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Id-1, ITF-2, and Id-2 Comprise a Network of Helix-Loop-Helix Proteins That Regulate Mammary Epithelial Cell Proliferation, Differentiation, and Apoptosis*

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Mammary epithelial cells proliferate, invade the stroma, differentiate, and die in adult mammals by mechanisms that are poorly understood. We found that Id-1, an inhibitor of basic helix-loop-helix transcription factors, regulates mammary epithelial cell growth, differentiation, and invasion in culture. Here, we show that Id-1 is expressed highly during mammary development in virgin mice and during early pregnancy, when proliferation and invasion are high. During mid-pregnancy, Id-1 expression declined to undetectable levels as the epithelium differentiated fully. Surprisingly, Id-1 increased during involution, when the epithelium undergoes extensive apoptosis. To determine whether Id-1 regulates both proliferation and apoptosis, we constitutively expressed Id-1 in mammary epithelial cell cultures. Id-1 stimulated proliferation in sparse cultures but induced apoptosis in dense cultures, which reflect epithelial cell density during early pregnancy and involution, respectively. To understand how Id-1 acts, we screened a yeast two-hybrid library from differentiating mammary epithelial cells and identified ITF-2, a basic helix-loop-helix transcription factor, as an Id-1-interacting protein. Overexpression of ITF-2 significantly reduced Id-1-stimulated proliferation and apoptosis. We show further that, in contrast to Id-1, Id-2 was expressed highly in differentiated mammary epithelial cells *in vivo* and in culture. In culture, Id-2 antisense transcripts blocked differentiation. Our results suggest that Id-1, ITF-2, and Id-2 comprise a network of interacting molecular switches that govern mammary epithelial cell phenotypes.

The mammary gland undergoes striking changes in morphology and function during development and puberty and adult life. During each menstrual cycle, and particularly during pregnancy, mammary epithelial cells undergo cycles of proliferation, invasion, differentiation, and apoptotic cell death. During pregnancy, mammary epithelial cells proliferate, invade the surrounding stromal extracellular matrix (ECM),[1] and form lobulo-alveolar structures that prepare the gland for lactation (1–3). At late pregnancy, prior to parturition, breast epithelial cells cease proliferation and invasion and functionally differentiate into cells that express and secrete milk proteins. Throughout lactation, epithelial cells remain quiescent but continue to express milk proteins (4). After weaning, the mammary gland undergoes involution, a phase of extensive remodeling characterized by degradation of ECM and epithelial cell death by apoptosis.

The molecular mechanisms that regulate and coordinate the changes in mammary epithelial cell phenotypes are poorly understood. Genes that are up- or down-regulated at different developmental stages have been identified, but little is known about how they are regulated and coordinated. The identification of tissue-specific transcriptional regulators in mammary epithelial cells is crucial, not only for deciphering the regulatory mechanisms of normal growth and differentiation but also for understanding how breast cancer develops.

We have shown that Id-1 is a crucial regulator of the growth, invasion, and differentiation of mammary epithelial cells in culture. Id proteins are members of a small family of low molecular mass polypeptides (13–20 kDa) that function as dominant negative regulators of basic helix-loop-helix (bHLH) transcription factors (5). Of the four mammalian Id proteins identified thus far, two (Id-1 and Id-3) are nearly ubiquitously expressed, whereas two (Id-2 and Id-4) show a restricted pattern of expression.

The targets of Id proteins, bHLH transcription factors, comprise a large family of sequence-specific DNA-binding proteins. Some bHLH proteins are ubiquitously expressed (*e.g.* E-proteins), whereas others are lineage-specific (*e.g.* MyoD) (for review, see Ref. 6). bHLH proteins activate transcription of tissue-specific genes and thus direct cell-lineage commitment and terminal differentiation. bHLH proteins act as obligate dimers; they heterodimerize through the HLH domains and bind to specific DNA sequences (E-boxes) through the composite basic

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[1] The abbreviations used are: ECM, extracellular matrix; bHLH, basic helix-loop-helix; ctl, control; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; PCR, polymerase chain reaction; ANOVA, analysis of variance; LH, lactogenic hormones.

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domains. Id proteins contain an HLH domain but lack a basic region. Id proteins can dimerize with bHLH proteins, but the resulting heterodimers cannot bind DNA. Thus, Id proteins negatively regulate bHLH transcription factors. Accordingly, Id proteins have been shown to inhibit the differentiation of trophoblasts (7), erythroid cells (8), B-lymphocytes (9, 10), myeloid cells (11), and mammary epithelial cells (12).

To understand the differentiation of mammary epithelial cells, we developed a line of murine mammary epithelial cells, SCp2, which originated from a mid-pregnant mouse mammary gland (13, 14). SCp2 cells do not grow in semi-solid media in culture or form tumors in nude mice. When given lactogenic hormones and ECM, SCp2 cells aggregate, arrest growth, and form alveolar structures that, within a few days, express and secrete several milk proteins (15, 16). The morphology of differentiated SCp2 cells is similar to the epithelial alveoli in pregnant and lactating mammary glands *in vivo*.

We found that Id-1 expression declines rapidly when SCp2 differentiate and that ectopic expression of Id-1 stimulates proliferation and blocks differentiation (12). Moreover, ectopic Id-1 expression stimulated SCp2 cell migration and invasion, at least in part because of its ability to induce a novel metalloproteinase (17). These studies suggest a potential role for Id-1 in the progression of some breast cancers toward aggressive malignant stages (18).

