[CANCER RESEARCH 60, 1332–1340, March 1, 2000]

A Role for Id-1 in the Aggressive Phenotype and Steroid Hormone Response of Human Breast Cancer Cells[1]

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ABSTRACT

The helix-loop-helix protein Id-1 inhibits the activity of basic helix-loop-helix transcription factors, and is an important regulator of cell growth and tissue-specific differentiation. We have shown (P. Y. Desprez *et al.*, Mol. Cell. Biol., *18:* 4577–4588, 1998) that ectopic expression of Id-1 inhibits differentiation and stimulates the proliferation and invasiveness of mouse mammary epithelial cells, and that there is a correlation between the levels of Id-1 protein and the aggressiveness of several human breast cancer cell lines. Here, we show that aggressive and metastatic breast cancer cells express high levels of *Id-1* mRNA because of a loss of serum-dependent regulation that is mediated by a 2.2-kb region of the human *Id-1* promoter. Three lines of evidence suggest that unregulated Id-1 expression may be an important regulator of the aggressive phenotype of a subset of human breast cancer cells: (*a*) a constitutively expressed *Id-1* cDNA, when introduced into a nonaggressive breast cancer cell line (T47D), conferred a more aggressive phenotype, as measured by growth and invasiveness; (*b*) Id-1 was an important mediator of the effects of sex steroid hormones on T47D cell proliferation. Estrogen stimulated proliferation and induced Id-1 expression, whereas progesterone inhibited proliferation and repressed Id-1 expression. Progesterone repressed Id-1 expression, at least in part by repressing transcription. Most importantly, an antisense oligonucleotide that reduced Id-1 protein levels reduced the ability of estrogen to stimulate cell proliferation, whereas constitutive Id-1 expression rendered cells refractory to growth inhibition by progesterone; and (c) using a limited number of breast cancer biopsies, we showed that Id-1 was more frequently expressed in infiltrating carcinomas compared with ductal carcinomas *in situ*. Our results suggest that Id-1 can control the malignant progression of breast cancer cells, particularly that mediated by sex steroid hormones. Moreover, Id-1 has the potential to serve as a marker for aggressive breast tumors.

INTRODUCTION

bHLH[4] transcription factors are key regulators of lineage- and tissue-specific gene expression in a number of mammalian and non-mammalian organisms. These transcription factors bind DNA as homo- or heterodimers, and activate the transcription of target genes containing E-boxes (CANNTG) or E-box-like sequences in their promoters. Dimerization occurs through the HLH domains, whereas DNA binding occurs through the two basic domains.

Id proteins ("*I*nhibitors of *D*ifferentiation or *D*NA binding") are HLH proteins that lack a basic domain. Id proteins act as dominant inhibitors of bHLH transcription factors by forming transcriptionally inactive heterodimers. Four *Id* genes (*Id-1* through *Id-4*) have been described. Although similar in their organization and HLH sequences, they localize to different chromosomes and show differences in their pattern of expression and function (1, 2). Constitutive expression of Id proteins has been shown to inhibit the differentiation of myoblasts (3), trophoblasts (4), erythroid cells (5), B-lymphocytes (6, 7), myeloid cells (8), and mammary epithelial cells (9).

We have shown that constitutive expression of Id-1 inhibits the functional differentiation and stimulates the proliferation and invasiveness of a murine mammary epithelial cell line (SCp2; Ref. 10), at least in part by inducing expression of a novel matrix metalloproteinase (11). These findings suggested that Id-1 controlled several aspects of breast epithelial cell growth, differentiation, and invasion. We, therefore, proposed that alterations in Id-1 expression may contribute to mammary carcinogenesis in humans and particularly to the malignant progression of some human breast cancers.

The development of a DCIS into a highly aggressive and metastatic breast tumor involves a series of sequential steps: breast epithelial cells must lose the ability to interact with other cells, acquire the ability to digest the surrounding basement membrane, migrate toward the blood stream, and survive and proliferate in ectopic sites. Invasiveness marks the onset of metastasis, which is a hallmark of often fatal malignant progression. Among the factors implicated in the regulation of malignant progression of breast cancer are the female sex steroid hormones E2 and Pg. Breast cancer cells that express ERs generally depend on circulating E2 for their ability to grow and invade surrounding tissue (12). The ERs and PRs are targets for antiestrogens and progestins, respectively, both of which suppress the growth and invasion of receptor-positive breast cancers (13, 14). The presence of ER and PR are good prognostic markers for breast cancer. However, there are a considerable number of ER- and PR-positive breast cancers that do not respond to hormone therapy (15). Little is known about the molecular mechanisms by which antiestrogens and progestins inhibit breast cancer progression. Even less is known about why some receptor-positive breast cancers fail to respond to hormone therapy. Alternative steroid hormone pathways have been proposed (16, 17), but definitive evidence is lacking. Breast cancers that do not respond to hormone therapy tend to be more aggressive and metastatic.

Our finding (9, 11) that Id-1 regulates the growth, differentiation, and invasion of mouse mammary epithelial cells led us to explore a possible link between Id-1 and human breast cancer progression. Our preliminary survey showed that some poorly differentiated, highly aggressive breast cancer cell lines expressed elevated levels of Id-1, compared with several well-differentiated, nonaggressive breast cancer cell lines (11). In this paper, we show that highly aggressive breast cancer cells have lost serum-dependent regulation of *Id-1* gene expression, which results in constitutively high levels of Id-1. We

Received 7/2/99; accepted 1/5/00.

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[1] Supported by a postdoctoral fellowship from the University of California Breast Cancer Research Program to C. Q. L.; by a grant (contract DE-AC03-76SF00098) from the United States Department of Energy (to J. C.); and by grants from the University of California Breast Cancer Research Program (1KB0274) and the National Cancer Institute, NIH (RO1 CA82548) as well as institutional funds from the California Pacific Medical Center (to P-Y. D.).

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[4] The abbreviations used are: bHLH, basic HLH; HLH, helix-loop-helix; E2, estradiol; Pg, progesterone; ER, E2 receptor; PR, Pg receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′6-diamidino-2-phenylindole; DCIS, ductal carcinoma(s) in situ; Egr, early growth response.

further show that ectopic Id-1 expression causes nonaggressive breast cancer cells to display a more aggressive phenotype. Strikingly, E2 and Pg both regulated *Id-1* gene expression, and the effects of these hormones on cell proliferation seemed to be mediated, at least in part, by Id-1. Using some tumor samples in a pilot study, we finally suggest that Id-1 may play a key role in the malignant progression of a subset of aggressive and invasive human breast cancers.

MATERIALS AND METHODS

pBabe-Id-1 Retroviral Vector and Virus Production. The full-length human *Id-1* cDNA was excised from CMV-*Id-1* (18) and cloned into pBabe-puro (19), a kind gift from Dr. Hartmut Land (ICRF, London, United Kingdom). Clones in which the *Id-1* cDNA was inserted in the sense orientation (pBabe-*Id-1*) were selected for use. pBabe-*Id-1* was transfected into the TSA54 packaging cell line (Cell Genesis; Foster City, CA) using calcium phosphate (20). Twenty-four h after transfection, culture medium (containing infectious virus) was harvested twice at 4 h intervals and was frozen at −80°C. Viral titers were determined by reverse-transcriptase activity. Briefly, thawed aliquots of harvested media were incubated with poly(A) (20 ng/μl), oligo dT (10 ng/μl), and [^{3}H]TTP (0.1 μCi/μl) in reaction buffer (50 mM Tris-HCl, 75 mM KCl, 0.5 mM EGTA, and 5 mM $MgCl_2$) for 30 min at 37°C. The reaction mixture was spotted on Whatman DE81 paper, which was washed with 2× SSC and counted in a scintillation counter. One unit of MMLV reverse transcriptase (Life Technologies, Inc.) was subjected to the same reaction, and the amount of incorporated [^{3}H]TTP was defined as 1 RT unit. The retroviral titer (RT units/ml) was determined by comparing the amount of [^{3}H]TTP incorporated by the virus-containing medium with that incorporated by MMLV reverse transcriptase.

