

Glucocorticoid-Induced Impairment of Mammary Gland Involution Is Associated with STAT5 and STAT3 Signaling Modulation

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The mammary epithelium undergoes cyclical periods of cellular proliferation, differentiation, and regression. During lactation, the signal transducer and activator of transcription factor (STAT)-5A and the glucocorticoid receptor (GR) synergize to induce milk protein expression and also act as survival factors. During involution, STAT3 activation mediates epithelial cell apoptosis and mammary gland remodeling. It has been shown that the administration of glucocorticoids at weaning prevents epithelial cell death, probably by extracellular matrix breakdown prevention. Our results show that the synthetic glucocorticoid dexamethasone (DEX) modulates STAT5A and STAT3 signaling and inhibits apoptosis induction in postlactating mouse mammary glands, only when administered within the first 48 h upon cessation of suckling. DEX administration right after weaning delayed STAT5A inactivation and degradation, preserving gene expression of target genes as β -casein (*bcas*) and prolactin induced protein (*pip*). Weaning-triggered GR down-regulation is also delayed by the hormone treatment. Moreover, DEX administration delayed STAT3 activation and translocation into epithelial cells nuclei. In particular, DEX treatment impaired the increment in gene expression of signal transducer subunit *gp130*, normally up-regulated from lactation to involution and responsible for STAT3 activation. Therefore, the data shown herein indicate that glucocorticoids are able to modulate early involution by controlling the strong cross talk that GR, STAT5, and STAT3 pathways maintains in the mammary epithelium. (*Endocrinology* 151: 5730–5740, 2010)

Mammary gland development is a complex and highly regulated multistep process. First, from pregnancy to lactation, lobuloalveolar growth is followed by the complete differentiation of the mammary epithelium, which is involved in the production and secretion of milk proteins. Second, at weaning, a dramatic switch from survival to death signaling occurs, leading to mammary gland involution. This process mainly involves extensive tissue remodeling characterized by massive programmed cell death of epithelial cells and mammary remodeling to a prepregnant state (1–3). The switch between lactation and involution in the mammary gland requires the integration of different signaling pathways. Convergence among these signals occurs at several levels, the regulation of gene expression being one of the main

targets. Gene expression is tightly regulated by the activation of distinct transcription factors that may directly bind to DNA and/or interact with other transcriptional regulators. Unraveling the molecular pathways leading to the activation of complex biological processes remains one of the great challenges of molecular biology today.

Two families of inducible transcription factors have been found to play a key role in mediating different processes in mammary gland development and involution: the glucocorticoid receptor (GR), and the signal transducers and activators of transcription (STATs). GR mediates glucocorticoid activity: upon ligand binding to the receptor, the activated GR translocates to the nucleus and modu-

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Abbreviations: DAB, 3,3'-Diaminobenzidine; DEX, dexamethasone; GR, glucocorticoid receptor; JAK, Janus kinase; LIF, leukemia inhibitory factor; p, phosphorylated; pip, prolactin-inducible protein; PRL, prolactin; qRT-PCR, quantitative real-time RT-PCR; STATs, signal transducers and activators of transcription.

lates the expression of target genes by direct DNA binding as well as by protein-protein interaction (4). STAT proteins are latent cytoplasmic transcription factors that require phosphorylation by the Janus kinase (JAK) for activation. Activated STAT molecules dimerize, translocate to the nucleus, and regulate gene transcription through the binding to specific DNA elements (5). Interestingly, it has been reported that different STAT family members interact physically and functionally with GR in several tissues and cell lines (6–16). Cross talk between both families of transcription factors has been found in the regulation of cell proliferation, differentiation, and survival, in a tissue-specific manner. For example, it has been reported that GR and STAT5 factors play antagonistic effects on cell death in hippocampal cells (12), whereas STAT3 behaves as a GR coactivator in hepatocytes and B cells (10, 17). On the other hand, in the mammary gland, GR synergizes STAT5 lactogenic and survival activities (15), whereas STAT3 antagonizes these effects, determining the end of lactation and apoptosis induction of mammary secretory cells (18, 19).