Here, we show that Id-1 is expressed highly during the initial stages of mammary gland development *in vivo*, when epithelial cells of the ductal trees proliferate and invade the stroma. Thereafter, Id-1 is down-regulated during mid-pregnancy and remains undetectable during much of lactation. Surprisingly, Id-1 is re-expressed early in involution, suggesting a role in apoptosis. In support of this idea, we show that ectopic expression of Id-1 in SCp2 cells triggers either proliferation or apoptosis, depending on the cell culture density. These effects of Id-1 were partly reversed by coexpression of the basic helix-loop-helix factor, ITF-2, which we isolated as an Id-1-interacting protein using the yeast two-hybrid system. We show further that, in contrast to Id-1, Id-2 is up-regulated during mammary epithelial cell differentiation in culture and during the second part of pregnancy *in vivo*. This up-regulation was crucial for full differentiation, because antisense Id-2 blocked milk production in culture. Our results suggest that Id-1, Id-2, and ITF-2 comprise a network of interacting HLH proteins in mammary epithelial cells and imply very different roles for Id-2 and Id-1 in determining breast epithelial cell phenotypes.

MATERIALS AND METHODS

Mammary Gland Extracts—Mammary tissues were obtained from BALB/C female mice, purchased either from Simonsen Laboratories, Inc. (Gilroy, CA) or Harlan (San Diego, CA). To obtain mammary tissue during pregnancy, lactation, and involution, 12-week-old virgin females were mated. Mammary gland involution was induced by removing the pups 20 days after birth. In all cases, the two fourth inguinal mammary glands were harvested. Virgin animals were sacrificed at 5, 7, and 12 weeks of age; pregnant animals were sacrificed at 2, 5, 12, and 18 days after onset of pregnancy; lactating animals were sacrificed at 2, 7, 12, and 20 days after onset of lactation; and involuting animals were sacrificed at 2, 3, and 5 days after weaning. Mammary glands were immediately frozen at −70 °C. RNA and proteins were extracted using TriPure isolation reagents (Roche Molecular Biochemicals).

Cell Culture and Retroviral Infection—The derivation of SCp2 cells has been described (15). SCp2-Id-1 cells were generated by transfecting SCp2 cells with the MMTV-Id-1 expression vector, as described (12). Cells were grown in Dulbecco's modified Eagle's and Ham's F-12 media (1:1) supplemented with 5% fetal bovine serum, insulin (5 μg/ml), and gentamicin (50 μg/ml; Life Technologies, Inc.) at 37 °C in a humidified CO_2 5% atmosphere. To induce apoptosis, cells were shifted to serum-free Dulbecco's modified Eagle's and Ham's F-12 media plus insulin and gentamicin for 5 days.

For the retroviral infection experiments, vectors were packaged in TSA-54 cells (Cell Genesis, Foster City, CA). Full-length human ITF-2 cDNA (obtained from Dr. T. Kadesch) was cloned in a LXSN vector. LXSN-control (LXSN-ctl) and LXSN-ITF-2 vectors were used to infect SCp2-Id-1 cells. Full-length mouse Id-2 cDNA was cloned in a sense, as well as an antisense, orientation in the same LXSN vector, and constructs were used to infect SCp2 cells.

In Situ Apoptosis Detection—Cells were plated at 3×10^4 (low density) or $3–5 \times 10^5$ (high density) on 22-mm^2 coverslips, allowed to grow in complete medium for 2 days, and then serum-starved as described above. Cells were washed once in PBS and fixed for 10 min in 1% paraformaldehyde; after two washes in PBS, 5-min each, cells were permeabilized in ethanol:acetic acid (2:1) for 5 min at −20 °C and washed twice for 5 min in PBS. TUNEL assay was performed using the indirect Apotag fluorescein kit (Oncor), according to the manufacturer's instructions. Nuclei were counterstained with DAPI (Sigma), and coverslips were mounted in glycerol-gelatin (Sigma) for viewing by fluorescence.

DNA Laddering—Cells were plated at 5 or 50% confluency, grown for 2 days in complete medium, and serum-deprived as described above. Cells and media were harvested on ice, washed once with PBS, and pelleted by centrifugation. Pellets were lysed in 1% Nonidet P-40, 20 mM EDTA, and 50 mM Tris-HCl, pH 7.5, for 10 s. Lysates were centrifuged, and supernatants were collected. Supernatants were treated with RNase A (5 μg/ml; Roche Molecular Biochemicals) for 2 h at 56 °C in 1% SDS and proteinase K (2.5 μg/ml; Sigma) at 37 °C for 2–4 h. The DNA was precipitated with 0.5 volume of 10 M ammonium acetate and 2.5 volumes of ethanol at −20 °C overnight. DNA was collected by centrifugation, pellets were resuspended in Tris-EDTA, and DNA was separated by electrophoresis on a 1.2% agarose gel containing ethidium bromide.

RNA Isolation and Northern Analysis—Total cellular RNA was isolated and purified as described (19), and poly(A)$^+$ mRNA was prepared according to the protocol published by New England BioLabs, Inc. Samples (20 μg for total RNA and 4 μg for poly(A)$^+$ mRNA) were electrophoresed through formaldehyde-agarose gels and transferred to a nylon membrane (Hybond N; Amersham Pharmacia Biotech). Membranes were hybridized with ^{32}P-labeled murine β-casein and Id-1 cDNA probes (17) and murine ITF-2 and Id-2 cDNA probes obtained by screening the SCp2 library. Membranes were washed and exposed to XAR-5 film for autoradiography, as described (20). 28 and 18 S ribosomal RNA are shown as controls for RNA integrity and quantitation.

Reverse Transcriptase PCR and Southern Analysis—Murine ITF-2 mRNA was detected by reverse transcriptase PCR, essentially as described (17). Briefly, cDNA was synthesized from total RNA, and 2 μg were amplified using 20 μM of the 5′ and 3′ primers, 5′-ACGACGACAAGAAGGATATC and 5′-ATAATACAGCTGTTAAGGAA. PCR was performed using 0.05 units/μl *Pfu* DNA polymerase and 18 cycles of 1-min denaturation at 94 °C, 1-min annealing at 55 °C, and 1-min extension at 72 °C. For Southern analysis, one-fifth of the PCR reaction product was separated on a 1.5% agarose gel, transferred to a nylon membrane (Hybond N+), and hybridized with the mouse ITF-2 cDNA.

