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Expression of Id and ITF-2 Genes in the Mammary Gland during Pregnancy

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Abstract

Id-1 is one of the four related helix-loop-helix Id proteins that act as inhibitors of the basic helix-loop-helix (bHLH) transcription factors that control cell differentiation, development and carcinogenesis. We previously found that Id-1 regulated the growth, differentiation, apoptosis and invasion of mouse mammary epithelial cells in culture. Using the techniques of immunohistochemistry and *in situ* hybridization, we now show that Id-1 gene expression is specifically detected in the epithelial cells of growing ductal structures during early pregnancy. Using adjacent sections, we determined that Id-1 was expressed in keratin 8/18 positive cells. We also demonstrated that the up-regulation of Id-2 during late pregnancy correlated with the down-regulation of Id-1. Using the yeast-two hybrid system, we identified the bHLH transcription factors, ITF-2A and ITF-2B, as the Id-interacting proteins. The levels of expression of these two splice variants did not change during the transition from growing ductal structures to differentiated alveoli. Therefore Id-1 and Id-2, but not the ubiquitous bHLH proteins, appear to represent the key factors whose expression is modulated during different stages of pregnancy in mouse mammary glands.

Keywords

Id proteins; development; pregnancy; proliferation; differentiation

Introduction

The controlled balance of cell growth/invasion and differentiation is essential to proper tissue development and homeostasis, and these events are regulated by the competing influence of transcriptional regulators. The path of breast cancer progression is determined by alterations in these regulatory mechanisms. Therefore, once the critical genes that control mammary epithelial phenotypes have been identified, reagents for the diagnosis and prognosis of breast cancer can be developed, and rational therapies become possible.

Id-1 is one of the four related helix-loop-helix (HLH) Id proteins (1) that act as inhibitors of the basic helix-loop-helix (bHLH) transcription factors that control cell differentiation, development and carcinogenesis. Studies of Id-1 expression in normal and cancerous breast cells, as well as in mouse mammary glands and in human breast cancer biopsies, demonstrated

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that Id-1 expression was associated with a proliferative and invasive phenotype (2,3). Id-1 was expressed at high levels in proliferating mouse mammary epithelial cells *in vitro*. When given lactogenic hormones and ECM, cells aggregated, growth arrested, formed alveolus-like structures, secreted milk proteins and rapidly down-regulated Id-1. Interestingly, the ectopic expression of Id-1 in the MECs blocked differentiation and stimulated cell proliferation as well as cell invasion (4,5).

In contrast, Id-2, another member of the Id protein family, was associated with a differentiated phenotype in both normal and transformed breast cells and seemed to play the opposite role from that of Id-1 (3,6,7). It has been reported that Id-2-deficient female mice displayed severe lactation defects (8,9). Indeed, Id-2 null mammary epithelial cells lost the capacity to form alveoli, showed a disorganized architecture, and were not able to undergo pregnancy-dependent differentiation. Interestingly, the yin-yang balance between Id-1 and Id-2 expressions appeared to be specific for the breast, and not for any other organ such as the prostate (10).

Both Id-1 and Id-2 proteins most likely regulate mammary gland growth and differentiation through their interaction with basic helix-loop-helix (bHLH). As demonstrated by the screening of a yeast two-hybrid library from differentiating mammary epithelial cells, Id-1 interacted with the HLH domain of the ubiquitously expressed Class I bHLH ITF-2 (6). Then using the same fragment corresponding to the HLH region of ITF-2 as a bait, we isolated Id-2 from our library prepared from differentiated cells. Therefore both Id-1 and Id-2 were able to interact with the same bHLH factor, a result that was difficult to reconcile with our hypothesis of an opposite role of Id-1 and Id-2.

However, two different mRNA corresponding to alternatively spliced forms of mouse ITF-2 have been reported (11). The first one, ITF-2A, is known to form active heterodimers with MyoD, a muscle-specific bHLH transcription factor, during muscle differentiation. The second one, ITF-2B, encodes a transcription factor that inhibits MyoD activity. ITF-2A and ITF-2B sequences only diverge in their N-terminal portion, while their HLH regions as well as their C-terminal parts are identical. The inhibitory activity of ITF-2B requires an extra sequence of 83 amino acids in the N-terminal part. We therefore hypothesized that Id-1 preferentially interacted with ITF-2A and Id-2 with ITF-2B.

Due to some controversies related to the type of cells expressing Id-1 in the mammary gland (12), we first confirmed in this manuscript the expression of Id-1 mRNA and protein in the epithelial cells of developing mouse mammary glands. We then show the expression of the bHLH factors ITF-2A and ITF-2B during different stages of pregnancy and demonstrate the specific interactions between Id proteins and ITF-2 proteins. Overall, our data provide a model for a role of helix-loop-helix proteins during the transition from proliferation to differentiation in the mammary gland of pregnant mice.

