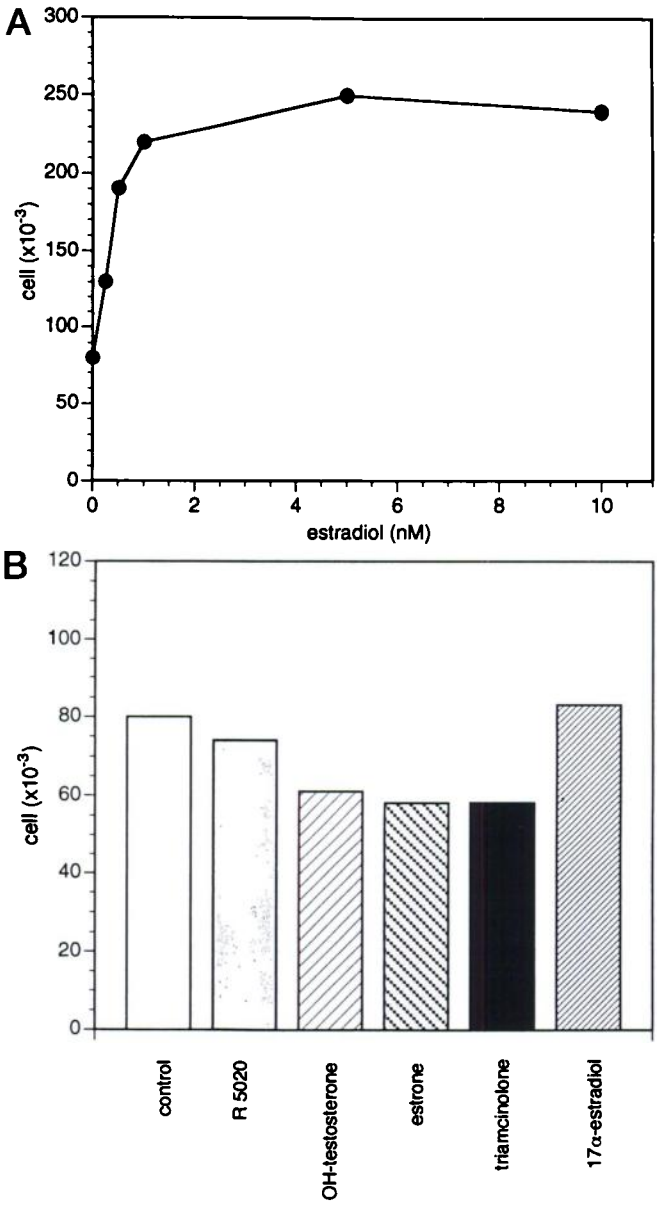

Fig. 2. Dose-dependent effect of estradiol on Caco-2 cell growth and specificity of this effect. Cells were maintained for 4 days in the absence (control) or in presence of either different concentrations of 17β-estradiol (*A*) or one of the following steroids (*B*): 10 nM R 5020, 50 nM OH-testosterone, 50 nM estrone, 50 nM triamcinolone, or 10 nM 17α-estradiol. Thereafter, cells of each well were counted.

Immunoprecipitation and Purification of c-src and c-yes. Cell lysates were prepared as described above. Two-ml lysate (~6 mg of protein) aliquots were incubated with 2 μg of mouse IgG for 1 h at 4°C under shaking. After incubation, 30 μl of a 50% suspension of protein G-Sepharose were added and incubated for an additional 45 min. Samples containing 3 mg of proteins were centrifuged, and supernatants were immunoprecipitated overnight at 4°C with about 1 μg/ml of either mouse monoclonal anti-c-src antibodies (clone 327) or rabbit polyclonal anti-c-yes antibodies. Parallel samples were incubated with control mouse or rabbit antibodies. At the end of the incubation, 40 μl of a 50% suspension of protein G-Sepharose were added to each sample, and incubation continued for an additional 30 min. The samples were divided into