Western Analysis—Cells were rinsed twice with PBS, scraped, and lysed in SSC and 0.25% SDS at 37 °C overnight. Lysates were heated at 95 °C for 10 min to inactivate proteases. Lysates were clarified by centrifugation, supernatants were collected, and protein concentration was measured. Proteins were separated by SDS-polyacrylamide gel electrophoresis using 10% acrylamide gels and blotted onto nitrocellulose membranes. Blots were probed with anti-β-casein (12), anti-Id-1 (18), anti-Id-2 (Santa Cruz Biotechnology), or anti-actin (Chemicon) antibodies. Membranes were incubated with horseradish peroxidase-labeled secondary antibodies, and binding was detected using enhanced chemiluminescence.

Immunohistochemical Analysis—Paraffin-embedded tissue sections (5 μm) from mouse mammary glands were deparaffinized in xylene, rehydrated with ethanol, rinsed in PBS, and incubated for 10 min at 37 °C with 0.1% trypsin. Sections were incubated with a rabbit polyclonal antibody against Id-1 (C-20; Santa Cruz Biotechnology), an antibody that works on paraffin sections (18). Normal rabbit IgG was used as a negative control. Tissue sections were washed and incubated with goat anti-rabbit IgG (Santa Cruz Biotechnology). Slides were then incubated for 30 min with an anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology), and the reaction was revealed by incubating with 3,3′-diaminobenzidine. Sections were briefly counterstained with Mayer's hematoxylin, dehydrated in graded alcohols, cleared in methyl cyclohexane, and mounted.

[^{3}H]Thymidine Labeling and Cell Growth—Cells cultured on coverslips were given [^{3}H]thymidine (10 μCi/ml, 60–80 Ci/mmol; Amersham Pharmacia Biotech) for the last 16 h of the experiments, unless other-

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wise indicated, were fixed with methanol/acetone (1:1), and were stained with DAPI. Radiolabeled nuclei were developed as described (17). The percentage of labeled nuclei was calculated by dividing 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. For cell growth experiments, SCp2-control and SCp2-Id-1 cells were plated at 5×10^4 (low density) onto 35-mm diameter dishes, allowed to attach overnight in complete medium, and then cultured in serum-free medium plus insulin. The cell numbers were determined by counting triplicates on a hemocytometer at daily intervals to 4 days.

Yeast Two-hybrid Screening—To isolate proteins that interact with Id-1, we used yeast two-hybrid screening (21), with components provided by CLONTECH (Palo Alto, CA) and Dr. S. Elledge (22). We constructed a cDNA library from SCp2 cells 12 to 24 h after they were induced to differentiate, which just precedes the appearance of β-casein mRNA. The cDNA library was fused to the yeast expression vector containing the Gal4 transactivation domain (pGAD424). The human Id-1 cDNA was cloned into the GAL-4 DNA-binding domain vector (pGBT9). The cDNA library in pGAD424 was transformed into yeast containing pGBT-Id-1, and screening was carried out as per CLONTECH guidelines. Colonies that grew in His medium containing 3-aminotriasol (>10 mM) were characterized. Interaction was confirmed by expression of the β-galactosidase reporter gene. Plasmids from colonies that grew in 3-aminotriazole and expressed β-galactosidase were isolated, and the inserts were sequenced.

RESULTS

Id-1 Expression during Mammary Gland Development—We examined Id-1 expression during normal mouse mammary gland development *in vivo*, using sequentially probed Northern blots of total RNA from virgin (*V*), pregnant (*P*), lactating (*L*), and involuting (*I*) BALB/C mice (Fig. 1*A*). As expected, β-casein mRNA was evident only during late pregnancy and lactation. Id-1 expression was inversely correlated with β-casein expression, suggesting a role for Id-1 *in vivo* similar to that observed in cultured SCp2 cells. To confirm that Id-1 was specifically expressed by epithelial cells *in vivo*, we performed immunohistochemistry using an Id-1-specific antibody (18). A strong signal was detected only in the epithelial cells at the end buds of growing ductal structures (Fig. 1*B*), confirming that proliferating and invading mammary epithelial cells express high levels of Id-1. Surprisingly, we found that Id-1 was re-expressed during involution (Fig. 1*A*), a time when mammary epithelial cells undergo extensive apoptosis (23). This re-expression raised the possibility that Id-1 may also regulate apoptosis in mammary epithelial cells.

Id-1 Induces SCp2 Cell Apoptosis in a Density-dependent Manner—Mammary epithelial cells undergo apoptosis when cultured in serum-free medium at confluency, and either insulin or epidermal growth factor can act as survival factors to prevent cell death (24). To determine whether Id-1 was important for apoptosis of mammary epithelial cells under these conditions, we plated control SCp2 cells (SCp2-ctl) or SCp2 cells that constitutively express Id-1 (SCp2-Id-1) (12) at low and high cell densities. We cultured the cells in serum-free medium containing insulin for 5 days and then assayed for apoptotic cells using TUNEL staining (Fig. 2*A* and 2B). SCp2-ctl cells did not undergo apoptosis at either density, and proliferation declined upon serum withdrawal (doubling time of about 30 h; see Fig. 2*C*). At low density, SCp2-Id-1 cells continued to proliferate (doubling time of 16 to 18 h; see Fig. 2*C*), confirming that ectopic expression of Id-1 stimulates proliferation even in the absence of mitogens (17, 25). However, at high density SCp2-Id-1 cells underwent apoptosis. To confirm these findings, we tested the same cell lines for non-random DNA fragmentation. SCp2-ctl cells showed no DNA fragmentation under any condition (low or high density) (Fig. 2*D*). Similarly, SCp2-Id-1 cells showed no fragmentation at low density. However, a characteristic apoptotic ladder was detected in the DNA of SCp2-Id-1 cells plated at high density.