Materials and Methods

Mammary gland preparation

Mammary gland tissues were obtained from Balb/c female mice, purchased from Simonsen Laboratories, Inc. (Gilroy, CA). To obtain tissues during pregnancy, 12-week old virgin females were mated. Pregnant animals were at 5, 12 and 18 d after onset of pregnancy. The two fourth inguinal mammary glands were harvested and immediately frozen at -70°C for RNA extraction or paraffin-embedded for immunohistochemistry and *in situ* hybridization.

RNA isolation and northern analysis

RNA was extracted using TriPure Isolation Reagents (Boehringer Mannheim). Samples (20 μg for total RNA) were electrophoresed through formaldehyde-agarose gels and transferred to

a nylon membrane (Hybond N, Amersham). Membranes were hybridized with ^{32}P -labeled murine β -casein, Id-1 and Id-2 cDNA probes (6). Membranes were washed and exposed to XAR-5 film for autoradiography. 28S and 18S ribosomal RNA are shown as controls for RNA integrity and quantitation.

Polymerase chain reaction

Transcripts for ITF-2A, ITF-2B and for GAPDH were reverse transcribed using Superscript Reverse Transcriptase II (Gibco-BRL), and polymerase chain reaction performed. PCR was performed in buffer containing 1 μM of each of the 5' and 3' PCR primer and 0.5 U of Taq polymerase using 28 cycles for amplification of ITF-2 cDNAs and 25 cycles for amplification of GAPDH cDNA. The 5' PCR primer was GTCCGAAAAGTTCCTCCGGGTTTGCCGTCT for ITF-2A and TCCAATCCTTCAACTCCTGTGGGCTCCCCT for ITF-2B; the 3' PCR primer was TTCCTTCTCGCGCTCAGCCTTCTG for both ITF-2A and ITF-2B. The 5' PCR primer for GAPDH was ACCACAGTCCATGCCATCAC and the 3' PCR primer was TCCACCACCCTGTT GCTGTA. The cycle conditions for ITF-2A and ITF-2B were 50 sec denaturation at 94°C, 50 sec annealing at 62°C, and 180 sec extension at 72°C. The cycle conditions for GAPDH were 30 sec denaturation at 94°C, 30 sec annealing at 60°C, and 60 sec extension at 72°C.

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 mouse antibody against keratin 8/18 (Sigma-2931), a mouse antibody against alpha-smooth muscle actin (Sigma-2547), a rabbit polyclonal antibody against keratin 14 (PRB-155P, Covance/Babco) or a rabbit polyclonal antibody against Id-1 (C-20, Santa Cruz Biotechnology) in the presence or absence of Id-1 blocking peptide. Tissue sections were washed and incubated with biotinylated secondary antibodies. Slides were then incubated for 30 min with anti-IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, CA), and the reaction 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.

In situ hybridization

To localize of Id-1 mRNA in the mammary glands, we used deparaffinized sections (5 μm) treated with proteinase K (5 $\mu\text{g}/\text{ml}$) and hybridized overnight with digoxigenin-labeled probes that were prepared from appropriately linearized plasmids. A 650bp mouse Id-1 cDNA sequence was used (PvuII-XhoI restriction sites). The cDNA sequences spanned protein coding regions outside of the helix-loop-helix consensus region were inserted into the (EcoRV-XhoI restriction sites) of a Bluescript plasmid (Stratagene). As a negative control, sense fragments were hybridized to adjacent sections. After hybridization at 47°C, sections were treated with RNase A for 10 minutes at 37°C, followed by stringent washes before autoradiography with NTB2 emulsion.

Yeast two-hybrid system

To determine the interactions amongst the different helix-loop-helix proteins, we used the yeast two-hybrid system (Parrinello et al., 2001). Parental yeast vector pGADT7 and pGBKT7 were purchased from Clontech (Palo Alta, CA). The 1.2 kb Id-1 fragment containing the entire coding region was cloned into pGADT7. The 600 bp Id-2 fragment containing the entire coding region was amplified by PCR and cloned into pGADT7. ITF-2A/Pgk and ITF-2B/Pgk were gifts from Dr. Skerjanc. The ITF-2A (1.5 kb) and ITF-2B (2 kb) fragments containing the entire coding region were cloned into pGBKT7. pGADT7 and pGBKT7 plasmids were

simultaneously co-transformed into Y187 yeast for colony lift filter assay to measure β -galactosidase activity. Each co-transformed Y187 strain was plated on selection plates. Fresh colonies were transferred to Duralose UVTM membranes (Stratagene) and frozen in liquid nitrogen. Filters were then thawed at room temperature, placed on the presoaked 3MM paper with Z buffer/X-gal solution and incubated at 30°C until blue color is detected.