During lactation, the successful differentiation of the secretory epithelium and milk production depend mainly on the action of prolactin (PRL) and glucocorticoids (20). The circulating PRL leads to activation of its receptor, resulting in tyrosine phosphorylation of STAT5A and STAT5B by JAK2 (21). These two members of the STAT family share more than 90% of their sequence identity and usually play redundant functions (22). However, it has been demonstrated that STAT5A is indispensable for mammary gland lactogenic differentiation, whereas STAT5B is not (23, 24). On the other hand, GR acts as a survival signal in the mammary gland (25) and participates in maintaining the secretory phenotype by inducing milk protein gene expression and milk secretion (26, 27). The prolactin-inducible protein (*pip*) is another PRL and GR target gene, which is expressed in normal exocrine glands and in benign and malignant breast tumors (28, 29). PIP has been described as an antiapoptotic factor and has been designated as a sensitive and specific marker for monitoring and defining apocrine differentiation in breast cancer (30).

During involution, two phases have been described according to their reversibility. The first phase, a reversible step lasting 48 h, starts immediately upon weaning (2, 31) and is mostly regulated by local factors (32, 33). The second phase begins between 48 and 72 h after weaning, when the descendent levels of circulating hormones induce massive apoptosis of epithelial cells and mammary gland remodeling (34). During the first stage of involution, milk stasis induces expression and secretion of the proinflammatory cytokine leukemia inhibitory factor (LIF) (35, 36). LIF binds to the specific LIF receptor, which forms a het-

erodimer by recruiting a specific subunit common to all members of that family of receptors, the glycoprotein 130 (Gp130) signal transducer subunit. This leads to activation of the JAK/STAT3 signaling pathway (35–37). It has been shown that LIF-dependent activation of STAT3 induces mammary epithelial cell death (35, 36). This process is accompanied by the rapid decline of STAT5 expression and activation (38, 39), suggesting a role for this transcription factor in preventing mammary gland involution (38). In fact, STAT5 directly protects cells from the STAT3-mediated death signals (40). In addition, STAT5-deficient mammary glands show elevated levels of apoptosis (41), whereas overexpression and activation of STAT5 delays cell death (42). Moreover, it has been shown that LIF treatment induces both, phospho-STAT3 increase and phospho-STAT5 decrease in mammary epithelial cells (43).

The present work investigates the effect of glucocorticoids in postlactating mouse mammary glands. It has been reported that glucocorticoid administration at weaning prevents mammary massive apoptosis and inhibits postlactational involution (44), probably by extracellular matrix breakdown prevention (45). However, the intracellular mechanisms involved in glucocorticoid-induced cell survival after weaning have not been fully determined. Is cross talk between GR and STAT families of transcription factors mediating the dramatic switch from survival to death signaling at weaning? Could the inhibition in mammary apoptosis generated by glucocorticoids be associated with modulation of STAT5 and STAT3 signaling pathways? The results presented in this study provide a novel insight into the molecular mechanisms modulating the switch between lactation and involution in mammary epithelial cells.

Materials and Methods

Animal and experimental treatments

BaLb/c mice were maintained in a pathogen-free, temperature-controlled environment on a 12-h light, 12-h dark cycle and given sterilized laboratory chow and water *ad libitum* in accordance with the standard international animal care protocols (Canadian Council of Animal Care's 1980 Guide to the Care and Use of Experimental Animals). Animal procedures, *i.e.* hormone injections and euthanasia described herein, were carried out at the Animal Facility of the School of Sciences of the University of Buenos Aires (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) following the guidelines provided by the University of Buenos Aires Institutional Animal Care and Use Committee, which oversees the university's animal care and use program and is responsible for reviewing all animal use protocols (U.S. Public Health Service Approved Animal Welfare Assurance: A5801-01). Mammary glands no. 4 were aseptically removed from the mice at lactation (7 d after delivery) and invo-

lution [24 h; 48 h and 72 h after weaning (pups were removed after 5–7 d of nursing)]. To assess the effect of glucocorticoids during involution, mice were injected sc with synthetic glucocorticoid dexamethasone (DEX; Sigma, St. Louis, MO) 0.5 mg per 100 g body weight or vehicle (1:2:32.5 ethanol-propyleneglycol-NaCl 0.9%) 0, 24, 48, and 72 h after weaning according to the experimental requirements.

