

## Molecular Cloning and Characterization of a Zinc Finger Protein Involved in Id-1-stimulated Mammary Epithelial Cell Growth\*

Received for publication, August 1, 2000, and in revised form, January 8, 2001  
Published, JBC Papers in Press, January 16, 2001, DOI 10.1074/jbc.M006931200

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**Id proteins are dominant negative regulators of basic helix-loop-helix transcription factors. Previous work in our laboratory has shown that constitutive expression of Id-1 in SCp2 mouse mammary epithelial cells inhibits their differentiation and induces proliferation, invasion, and migration. Id-1 expression also correlates with the invasive and aggressive potential of human breast cancer cells. However, little is known about Id-1 target genes that are important for regulating normal and transformed breast epithelial cell phenotypes. Now we report the cloning of a novel zinc finger protein, Zfp289, using degenerate primers to specifically amplify cDNAs from Id-1-transfected SCp2 cells. Zfp289 has homology with a yeast zinc finger protein, the GTPase-activating protein Gcs-1, which was initially identified as a gene required for the re-entry of cells into the cell cycle after stationary phase growth. Zfp289 mRNA expression pattern correlates with Id-1 expression in SCp2 mammary epithelial cells under various experimental conditions as well as in the mouse mammary gland at different stages of development. It is predominantly present in the cytoplasm of the cells as evident from green fluorescent protein fusion protein localization. SCp2 mammary epithelial cells with constitutive expression of Zfp289 have a higher S-phase index, compared with control cells, when cultured in a serum-free medium. We conclude that the novel zinc finger protein Zfp289, which may represent the mammalian homologue of Gcs-1, is potentially an important mediator of the Id-1-induced proliferation pathway in mammary epithelial cells.**

Basic helix-loop-helix (bHLH)<sup>1</sup> factors are transcription factors that bind DNA as homo- or heterodimers and regulate transcription of target genes containing E-boxes (CANNTG) or E-box-like sequences in their promoters. Dimerization occurs

through interactions of the HLH domains, while binding to DNA is mediated by the basic domain. These factors have been shown to regulate the expression of tissue-specific genes in a number of mammalian and nonmammalian organisms (1).

Id proteins (for “inhibitors of differentiation or DNA binding”) are dominant negative regulators of the bHLH transcription factors. Id proteins contain an HLH domain, allowing them to form dimers with bHLH proteins, but they lack the basic domain, and therefore such dimers, Id/bHLH, do not bind DNA (2). Therefore, Id proteins do not regulate transcription directly, but indirectly, by preventing bHLH proteins from interacting with the promoter of various target genes. The role of Id proteins in the tissue-specific regulation of growth and differentiation has been examined in several systems. For example, Id-1 has been found to inhibit differentiation in myoblast (2), trophoblast (3), erythroid (4), B-lymphocyte (5, 6), and myeloid cells (7).

Previous studies in our laboratory have shown that constitutive expression of Id-1 results in the inhibition of differentiation of SCp2 mouse mammary epithelial cells (8). It also induces proliferation, invasion, and migration of the same cells (9) and increased secretion of a 120-kDa matrix metalloproteinase, the level of which correlates well with the invasive ability of these cells. In addition, Id-1 is highly expressed in aggressive and invasive human breast cancer cell lines as compared with noninvasive cell lines (9) and in biopsies from invasive ductal carcinomas as compared with ductal carcinomas *in situ* (10).

Investigations have shown that Id-1 is a positive regulator of G<sub>1</sub>-S phase transition during cell cycle progression and is also involved in inducing apoptosis (11–16). A recent report demonstrated that Id-1 and Id-3 might also control angiogenesis by regulating the growth and invasion of endothelial cells (17). However, little is known about Id target genes, which are important for regulating growth, differentiation, invasion, and apoptosis of normal and transformed mammary epithelial cells.

In this paper, we report the cloning of a novel Id-1-induced zinc finger protein, Zfp289, which is predominantly localized in the perinuclear compartment of the cells and which appears to function as a GTPase-activating protein (GAP). Zfp289 expression is correlated with the proliferative stages of mammary epithelial cells in culture and during mammary gland development, and this novel zinc finger protein is able to induce higher S-phase entrance when constitutively expressed in epithelial cells.

### MATERIALS AND METHODS

**cDNA Cloning of Zfp289**—We used PCR amplification to isolate genes specifically regulated by Id-1, as indicated by their up-regulation in SCp2 cells transfected with Id-1. Our rationale for selecting the degenerate primers was that we previously demonstrated a novel matrix metalloproteinase family member to be up-regulated in SCp2 cells transfected with Id-1 (9). Since we were particularly interested in cloning this novel metalloproteinase, and since most of the known metalloproteinases have a “Cys” motif and a “zinc” binding motif in

\* This work was supported by Postdoctoral Fellowship 5FB-0112 from the University of California Breast Cancer Research Program (to J. S.), by a fellowship from Fondazione Bonino-Pulejo (Italy) (to S. P.), by Grant 31B-0123 from the University of California Breast Cancer Research Program, and by the National Institutes of Health-NCI Grant RO1 CA82548 (to P.-Y. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF229439.

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<sup>1</sup> The abbreviations used are: bHLH, basic helix-loop-helix; GAP, GTPase-activating protein; PCR, polymerase chain reaction; kb, kilobase(s); GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; ARF, ADP-ribosylation factor; ANOVA, analysis of variance.