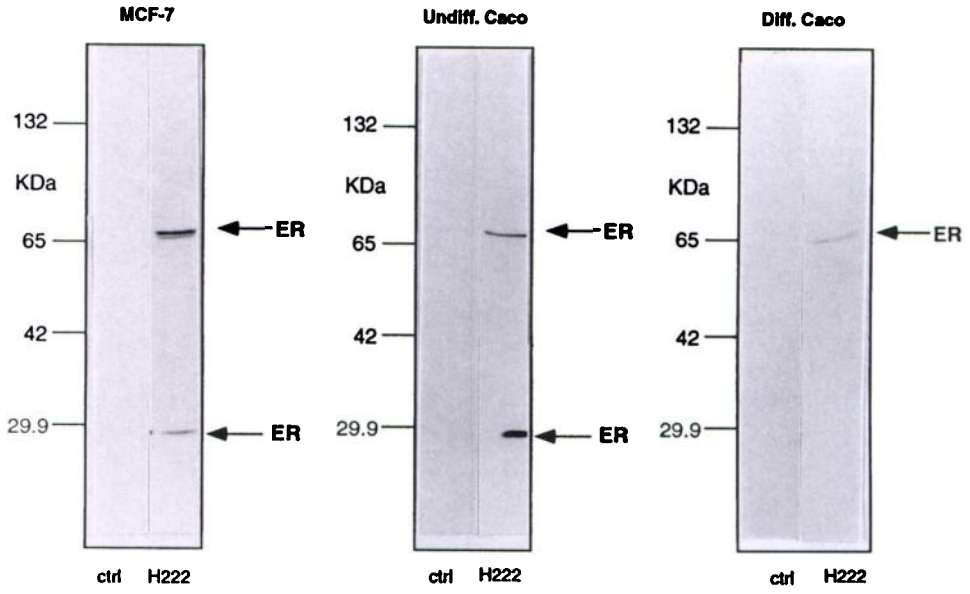

Fig. 3. Estrogen receptor in undifferentiated (subconfluent) and differentiated (confluent) Caco-2 cells. Cells were lysed, and lysate aliquots containing about 100 μg of proteins from undifferentiated Caco-2 cells (*Undiff. Caco*), MCF-7 cells (*MCF-7*), and differentiated Caco-2 cells (*Diff. Caco*) were run in duplicate on SDS-PAGE, transferred on nitrocellulose filters, and probed with nonimmune (*ctrl*) or H222 antibodies. *Arrows* show the M_r 67,000 receptor as well as a M_r 29,000 proteolytic fragment. *Left*, positions of molecular weight markers.

two portions of 300 and 700 μl, respectively, and centrifuged; then pellets were washed with 1 ml of lysis buffer four times. The pellets from the 300-μl aliquots were used for the kinase assay, and the 700-μl aliquot pellets were submitted to SDS-PAGE; proteins were transferred on filters and blotted with either anti-c-src or anti-c-yes antibodies.

c-src and c-yes Kinase Assay. The assay was performed in 100 mM PIPES-NaOH (pH 6.8) containing 20 mM $MnCl_2$ by mixing 20 μl of either anti-c-src or anti-c-yes immunoprecipitates and 2.5 μl of substrate, the acid-treated enolase. The reaction was started by adding 10 μM [γ-^{32}P]ATP, held at 30°C for 10 min, and stopped when 2 mM ATP were added. The samples were reduced with an equal volume of 2× SDS Laemmli sample buffer, and aliquots (25 μl) were submitted to SDS-PAGE (10% acrylamide). The dried gel was exposed to hyperfilm (Amersham) for 12 h.

Electrophoresis and Immunoblotting. Samples were submitted to SDS-PAGE (7.5–13.5%, acrylamide:BIS ratio 37.5:1). Electrophoretically separated proteins were transferred overnight at room temperature on nitrocellulose filters at 25 V using a transfer buffer containing 50 mM Tris, 380 mM glycine, 0.1% SDS, and 20% methanol. The filters were soaked for 2 h in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 8.0) containing 3% BSA to block nonspecific binding sites (blocking solution). They were then hybridized for 2–3 h with anti-c-yes antibodies or anti-c-src antibodies or anti-receptor antibodies (1 μg of each antibody/ml in blocking solution). Thereafter, filters were washed with TBST buffer at least three times for 10 min. After washing, the filters probed with anti-c-src antibodies were incubated with AP-linked antimouse IgG antibodies (1:4000 dilution in TBST), and the filters treated with anti-c-yes antibodies were incubated with AP-linked antirabbit IgG antibodies (1:7500 dilution in TBST). The filters trated with anti estrogen receptor (H222) antibodies were incubated with AP-liked antirat IgG antibodies (1:4000 dilution in TBST) for 45 min at room temperature. Finally, filters were washed again after the procedure described above, and protein-antibody complexes were revealed according to the manufacturer's instructions.