Cell Culture and Retroviral Infection. Human breast cancer cell lines MCF7, T47D, and MDA-MB-231 were purchased from the American Tissue Culture Collection (ATCC). Metastatic MDA-MB-435 cells from ATCC were selected for a highly aggressive phenotype by passage in immunodeficient mice.[5] Briefly, cells were injected into nude mice and, 3–4 weeks later, fast growing tumors were harvested and processed for *in vitro* cultivation. Fibroblasts were eliminated from the culture by differential trypsinization, and the tumor cells were expanded and cryopreserved for future use.

Breast cancer cell lines were grown in DMEM or RPMI 1640 (from University of California-San Francisco) containing 10% fetal bovine serum and insulin (5 μg/ml, Sigma). For experiments using serum-free medium, fetal bovine serum was omitted. Where indicated, hormones (E2 or Pg) were added at 10 nM in medium that contained insulin.

Approximately eight RT-units of either pBabe-puro or pBabe-*Id-1* retrovirus were mixed with 5 ml of medium containing 4 μg/ml polybrene and were added to T47D cells in 100-mm dishes. Cells expressing the retroviral genes were selected in 0.6 μg/ml puromycin, which killed all of the mock-infected cells within 3 days, whereas 80 or 30% of the pBabe-puro- or pBabe-*Id-1*-infected cells, respectively, survived. These puromycin-resistant cells are referred to as T47D-pB0 or T47D-*Id-1*. To establish single-cell clones, the T47D-*Id-1* population was plated at 1–2 cells/well in 24-well tissue culture plates. Clones that grew in the wells were expanded.

***Id-1* Promoter-Reporter Assays.** A *Sac*I-*Bsp*HI fragment containing 2.2 kb of the human *Id-1* 5′ upstream region driving a luciferase reporter gene (*Id-1*-luc) has been described (21). T47D and MDA-MB-231 cells were plated in six-well dishes at a density of 3×10^5 per well in medium supplemented with 10% FCS and 5 μg/ml insulin. After 24 h, cells were cotransfected with 6 μg of luciferase reporter plasmids and 2 μg of pCMVB (Clontech) using SuperFect reagent (Qiagen). CMVB contains bacterial β-galactosidase driven by the constitutive CMV promoter (pCMVB), and served to control for variation in transfection efficiency. Three h after transfection, the cells were rinsed twice with PBS and were cultured in the indicated media. In a first set of experiments, cells were cultured for 48 h in serum-containing or serum-free medium before harvest.

In a second set of experiments, cells were cultured for 48 h in serum-containing medium and hormone or ethanol. Cells were treated for a further 2, 4, 6, or 8 h with either Pg (10 nM) or ethanol before harvest. Cells were scraped into 1 ml of PBS and collected by centrifugation (13,000 rpm, 10 min), and the cell pellets were stored at 70°C. Cell pellets were thawed and resuspended in 80 μl of reporter lysis buffer (Promega) for 10 min at room temperature. After centrifugation (13,000 rpm, 5 min), supernatants were harvested. Luciferase and β-gal assays were performed using a Luciferase Assay System (Promega), a β-Gal Assay Kit (Clontech), and a 2010 luminometer. Luciferase activities were normalized for β-gal activity.

RNA Isolation and Northern Analysis. Total cellular RNA was isolated and purified as described by Chomczynski and Sacchi (22). Twenty μg were separated by electrophoresis through formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N; Amersham). The membrane was hybridized to a ^{32}P-labeled human *Id-1* cDNA probe (18)and was washed and exposed to XAR-5 film for autoradiography, as described previously (23). The same blot was hybridized to a 28S rRNA probe to control for RNA integrity and quantitation.

Isolation of Genomic DNA and Southern Analysis. Genomic DNA was prepared using standard methods (23). Briefly, cells were lysed in 100 mM NaCl, 10 mM Tris, 25 mM EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K. After phenol/chloroform extraction, the DNA was precipitated with ethanol, solubilized in TE, and quantified by absorbance ($A_{260\ nm}$). Equal amounts of DNA (10 μg) were digested with *Pst*I, separated on a 1% agarose gel, transferred to a nylon membrane (Hybond N+, Amersham) and hybridized to a ^{32}P-labeled human *Id-1* cDNA probe (18). The blot was then stripped and reprobed with a *GAPDH* cDNA probe to control for DNA integrity and quantitation.

Western Analysis. Cells were lysed in 2× Laemmli buffer (23) and stored at −70°C. Protein concentration was determined by the DC protein assay (Bio-Rad, Hercules, CA). Samples (20–30 μg) were separated by SDS-PAGE and were transferred to a Immobilin-P filter (Millipore) by standard methods (23). The membrane was blocked for 1 h at room temperature with TBST (20 mM Tris Base, 137 mM NaCl, 3.8 mM HCl, and 0.1% Tween 20) containing 5% nonfat milk, and incubated with a rabbit polyclonal antibody against human Id-1 (C-20; Santa Cruz Biotechnology) or with a rabbit polyclonal antibody specific for the PR-A and PR-B forms of the Pg receptor (C-20; Santa Cruz Biotechnology) for 1.5 h. The membrane was washed, incubated with secondary antibody (goat antirabbit IgG-horseradish peroxidase; Santa Cruz Biotechnology), washed again, and developed for enhanced chemiluminescence using the Amersham ECL kit, according to the supplier's instructions.

Boyden Chamber Invasion Assays. Invasion assays were performed in modified Boyden chambers with 8 μm pore filter inserts for 24-well plates (Collaborative Research). Filters were coated with 10–12 μl of ice-cold Matrigel (8 mg/ml protein; Collaborative Research). Cells (80,000 per well) and were added to the upper chamber in 200 μl of the appropriate medium containing 0.1% BSA. Cells were assayed in triplicate or quadruplicate, and the results were averaged. The lower chamber was filled with 300 μl of NIH-3T3 cell-conditioned medium (24). After a 20-h incubation, cells were fixed with 2.5% glutaraldehyde in PBS and were stained with 0.5% toluidine blue in 2% Na_2CO_3. Cells that remained in the Matrigel or attached to the upper side of the filter were removed with cotton tips. Cells on the lower side of the filter were counted using light microscopy.

[^{3}H]Thymidine Labeling. Cells cultured on coverslips were given [^{3}H]thymidine (10 μCi/ml; 60–80 Ci/mmol; Amersham) for the last 16 h of the experiments, unless otherwise indicated, whereupon they were fixed with methanol/acetone (1:1) and stained with DAPI. [^{3}H]-thymidine-labeling was developed as described previously (11). The percentage of labeled nuclei was calculated by comparing the number of [^{3}H]thymidine-labeled nuclei with the number of DAPI-stained nuclei in a given field, using phase contrast and fluorescence microscopy.

Antisense Oligonucleotide Treatment. Phosphorothiolated oligonucleotides were made by Life Technologies, Inc. The *Id-1* antisense oligonucleotide and nonspecific control oligonucleotide were described previously (18). T47D cells were cultured on coverslips in serum-free medium for 2 days. On days 3 and 4, the medium was changed in the morning to serum-free medium containing either E2 (10 nM), or E2 and the oligonucleotides (10 μM). On the evening of day 4, protein was extracted from one set of dishes, whereas [^{3}H]thymidine was added to the other set for an additional 16 h. Cells were fixed on day 5 and assessed for labeled nuclei as described above.