DNA fragmentation

DNA from the mammary gland was obtained as previously described (46). Briefly, 200–300 mg of mammary tissue was homogenized in digestion buffer containing 100 mM NaCl, 4 mM EDTA, 50 mM Tris-HCl, and 0.5% sodium dodecyl sulfate (SDS; pH 8) supplemented with proteinase K (100 μ g/ml; Sigma) at 55 C for 4 h to facilitate membrane and protein disruption. After incubation, samples were cooled 30 min on ice in 1 M potassium acetate and 50% chloroform to initiate protein precipitation and centrifuged at 9000 \times g for 8 min at 4 C. Supernatants were then precipitated 30 min in 2.5 volumes of ethanol at –70 C and centrifuged 20 min at 5000 \times g at 4 C. Finally, the samples were extracted in 70% ethanol and resuspended in water. The DNA content was measured by reading the absorbance at 260 nm and incubated 1 h with ribonuclease (10 μ g/ml) at 37 C. DNA samples were electrophoretically separated on 2% agarose gels containing ethidium bromide (0.4 μ g/ml) and visualized in an UV transilluminator (DyNA light; Labnet, Edison, NJ) and photographed with a Kodak DC290 camera system (Rochester, NY).

Histology and immunohistochemical assays

For histological examination, glands were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 7 μ m, and routinely stained with hematoxylin-eosin for the histological analysis. For immunohistochemical assay, mammary tissue were deparaffinized with xylene, hydrated in a series of alcohols, and in Tris-buffered saline containing 0.1% Tween 20 (pH 7.4). For immunodetections, mammary sections were incubated 10 min in 10 mM sodium citrate buffer (pH 6.0) at a subboiling temperature. Endogenous peroxidase activity and nonspecific binding were blocked by incubation in 5% hydrogen peroxide for 5 min and in 1% BSA in phosphate buffer, 50 mM; ClNa, 0.9%; Triton X-100, 0.1% for 1 h at room temperature, respectively. Sections were incubated 24 h at 4 C with the following primary antibodies: antihuman cleaved caspase-9 (catalog no. 9507) and caspase-3 (catalog no. 9661) (Cell Signaling Technology, Inc., Beverly, MA); antimouse STAT5a (phosphor Y694) (ab 30648–100; Abcam, Cambridge, MA); and antimouse Stat3 (sc-482; Santa Cruz Biotechnology, Santa Cruz, CA). Then sections were subsequently incubated at room temperature, for 1 h each, with antirabbit or antimouse biotinylated antibody (Jackson ImmunoResearch, West Grove, PA) and streptavidin-peroxidase complex (catalog no. 43-8323; Zymed Laboratories, San Francisco, CA). Immunoreactivity was detected by exposure to 3,3'-diaminobenzidine (DAB; Dako Cytomation, Carpinteria, CA), and after the precipitation product had appeared, the reaction was stopped. Sections were counterstained with hematoxylin and mounted for conventional light microscopy. The specificity of the immunostaining was tested by omission the primary antiserum. Controls gave negligible background staining (data not shown).

Protein analysis

Total proteins were extracted from mammary tissue in RIPA buffer (50 mM Tris-HCl; 150 mM NaCl; 1% Triton X-100; 0.25% sodium deoxycholate; 1 mM EDTA, pH 7.4) supplemented with protease cocktail set I (Calbiochem, San Diego, CA) and phosphatase inhibitors (1 mM NaF, 1 mM Na₂VO₄). The lysate was centrifuged at 13,000 \times g and 4 C for 20 min and the pellet discarded. Cleared lysates were combined with SDS sample buffer (50 mM Tris, pH 6.8; 1% SDS; 0.1% bromophenol blue; 10% glycerol; 100 mM dithiothreitol). Protein concentration was determined by the method of Bradford (47) using BSA as a standard. For Western blot, samples were boiled for 5 min and electrophoresed for 3 h at 100 V in a 15 or 12%, SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electroblotting in transfer buffer containing 20% methanol (vol/vol), 0.19 M glycine, and 0.025 M Tris-base (pH 8.3) at 300 mA for 1.5 h at 0 C. Blots were blocked 1 h at room temperature in TBS (20 mM Tris-Cl, pH 7.5; 500 mM NaCl) containing low-fat 5% powdered milk and 0.1% Tween 20. The incubations with primary antibodies were performed at 4 C for 12 h in blocking buffer (3% skim milk, 0.1% Tween 20, in Tris-buffered saline). The membranes were then incubated with the corresponding counterantibody and the proteins evidenced by enhanced chemiluminescence detection (ECL+Plus system; GE Health Care, Little Chalfont, Buckinghamshire, UK). The following primary antibodies were used: GR (BuGR2 clone; Affinity Bioreagents, Golden, CO); antimouse STAT5a (phosphor Y694) (ab 30648–100; Abcam); antimouse STAT5A (sc-1081); antimouse STAT3 (sc-482); antimouse phosphorylated STAT3 (pSTAT3) (sc-8059); and α -actin (sc-1616-R; Santa Cruz Biotechnology). The following secondary antibodies were used; a peroxidase-labeled α -rabbit IgG and an α -mouse IgG (Bio-Rad). The immunoreactive protein bands were detected using the enhanced chemiluminescence system (ECL+Plus system; GE) and the FujiFilm ImageReader LAS-1000; Fuji Film, Tokyo, Japan). Densitometric analysis of protein levels was performed with ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, <http://rsb.info.nih.gov/ij/>). For band quantification, the obtained images were converted to gray scale, and equal areas encompassing each band were drawn. Then the integrated density in each rectangle was obtained and the background noise was subtracted for each band. In each case, the obtained value was normalized as indicated in each experiment.