RESULTS

Estradiol Stimulation of Caco-2 Cell Growth. Caco-2 cells maintained in a medium lacking both serum and phenol red, a weak estrogenic compound, grew very slowly. The addition of 10 nM estradiol induced a large increase of cell number. OH-Tamoxifen and ICI 182,780, two well-known antiestrogens, prevented the estradiol proliferative effect (Fig. 1). Estradiol stimulates cell growth in a concentration-related manner with half-maximal stimulation occurring at about 0.4 nM (Fig. 2*A*). The progestin R 5020, OH-testosterone, and the glucocorticoid triamcinolone, as well as estrone and 17α-estradiol, two estrogens biologically less active than estradiol, do not stimulate cell growth (Fig. 2*B*).

Estradiol Receptor Levels in Caco-2 Cells. The presence of estradiol receptor in subconfluent, proliferating Caco-2 cells was verified by different methods. H222 antibodies directed against the carboxyl-terminal portion of the receptor, the rat H222, were used. Lysates were prepared from Caco-2 cells and, for comparison, from MCF-7 cells, which are classic estradiol-responsive cells and are derived from human mammary cancers. Electrophoretically separated proteins were blotted with anti-receptor antibodies as well as control antibodies (rat IgG). The anti-receptor antibodies detected the M_r 67,000 receptor and its proteolytic fragments (Fig. 3). Although the ratio of M_r 67,000 receptor to proteolytic fragments is different in the two cell lines, the total amount of the receptor in subconfluent Caco-2 cells and MCF-7 is similar. In contrast, in confluent, nonproliferating Caco-2 cells, the amount of the estradiol receptor detectable by antibodies is much lower (Fig. 3).

Estradiol receptor was also assayed by hormone binding to whole subconfluent Caco-2 cells; 1.5 fmol/μg DNA was the value found using a single-point saturation assay. When the estradiol binding of cytosol protein was analyzed by Scatchard plot using the dextran-coated charcoal assay, 250 fmol receptor/mg protein were detected in subconfluent cells. The K_d values of the estradiol binding was ~1 nM (data not shown). About 24 fmol receptor/mg of protein were found in cytosol of confluent Caco-2 cells.

Estradiol Stimulation of c-src and c-yes Kinase Activity in Subconfluent Caco-2 Cells. Subconfluent Caco-2 cells were treated with estradiol for different lengths of time and then lysed. Cell lysates were incubated with monoclonal 327 anti-c-src antibodies or polyclonal anti-c-yes antibodies. A part of the c-yes immunoprecipitates was used to assay the enolase phosphorylating activity (Fig. 4*A*, *lower panel*). The enolase phosphorylation was quantified by laser scanning of autoradiographic films. After 2 and 5 min of estradiol treatment, the activity of c-yes is stimulated over the basal levels by 40 and 90%, respectively. The activity decreases to the basal levels after 10 min. Immunoprecipitates were also analyzed for the c-yes content by blot with anti-c-yes antibody. Similar amounts of this kinase are found in lysates from cells treated with estradiol for different lengths of time (Fig. 4*A*, *upper panel*).

The behavior of c-src in Caco-2 cells in response to estradiol mirrors that of c-yes. There was a 30% increase in activity after 2 min

and, in a different experiment, a 20% increase (data not shown). By 5 min, there was a 90% increase. In a different experiment, there was a 120% increase (data not shown). Conversely, after 10 min, the activity was lower than that seen under basal conditions (Fig. 4*B, lower panel*). Also in this experiment, similar amounts of c-src were detected by blot with anti-c-src antibody of the immunoprecipitated proteins (Fig. 4*B, upper panel*). Furthermore, estradiol activation of c-src and c-yes is prevented by the pure antiestrogen ICI 182,780 (Fig. 4*C*). Identification of the kinase stimulated by estradiol with c-yes and c-src is confirmed by the absence of both enzymic activity and c-yes and c-src in proteins immunoprecipitated by control antibodies (Fig. 4, *A, B, lower* and *upper panels*, respectively).