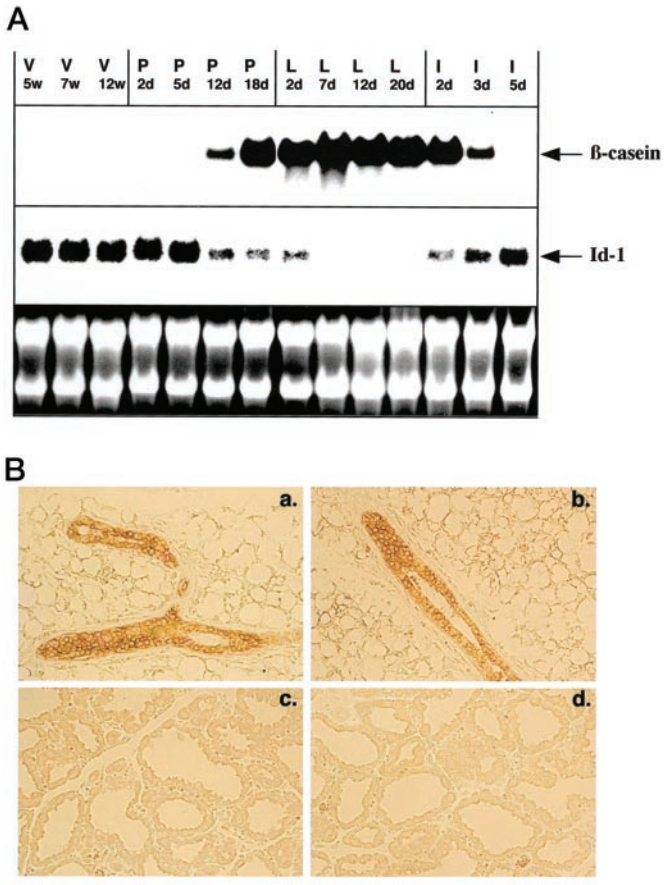

FIG. 1. *A*, β-casein and Id-1 expressions during mammary gland development *in vivo*. Total RNA was isolated from mammary glands at different stages and analyzed on Northern blots. *V*, virgin mice 5, 7, and 12 weeks after birth; *P*, mice 2, 5, 12, and 18 days after onset of pregnancy; *L*, mice 2, 7, 12, and 20 days after onset of lactation; *I*, mice 2, 3, and 5 days after onset of involution. *B*, Id-1 protein expression detected by immunohistochemistry. *a* and *b* show sections from a 5-day pregnant mouse whereas *c* and *d* show sections from a 12-day lactating mouse.

These results indicate that Id-1 can mediate the apoptotic response, in addition to the proliferation response, of mammary epithelial cells. Moreover, proliferation was favored in sparse cultures, which more closely resembles the virgin or early pregnant gland, whereas apoptosis was favored in dense cultures, which more closely resembles the gland at the start of involution.

Identification of Id-1-interacting Proteins—Because Id proteins function by binding bHLH transcription factors, Id-1-interacting proteins are very likely important transcriptional regulators of mammary epithelial cell properties. To identify Id-1-interacting proteins in mammary epithelial cells, we used the yeast two-hybrid system (21) to screen a cDNA library constructed from SCp2 cells in early stages of differentiation (12 to 24 h after addition of ECM, a time just preceding the appearance of β-casein mRNA). We screened the library with the human Id-1 cDNA fused to the yeast Gal4 DNA-binding domain. From a screen of about 300,000 clones, we isolated two positive clones carrying the same 950-base pair cDNA insert. The sequence of this insert was identical to the ubiquitous bHLH transcription factor ITF-2. This mouse ITF-2 insert contained a 950-base pair open reading frame encoding the bHLH and C-terminal domains of ITF-2 but missing the N-terminal region.

Because of its low abundance, we used semi-quantitative reverse transcriptase PCR to examine the expression of ITF-2

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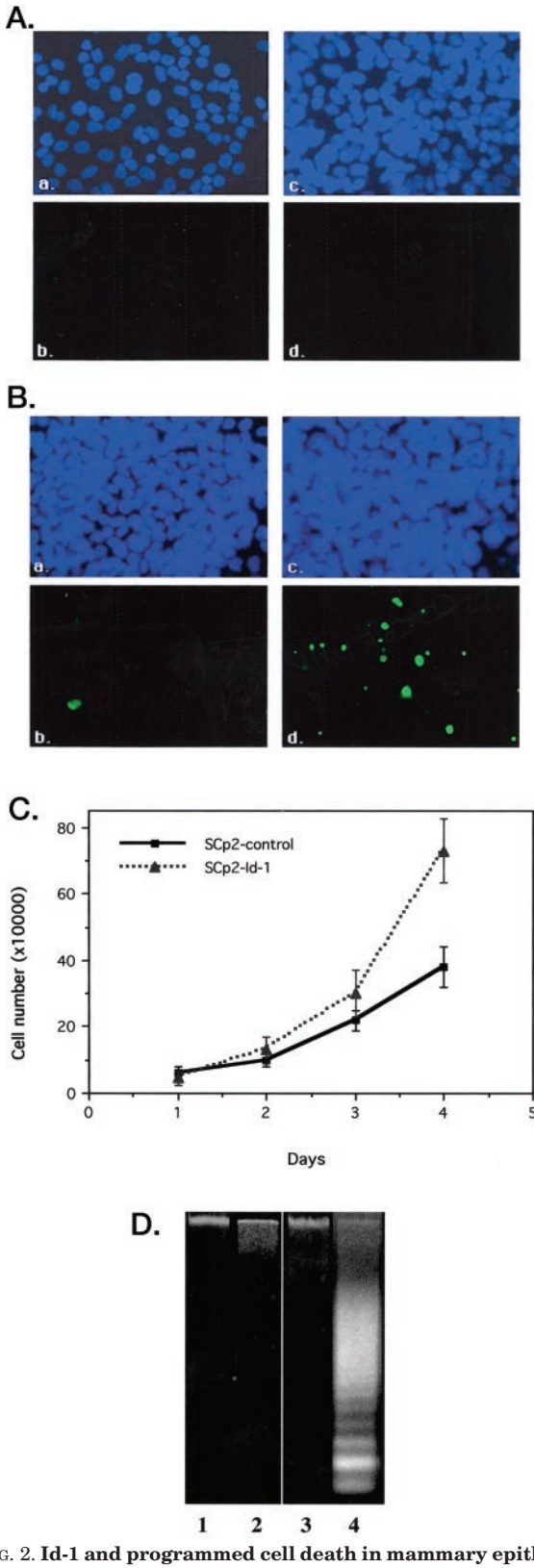