Immunohistochemistry. Formalin-fixed paraffin-embedded tumor tissue sections (obtained from the ICRF Breast Pathology Laboratory, Guy's Hospi-

[5] R. Shalaby and G. Colbern, personal communication.

tal, London, United Kingdom) were used to determine Id-1 protein expression in both DCIS (10 cases) and infiltrating grade III ductal carcinoma (12 cases). Slides were dewaxed, rehydrated, and placed in a container containing 1 liter of 0.01 M citrate buffer (pH 6.0); they were then microwaved at 700 W for 20 min, allowed to remain in the hot citrate buffer for 15 min, and cooled down in running cold water. The slides were washed in deionized water and incubated in 10% nonfat dry milk for 30 min at room temperature, washed in TBS, and incubated with 1 μg/ml of anti Id-1 antibody overnight at 4°C. Control slides were incubated with rabbit immunoglobulin. The slides were washed in TBS and incubated with biotinylated swine antirabbit $F(ab')_2$ fragments (1:400) for 30 min. After washing in TBS, endogenous peroxidase was blocked with 0.3% hydrogen peroxide and 0.1% sodium azide for 10 min. The slides were washed in TBS and incubated with 1:500 streptavidin-horseradish peroxidase for 30 min. After washing in TBS, peroxidase was visualized by incubating in 0.5 mg/ml diaminobenzidine-4-HCl and 0.03% hydrogen peroxide in TBS for 3 min. The slides were washed in TBS and water before mounting.

RESULTS

Highly Aggressive Human Breast Cancer Cells Have Lost Serum Regulation of *Id-1* Expression. We previously showed that *Id-1* expression was undetectable in several differentiated, noninvasive breast cancer cell lines, including T47D and MCF-7, cultured in serum-free medium. Under the same conditions, however, *Id-1* was highly expressed by the poorly differentiated, highly aggressive cell lines, MDA-MB-231 and a subpopulation of MDA-MB-435 (11).

In some cells, *Id* expression is induced by mitogens, including serum (3, 25–27). We, therefore, examined the regulation of *Id-1* mRNA by serum in T47D, MCF-7, MDA-MB-231, and MDA-MB-435 cells. T47D and MCF-7 cells expressed high levels of *Id-1* mRNA when cultured in serum but undetectable levels when cultured in serum-free medium for 2 days (Fig. 1*A*). In contrast, MDA-MB-231 and MDA-MB-435 cells constitutively expressed *Id-1* mRNA, regardless of the presence of serum. Southern analysis showed that the constitutively high levels of Id-1 in MDA-MB-231 and MDA-MB-435 cells was not due to gene amplification (Fig. 1*B*). The *Id-1* hybridization signal (locus on chromosome 20q11) was about the same in all of the four cell lines when normalized to the hybridization signal from the *glyceraldehyde phosphate dehydrogenase* gene (locus on chromosome 12p13), a commonly used control for potential gene amplification (28). As an additional control, the blots were rehybridized to an actin probe, which produced a hybridization signal that was very similar in intensity to that produced by the *Id-1* and *GAPDH* probes (data not shown).

Thus, MDA-MB-231 and MDA-MB-435, two highly aggressive breast cancer cell lines, lost serum-dependent *Id-1* gene expression and, consequently, constitutively express *Id-1* mRNA.

Id-1 Promoter Analysis in Breast Cancer Cell Lines. To determine whether this pattern of Id-1 expression was due to transcriptional regulation, we used the recently cloned and characterized human *Id-1* promoter (−2.2 kb to +1 bp) fused to a luciferase reporter gene (21). This construct was transfected into T47D and MDA-MB-231 cells, proliferating in the presence of serum or serum-starved for 48 h. Cells were harvested and assayed for luciferase activity.

The 2.2-kb *Id-1* upstream region contained most of the regulatory sequences needed for the serum responsiveness of T47D and MDA-231 cells (Fig. 1*C*). Luciferase activity was high in proliferating T47D cells but decreased about 6-fold (from 100 to 17%) on serum-deprivation. Luciferase activity was also high in proliferating MDA-MB-231 cells but, compared with T47D cells, declined to a much lesser extent on serum deprivation (from 100 to about 80%). These results suggest that changes in *Id-1* promoter activity can, in large part, account for the observed changes in *Id-1* mRNA levels. However, we cannot dismiss the possibility that additional elements, missing in the 2.2-kb construct that was used in these experiments, contribute to the regulation of *Id-1* transcription. For example, Saisanit and Sun (29) showed in B lymphocytes that *Id-1* transcription is governed primarily by a pro-B-cell enhancer located 3 kb downstream of the transcription start site.

We conclude that a subset of aggressive human breast cancer cell lines have lost serum-dependent *Id-1* regulation, and that, at least in some cases, this is due to unregulated transcription.

Constitutive Id-1 Expression Converts a Nonaggressive Breast Cancer Cell Line into a More Aggressive Cell Line. To test the hypothesis that unregulated Id-1 expression contributes to the aggressive phenotype of human breast cancer cells, we asked whether constitutive Id-1 expression converts a nonaggressive cell into an aggressive one. The human *Id-1* cDNA was expressed in nonaggressive T47D cells using an amphotropic retrovirus (pBabe-*Id-1*). Puro-

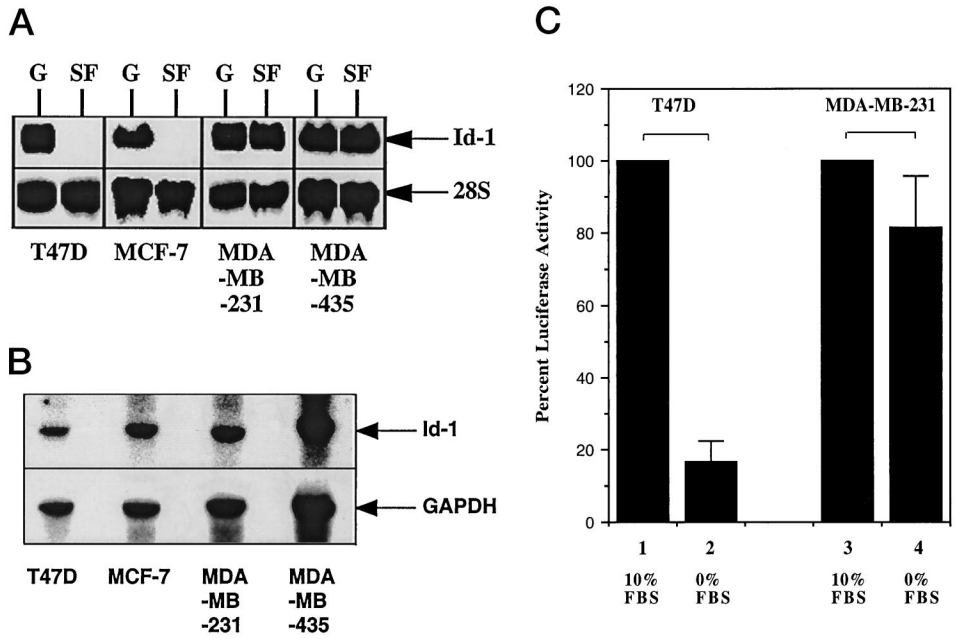

Fig. 1. Loss of serum-regulated *Id-1* expression in aggressive breast cancer cell lines. In *A*, RNA was isolated from T47D, MCF-7, MDA-MB-231, and MDA-MB-435 cells that were either growing in 10% serum (*G*) or incubated in serum-free medium (*SF*) and was analyzed by Northern blotting as described in "Materials and Methods." *Upper panel*, position of the *Id-1* mRNA (1.2 kb); *lower panel*, position of the ribosomal 28S RNA used to control for RNA integrity and quantitation. In *B*, genomic DNA was isolated from the indicated cell lines and digested with *Pst*I. The digested DNA (10 μg) was analyzed by Southern blotting as described in "Materials and Methods." Hybridization signals specific for *Id-1* and *GAPDH* are indicated. *C*, T47D and MDA-MB-231 breast cancer cells were transfected with a *Id-1*-promoter-driven luciferase vector and a normalization vector, as described in "Materials and Methods." Transfected T47D (*Lanes 1* and *2*) and MDA-MB-231 (*Lanes 3* and *4*) cells were either maintained in high serum (*Lanes 1* and *3*) or deprived of serum for 48 h (*Lanes 2* and *4*) prior to harvesting. The results represent the average of at least three independent experiments. Each transfection was done in triplicate.