RNA analysis

RNA was extracted from homogenized fragments of mammary glands with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For reverse transcription, 1 g of total RNA was used. The first cDNA strand was synthesized with 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI); 25 ng/liter random primers (Invitrogen); 2 g/liter Rnasin (Promega) and 1.5 mM deoxynucleotide triphosphates (Invitrogen). Retrotranscription was performed at 37 C for 60 min followed by 15 min at 72 C. For the quantitative real-time RT-PCR (qRT-PCR), an aliquot of 0.2 l cDNA was used. All reactions were conducted in a volume of 25 μ l containing 4 mM MgCl₂ (Invitrogen), 0.2 mM deoxynucleotide triphosphates (Invitrogen), 0.75U Taq polymerase (Invitrogen), 1:30,000 Sybr Green (Roche, Indianapolis, IN), and specific oligonucleotides for each gene (as shown below) in a DNA Engine Opticon instrument (MJ Research, Waltham, MA). The oligonucleotides used for each

gene: *b-cas*, 5'-TCCCACAAAACATCCAGCC-3' and 5'-ACGGAATGTTGTGGAGTGG-3'; *gr*, 5'-AGATTCGAAGCAGCAGAGG-3' and 5'-GGTAGCCCAAGTCATTCCC-3'; *lif*, 5'-GGCAACCTCATGAACCAGATCA-3' and 5'-GCAAAGCATTGCTGAGGAGG-3'; *>gp130*, 5'-ACCACCACTGCTC-CACCTACG-3' and 5'-ATGCCACCCTTCAACAAAACACC-3'; *pip*, 5'-TCTGGTGTCTGACTTCTCC-3' and 5'-TTTCCTGTGCTGTTGATGG-3'; and *actin*, 5'-GTGGGCCGCTCT-AGGCACCA-3' and 5'-CGGTTGCGCTTAGGGTTCAGG-GGGG-3'.

Reactions were run for 35 cycles under the following conditions: 15 s at 94 C, 20 sec at 58–65 C (according to the primers), and 25 sec at 72 C. The amplification of unique products in each reaction was verified by melting curve and ethidium bromide (Sigma Aldrich)-stained agarose gel electrophoresis. The expression level of each gene was normalized to *actin* expression level using standard curve method and specific primers (the oligonucleotide for *actin* is shown above). Means and SEs from at least three experiments were calculated and shown as fold changes respect to lactation group.

Statistical analysis

Results were expressed as means ± SE as it is indicated in the figure legends. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). Differences were regarded as significant at *P* < 0.05.