FIG. 2. **Id-1 and programmed cell death in mammary epithelial cells.** *A*, SCp2-control cells plated at low density for 5 days (*a*, DAPI; *b*, TUNEL). SCp2-control cells were plated at high density for 5 days (*c*, DAPI; *d*, TUNEL). *B*, SCp2-Id-1-transfected cells plated at low density for 5 days (*a*, DAPI; *b*, TUNEL). SCp2-Id-1-transfected cells were plated at high density for 5 days (*c*, DAPI; *d*, TUNEL). *C*, growth curves of SCp2-control *versus* SCp2-Id-1-transfected cells plated at low density. Each point is the mean value of triplicate experiments. *D*, high density SCp2-control cells (*lane 2*) show intact genomic DNA in comparison with SCp2-Id-1-transfected cells (*lane 4*). Both cell populations show intact genomic DNA when cultured at low density (*lane 1*, SCp2-ctl cells; *lane 3*, SCp-2-Id-1 cells).

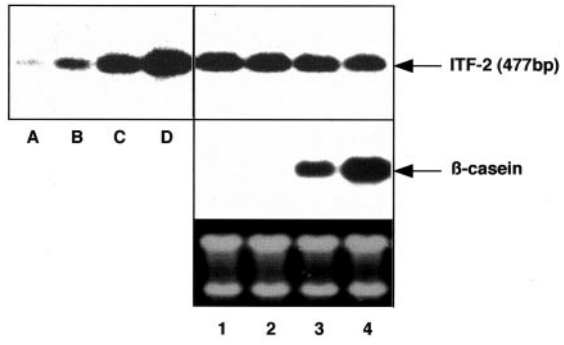

FIG. 3. SCp2 cells were grown in 2% serum (*lane 1*) or treated with ECM and lactogenic hormones (LH) for 1, 2, or 3 days (*lanes 2–4*, respectively). RNA was extracted and analyzed by PCR for ITF-2 (477-base pair (*bp*) band, produced from 100 ng of cDNA) (*upper panel*) or subjected to Northern blotting for β-casein mRNA (*lower panel*). To control for the PCR semi-quantitation, we used 25, 50, 100, and 200 ng of cDNA from one sample (*lanes A–D*, respectively; *upper panel*). To control for RNA quantity and integrity, the ethidium bromide-stained gel is shown in the *lower panel*.

in proliferating and differentiating SCp2 cells (Fig. 3). ITF-2 mRNA did not fluctuate, regardless of whether cells were proliferating or induced to differentiate by ECM plus lactogenic hormones for 1, 2, or 3 days. Thus, although Id-1 expression fluctuated during mammary epithelial cell growth and differentiation, ITF-2 appeared to be expressed constitutively.

ITF-2 Reverses Phenotypes Induced by Constitutive Id-1 Expression—We used the full-length human ITF-2 cDNA to explore ITF-2 function in mammary differentiation. We cloned the hITF-2 in an LXSN retroviral vector, and LXSN-ctl or LXSN-ITF2 retroviruses were used to infect SCp2-Id-1 cells. SCp2-Id-1-LXSN-ctl and SCp2-Id-1-LXSN-ITF-2 cell populations expressed Id-1 protein at the same level (Fig. 4*A*). However, only SCp2-Id-1-LXSN-ITF2 cells expressed high levels of ITF-2 (Fig. 4*B*). When induced to differentiate, SCp2-Id-1-LXSN-ITF2 cells were capable of expressing β-casein (Fig. 4*C*), the marker of differentiation that SCp2-Id-1 cells failed to express (12). When plated on plastic at low density and cultured in serum-free medium, the ITF-2-expressing cells lost the high proliferative capacity characteristic of SCp2-Id-1 (12) or SCp2-Id-1-LXSN-ctl cells (Fig. 4*D*). When plated at high density and serum-starved, the same ITF-2-expressing cells showed much less apoptosis, compared with SCp2-Id-1-LXSN-ctl cells (Fig. 4*E*). These data suggest that these effects of Id-1 on mammary epithelial cell phenotypes occur, at least partly, through its interaction with the basic helix-loop-helix E-protein ITF-2.