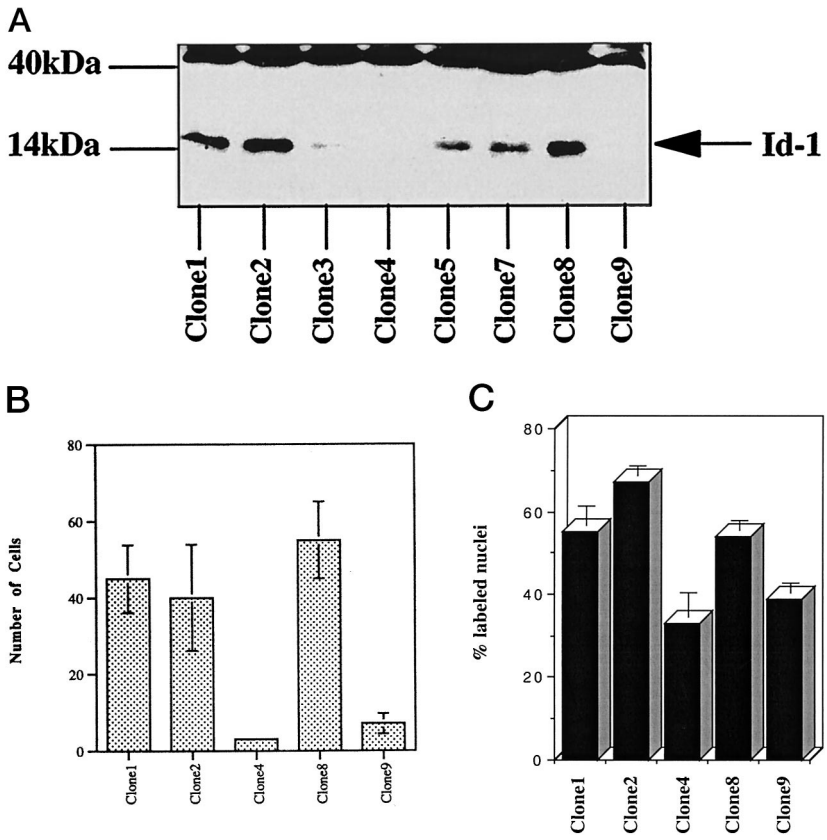

Fig. 2. Id-1 protein levels, invasiveness, and proliferation in T47D-*Id-1* cell clones. In *A*, T47D cells were infected with the pBabe-*Id-1* retrovirus, and single-cell-derived clones were obtained as described in "Materials and Methods." Clones were cultured in serum-free medium for 2 days before protein extraction and Western analysis using a polyclonal antibody against human Id-1. Position of the Id-1 protein and molecular weight markers are indicated. Cross-reactive bands around M_r 40,000 (*40kDa*) indicate loading and transfer efficiency. *B*, Boyden chamber invasion assay for T47D-*Id-1* cell clones. Cells were cultured in serum-free medium for 2 days before they were placed in the upper chamber of Matrigel-coated *trans*-well filters. The invasion assay was carried out for 20 h in serum-free medium, and cells that migrated through the filter were stained and counted. Three to four wells were used for each cell line in each experiment; the results were averaged, and SDs were calculated. Shown is the result of one experiment of a total of three experiments showing similar results. In *C*, the cells were cultured in serum-free medium for 32 h before incubation with [^{3}H]thymidine for an additional 16 h and then were processed for autoradiography. Cells that incorporated thymidine were calculated as a percentage of total DAPI-stained nuclei. Shown is the average from four independent experiments; *error bars*, SDs. Thymidine labeling index for clones 1, 2, and 8: mean of 59% and SD of 7%; for clones 4 and 9: mean of 36% and SD of 6%. One-way ANOVA comparing clones 1, 2, and 8 *versus* clones 4 and 9 was significantly different at $P < 0.0001$.

mycin was used to select for virus-expressing cells. Uninfected cells (Fig. 1*A*) and cells infected with a control virus pB0 (not shown) expressed undetectable levels of Id-1 mRNA in serum-free medium. The pBabe-*Id-1*-infected population (T47D-*Id-1*) expressed only a slightly higher level of Id-1 under these conditions (data not shown). Because many fewer puromycin-resistant cells were recovered after pBabe-*Id-1* infection relative to infection by the insertless control virus (pB0; 30% *versus* 80%), Id-1 may be toxic when expressed at too high a level. We, therefore, isolated nine single-cell-derived clones from the T47D-*Id-1* population, one of which, clone 6, was lost before it could be analyzed. Each of the eight surviving clones expressed a different level of Id-1 protein, determined by Western analysis (Fig. 2*A*). Clones 1, 2, and 8 expressed relatively high levels of Id-1 in serum-free medium, whereas clones 4 and 9 expressed very low levels of Id-1 under these conditions. The other clones expressed Id-1 at intermediate levels.

Five T47D-*Id-1* clones, expressing either high or low levels of Id-1 in serum-free medium, were examined for invasiveness using the Boyden chamber assay. The invasive activity of each clone was approximately proportional to the level of Id-1 protein expression (Fig. 2*B*). Thus, clones with constitutively high levels of Id-1 (clones 1, 2, and 8) were more invasive than clones expressing low levels of Id-1 (clones 4 and 9). The invasive activity of the low-expressing clones resembled that of the uninfected parental T47D cells (not shown).

Ectopic Id-1 expression also conferred a growth advantage in serum-free medium, as measured by the percentage of cells incorporating [^{3}H]thymidine. The three T47D-*Id1* clones that expressed Id-1 at higher levels had a greater thymidine-labeling index than two clones in which Id-1 expression was lower (Fig. 2*C*).

We conclude that when normal *Id-1* regulation is lost and Id-1 is constitutively expressed, human breast cancer cell lines acquire increased invasiveness and a proliferative advantage in growth factor-deficient media. Therefore, ectopic Id-1 expression converted a relatively nonaggressive breast cancer cell line into a relatively aggressive one, as determined by these criteria.

Regulation of *Id-1* Expression by Sex Steroid Hormones. E2 and Pg are important regulators of normal breast development, as well as breast cancer progression. In breast cancer, E2 tends to stimulate ER+ cells to grow and invade, whereas Pg tends to inhibit the growth and invasiveness of PR+ cells. Because Id-1 can regulate many aspects of breast epithelial cell behavior that are also regulated by hormones, we investigated the possibility that *Id-1* might be a downstream effector of E2 and/or Pg in breast cancer cells.