Estradiol Activation of MAP Kinases in Subconfluent Caco-2 Cells and Inhibitory Effects of Genistein. Lysates from cells treated or not with estradiol for different lengths of time were immunoprecipitated using two different antibodies specifically raised against either erk-1 or erk-2. Fig. 5 shows that estradiol rapidly and reversibly stimulates the MBP phosphorylating activity of both immunoprecipitates. erk-1 is stimulated by 5 min treatment (Fig. 5*A*) and erk-2 by 2 min treatment (Fig. 5*B*). Identification of erk-1 and erk-2 with the estradiol-activated kinases is corroborated by the finding that no stimulation is observed when immunoprecipitation is performed by control antibodies (Fig. 5*A*) or by the anti-erk-2 antibodies in the presence of an excess of the erk-2 peptide (SC-154P), against which these antibodies have been raised (Fig. 5*B*). The pure antiestrogen, ICI 182,780, prevents estradiol activation of both erk-1 and erk-2 (Fig. 5*C*), indicating that occupancy of the receptor by estradiol is required for MAP kinase activation. The dependence of erk-2 and cell proliferation activation on c-src kinase family member stimulation by estradiol is corroborated by the inhibitory effect of the specific tyrosine kinase inhibitor, genistein, on erk-2 activity and growth of Caco-2 cells treated with estradiol (Fig. 6).