Id-2 Expression during Mammary Epithelial Cell Differentiation—Because E-proteins dimerize with either another E-protein or a tissue-specific bHLH transcription factor, we used yeast two-hybrid screening to identify ITF-2-interacting proteins expressed in differentiated epithelial cells. Surprisingly, this screen yielded cDNA inserts encoding the helix-loop-helix protein Id-2. We therefore examined Id-2 expression in proliferating and differentiating SCp2 cells treated with ECM and lactogenic hormones. Western analysis (Fig. 5) showed that differentiated SCp2 cells expressed high levels of the 16-kDa Id-2 protein, whereas Id-2 was not detectable in proliferating cells. In comparison, Id-1 was detectable only in proliferating cells as reported previously (12). We confirmed this inverse expression pattern of Id-1 and Id-2 by Northern analysis of mRNA isolated from cells proliferating or treated with laminin (the major component of ECM required for mammary epithelial cell differentiation) and lactogenic hormones for 24 and 48 h (Fig. 6*A*). Thus, in contrast to Id-1, Id-2 appeared to be associated with differentiation in mammary epithelial cells.

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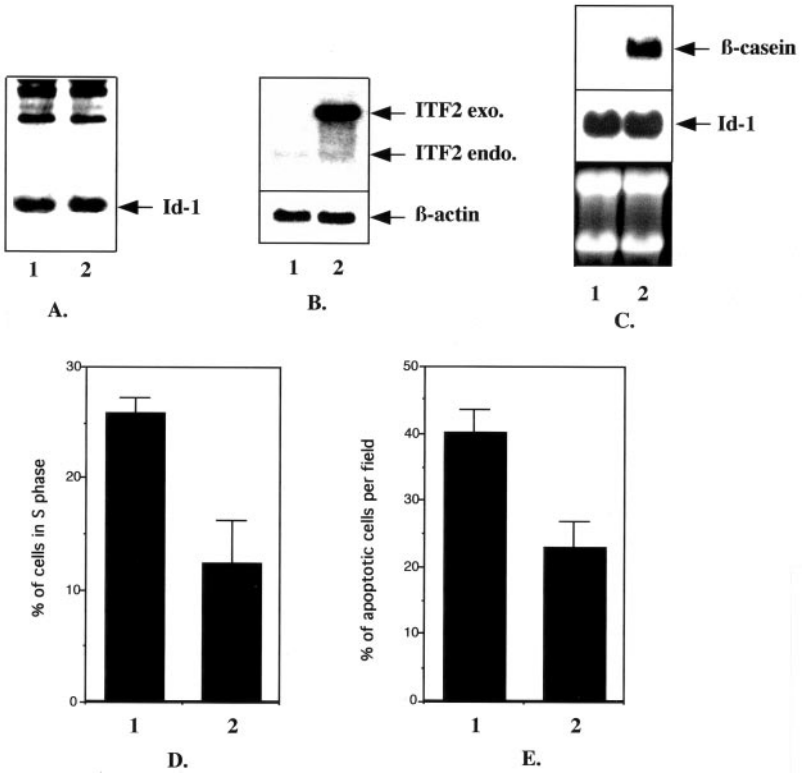

FIG. 4. **ITF-2 counterbalances Id-1 effects on mammary epithelial cell phenotypes.** *A*, Id-1 protein levels in SCp2-Id-1-LXSN-ctl (*lane 1*) and SCp2-Id-1-LXSN-ITF2 (*lane 2*) cells. *B*, ITF-2 mRNA levels in SCp2-Id-1-LXSN-ctl (*lane 1*) and SCp2-Id-1-LXSN-ITF2 (*lane 2*) cells. poly(A)$^+$ mRNA was used for this Northern blot. *C*, differentiation of SCp2-Id-1-LXSN-ctl (*lane 1*) and SCp2-Id-1-LXSN-ITF2 (*lane 2*) cells treated with ECM and LH for 4 days. β-Casein mRNA expression is indicated. *D*, percentage of cells in S-phase in SCp2-Id-1-LXSN-ctl (*lane 1*) and SCp2-Id-1-LXSN-ITF2 (*lane 2*) cells plated at low density, cultured in serum-free medium, and determined by [^{3}H]thymidine incorporation and autoradiography. One-way ANOVA comparing *lane 1 versus lane 2* was significantly different at $p < 0.0001$. *E*, percentage of apoptotic cells in SCp2-Id-1-LXSN-ctl (*lane 1*) and SCp2-Id-1-LXSN-ITF2 (*lane 2*) cells plated at high density, cultured in serum-free medium for 5 days, and determined by TUNEL assay. One-way ANOVA comparing *lane 1 versus lane 2* was significantly different at $p < 0.0001$.

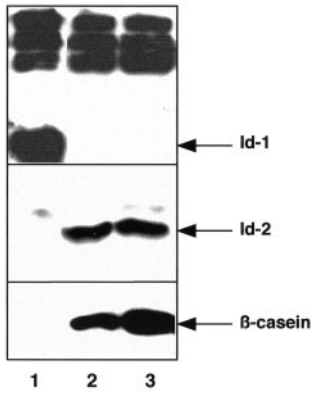

FIG. 5. Inverse correlation between Id-1 and Id-2 protein expression in growing (*lane 1*) and differentiated SCp2 mammary epithelial cells treated with ECM + LH for 48 (*lane 2*) and 72 h (*lane 3*). Protein was extracted and analyzed using antibodies specific for Id-1, Id-2, and β-casein.