First, we examined the effects of E2 and Pg on *Id-1* expression. T47D cells were cultured in serum-free medium for 2 days, after which time *Id-1* mRNA was barely detectable (Fig. 1*A*). When the serum-deprived cells were treated with E2 (10 nM), *Id-1* mRNA rose to easily detectable levels within 24 h (Fig. 3*A*, *Lane 2*). This rise did not occur in control cells treated with solvent lacking E2 (Fig. 3*A*, *Lane 1*). Treatment with E2 (10 nM) for only 6 h had no detectable effect on *Id-1* expression (data not shown). One interpretation of these results is that E2 may increase *Id-1* mRNA level via an indirect mechanism. Pg had the opposite effect on *Id-1* expression and opposed the effect of E2. When cells were treated with E2 for 22 h and then with the combination E2 and Pg (10 nM) for an additional 2 h, *Id-1* expression declined to control levels (Fig. 3*A*, *Lane 3*). The effects of E2 and Pg on *Id-1* expression were confirmed at the protein level by Western analysis (Fig. 3*B*). Thus, E2 and Pg regulated Id-1 expression in opposite directions, and the inhibitory effect of Pg was dominant over the stimulatory effect of E2.

Pg not only antagonized the induction of *Id-1* by E2, it also antagonized serum-dependent *Id-1* expression. In T47D cells growing in 10% serum, Pg reduced *Id-1* mRNA about 4-fold within 1 h (Fig.

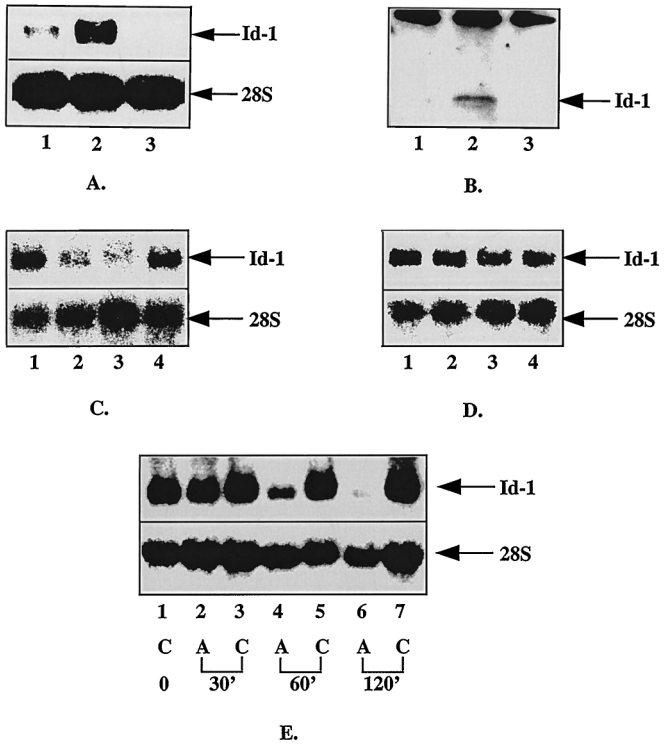

Fig. 3. The effects of E2 and Pg on *Id-1* expression. In *A*, T47D cells were cultured in serum-free medium for 2 days before they were treated with solvent only (*Lane 1*) for 24 h, with E2 for 24 h (*Lane 2*), or with E2 for 22 h followed by a combination of E2 and Pg for another 2 h (*Lane 3*). RNA extraction and Northern analysis were performed as described in "Materials and Methods." The position of *Id-1* mRNA (1.2 kb) and 28S rRNA control are indicated. In *B*, T47D cells were treated with solvent (*Lane 1*), with E2 alone (*Lane 2*), or with a combination of E2 and Pg (*Lane 3*) for 2 days in serum-free medium. Western analysis of Id-1 protein (M_r 15,000) was performed as described in "Materials and Methods." In *C*, RNA was extracted from T47D cells cultured in 10% serum without Pg (*Lane 1*), 1 or 2 h after the addition of Pg (*Lanes 2* and *3*, respectively), or 2 h after the addition of solvent (*Lane 4*), and was analyzed by Northern blotting. The positions of *Id-1* mRNA (1.2 kb) and 28S rRNA control are indicated. *D*, RNA was extracted from MCF-7 cells cultured in 10% serum without Pg (*Lane 1*), 1 or 2 h after the addition of Pg (*Lanes 2* and *3*, respectively), or 2 h after the addition of solvent (*Lane 4*), and analyzed by Northern blotting. In *E*, T47D cells cultured in 10% serum were treated with either actinomycin D (*Lanes 2*, *4*, and *6*: *A*) or control solvent (*Lanes 3*, *5*, and *7*: *C*) for 30, 60, or 120 min before being harvested for RNA extraction.

3*C*, *Lane 2*). *Id-1* mRNA was barely detectable 2 h after Pg addition (*Lane 3*). In comparison, treatment with ethanol had no effect on *Id-1* mRNA after 2 h (*Lane 4*). This result suggests that Pg suppresses the activity of positive regulators of *Id-1* expression, such as E2 and serum factors, in a dominant fashion. Moreover, in contrast to E2, which required >6 h to induce *Id-1*, Pg acted rapidly, suppressing *Id-1* mRNA within 2 h.

MCF-7 cells, in contrast to T47D cells, are not growth- inhibited by progestins (30), very likely because of their reduced levels of PRs. We failed to detect PR-B and PR-A on Western blots (data not shown) of protein extracts from MCF-7 cells. Consistent with this result, there was no decline in *Id-1* mRNA expression in MCF-7 cells treated with Pg (Fig. 3*D*).

We also measured the *Id-1* mRNA half-life using actinomycin D (Fig. 3*E*). After normalizing for 28S rRNA, we estimate that the *Id-1* mRNA half-life is only 20–30 min. These data, together with that in Fig. 3, *A* and *C*, support the idea that Pg down-regulates *Id-1* expression, at least in part, by suppressing transcription.

Id-1 Mediates Hormone Action on Cell Proliferation. We next explored the possibility that E2 and Pg regulate the growth of human breast cancer cell lines via their effects on *Id-1* expression. We first examined the effect of E2 and Pg on T47D cell proliferation. T47D cells were deprived of serum for 48 h, after which the cells were given E2 (10 nM) or the combination E2 and Pg (10 nM each) for an additional 48 h. [^{3}H]thymidine was added for the last 16 h of hormone treatment. The percentage of nuclei that incorporated radiolabeled thymidine was proportional to the level of Id-1 protein expressed by the cells (see Fig. 3*B*). Thus, control cells (Fig. 4*A*, *Lane 1*) had a labeling index of about 20%, whereas E2, which increased Id-1 protein, increased the labeling index to about 60% (*Lane 2*). The combination of E2 and Pg, which reduced Id-1 protein to the control level, reduced the labeling index to the control level (*Lane 3*).

To more directly test the possibility that E2 and Pg regulate breast cancer cell proliferation through regulation of *Id-1*, we used an *Id-1* antisense oligonucleotide that had been previously shown to reduce Id-1 protein levels in human fibroblasts (18). When the *Id-1* antisense oligonucleotide was incubated with serum-deprived T47D cells together with E2, the stimulatory effect of E2 on DNA synthesis was reduced by 50% (Fig. 4*B*, *Lane 3*). A control oligonucleotide had no effect on E2-stimulated DNA synthesis (*Lane 4*). As expected, the *Id-1* antisense oligonucleotide reduced the Id-1 protein level by 2- to 3-fold, in comparison with the control oligonucleotide (Fig. 4*B*, *insert*). Thus, the 50% reduction in labeling index caused by the antisense oligomer could be accounted for by the reduction in Id-1 protein. There was no further reduction in Id-1 protein or labeling index caused by a higher concentration of *Id-1* antisense oligomer (10 *versus* 20 μM), which suggests that 50% was the maximum reduction achievable under these conditions. This result suggests that *Id-1* is a downstream effector of E2.