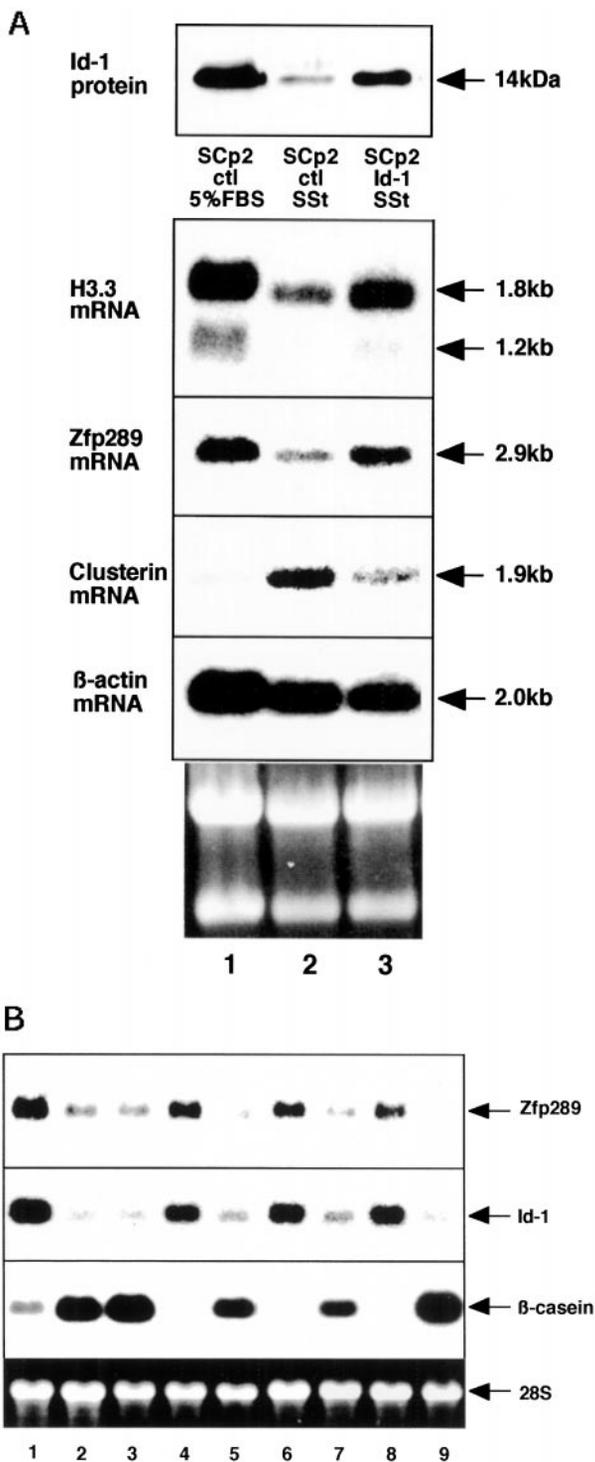


FIG. 1. A, correlation between Id-1 expression and expression of two other genes. Cells were cultured under different conditions, and RNA extraction and Northern analysis were performed as described under "Materials and Methods." Lane 1, SCp2 control cells (pool population) growing in 5% fetal bovine serum; lane 2, SCp2 control cells (pool population) in serum-free medium for 2 days; lane 3, Id-1 sense-transfected SCp2 cells (pool population) in serum-free medium for 2 days. B, Northern blot analysis of Zfp289, Id-1, and  $\beta$ -casein comparing nine different clones expressing variable amounts of Id-1. These clones were isolated from a pool population of Id-1 antisense transfected SCp2 cells.

their sequence, we designed degenerate primers against two regions of interest, one containing a cysteine residue Cys (PRCGXPD), the other a catalytic domain binding zinc ions (VAAHEFGHALGLH). Cys and zinc sequences were as followed: 5'-S(c/g)GR(g/a) TGT GGY(c/t) S(c/g)W (a/t)R(g/a) CCN(a/c/g/t) GA-3' and 5'-GCR(g/a) TGS(g/c) CCV(a/c/g) AAY(c/t) TCR(g/a) TGS(c/g) GC-3'.

Total RNA was isolated from SCp2-Id-1-transfected cells and SCp2 control cells, and cDNA was prepared. Specific AP-1 adaptors (Marathon cDNA amplification kit) were ligated at both ends of the cDNAs, and a first round of PCR amplification was performed using adaptor-specific primers on one side and zinc primers on the other. A second round of amplification was performed using the PCR product from the first round and using Cys and zinc primers. Only two amplified products of 0.8 and 2.6 kb were visible. We used annealing temperatures of 45 and 50 °C to obtain sharp bands at 0.8 and 2.6 kb, respectively. Both of these bands were extracted and cloned into a TOPO vector for sequencing.

**Cell Culture**—SCp2 mouse mammary epithelial cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (Dulbecco's modified Eagle's medium-F-12) containing 5% heat-inactivated fetal bovine serum and insulin (5  $\mu$ g/ml), at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere, as described previously (18). For experiments in serum-starved conditions, fetal bovine serum was omitted. Pool populations of SCp2 cells transfected with an empty vector or with the murine Id-1 sense cDNA driven by the mouse mammary tumor virus promoter were as described previously (8). Single cell-derived clones from SCp2 cells transfected with an Id-1 antisense cDNA (8) were derived by plating cells at limiting dilutions in 24-well plates.

**Plasmids Construction and Transfection**—The Zfp289 encoding region including the Kozak sequence was amplified from the BamHI site at the 5'-end, to either BamHI (for LXSN vector) or SalI site (for pBabe vector) at the 3'-end. The restriction digested fragments were then cloned into appropriate sites of LXSN and pBabe vectors. These viral vectors were then packaged in TSA-54 cells (Cell Genesis, Foster City, CA). After infecting the SCp2 cells with control or Zfp289 vectors, stably transfected cells were selected with neomycin and puromycin (for LXSN and pBabe vectors, respectively).