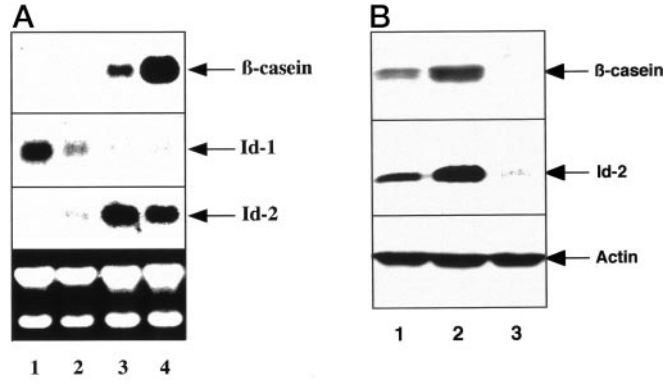

FIG. 6. *A*, inverse correlation between Id-1 and Id-2 mRNA expression in growing (*lane 1*), serum-starved (*lane 2*), and SCp2 cells treated with laminin + LH for 24 (*lane 3*) and 48 h (*lane 4*). Total RNA was extracted and analyzed using probes specific for Id-1, Id-2, and β-casein. *B*, increased β-casein expression in SCp2 cells infected with an LXSN-Id-2-sense expression vector (*lane 2*) and inhibition of β-casein expression infected with an LXSN-Id-2-antisense expression vector (*lane 3*). *Lane 1* shows cells infected with an LXSN-control vector. All cell populations were treated with laminin + LH for 48 h. Actin protein was used as a control for loading and integrity.

Determination of Id-2 Up-regulation as a Necessary Step for Differentiation—To determine whether Id-2 was essential for mammary epithelial cell differentiation and milk production, we infected SCp2 cells with LXSN-control, LXSN-Id-2-sense, or LXSN-Id-2-antisense retroviruses (Fig. 6*B*). After selection for infected cells, the cells were induced to differentiate using laminin and lactogenic hormones. β-Casein expression was significantly higher in SCp2-LXSN-Id-2-sense cells compared with control cells. Moreover, β-casein expression was almost undetectable in SCp2-LXSN-Id-2-antisense cells, which expressed much lower levels of Id-2 protein compared with control cells (Fig. 6*B*). These results suggest that ectopic expression of Id-2 accelerates differentiation, whereas depletion of Id-2 protein inhibits differentiation.

Id-2 Expression during Mammary Gland Development in Vivo—To determine whether Id-2 expression changed during mammary gland development *in vivo*, we analyzed mammary gland protein extracts by Western blotting (Fig. 7). The rationale for determining Id-2 expression at a protein level is that some feedback loops have been shown to be present specifically in Id-2 transcriptional regulation (26). Therefore the presence of Id-2 protein is more indicative of Id-2 activity and function. Id-2 was barely detectable in virgin glands and at the beginning of pregnancy. Expression was apparent first at day 12 of pregnancy, when the epithelial cells begin producing the milk protein β-casein. Id-2 expression continued to increase, reaching its highest level at the end of pregnancy (day 18) and throughout lactation, when the epithelial cells had fully differentiated. Thus the expression pattern of Id-2 was quite different from that of Id-1, confirming the data obtained from SCp2 cells in culture.

DISCUSSION

In vivo, the growth and differentiation of the mammary gland progress through several stages during puberty. Ulti-

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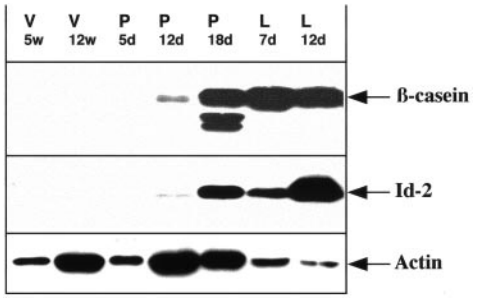

FIG. 7. **Id-2 expression during mammary gland development.** Protein was extracted from glands at different stages of development (*V*, virgin 5 and 12 weeks; *P*, pregnant 5, 12, and 18 days; *L*, lactating 7 and 12 days). Western analysis was performed using antibodies specific for β-casein, Id-2, and actin.

mate function (expression and secretion of milk proteins) is achieved by the epithelial cells during lactation. Mammary epithelial cells lose their ability to proliferate as they progress toward functional differentiation. Once lactation terminates, most of the epithelial cells undergo apoptosis. Very little is known about the molecular mechanisms that coordinate growth, differentiation, and the tightly regulated, self-limited, and transient invasive behavior of normal epithelial cells during mammary gland development and remodeling (27).

A role for helix-loop-helix Id proteins in the differentiation of mammary epithelial cells was suggested first by our finding that Id-1 expression declined when murine SCp2 cells were induced to differentiate in culture and that constitutive Id-1 expression blocked differentiation (12). Our findings also implicated Id-1 in the invasive phenotype of mammary epithelial cells and breast carcinoma cell lines (17).

Id-1 Expression during Mammary Gland Development in Vivo—Here, we show that Id-1 expression in the mammary gland follows a pattern expected from the cell culture studies. In the mammary gland, Id-1 expression was inversely correlated with that of β-casein. β-Casein mRNA was evident during mid- and late pregnancy and throughout lactation but declined as the gland involuted. By contrast, Id-1 mRNA was expressed highly in the developing gland but was reduced during pregnancy and completely abolished during lactation. Interestingly, Id-1 was also expressed highly during involution. This expression corresponds to the onset of mammary epithelial apoptosis (23). Thus, Id-1 may not only be important for the proliferation of mammary epithelial cells in virgin and early pregnant mice but also for the onset of programmed cell death when the gland involutes.

Id-1 and Programmed Cell Death in Breast Epithelial Cells—Id proteins have been implicated in the regulation of apoptosis in non-epithelial cells. Ectopic expression of Id-3 induced apoptosis in serum-deprived fibroblasts (28). Similarly, Id-2 induced apoptosis in both myeloid precursors and osteosarcoma cells (29). In both cases, Id-2 appeared to act by an HLH-independent mechanism, and the death-promoting function was localized to the N terminus. In rat embryo fibroblasts, Id proteins overcame the suppression of adenovirus E1A-induced apoptosis by mutant p53 (30). In addition, expression of Id-1 caused apoptosis in cardiac myocytes through a redox-dependent mechanism (31), and Id-4 expression induced apoptosis in astroglial primary cultures (32).