Id-1 also appeared to be an effector of Pg. Constitutive expression of Id-1 from the retroviral promoter in T47D-*Id-1* cells rendered them refractory to growth inhibition by Pg. T47D-*Id-1* clones that expressed different levels of Id-1 in serum-free medium all expressed relatively high levels of Id-1 in the presence of serum, as expected because the endogenous *Id-1* is stimulated by serum (data not shown). Consistent with this finding, all of the clones had similar [^{3}H]thymidine labeling indexes (Fig. 4*C*, ■) when cultured in serum. Pg (10 nM) significantly reduced the proliferation of the parental T47D cells as well as that of the low-Id-1-expressing clones 4 and 9 (Fig. 4*C*, ▨). By contrast, the high-Id-1-expressing clones (1, 2, and 8), like the aggressive breast cancer cells MDA-MB-231 and MDA-MB-435, which lack PR (31), were refractory to growth inhibition by Pg.

To rule out the possibility of clone-dependent loss of steroid receptor expression, which could explain the differences in growth inhibition by Pg, cells were analyzed for PR expression using Western blotting and an antibody specific for the PR-A and PR-B forms of the PR (M_r 94,000 and 120,000, respectively). The T47D clones maintained the same level of PR expression as parental T47D, whereas these receptors were undetectable in the MDA cell lines (Fig. 4*D*).

These result suggest that Id-1 is a downstream mediator of the effect of Pg on cell proliferation, and that the *Id-1* promoter may be repressed by Pg.

To test this idea, we transfected T47D and MDA-MB-231 cells with the *Id-1* promoter-luciferase construct, and examined the effects of Pg on luciferase activity (Fig. 4*E*). In T47D cells, Pg reduced *Id-1* promoter activity in T47D in a time-dependent manner, with maximal repression evident after about 6 h. The kinetics of reporter activity in this type of experiment may be delayed and/or reduced, when compared with regulation of the endogenous gene, for a variety of reasons. For example, the half-life of luciferase is about 90 min (32), whereas it is only 20–30 min for *Id-1* mRNA (see Fig. 3*E*). In contrast to the significant effect of Pg on *Id-1* promoter activity in T47D cells, *Id-1* promoter activity was not altered by Pg in the PR-negative MDA-MB-231 cells (Fig. 4*E*). Although we cannot rule out the possibility that the rapid down-regulation of Id-1 by Pg is enhanced by acceler-