Results

DEX treatment delays STAT5A deactivation and maintains a mammary secretory epithelium

Activated STAT5A is indispensable for mammary gland lactogenic differentiation (23, 24); the effect of DEX administration on STAT5A activation levels was addressed. Figure 1A shows that DEX inhibited the abrupt decrease of pSTAT5A levels observed in the involuting glands at 48 h. In fact, the ratio between pSTAT5A and total STAT5A determined in mammary glands from DEX-treated animals at that time, was similar to the observed in nursing females. However, no differences in this ratio were observed at 72 h, when comparing involuting glands from control and DEX-treated animals. Similar results were obtained with total STAT5A expression levels: the dramatic decrease observed in involuting glands was delayed by DEX treatment. In agreement with these observations, immunostaining assays showed that pSTAT5A was localized in nuclei of mammary epithelium from lactating and DEX-treated (48 h) mice (Fig. 1B and Supplemental Fig. 1, left and right panels, published on The Endocrine Society's

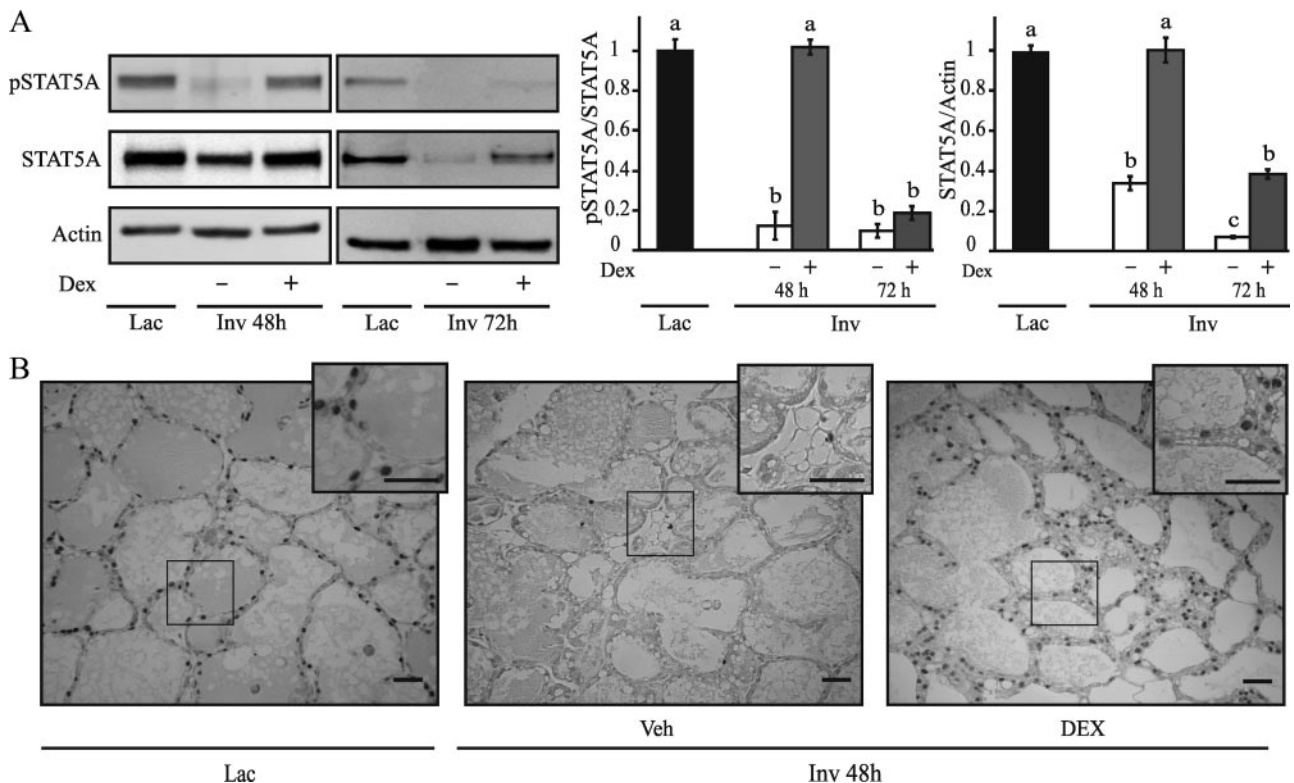


FIG. 1. DEX delays STAT5A inactivation during involution. A, Protein extracts prepared from lactating mammary glands (Lac) and from 48 (Inv 48 h) or 72 (Inv 72 h) hours of mammary involution from mice treated (+) or not (–) with DEX. Western blots were performed with antibodies against pSTAT5A and total STAT5A. Protein levels were quantified by using Image J software. STAT5A activation levels were calculated as the ratio pSTAT5A/STAT5A and total STAT5A levels were normalized against actin expression. Values are expressed as fold induction relative to lactation. The means ± SE from three independent experiments are shown. Bars with different superscript letters are significantly different from each other (*P* < 0.05). B, STAT5A activation analysis of mammary glands from normal lactating (left panel) or 48 h of mammary involution mice treated with DEX (right panel) or with vehicle (Veh) (middle panel). Tissue was fixed and immunohistochemical assays were performed by using a specific antibody against the pSTAT5A followed by DAB visualization. Bar (amplified section), 50 μm. Images correspond to one representative experiment (n = 3).