For intracellular localization studies, the full-length coding sequence of Zfp289 was cloned into a pEGFP vector (CLONTECH) between the SalI and BamHI sites. The pEGFP vector was transfected using Superfect transfection reagent (Qiagen). Neomycin-resistant cells were subcultured, and localization of the GFP fusion protein was determined under inverted fluorescent microscopy.

**RNA Isolation and Northern Analysis**—Total cellular RNA was isolated and purified as described by Chomczynski and Sacchi (19). RNA (15  $\mu$ g) was size-fractionated by electrophoresis through denaturing formaldehyde-agarose gels and transferred to Nylon membrane (Hybond-N from Amersham Pharmacia Biotech). The blots were hybridized with <sup>32</sup>P-labeled probes prepared by random oligonucleotide priming, washed, and exposed to Kodak XAR-5 film for autoradiography (20). The multiple tissue Northern blot was purchased from CLONTECH and probed as above. The Zfp289 probe was the PCR-amplified 2.6-kb fragment described under "cDNA Cloning of Zfp289," whereas  $\beta$ -casein and Id-1 probes were as described previously (8). The clusterin probe was obtained by subtractive hybridization,<sup>2</sup> and the  $\beta$ -actin probe was obtained from CLONTECH.

**Preparation of Mammary Gland RNA**—BALB/c wild type virgin female mice were purchased from Simonsen Laboratories, Inc. (Gilroy, CA) and some were mated at the age of 12 weeks. The animals were sacrificed, and biopsies of the fourth inguinal mammary gland were performed at 3, 7, and 12 weeks of age for the virgin stage; at days 2 and 12 of pregnancy; and at days 2, 7, 20, and 21 of lactation. Mammary glands were immediately frozen at -70 °C until utilized for total RNA isolation. Total RNA was isolated using TriPure Isolation reagent (Roche Molecular Biochemicals).

**DNA Synthesis and Autoradiography**—Cells (10<sup>4</sup> or 5  $\times$  10<sup>4</sup>) plated on coverslips were labeled with [<sup>3</sup>H]methylthymidine (10  $\mu$ Ci/ml; 60–70 Ci/mmol) for at least 7 h, washed twice with phosphate-buffered saline, then fixed for 5 min with 1:1 (v/v) mixture of acetone and methanol kept at -20 °C. Nuclei were stained with 4,6-diamidino-2-phenylindole-diluted 1:10,000 in phosphate-buffered saline for 2 min. The coverslips were air-dried, coated with Kodak NTB2 emulsion (1:2 dilution), and exposed for 16–24 h. The coverslips were developed with D19, fixed with Rapid-fix, and viewed by phase contrast microscope.

**Purification of Zfp289 and ARF-1**—The coding region of Zfp289 was amplified by PCR and then cloned into a bacterial expression vector pTrcHis A (Invitrogen). The 6 $\times$ His-tagged protein was expressed in the *Escherichia coli* strain DH5 $\alpha$ . After inducing protein expression for 5 h with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside, bacteria were pelleted and lysed under denaturing conditions in buffer containing 6 M guanidine hydrochloride, 10 mM Tris-HCl, 100 mM Na<sub>2</sub>PO<sub>4</sub>, pH 8.0, for 1 h at

<sup>2</sup> J. Singh and P. Y. Desprez, unpublished data.