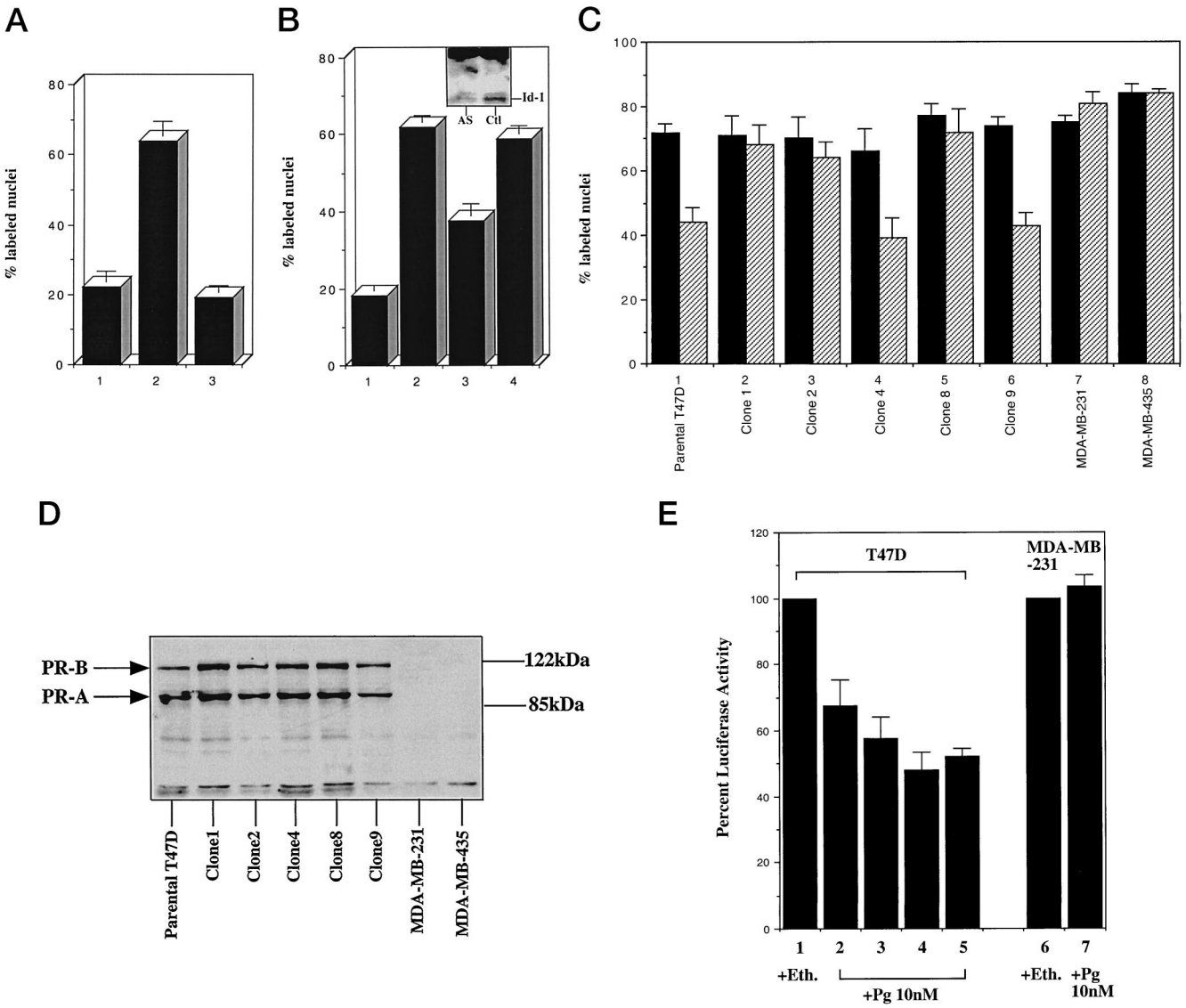

Fig. 4. Id-1 mediates the effects of E2 and Pg on cell proliferation. In *A*, T47D cells, cultured in serum-free medium, were treated with solvent (*Lane 1*), with E2 alone (*Lane 2*), or with a combination of E2 and Pg (*Lane 3*). [^{3}H]thymidine was included in the medium for the last 16 h of the experiment. Cells that incorporated thymidine were calculated as a percentage of total DAPI-stained nuclei. Shown is the average from 4 independent experiments; *error bars*, SDs. *B*, T47D cells, cultured in serum-free medium, were given solvent (*Lane 1*) or E2 (*Lanes 2–4*) as described in *A*. The *Id-1* antisense oligonucleotide or control oligonucleotide was added with E2 (*Lane 3* and *Lane 4*, respectively). [^{3}H]thymidine was added for the last 16 h. Shown is the average from three independent experiments. *Insert*, the Western analysis for Id-1 protein (M_r 15,000) in the antisense oligomer-treated cells compared with control oligomer-treated cells. One-way ANOVA comparing *Lane 2* (or *4*) *versus Lane 3* was statistically different at $P < 0.0001$, whereas one-way ANOVA comparing *Lane 2 versus Lane 4* was not statistically different ($P = 0.09$). In *C*, cells were cultured in 10% serum either without (■) or with (▨) Pg treatment for 48 h. [^{3}H]thymidine was included in the last 16 h. *Columns 1*, parental T47D cells; *Columns 2*, T47D-*Id-1* clone 1; *Columns 3*, clone 2; *Columns 4*, clone 4; *Columns 5*, clone 8; *Columns 6*, clone 9; *Columns 7*, MDA-MB-231 cells; *Columns 8*, MDA-MB-435 cells. The percent labeled nuclei was quantified and plotted as described for *A*. The labeling index in the Pg-treated cells were subjected to statistical analysis: T47D and clones 4 and 9 had a mean of 42%, SD of 6%, and no statistical difference ($P = 0.66$) among them; clones 1, 2 and 8 had a mean of 68%, SD of 7%, and no statistical difference ($P = 0.47$) among them. One-way ANOVA comparing the first group (parental T47D cells and clones 4 and 9) *versus* the second group (clones 1, 2, and 8) was significantly different ($P < 0.0001$). In *D*, parental T47D, T47D-*Id-1* clones (1, 2, 4, 8, and 9), MDA-MB-231, and MDA-MB-435 cells were cultured in serum-free medium for 2 days before protein extraction and Western analysis using a polyclonal antibody against human PR-A and PR-B forms of the Pg receptor. Position of the two forms and molecular weight markers are indicated. *E*, the effect of Pg on *Id-1* promoter activity. T47D (*Lanes 1–5*) and MDA-MB-231 (*Lanes 6* and *7*) were transfected with an *Id-1*-promoter driven luciferase vector. Transfected cells, maintained in 10% serum, were treated with ethanol solvent (*Lanes 1* and *6*) or Pg at 10 nM (*Lanes 2–5* and *7*), as described in "Materials and Methods." T47D cells were harvested at 2, 4, 6, and 8 h (*Lanes 2, 3, 4*, and 5 respectively) and MDA-MB-231 cells were harvested at 6 h (*Lane 7*). The results represent the average of three independent transfection experiments. Each transfection experiment was done in triplicate.

ated mRNA degradation, the simplest hypothesis is that Pg down-regulates transcription of *Id-1* gene in T47D cells.

Id-1 Expression in Breast Cancer Biopsies. The above findings suggested that Id-1 expression might serve as a prognostic marker for at least a subset of aggressive breast cancers. As a first step toward testing this idea, we examined a limited number of breast cancer biopsies for Id-1 expression. Immunohistochemistry was carried out using a specific batch of anti-Id-1 antibody, which showed no cross-reactive bands on Western blots (Fig. 5*A*). Of a total of ten DCIS, 8 were negative for Id-1 staining (two examples are shown in Fig. 5*B*, panels *a* and *b*). One DCIS was clearly positive (part of the large duct is represented in Fig. 5*B*, panel *c*), and the other was weakly positive. Of a total of 12 infiltrating grade III carcinomas of ductal origin, 7 were strongly Id-1-positive. Three showed Id-1 in all of the invasive cells, and four showed positivity in most areas of invasion (two examples are shown in Fig. 5*B*, panels *d* and *e*; Table 1). One infiltrating tumor showed moderate Id-1 immunoreactivity, whereas four were negative (one example is shown in Fig. 5*B*, panel *f*).

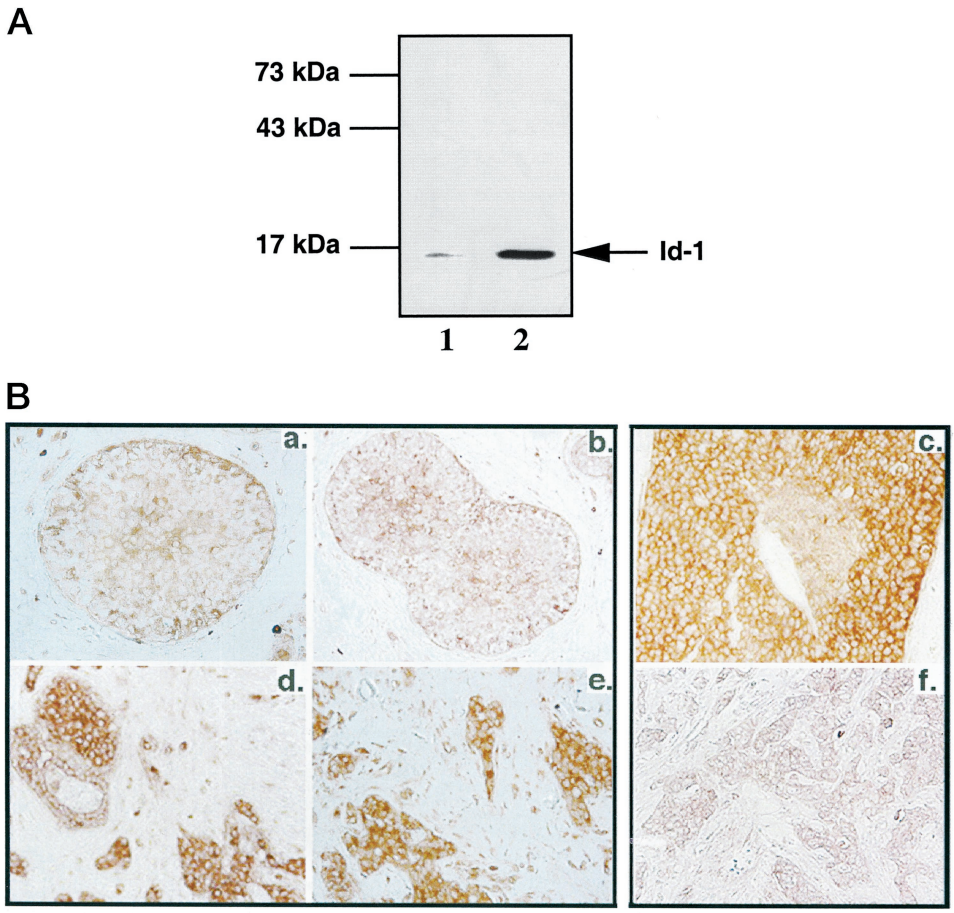

Fig. 5. Id-1 expression in human breast cancer biopsies. *A*, Western analysis showing the specificity of the Id-1 antibody used for immunohistochemistry. T47D *(Lane 1)* and MDA-MB-231 *(Lane 2)* cells were cultured in serum-free medium for 48 h. Position of the Id-1 protein is indicated, and no cross-reactive band is detectable. In *B*, representative sections from DCIS (panels *a*, *b*, and *c*) and grade III invasive carcinomas (panels *d*, *e*, and *f*) were analyzed by immunohistochemistry using an antiserum directed against Id-1. The majority of DCIS were negative (panels *a* and *b*), whereas only one showed strong positivity in its large ductal structure (panel *c*). The majority of the infiltrating carcinomas showed strong Id-1 immunoreactivity (panels *d* and *e*), whereas the minority of the invasive tumors were negative (panel *f*). In panel *d*, a differentiated glandular structure is negative, whereas infiltrating cells show strong immunoreactivity.

Id-1-positive cells displayed prominent cytoplasmic Id-1 staining, as previously reported for all Id proteins (33). This is expected because Id-1 is not a transcription factor *per se*, and Id-1 proteins lack the nuclear localization signals found on many bHLH proteins. Cytoplasmic staining for Id proteins has been found in human seminomas (33), pancreatic cancers (34), and cultured human breast cancer cells (data not shown).