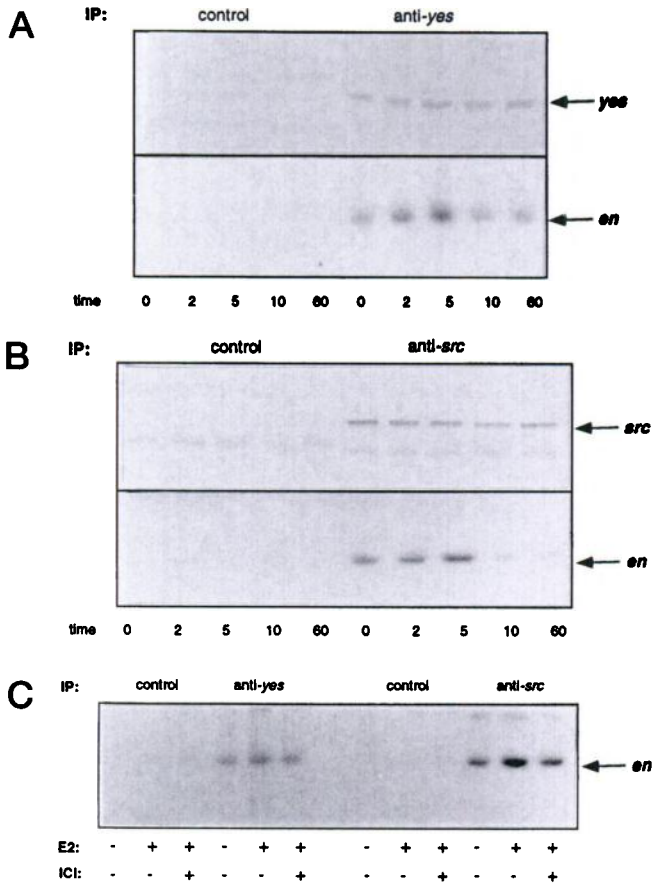

Fig. 4. Effect of 17β-estradiol on c-yes and c-src kinase activity in subconfluent Caco-2 cells. In *A*, cells were treated with 10 nM 17β-estradiol for the indicated times (in min) and lysed, and lysates were incubated with either control or rabbit anti c-yes antibodies. *Upper panel*, aliquots from each immunoprecipitate were run on SDS-PAGE, transferred on nitrocellulose filters, and probed with anti-c-yes antibodies. *Arrow*, the c-yes (*yes*) position. *Lower panel:* parallel aliquots from immunoprecipitates were incubated with acid-treated enolase and [γ-^{32}P]ATP. The reaction wa stopped by adding Laemmli SDS-sample buffer, and samples were submitted to SDS-PAGE followed by autoradiography. *Arrow*, the enolase (*en*) position. In *B*, cells were treated with 17β-estradiol for the indicated times (in min) and lysed, and lysates were incubated with control or 327 anti-src antibodies. *Upper panel:* aliquots from each immunoprecipitate were run on SDS-PAGE, transferred on nitrocellulose filters, and probed with anti c-src antibodies. *Arrow*, the c-src (*src*) position. *Lower panel:* parallel aliquots from each immunoprecipitate were incubated with acid-treated enolase and [γ-^{32}P]ATP. The reaction was stopped by adding Laemmli SDS-sample buffer, and samples were submitted to SDS-PAGE, followed by autoradiography. *Arrow*, the enolase (*en*) position. In *C*, cells were treated with 17β-estradiol (*E2*) for 5 min either in the absence or in presence of 1 μM ICI 182,780 (*ICI*) and lysed, and lysates were incubated with either control, anti-c-src, or anti-c-yes antibodies. Immunoprecipitates were incubated with acid-treated enolase and [γ-^{32}P]ATP. The reaction was stopped by adding Laemmli SDS-sample buffer, and samples were submitted to SDS-PAGE, followed by autoradiography. *Arrow*, the enolase (*en*) position.

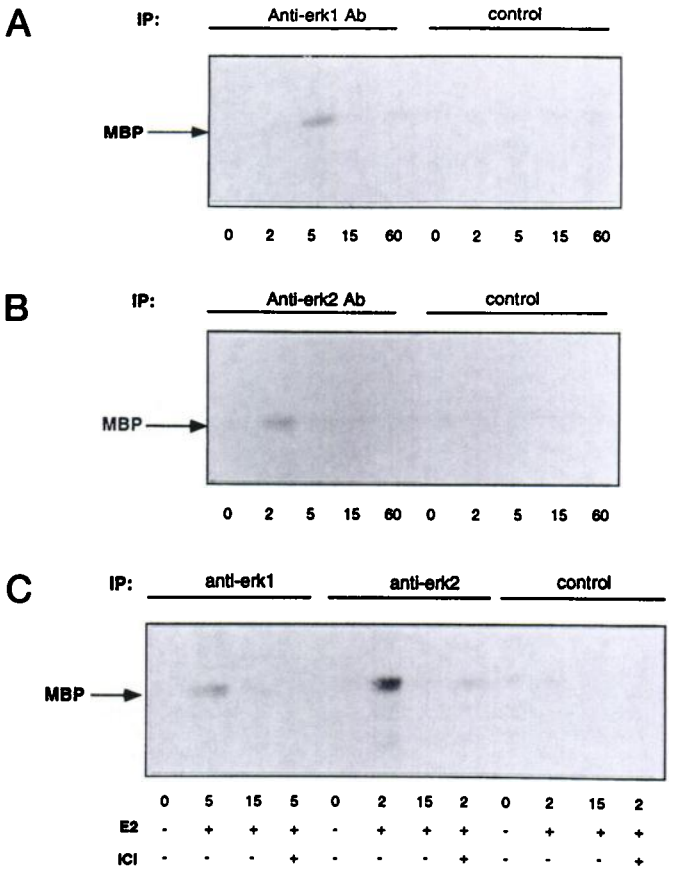

Fig. 5. Activation of erk-1 and erk-2 in estrogen-treated subconfluent Caco-2 cells. In *A*, cells were not treated or were treated for the indicated times (in min) with 10 nM 17β-estradiol. Cells were lysed, and lysates were immunoprecipitated using either anti erk-1 or control rabbit antibodies. In *B*, Caco-2 cells were not treated or were treated for the indicated times (in min) with 10 nM 17β-estradiol. Cells were lysed, and lysates were immunoprecipitated using anti erk-2 antibodies in the absence or in presence of the SC-154P control peptide (*control*). In *C*, Caco-2 cells were not treated (0) or were treated for the indicated times with 10 nM 17β-estradiol (*E2*) either in the absence or in the presence of 1 μM ICI 182,780 (*ICI*). Lysates in *A*, *B*, and *C* were incubated with either anti-erk-1 or anti-erk-2 in the absence or in the presence of the SC-154P control peptide (*control*). The immunoprecipitates were assayed for MAP kinase activity using MBP as a substrate. Phosphorylated proteins were resolved on 15% SDS-PAGE and revealed by autoradiography. *Arrows*, the MBP position.