## Novel Id-1-induced Zinc Finger Protein

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1 CGCGGTTGTGTCTGGAAGGAGAGAAAA ATG GCG GCG AGC CCG AGC AAG ACC GAA ATC CAG
M A A S P S K T E I Q
61 ACT ATT TTT AAG AGA CTT CGC GCA ATT CCC ACC AAC AAG GCC TGT TTC GAT TGT GGC GCC
T I F K R L R A I P T N K A C F D C G A
121 AAG AGT CCT AGT TGG GCC AGC ATC ACT TAC GGT GTA TTT TTG TGT ATT GAC TGC TCC GGA
K S P S W A S I T Y G V F L C I D C S G
181 GTG CAT CGC TCC TTG GGT GTC CAT CTC AGC TTT ATC AGG TCC ACA GAG TTG GAT TCG AAC
V H R S L G V H L S F I R S T E L D S N
241 TGG AGC TGG TTG CAG CTG AGG TGT ATG CAG GTC GGC GGG AAT GCC AAT GCG ACT GCT TTT
W S W L Q L R C M Q V G G N A N A N A T F
301 TTC CGC CAA CAC GGA TGT ATG GCC AAT GAT GCT AAC ACC AAA TAT ACG AGC CGA GCT GCC
F R Q H G C M A N D A N T K Y T S R A A
361 CAG ATG TAC CGA GAG AAG ATC CCG CAG TTA GGG AGT GCA GCT CTG ACT AGG CAT GGT ACT
Q M Y R E K I R Q L G S A A L T R H G T
421 GAT CTT TGG ATA GAC AGT ATG AAC AGT GCT CCA AGT CAC TCT CCA GAG AAG AAA GAC TCT
D L W I D S M N S A P S H S P E K K D S
481 GAT TTC TTC ACA GAA CAT ACT CAG GCC CCT GCT TGG GAT ACA GCA GCC ACT GAT CCT TCA
D F F T E H T Q A P A W D T A A T D P S
541 GGG ACT CAG CAA CCA GCC CTA CCT TCA GAG AGC AGT AGC CTG GCA CAA CCT GAA CAA GGC
G T Q Q P A L P S E S S S L A Q P E Q G
601 CCT AAC ACT GAT CTG CTC GGA ACC TCA CCC CAA GCC TCT CTG GAA CTG AAA AGC TCC ATT
P N T D L L G T S P Q A S L E L K S S I
661 ATT GGC AAG AAG AAG CCA GCA GCC GCT AAG AAA GGG CTG GGT GCC AAG AAA GGC CTG GGA
I G K K K P A A A K K G L G A K K G L G
721 GCT CAG AAG GTG AGC AAC CAG AGC TTT ACT GAG ATT GAG CGG CAG GCC CAG GTG GCA GAG
A Q K V S N Q S F T E I E R Q A Q V A E
781 AAG CTC CGA GAG CAG CAG GCA GCT GAT GCC AAG AAG CAG GCA GAA GAG TCC ATG GTT GCC
K L R E Q Q A A D A K K Q A E E S M V A
841 TCC ATG CGT CTG GCC TAC CAG GAG CTC CAG ATT GAT CGC AAG AAG GAG GAG AAA AAG CTC
S M R L A Y Q E L Q I D R K K E E K K L
901 CAG AAT CTT GAA GGA AAG AAG CGA GAG CAG GCT GAA AGG CTG GGC ATG GGC TTG GTG TCC
Q N L E G K K R E Q A E R L G M G L V S
961 CGA AGC TCC ATC TCC CAT TCT GTG CTG TCT GAG ATG CAG ATG ATT GAG CAG GAA ACC CCG
R S S I S H S V L S E M Q M I E Q E T P
1021 CTG AGT GCG AAA TCT TCC CGT TCA CAG CTT GAC TTG TTT GAT GAT GTT GGA ACA TTT GCA
L S A K S S R S Q L D L F D D V G T F A
1081 TCT GGA CCC CCA AAG TAC AAG GAC AAC CCC TTT TCC TTG GGA GAA ACT TTT GGT TCC CGC
S G P P K Y K D N P F S L G E T F G S R
1141 TGG GAT TCA GAT GCA GCC TGG GGT ATG GAC AGG GTA GAA GAG AAG GAA CCT GAA GTT ACC
W D S D A A W G M D R V E E K E P E V T
1201 ATC TCC AGC ATC CGG CCT ATT TCA GAA AGA ACT GCG AGT CCG AGG GAA GTG GAG ACT CGG
I S S I R P I S E R T A S R R E V E T R
1261 AGC TCA GGC CTT GAG TCC AGT GAA GCA CCG CAG AAG TTT GCA GGA GCC AAA GCC ATC TCG
S S G L E S S E A R Q K F A G A K A I S
1321 TCC GAC ATG TTC TTT GGT CGT GAG GTG GAT TCT GAG TAT GAA GCT AGG TCT CGG TCA CAG
S D M F F G R E V D S E Y E A R S R L Q
1381 CAG CTT TCG GGC AGC AGT GCC ATC AGC TCT TCA GAC CTT TTT GGA GAC ATG GAT GGA GCT
Q L S G S S A I S S S D L F G D M D G A
1441 CAT GGA GGA GGA ACT GTA TCT CTG GGG AAT GTA CTT CCT ACA GCT GAC ATT GCC CAG TTT
H G G G T V S L G N V L P T A D I A Q F
1501 AAG CAG GGT GTC AAG TCT GTG GCT GGC AAG ATG GCT GTG CTG GCC AAT GGT GTG ATG AAT
K Q G V K S V A G K M A V L A N G V M N
1561 TCC TTA CAG GAT CGC TAC GGT TCC TAC TGATCCAGGCTCCATGGCTCAGGCCTGTGGTGACGACAGCAAG
S L Q D R Y G S Y
1631 AACCTGAGTCCCAGGCTGGAGATGCCGGTCTTGTGGGCTGCGGGAGTTGTTGACTTTGTGTGTGGTGTGAGTGG
1711 ATGACTTGGAGGATCTTGGAGCGGGGAATGGTCGGCATCAGCCCTTGTCTCAGCCTGTGATCGAGTCCTTGCCTCA
1791 GCCCTCACACTCCCTCCATGCTAGTGTCTTCTCCAGTCTCCGCCAAAGCTGTCTGCTGTGGCCTGCTGCACTGG
1871 TACTGAGGGGGAGCTCCATCAGGATGGCTTTATGGATCTAATTTCTCCCAAGGAGGGGAGGACACAAGGCTCTCGAG
1951 CTCTAGGATCCTTGTAGGTGGTCTGGGGCTTGGGGCTCCACCTTCTGGAACAGGGGAGGCTAGCAAGAGTTGTGCTG
2031 TATCAGCCCTCAGGGAGAAGTTCTGGACTCGTCACTGTGTTCTGTCAGTGGCCTCTCTGGGTTCCAGGAAAGGTTA
2111 CCGAAGGATCCTGTAGCTACCAAGAGCTAGAATTTTGTGGTACCCAAAGGTTTATTTGGGATAGCCCGGAGG
2191 GGTAGCACTCTTCAATTTGACAGCTGTCCCTTGAAGGCTGACTTCTGCTAGTGTTTGAGAGCCTTTATCTACCAAT
2271 ACTGTGGGTCAAGCGCCCTGTCTATGCTTGAAGTCTCTTGTGTCTCTTGGCCCTATGGTGTCTCATCTGTG
2351 TGTCTGCTGGATCTGTGAACCTGTTAGCTGAGGAAGCTAAGACTGAGAGAAAGCAGCCCTGGAGCTCAGCTTTTATC
2431 ATCTGCATCATCTGGTTTGGGGTCTGGACAGCACCCCAATCAACAGTGAAGAGTGTGAGGGCTGACGCTGACGGCTC
2511 CACCCACTGGCAGCTGGGCCAGCACTGTGTCCTTCTTGGTGCCATCTCACTGCCACTGGCAGTCTGGCCCACTC
2591 TACCTGTGGATTGCAAGTGGGGCTCCCAAAACAACAGACCACTCTCCCTCATCCCTCCCAAGGGGACATGACTTTCT
2671 TTCAGCACTGTTTGTATTGAAACAAGTGGTCAAAATAAGGCCCAAGGGCCCTGTGGGTCAAAAAAAAAAAAAA
2751 AA