Results

Id-1 expression during early pregnancy

We previously examined Id-1 mRNA expression during normal mouse mammary gland development *in vivo*, using northern blots of total RNA from virgin, pregnant, lactating and involuting Balb/c mice (6). Id-1 expression was inversely correlated with β -casein expression, suggesting a role for Id-1 *in vivo* similar to that observed in cultured mammary epithelial cells (5). However, it was claimed that Id-1 was not expressed in epithelial cells in mouse mammary gland, but in the cytoplasm of myoepithelial cells (12). However, this staining did not appear to correspond to Id-1, because it was also observed in mammary myoepithelial cells from Id-1 null mice.

Therefore, in order to confirm that Id-1 was indeed expressed by epithelial cells in mouse mammary glands, we performed immunohistochemistry using an Id-1-specific antibody (13). No signal was obtained in resting or fully differentiated glands, which should be Id-1 negative (data not shown). However, a strong signal was detected in the epithelial cells of growing ductal structures at day 5 of pregnancy (Fig. 1A and B), showing that proliferating and invading mammary epithelial cells indeed expressed high levels of Id-1.

Using adjacent sections of day 5 pregnant mammary glands, we determined the co-localization of Id-1 protein expression (Fig. 1C) specifically with markers of epithelial cells (K8/18 in Fig. 1D), but not with markers of myoepithelial cells and stroma cells (K14 in Fig. 1E and SMA in Fig. 1F). In addition, we confirmed Id-1 mRNA expression in epithelial cells using *in situ* hybridization (digoxigenin-labeled probes) on mammary gland sections, also at day 5 of pregnancy (Fig. 2A). Using the antisense probe, a signal was detected in ductal epithelial cells and not in any other cell types, including myoepithelial cells or stroma cells. As expected, no signal was detected using sense probes (Fig. 2B).

Identification of Id-1 and Id-2-interacting bHLH proteins

We previously examined Id-2 expression during normal mouse mammary gland development *in vivo* using Western analysis (6). Id-2 protein expression was correlated with β -casein and was inversely correlated with Id-1 protein expression, i.e., Id-2 was expressed when cells started to differentiate during the second part of pregnancy.

When Id-1 and Id-2 mRNA expression was investigated during early (5d), middle (12d) as well as late pregnancy (18d), Id-1 mRNA expression decreased at day 12, at a time Id-2 mRNA expression increased (Fig. 3A). A peak of Id-2 mRNA expression was detectable at day 18 of pregnancy when Id-1 mRNA expression was almost undetectable. The decrease in Id-1 mRNA expression and the increase in Id-2 mRNA expression during mid-pregnancy corresponded to a time when mammary epithelial cells stopped proliferation, and when differentiation occurred and milk proteins such as β -casein began to be expressed.

Due to their low abundance and to the absence of specific antibodies, we used semi-quantitative RT-PCR to examine the expression of ITF-2A and ITF-2B during early, middle and late pregnancy (Fig. 3B). We used a minimum number of cycles that gave detectable bands for both genes. As expected for factors that belong to the class I of ubiquitous bHLH regulators, levels of mRNAs did not fluctuate regardless the origin of RNA extracts, i.e., from 5d, 12d or

18d pregnant mammary glands. Thus, although Id-1 and Id-2 expression fluctuated during mammary epithelial cell growth and differentiation, ITF-2A and ITF-2B genes appeared to be uniformly expressed.

Using the yeast two-hybrid system, we then determined whether Id-1 and Id-2 interacted preferentially with one of the two ITF-2 splice variants. The 1.2 kb Id-1, 600 bp Id-2, 1.5 kb ITF-2A and 2 kb ITF-2B fragments containing the entire coding region were cloned into the yeast vectors pGADT7 and pGBKT7. Each of the four constructs was individually transformed into Y187 yeast strain. Proteins were extracted and analyzed by Western blotting to confirm the expression of the transgenes (anti-HA tag antibody against pGADT7-derived protein products and anti-Myc antibody against pGBKT7-derived protein products) (data not shown).

Yeast co-transformations were then performed according to manufacturer's protocol (Clontech, Matchmaker GAL4 Two-Hybrid System 3). Potential interacting proteins in either pGADT7 or pGBKT7 were simultaneously co-transformed into Y187 for colony lift filter assays to measure beta-galactosidase activity (Fig. 3C). As a positive control, we used yeasts co-transformed with p53 and TAg. As a negative control, we used yeasts co-transformed with lamin and TAg. Blue colonies were detected when Id-1 was co-expressed with either ITF-2A or ITF-2B. However, blue colonies were only detected when Id-2 was co-expressed with ITF-2B, but not with ITF-2A.