We found that ectopic Id-1 expression stimulated proliferation in SCp2 mammary epithelial cells when the cells were cultured at subconfluence in a serum-free medium. However, the same cells underwent apoptosis when cultured at high density in serum-free medium. These findings suggest that high density SCp2 cells in serum-free medium may be a good cell culture model for mammary gland involution. Id-1 was re-expressed at the beginning of involution when mammary epithelial cells, packed inside the fully differentiated alveoli, undergo apoptosis. Depending on the cell context, Id-1 protein can promote either growth or apoptosis.

Effects of Id-1 through Its Interaction with ITF-2—bHLH factors, whose activities are inhibited by Id-1, are very likely important regulators of normal mammary epithelial cell functions and malignant transformation. Basic HLH proteins have been implicated in the regulation of cell-lineage specification and tissue-specific differentiation. However, little is known about this family of transcription factors and their functions in the mammary gland. We used the yeast two-hybrid system to identify bHLH proteins that interact with Id-1 in mammary epithelial cells. We identified ITF-2, a member of the ubiquitous bHLH protein family, which, in other systems, binds both tissue-specific bHLH proteins and Id proteins.

There are two classes of simple bHLH transcription factors. The first class is the ubiquitous E-box binding factors, found in almost all tissues examined. To date, this class includes the differentially spliced E2A gene products (E12 and E47), E2–5/ITF-1 (33), E2–2/ITF-2 (34), and HEB (35). The second class is the tissue-specific bHLH proteins exemplified by MyoD. ITF-2 belongs to the ubiquitous E-box binding factors, which are expressed in most tissues examined. There was no change in its level of expression in SCp2 cells, whether growing or differentiated.

Nonetheless, overexpression of ITF-2 counterbalanced the effects of Id-1 on differentiation, proliferation, and apoptosis in SCp2 mammary epithelial cells. It was reported previously that even modest changes in the activities of bHLH proteins can have a dramatic effect on cellular function. E2A regulation of B cell development provides one such example. A 2-fold reduction in bHLH activity was associated with an equivalent reduction in the numbers of B cells (36, 37).

High levels of ITF-2 may trigger a switch in the balance between bHLH proteins and their negative regulator Id-1. Indeed, ITF-2 overexpression reduced the apoptotic response of ectopic Id-1 expression. These findings suggest that Id-1 functions in mammary epithelial cells by the mechanism described by Norton and Atherton (28), *i.e.* Id-1 triggers apoptosis through its HLH domain and interaction with a bHLH transcription factor.

Role of Id-2 during Mammary Epithelial Cell Growth and Differentiation—Unlike Id-1, which is expressed during proliferation and suppressed during differentiation in all cell types examined, the data on Id-2 are less consistent. The isolation and characterization of Id-2 was first reported by Sun *et al.* (38). Id-2 contained a helix-loop-helix motif similar to that of Id-1 (90% identity (39)), but the two proteins differed markedly elsewhere. Id-1 and Id-2 are encoded by unlinked genes. Id-2, like Id-1, was identified first as an inhibitor of differentiation, because it was down-regulated during the differentiation of a variety of cell types. For example, in the B cell lineage, Id-2 is expressed in pro-B cells and down-regulated during differentiation (9). In addition, overexpression of Id-2 inhibited the differentiation of myoblasts (40) and led to a stage-specific developmental block early in thymopoiesis (41).

However, other data are inconsistent with a role for Id-2 as an inhibitor of differentiation. For example, Id-2 mRNA levels increase during the differentiation of myeloid precursors (such as HL-60) (42). Id-2 gene expression was also maintained during the hematopoietic differentiation of embryonic stem cells (43). It was reported recently that mice deficient in Id-2 lack lymph nodes and Peyer's patches, because they are deficient in a cell population essential for the generation of peripheral

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lymphoid organs (44). In addition, Id-2 null mutant mice displayed disturbed natural killer cell differentiation. Id-2-deficient mice also exhibited a lactation defect (45). Mori and collaborators (45) concluded that the function of Id-2 in the pregnant mammary gland was to stimulate cell cycle progression by negatively regulating potential bHLH factors important for differentiation. However, they did not exclude the possibility that Id-2 may alternatively promote differentiation of mammary gland by inhibiting the activity of bHLH involved in stimulation of cell proliferation.

Our data in mammary epithelial cells in culture and *in vivo* provide support for the alternative hypothesis. We propose that Id-2 is an activator, rather than an inhibitor, of mammary differentiation. Thus, both Id-1 and Id-2 may be involved in regulating cell growth and differentiation during normal development of the breast but play opposite roles. To reach full differentiation, mammary epithelial cells may need to switch off Id-1 and turn on Id-2, and these two events may be coordinated.

To our knowledge, the mammary gland represents the first organ in which the importance of the coordination of Id-1/Id-2 is suggested. The Id-2 promoter might be up-regulated by Id-1 protein down-regulation, which presumably would release bHLH factors. In agreement with this hypothesis, Neuman *et al.* (26) isolated the 5′ regulatory region of the Id-2 gene and demonstrated that it contained several E-box clusters. They also showed that bHLH transcription factors regulated Id-2 gene expression in F9 cells. We identified one of these bHLH factors, ITF-2. We are currently searching for ITF-2-bHLH interacting proteins. The identification of these factors will broaden our understanding of the complex network of HLH factors that regulate mammary epithelial cell phenotypes.

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Id-1, ITF-2, and Id-2 Comprise a Network of Helix-Loop-Helix Proteins That Regulate Mammary Epithelial Cell Proliferation, Differentiation, and Apoptosis

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