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FIG. 2. Nucleotide sequence and deduced amino acid sequence of murine Zfp289. The N-terminal zinc finger domain is underlined, and the putative polyadenylation signal is shaded.

room temperature with continuous shaking. Cellular debris was then pelleted by centrifugation of cell lysate at  $10,000 \times g$  for 30 min at room temperature. The supernatant was mixed with nickel-nitrilotriacetic acid beads (Qiagen) and stirred for 1 h at room temperature. The beads were then transferred to a column and sequentially washed with Buffer

1 (6 M urea, 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0) and Buffer 2 (20 mM Tris-HCl, 0.15 M NaCl, pH 8.0) to remove the nonspecific proteins and to renature the nickel-nitrilotriacetic acid-bound proteins (21). The  $6 \times$ His-tagged ZFP-289 protein was eluted with Buffer 2 containing 50 mM EDTA. The eluted protein was concentrated by using centricon

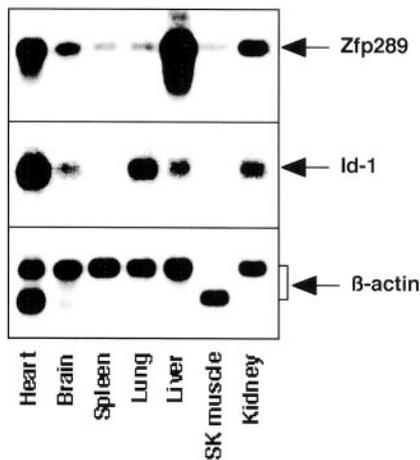


FIG. 3. Tissue distribution of Zfp289 and Id-1 in multiple tissue Northern blot of mice.  $\beta$ -Actin was used as an internal control.

column, and the purity of the sample (75–80%) was determined by SDS-polyacrylamide gel electrophoresis. Full-length coding region of mouse ARF-1 (ADP-ribosylation factor-1) was PCR-amplified and cloned into the bacterial expression vector pTrcHis A. After induction of recombinant ARF-1 for 5 h with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside, the bacteria were lysed and supernatant mixed with nickel-nitrilotriacetic acid beads as described above. The beads were transferred to a column and washed with Buffer 3 (8 M urea, 10 mM Tris-HCl, 0.1 M  $\text{Na}_2\text{PO}_4$ , pH 6.3). The bound 6 $\times$ His-tagged ARF-1 was eluted with Buffer 4 (8 M urea, 10 mM Tris-HCl, 0.1 M  $\text{Na}_2\text{PO}_4$ , pH 5.9) and then again with Buffer 4 containing 250 mM imidazole. The eluted ARF-1 was renatured by sequential dialysis against 4 M urea, 100 mM  $\text{Na}_2\text{PO}_4$ , 10 mM Tris-HCl, pH 8.0, then against 2 M urea 10 mM Tris-HCl, pH 8.0, and finally twice against 10 mM Tris-HCl, pH 8.0. The renatured ARF-1 was concentrated, and its purity (about 80–85%) was determined by SDS-polyacrylamide gel electrophoresis analysis.

**ARF-GAP Assay**—ARF-GAP activity was assayed by measuring the effects of the putative GTPase-activating protein (Zfp289) on the hydrolysis of ARF-bound GTP to GDP, with some modifications of the assay previously described by Goldberg (22). Briefly, ARF-1 was incubated with [ $\gamma$ - $^{32}\text{P}$ ]GTP for 30 min in binding buffer (20 mM Tris-HCl, 5 mM  $\text{MgCl}_2$ , 0.1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1  $\mu\text{g}/\text{ml}$  bovine serum albumin, pH 7.5) containing 0.3 mM [ $\gamma$ - $^{32}\text{P}$ ]GTP. ARF ([ $\gamma$ - $^{32}\text{P}$ ]GTP) was then diluted 10 times with binding buffer. GTPase assay was done by incubating GTP-bound ARF-1 with or without Zfp289 at room temperature for 20 min in a 25- $\mu\text{l}$  reaction mixture. After incubation, the samples were spotted on nitrocellulose membrane, washed four times with Tris buffer saline, and radioactivity assayed by scintillation counting. The potential GTPase-activating protein activity of Zfp289 was determined as the decrease of label associated with ARF-1.

## RESULTS

**Isolation of Two Id-1-inducible Genes in SCp2 Cells**—Using degenerate primers as described under "Materials and Methods," we isolated two cDNA clones (0.8 and 2.6 kb) preferentially up-regulated in SCp2-Id-1-transfected cells. A partial sequencing of the 0.8-kb band revealed that it corresponded to a known gene encoding histone H3.3. Since we only performed a partial sequencing of this clone and did not analyze it further, we could not ascertain the presence of the zinc and Cys motifs. Using this cDNA as a probe, two mRNAs of 1.2 and 1.8 kb were detected in SCp2 cells cultured in 5% serum (Fig. 1A, lane 1) and in SCp2-Id-1 serum-starved for 2 days (Fig. 1A, lane 3).

The other 2.6-kb band corresponded to a gene encoding a mRNA of about 2.9 kb. We found a higher expression of this 2.9-kb transcript in serum-starved Id-1-transfected cells (Fig. 1A, lane 3) as compared with control cells, which were also serum-starved for 2 days and which showed a reduced amount of Id-1 protein (Fig. 1A, lane 2).