Discussion

The helix-loop-helix Id-1 and Id-2 proteins play an essential role in the regulation of breast epithelial cell differentiation and proliferation (2) and the coordination of the Id-1/Id-2 balance may be important for normal mammary gland development. Based on the data presented in this manuscript, we propose that the effects of both Id proteins on mammary epithelial cell phenotypes are mediated through their interaction with the two splice variants of ITF-2. A model for mammary epithelial cell growth and differentiation during pregnancy is presented in Figure 4.

The initial down-regulation of Id-1 would lead to the release of ITF-2A. ITF-2A as a homodimer, or as a heterodimer with another bHLH (another E-protein), would then induce the up-regulation of Id-2 gene expression, leading to the sequestration of ITF-2B by Id-2 and the release of a putative tissue-specific (TS) bHLH. We propose that, as in the muscle, ITF-2B inhibits the TS bHLH activity by forming an inactive heterodimer. After its release from ITF-2B, this TS bHLH then dimerizes with ITF-2A, and consequently activates the transcription of genes involved in the functional and morphological differentiation of mammary epithelial cells during mammary gland development.

The Id-2 gene contains several E-boxes within its promoter, and bHLH factors regulate Id-2 expression in F9 cells (14). Since Id-2 also appears to be involved in the normal process of differentiation and the expression of Id-2 is up-regulated when Id-1 is absent, Id-1 could be the inhibitor of Id-2 gene expression through interaction with a specific bHLH such as ITF-2A. Therefore, Id-1, Id-2 and ITF-2 proteins are acting within a network governing mammary epithelial cell phenotypes and potentially acting as molecular switches from proliferation towards differentiation.

ITF2-A and ITF-2B are ubiquitously expressed basic helix-loop-helix transcription factors that belong to the E-box protein family. Even if we confirmed their respective interactions with Id-1 and Id-2, these bHLH factors may only represent minor players in the HLH network regulating breast epithelial cell phenotypes. We have not been successful in isolating what could be the major player in this network, i.e., a breast tissue specific bHLH equivalent to MyoD. In collaboration with another group (15), we recently determined that the bHLH

transcription factor Mist1 was expressed in mouse mammary epithelial cells. Mist1 protein is present only in cell types that are secretory in nature. We found that Mist1 expression was up-regulated when mammary epithelial cells differentiated upon treatment with extracellular matrix and lactogenic hormones. We concluded that Mist1 could be involved in the HLH network regulating mammary epithelial cell phenotypes. However, we were not able to determine a specific interaction of Mist1 with either ITF-2A or ITF-2B (unpublished data), suggesting that the involvement of Mist1 in the regulation of differentiation and milk production may happen in an independent manner from ITF-2 proteins.

Since Id proteins exert their function through the sequestration of bHLH factors, Id-interacting bHLH proteins may play an important role in mammary gland development and breast cancer. We suggest that Id-1 and Id-2, as well as their interacting bHLH partners, determine the fate of normal mammary epithelial cells and that coordination amongst these HLH proteins is disrupted in aggressive breast cancer. Thus, studies in normal mammary gland development will give valuable information for cancer progression and for the identification of key targets for cancer therapy as well as markers for tumor progression.

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Abbreviations

ECM	extracellular matrix
MEC	mammary epithelial cell
HLH	helix-loop-helix proteins
bHLH	basic helix-loop-helix proteins
Id	inhibitor of DNA binding

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Figure 1.

Id-1, K8/18, K14 and SMA expression detected by immunohistochemistry. Panels A-F show mammary gland sections from a 5-day pregnant mouse. In panels A and C we used an antibody specific for Id-1, in panel D an antibody against K8/18, in panel E an antibody against K14 and in panel F an antibody against SMA. In panel B, section was incubated with a mixture of Id-1 blocking peptide and anti-Id-1 antibody.

Figure 2. Id-1 mRNA expression detected by *in situ* hybridization. Panels A and B show mammary gland sections from a 5-day pregnant mouse. In panel A we used an antisense Id-1 probe whereas in panel B we used a sense probe.

Figure 3.

Id and ITF-2 expression during pregnancy. Panel A shows β -casein, Id-1 and Id-2 mRNA expression using total RNA extracted from early (5d), middle (12d) and late (18d) pregnancy. Panel B shows ITF-2A, ITF-2B and GAPDH mRNA expression in the same mammary gland extracts using RT-PCR. Panel C shows interactions between Id and ITF-2 proteins using the yeast two-hybrid technique.

Figure 4.
Model representing a potential link between Id-1 down-regulation and Id-2 up-regulation through interactions with ITF-2 proteins during different stages of pregnancy.