DISCUSSION

The presence as well as the role of estradiol receptor in normal and neoplastic intestinal cells has been analyzed by different groups to

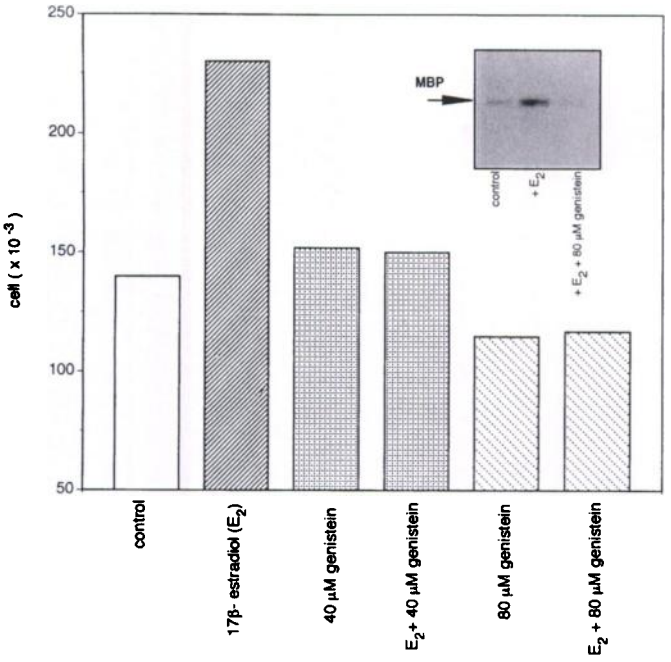

Fig. 6. Effect of the specific tyrosine kinase inhibitor, genistein, on estradiol stimulation of both growth and erk-2 activity of Caco-2 cells. Cells were maintained for 2.5 days in the absence (*control*) and in the presence of 10 nM 17β-estradiol. Parallel samples of cells were maintained in the presence of 40 or 80 μM genistein either alone or with 10 nM 17β-estradiol. Thereafter, cells of each well were counted. *Inset*, cells not treated or treated for 2 min with 10 nM 17β-estradiol were lysed, and lysates were immunoprecipitated with anti-erk-2 antibodies. Cells preincubated with 80 μM genistein for 18 h were also stimulated with 17β-estradiol for 2 min. The immunoprecipitates were assayed for MAP kinase activity.

determine whether these cells are estradiol responsive (1, 2, 16). In particular, it has been suggested that estradiol can stimulate intestinal epithelial cell proliferation during the estrous cycle (17). Furthermore, it has been reported that reduction of estradiol receptor expression abolishes the growth-stimulatory effect of estradiol on a mouse colon cancer cell line (18). However, under the conditions described, the hormone stimulatory effect is very small, whereas the growth rate in the absence of estradiol is very fast.

We now observe that, after exposure to a medium lacking serum and phenol red, subconfluent, undifferentiated Caco-2 cells grow at a very slow rate in the absence of estradiol and respond to physiological concentrations of estradiol with active proliferation. The intensity of the stimulatory effect of estradiol and its complete inhibition by different antiestrogens is reminiscent of the estradiol responsiveness of MCF-7 cells, a classic target of this hormone. Therefore, it is not surprising that proliferating, subconfluent Caco-2 cells and MCF-7 cells have a similar receptor content, whereas nonproliferating, confluent Caco-2 cells have much less receptor. This difference in estradiol receptor levels suggests a role for this receptor in cell growth. There is increased importance for this role, even in the absence of estradiol, because tyrosine phosphorylation of the estradiol receptor (19) induced by a tyrosine phosphatase inhibitor was not only seen to activate receptor hormone binding but also to trigger MCF-7 cell growth proliferation (15).