We detected a high level of expression of this 2.9-kb transcript in control SCp2 cells cultured in the presence of 5%

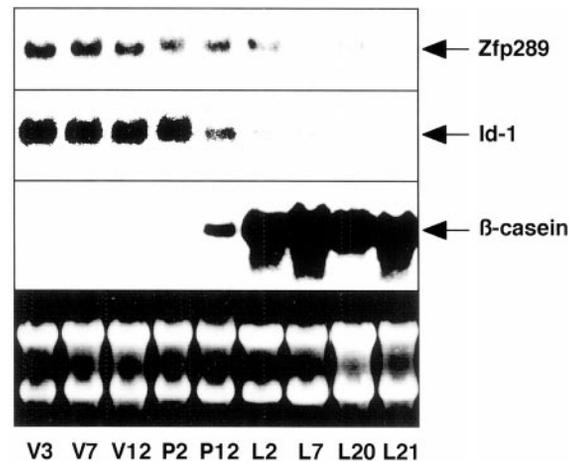


FIG. 4. Northern blot analysis of Zfp289, Id-1, and  $\beta$ -casein during mammary gland development in mice. V3, V7, and V12: glands from virgin 3-, 7-, and 12-week-old mice, respectively; P2 and P12, glands from 2- and 12-day pregnant mice, respectively; L2, L7, L20, and L21: glands from 2-, 7-, 20-, and 21-day lactating mice, respectively.

serum (Fig. 1A, lane 1). This level of expression was not further increased in Id-1-transfected SCp2 cells also cultured in 5% serum (data not shown). This may be due to the large amount of endogenous Id-1 proteins, up-regulated by serum in control cells, which may be sufficient to interact with all bHLH proteins present. The level of expression of target genes, such as the one encoding the 2.9-kb transcript, may then correspond to a maximum.

As a control of the loading, we used ethidium bromide staining of ribosomal RNA as well as  $\beta$ -actin, which did not show a difference of expression at the mRNA level between control and Id-1-transfected cells in serum-starved conditions for 2 days. However, mRNA levels of another gene, clusterin, a glycoprotein involved in cell-cell interaction, were down-regulated in the presence of Id-1.

To establish further that Id-1 up-regulates expression of Zfp289, we analyzed mRNA levels of both genes in nine different clones from SCp2 cells transfected with Id-1 antisense vectors (8) and treated with growth factors, lactogenic hormones, and extracellular matrix. As shown in Fig. 1B, these clones expressed variable amounts of Id-1. In each of these clones, the levels of Zfp289 strongly correlated with that of Id-1. Four clones (lanes 1, 4, 6, and 8) expressed high levels of Id-1 and Zfp289, whereas in five clones (lanes 2, 3, 5, 7, and 9) Id-1 expression was considerably reduced, and therefore Zfp289 mRNA levels were significantly down-regulated. As described previously (8, 9), the expression of  $\beta$ -casein (a differentiation-related gene) was inversely correlated with that of Id-1.

**Sequence Analysis**—The nucleotide sequence analysis of the 2.6-kb fragment (plus the sequence of overlapping expressed sequence tags at the 5'- and 3'-ends) revealed an open reading frame of 1560 base pairs, encoding a 519-amino acid polypeptide with a predicted molecular mass of about 57 kDa (Fig. 2). We could clearly detect a sequence on this cDNA that was homologous to the zinc degenerate primer. We attempted to determine any sequence homology to the Cys motif, but due to the level of degeneracy of this Cys primer, we were not able to locate any region of clear homology. A search of the protein data base revealed that this predicted 57-kDa protein was a unique sequence, having, however, a 48% homology and 32% identity with the yeast zinc finger protein Gcs-1 (23). This putative mouse protein, which we called Zfp289 after submission to the nomenclature committee, has one zinc finger domain at the N terminus, with a CxxC<sub>(16)</sub>CxxC motif encompassing 26–49 amino

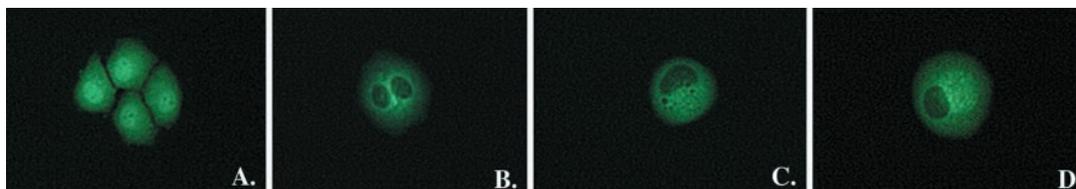


FIG. 5. **Cellular localization of Zfp289.** SCp2 cells were transfected with an empty GFP plasmid (A) or with a GFP plasmid containing the Zfp289 coding region (B, C, and D) and analyzed using fluorescent microscope.

