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

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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 upstream 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 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. (Manhasset, NY). Anti-er-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'-tetramethylethylene diamine, ammonium persulfate, SDS, Tween 20, and protein assay kit were from Bio-Rad (Richmond, CA). Prestained molecular weight markers for protein electrophoresis and [γ-32P]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 (1,3,5(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% CO2 in an 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-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 triplicate.

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1 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 [³H]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 MgCl₂, 1 mM DTT, 1 mM benzamidine, 0.3 mg/ml MBP, and 50 μM [γ-³²P]ATP (10 μCi). Reactions were stopped with 2X SDS sample buffer, and samples were analyzed by SDS-polyacrylamide gel electrophoresis (13.5% acrylamide) and gel autoradiography.

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
Thereafter, filters were washed with TBST buffer at least three times for 10 min. After washing, the filters treated with anti-c-src antibodies were incubated with AP-linked antirabbit IgG antibodies (1:7500 dilution in TBST). The filters (rated with anti-receptor antibodies (1:4000 dilution in blocking solution). They were then hybridized for 2 to 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 were probed with anti-c-src antibodies. Similar amounts of this kinase are found in subconfluent Caco-2 cells and MCF-7 in confluent, nonproliferating cells. The amount of the estradiol receptor detectable by antibodies is much lower (Fig. 3).

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, 67,000 receptor and its proteolytic fragments (Fig. 3). Although the ratio of M, 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 in subconfluent (undifferentiated) 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, 67,000 receptor as well as a M, 29,000 proteolytic fragment. Left, positions of molecular weight markers.

### RESULTS

**Estradiol Stimulation of Caco-2 Cell Growth.** Caco-2 cells maintained in a medium lacking both serum and phenol red, a weak estrogem 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. 2A). The progestin R 5020, OH-testosterone, and the glucocorticoid triamcinolone, as well as estrone and 17α-

### Estradiol Receptor in subconfluent (undifferentiated) 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, 67,000 receptor as well as a M, 29,000 proteolytic fragment. Left, positions of molecular weight markers.

**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. 4A, 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. 4A, 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 of estradiol, two estrogens biologically less active than estradiol, do not stimulate cell growth (Fig. 2B).
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. 4B, 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. 4B, upper panel). Furthermore, estradiol activation of c-src and c-yes is prevented by the pure antiestrogen ICI 182,780 (Fig. 4C). 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 immunoprecipitated erk-1 is stimulated by 5 min treatment (Fig. 5A) and erk-2 by 2 min treatment (Fig. 5B). 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. 5A) 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. 5B). The pure antiestrogen, ICI 182,780, prevents estradiol activation of both erk-1 and erk-2 (Fig. 5C), 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).

DISCUSSION

The presence as well as the role of estradiol receptor in normal and neoplastic intestinal cells has been analyzed by different groups to
cells have a similar receptor content, whereas nonproliferating, con-
different antiestrogens is reminiscent of the estradiol responsiveness
concentrations of estradiol with active proliferation. The intensity of
the absence of estradiol is very fast.

hormone stimulatory effect is very small, whereas the growth rate 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, con-
fluent 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
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 stimu-
lates c-src and c-yes of Caco-2 cells. The src family of genes
consists of several members that encode highly conserved nonre-
ceptor-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 tumori-
genesis. 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 expres-
sion, 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 intes-
tinal 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 immuno-
precipitated 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 NH₂-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 M₉, 90,000 heat
shock protein, are targets of both estradiol receptor and c-src?
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