The mechanism by which estradiol activates cell multiplication is debatable. It has been observed that estradiol stimulates secretion of specific growth factors from hormone-dependent breast cancer (20). It has also been proposed that estrogens activate proliferation through interaction of its own receptor with specific DNA sequences regulating the expression of genes required for cell multiplication (20, 21). We now observe that estradiol stimulates c-src and c-yes of Caco-2 cells. The src family of genes consists of several members that encode highly conserved nonreceptor-tyrosine kinases (22). These enzymes are involved in signal transduction processes initiated by growth factors (23). *src* and *yes*, two members of this family, have also been identified as transforming genes (24). Activation of human c-src has been observed in a large proportion of human breast and colon carcinomas (25, 26), and the importance of c-src in tumorigenesis has been directly tested in the mammary epithelium (26). Different findings also indicate that activation of c-yes has a role in mammary tumorigenesis. Mammary epithelial expression of polyomavirus middle T antigen results in increased activity of both c-src and c-yes in mammary tumors (27), and c-yes activity is elevated in Neu-induced mammary tumors (28). Therefore, it is likely that estradiol activation of c-src and c-yes kinases is crucial in triggering cell proliferation and in carcinogenesis. The inhibition of the estradiol stimulation of Caco-2 cell growth by a specific tyrosine kinase inhibitor, genistein, indicates that hormonal stimulation of these enzymes is relevant to the proliferative activity of estradiol.

In Caco-2 cells, estradiol triggers a rapid and transient activation of the MAP kinases, erk-1 and erk-2. This activation, like that of c-src and c-yes, is mediated by the estradiol receptor since it is inhibited by the pure antiestrogen, ICI 182,780. Activated erk-1 and erk-2 phosphorylate nuclear proteins and induce c-fos expression, thereby contributing to the stimulation of AP-1 (29). The observation of the rapid and reversible activation of MAP kinases by estradiol in Caco-2 cells is in agreement with the recent finding that estradiol rapidly and reversibly induces c-fos mRNA in intestinal epithelial cells (30). erk-1 and erk-2 are activated by receptor- and nonreceptor-tyrosine kinases. Therefore, like peptide growth factors, it appears that estradiol activates MAP kinases in Caco-2 cells through stimulation of c-src kinase family members. This possibility is strongly corroborated by the finding that genistein, a src family kinase inhibitor (13), abolishes the erk-2 stimulation induced by estradiol in Caco-2 cells. The possibility that estradiol acts on Caco-2 cells through binding to erb-B2, as reported in a different system (31), has been investigated and excluded. In fact, we did not observe tyrosine phosphorylation of erb-B2 immunoprecipitated from 5-min estradiol-treated Caco-2 cell lysates (data not shown). We have observed recently that estradiol activates tyrosine kinases and MAP kinases in MCF-7 cells (14, 32). The fact that two cell lines, one of which originates from human colon cancer, the other derived from human mammary cancer, both respond to estradiol by activating the same proliferative pathway (although with slightly different kinetics) is additional evidence of the relationship between these two tumors and strengthens the role of the activation of this pathway in the proliferation of estradiol-responsive cells. Our observation that estradiol stimulates MAP kinase activity in target cells is made even more interesting by the recent report on stimulation of the transcriptional activity of the estrogen receptor by MAP kinase-induced phosphorylation of this receptor at serine 118 (33). Since estradiol causes phosphorylation of this serine (34), our findings suggest that this hormone confers the transcriptional function located in the NH_2-terminal A/B region (AF-1) of the estradiol receptor through MAP kinase. The present report raises many important questions: is the estradiol receptor directly responsible for activation of the membrane-associated c-src and c-yes? Is the estradiol receptor associated with the membrane stimulating these tyrosine kinases? What is the role in this process, if any, of other proteins that, like the M_r 90,000 heat shock protein, are targets of both estradiol receptor and c-src?

ACKNOWLEDGMENTS

The generous gift of Caco-2 cells from Dr. G. Tritto (Cattedra di Gastroenterologia, Università di Napoli Federico II, Napoli, Italy) is gratefully acknowledged. We thank Dr. S. A. Courtneidge (Sugen, Inc., Redwood City, CA) for rabbit polyclonal anti-c-yes antibodies, Dr. Darlene Cotter (Abbott Labs, Abbott Park, IL) for the H222 antiestrogen receptor monoclonal antibodies, and Dr. Giulio Isola (Zeneca, Basiglio, Milano, Italy) for ICI 182,780. We are indebted to Domenico Piccolo for technical assistance and Gian Michele La Placa for editorial work.

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