DISCUSSION

Our results implicate Id-1 as a critical downstream target of steroid hormones and critical mediator of the aggressive phenotype in a subset of human breast cancer cells. Certain aggressive breast cancer cells expressed constitutively high levels of Id-1, apparently due to the loss of serum-dependent regulation. The human promoter contains a classic TATA box, an Egr-1 binding site (at −1 kb), and two binding sites (at −1.5 kb and −1 kb) for the transcription factor ATF/CREB (21). The Egr-1 binding site may mediate the serum response of the promoter, as was reported for the mouse *Id-1* promoter (27). The promoter does not contain a cannonical PR binding site. However, several studies have shown that steroid hormone receptors regulate gene expression through interaction with ATF/CREB (35, 36). The ATF/CREB sites are, therefore, candidates for mediating the effects of Pg on *Id-1* expression. We are currently analyzing the human *Id-1* promoter in detail to determine the serum-Pg-responsive regions.

Nonaggressive breast cancer cells acquired a more aggressive phenotype, as judged by increased growth and invasive activity in serum-free medium, when Id-1 was constitutively expressed from a retroviral vector. Thus, the loss of regulated *Id-1* expression may be an important factor in the progression of some human breast cancers toward a less differentiated, more aggressive phenotype. Consistent with this idea, *Id-1* expression was regulated by both E2 and Pg. Our results indicate that Id-1, at least in part, mediates the effect of these hormones on breast cancer cell proliferation. Therefore, *Id-1* is very likely an important downstream target for steroid hormone action. Because Id-1 is a regulator of transcription, it may be responsible for some of the changes in gene expression caused by steroid hormones that lead to increased growth and invasion (12).

Id-1 was the only member of the Id family the expression of which correlated with the aggressive phenotype in the four human breast cancer cell lines that we studied.[6] These cells expressed Id-1, Id-2, and Id-3 but not Id-4. Although *Id* gene family members share high homology in the HLH domain, regions outside this domain are less homologous and are subject to different posttranslational modifications. These non-HLH domains may determine the tissue-specificity of *Id* gene action and its binding specificity for bHLH partners. During mouse development, different *Id* genes are expressed in different regions of the embryo and during different stages of embryogenesis (1, 2). Id-2, but not Id-1, has been shown to bind the

Table 1 *Id-1 protein immunoreactivity levels in breast cancer biopsies*

Breast cancer biopsies were obtained and stained for Id-1 protein expression by immunohistochemistry, as described in "Materials and Methods."

	Negative	Weakly positive	Strongly positive
DCIS	80% (8/10)	10% (1/10)	10% (1/10)
Infiltrating	33% (4/12)	8% (1/12)	58% (7/12)

[6] C. Q. Lin, J. Campisi, and P-Y. Desprez, unpublished data.

retinoblastoma tumor suppressor protein, pRB (37). Additionally, cyclin-dependent kinase 2 was found to phosphorylate Id-2, Id-3, and Id-4 on a site that is missing from Id-1 (38). These differences among Id proteins, and our results, suggest a unique role for Id-1 in regulating the growth and invasion of breast epithelial cells.

Our results on the relationship between Id-1 expression and steroid hormones provide a possible mechanism for their well-known, yet poorly understood, coordinated actions on breast cancer cell growth and invasion (12). It is widely accepted that the ER+/PR+ breast cancers rely on circulating E2 for their aggressiveness. Antiestrogens such as tamoxifen are used to reduce cancer growth and invasion. Pg and many of its derivatives, although found to promote the proliferation of normal breast epithelial cells (39), are known to reduce tumor growth (40). Several possible downstream effectors of E2 and Pg have been investigated (41, 42). For example, transforming growth factor α has been shown to be an important mediator of the E2-stimulated growth of T47D cells (43). Prat *et al.* (44) showed that estrogen induces expression of the *Egr-1* gene in breast cancer cells. Because the murine *Id-1* promoter is regulated by Egr-1 (27), E2 may stimulate *Id-1* gene expression indirectly by inducing Egr-1. We think it is unlikely that the rise in *Id-1* expression after E2 treatments reflects merely growth stimulation. Several lines of evidence suggest that Id-1 is a critical cell cycle regulator. Hara *et al.* (18) showed that human fibroblasts treated with *Id-1* antisense oligonucleotides cannot enter the S phase of the cell cycle. Our data (Fig. 4*B*) suggest that this is at least partly the case for human cells of epithelial origin as well. Moreover, there is now substantial evidence for a regulatory role for Id proteins in coordinating differentiation and cell-cycle control in response to extracellular signals [reviewed in Norton *et al.* (45)].

Musgrove *et al.* (46) showed that growth suppression of T47D cells by Pg was preceded by a decline in G_1 cyclin-dependent kinase activities and phosphorylation of pRB and p107. However, these changes were not evident until 18 h after Pg treatment, which suggests that they were a secondary effect. Our results suggest that the repression of *Id-1* gene expression by Pg may be a primary effect, inasmuch as it was detectable within 1 h of Pg addition. The half-life of *Id-1* mRNA by an actinomycin D experiment appeared to be around 20–30 min (Fig. 3*E*). Because Pg reduced *Id-1* mRNA to control levels within 2 h and also reduced *Id-1* promoter expression in transfected cells, we favor the hypothesis that Pg down-regulates *Id-1* expression via a transcriptional mechanism. *Id-1* repression may be an early downstream event that eventually leads to the G_1 growth arrest caused by Pg. Inappropriate *Id-1* expression may be responsible for the lack of steroid responsiveness shown by some breast cancers.

Our preliminary data on the expression of Id-1 protein in breast cancer biopsies suggest that Id-1 may be a useful marker for infiltrating breast cancer. In this limited sample of biopsies, only 1 of 10 DCIS was strongly positive for Id-1 expression, whereas nearly 60% of invasive tumors were positive. The strongly positive DCIS was from a 40-year old premenopausal woman, whereas most of the negative DCIS were from postmenopausal women >50 years of age. This is of interest because breast tumors tend to be more aggressive and to grow faster in younger patients, in whom estrogen is present (47). However, because patients diagnosed with DCIS are not evaluated for steroid receptor status, we do not know whether this particular biopsy is ER+, and therefore whether *Id-1* expression is elevated because of estrogen action. The infiltrating grade III carcinomas of ductal origin were of no special type. Our hypothesis and data on aggressive breast cancer cell lines suggest that Id-1 may be constitutively expressed, regardless of the steroid hormone receptor status. Indeed, we saw no correlation between ER/PR status and Id-1 expression in these tumors. However, there was a strong correlation between Id-1 expression and vascular invasion. All of the seven Id-1-positive grade III tumors showed invasion of the vasculature. The weakly Id-1-positive tumor, as well as two Id-1-negative tumors, showed no vascular invasion. Because of the absence of data on several of the grade III tumors (patients who did not have axillary dissection), we could not correlate Id-1 expression and nodal status. We have now begun a much more extensive study to test the utility of Id-1 as a reliable prognostic marker for breast cancer invasion and metastasis.

Finally, two recent reports underscore the importance of Id proteins in cancer progression. Lyden *et al.* (48) found that Id proteins were required for the proliferative and invasive phenotype of endothelial cells during angiogenesis. These authors suggest that Id proteins may be important new targets for antiangiogenic drug design. However, Maniotis *et al.* (49) suggest that targeting endothelial cells may not be sufficient. Some aggressive cancers can apparently make new blood supply channels themselves. In this case, tumors may circumvent antiangiogenic drugs directed at endothelial cells if these drugs target characteristics of endothelial cells not shared by the tumor cells. Therefore, according to Lyden *et al.* (48), and supported by our data, drugs that interfere with Id-1 may target endothelial cells as well as aggressive cancer cells and, thus, provide effective tools for cancer treatment.

ACKNOWLEDGMENTS

We thank Dr. Robert Debs for his critical review of this manuscript, Dr. Dan H. Moore for help with the statistical analysis, Dr. Lucien Frappart for advice on immunohistochemistry, Dr. Nancy M. Lee for helpful scientific discussion, and Paul Chang and Yoko Iritani for technical assistance.

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