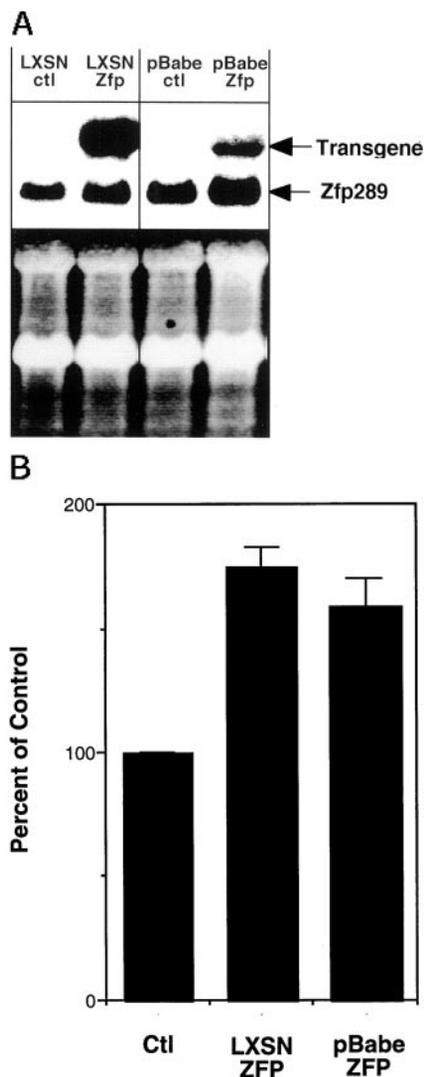


FIG. 6. **A**, Northern blot analysis showing expression of Zfp289 transgene in SCp2 mammary epithelial cells. **B**, thymidine incorporation in SCp2 cells transfected with either control plasmid (LXSN or pBabe) or plasmid containing full-length coding region of Zfp289 gene. Data represent the average of four independent experiments and is presented as percentage of control. One-way ANOVA comparing Zfp289-transfected cells with control cells was significantly different at  $p < 0.0001$ .

acids. This zinc finger domain has 66% homology with the zinc finger domain in the yeast protein Gcs-1.

**Tissue Distribution**—Northern blot analysis of Zfp289 revealed a predominantly 2.9-kb transcript in all the major murine tissues (Fig. 3), although the level of expression varied widely. Zfp289 mRNA was expressed at very high levels in liver, followed by heart and kidney. Skeletal muscle and spleen had the lowest levels of mRNA expression.

During mammary gland development in mice, Zfp289 mRNA expression pattern was closely correlated with Id-1 expression (Fig. 4). We detected high levels of expression of Zfp289 as well as Id-1 in the mammary gland from virgin (V) mice and during

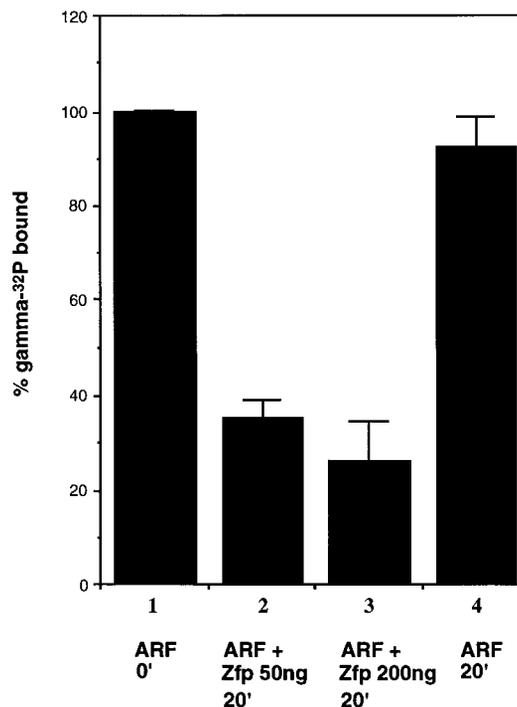


FIG. 7. **Analysis of the GTPase activity of ARF-1 in the presence of two different amounts of recombinant Zfp289 protein after 20-min incubation.** The data shown are from one of three independent experiments which showed similar differences. One-way ANOVA comparing lane 4 versus lane 1 was not statistically different ( $p = 0.074$ ), whereas one-way ANOVA comparing lane 2 (or lane 3) versus lane 4 was statistically different at  $p < 0.0001$ .

pregnancy (P) when there is extensive ductal cell proliferation and lobulo-alveolar development, respectively. The expression of both genes declined at the beginning of lactation (L) when the glands fully differentiate and express the milk product  $\beta$ -casein. Zfp289 and Id-1 were almost undetectable after day 2 of lactation until day 21.

**Cellular Localization**—Localization studies using EGFP vector-transfected SCp2 cells showed that GFP-Zfp289 fusion protein was predominantly present in the cytoplasm with a high proportion of cells showing perinuclear staining (Fig. 5, B, C, and D). These data suggest that Zfp289 is not a transcription factor, which is corroborated by the fact that it contains only one zinc finger domain and not several like typical transcription factors belonging to the zinc finger protein family. This also suggests a function in the regulation of exocytic and/or endocytic vesicular transport pathways at the periphery of the nuclei. The control EGFP plasmid-transfected cells showed homogenous nonspecific staining all over the cell cytoplasm as well as nucleus (Fig. 5A).

**Functional Analysis of Zfp289**—To investigate the functional role of Zfp289, we stably transfected the SCp2 mouse mammary epithelial cells with two different mammalian expression vectors (LXSN and pBabe) containing the full-length coding region of Zfp289. Northern blot analysis of total RNA from these cells confirmed the constitutive transcription of

transfected Zfp289, which displayed a larger size than the endogenous Zfp289 mRNA (Fig. 6A). In low serum conditions, constitutive expression of Zfp289 resulted in higher S-phase rate of mammary epithelial-transfected SCp2 cells as compared with control plasmid-transfected cells (Fig. 6B). The difference was more significant in the case of cells transfected with LXS<sub>N</sub> (170% of the control) than with pBabe (150% of the control). This may be due to a higher level of the transgene in the LXS<sub>N</sub> vector than in the pBabe vector.

Zfp289 does not appear to play a role in the invasive behavior of the cells. Even after 2 weeks on extracellular matrix, both control as well as Zfp289-transfected cells remained associated within compact spheres (data not shown). In contrast, constitutive expression of Id-1 was able to induce invasion in SCp2 cells (9). We conclude that Zfp289 may be a downstream gene under the control of the transcriptional regulators of the helix-loop-helix family. This novel zinc finger protein is potentially an important mediator of the Id-1-induced proliferation pathway, but not of Id-1-induced invasiveness and/or migration, in mammary epithelial cells.

*Zfp289 Appears to Be a GAP Protein*—We noted above the homology of sequence between Zfp289 and the yeast GAP Gcs-1. Since Zfp289 also contains the zinc finger motif shared by most of the GAP proteins, we examined whether Zfp289 was functionally a GTPase-activating protein. We analyzed the GTPase activity of ARF-1 in the presence of two different amounts of recombinant Zfp289 protein. ARF (ADP-ribosylation factor) proteins are 20-kDa guanine nucleotide-binding proteins that are active when GTP is bound. However, hydrolysis of bound GTP requires interaction with a GAP protein, as ARF itself has no detectable GTPase activity. Zfp289 displays a strong GAP activity as demonstrated by the decrease of label associated with ARF-1 (Fig. 7). This is particularly significant at 200 ng of Zfp289 and already detectable at 50 ng. Zfp289 may therefore correspond to the mammalian counterpart of the yeast GAP protein, Gcs-1.

#### DISCUSSION

We have previously reported that Id-1, a dominant negative regulator of bHLH transcription factors, is not only involved in the inhibition of differentiation, but also induces proliferation, migration, and invasion in SCp2 mouse mammary epithelial cells (8, 9). To learn more about its action, we sought here to identify transcripts up-regulated in cells transfected with Id-1. Of the two transcripts we identified, one was the histone H3.3. This is consistent with the up-regulation of H3.3 during the G<sub>0</sub> to S-phase transition in mouse kidney cells (24) and suggests that histone H3.3 is one of the mediators of Id-1-controlled proliferation.

Of more interest was the second up-regulated transcript, Zfp289, which encodes for a zinc finger protein with homology to the yeast zinc finger protein Gcs-1 and its related protein Glo-3. Gcs-1 was first identified because of its requirement for transition of yeast cells from stationary to proliferation phase (23). Reports have also shown that Gcs-1 protein is a GAP for the ARF (25) and is involved in regulation of vesicular trafficking and actin cytoskeleton network (26). Yeast cells containing a functionally mutant Gcs-1 gene were unable to transit from the stationary phase to growth phase (23) and exhibited vesicle trafficking defects at the nonpermissive 15 °C temperature (27).

Zfp289 seems to be evolutionarily a well conserved protein, with about 50% homology between mammalian and yeast sequences, suggesting a conservation of function as well. The GAP activity of Gcs-1 has been localized in the N-terminal zinc finger domain (28), and the Zfp289 sequence in this finger domain (CxxC<sub>(16)</sub>CxxC) is 66% homologous to that of yeast. We found that Zfp289 appears to have similar function, as it acti-

vates ARF-1-GTPase activity. The intracellular localization experiments in SCp2 cells confirmed that Zfp289 functions predominantly in the cytoplasm, and in the majority of the cells, Zfp289-GFP fusion proteins seemed to be concentrated around the perinuclear region.

When we compared mRNA expression of Zfp289 with that of Id-1 in the multiple tissue Northern blot, we found a direct correlation in five out of seven tissues (Fig. 3). The liver exhibited a relatively low level of Id-1 message compared with that of Zfp289, while in lung, Id-1 expression was quite high as compared with that of Zfp289. In these tissues, there may be additional or other types of controls than Id-1 for regulating Zfp289 gene expression. However, Zfp289 expression paralleled that of Id-1 during mammary gland development, with Zfp289 expressed during ductal (virgin) as well as lobuloalveolar (pregnant) morphogenesis, when there is extensive proliferation of mammary epithelial cells. Its down-regulation followed the decrease in Id-1 expression in differentiated, growth-arrested lactating epithelial cells.

The data presented in Fig. 1, A and B, provide indirect evidence that Zfp289 mRNA expression may be controlled by Id-1 levels. To establish this relationship directly, it will be necessary to sequence the Zfp289 promoter, to determine the presence of E-box motifs and to isolate the Id-1-interacting bHLH proteins, work now in progress. Nevertheless, this novel zinc finger protein Zfp289 appears to mediate some of the Id-1-dependent phenotypic effects on mouse mammary epithelial cells. Functional analysis using retroviral-mediated transfection in SCp2 cells indicates that it may be an important mediator of Id-1-dependent S-phase entrance (Fig. 6B), a conclusion consistent with the known function of the yeast protein. The increased expression of Zfp289 may confer an advantage in cell cycle entrance to Zfp289-transfected cells in comparison with control cells. This may represent an example of the involvement of ARF-GAP proteins in many fundamental cellular processes such as cell growth and survival, as well as vesicular trafficking and cytoskeletal organization.

Although we have no direct evidence that Zfp289 plays a role in migration or invasion of cells, this question should remain open. Besides the role of the yeast homologue Gcs-1 in the cytoskeletal organization, it has been found that members of the GTPase family (such as Rho and Rac) and their activators can regulate cell migration through their control of actin polymerization and cytoskeletal distribution (29). Our negative results obtained from invasion assays in Zfp289-transfected cells may be due to the fact that constitutive expression of Zfp289 alone is not sufficient to induce the invasive phenotype, an event that may also require the induction of some other genes, such as matrix metalloproteinases.

*Acknowledgments*—We thank Dr. Judith Campisi for her critical review of this manuscript and help in preparing the retrovirus, Dr. Andrew P. Smith for editing, Dr. Dan H. Moore for help with the statistical analysis, Dr. Ling-Chun Chen for advice on intracellular localization studies, Dr. Udo Greiser for advice on ARF-GAP assays, and Yoko Iritani for technical assistance.

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