Journals Online web site at <http://endo.endojournals.org>), while very low positive staining was detected in control glands 48 h after weaning (Fig. 1B and Supplemental Fig. 1, *middle panel*). These results suggest that DEX delays STAT5A inactivation in the mammary gland, at least for 48 h, by maintaining its phosphorylation status, nuclear localization, and total protein level.

To analyze whether DEX treatment also maintains STAT5A transcriptional activity, mRNA levels corresponding to two target genes, *b-cas* and *pip*, were determined. Figure 2A shows that *b-cas* expression significantly decreased in a time-dependent manner throughout involution, being near undetectable at 72 h after weaning, whereas DEX treatment delayed this decay for at least 24 h. In a similar way, Fig. 2B shows that *pip* down-regulation was also delayed by DEX treatment, at least for 24 h. These results indicate that glucocorticoid administration may contribute to mammary differentiation maintenance by the impairment of STAT5A deactivation.

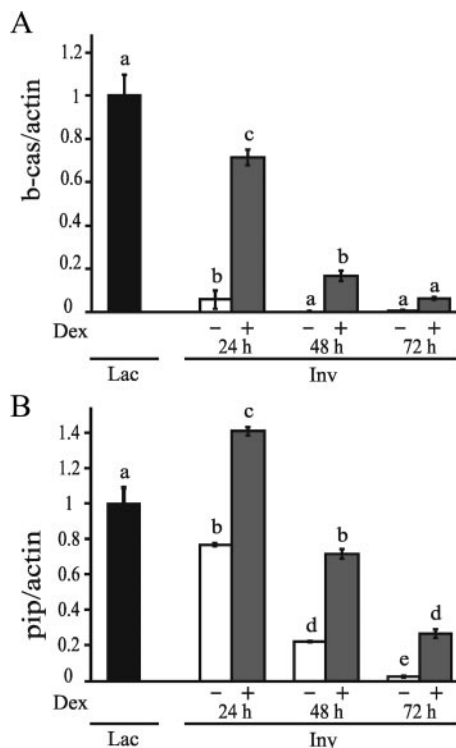


FIG. 2. DEX delays the loss of mammary gland differentiation and antiapoptotic markers. Total RNA was extracted from lactating mammary glands (Lac) and from 24 (Inv 24 h), 48 (Inv 48 h), or 72 h (Inv 72 h) of mammary involution from mice treated (+) or not (–) with DEX. Then qRT-PCR for *b-casein* (A) and *pip* (B) was performed with specific primers as described in *Materials and Methods*. Gene expression was normalized against *actin* expression, and values are expressed as fold induction relative to lactating glands. The means \pm SE from three independent experiments are shown. Bars with different superscript letters are significantly different from each other ($P < 0.05$).

DEX impairs STAT3 activation and *gp130* up-regulation independently of *lif* expression

STAT3 activation within 48 h after weaning is required for the normal mammary involution process (39). Therefore, STAT3 phosphorylation levels were analyzed to determine whether DEX treatment affected this pathway. Figure 3A shows that DEX completely blocked STAT3 phosphorylation observed in 48 h involuted control glands. This effect was lost 72 h after glucocorticoid administration. In agreement with these results, STAT3 immunostaining showed nuclear localization in the mammary epithelium at 48 h of involution (Fig. 3B and Supplemental Fig. 2, *middle panel*), whereas it was localized in the cytoplasm of epithelial cells from both lactating (Fig. 3B and Supplemental Fig. 2, *left panel*) and 48 h DEX-treated glands (Fig. 3B and Supplemental Fig. 2, *right panel*). These results indicate that glucocorticoids delay STAT3 activation, keeping STAT3 out of the epithelial nuclei for, at least, 48 h after weaning.

During the first stage of involution, milk stasis induces expression and secretion of cytokine LIF (35, 36), which leads to activation of the JAK/STAT3 signaling pathway (35–37). To figure out the mechanisms by which glucocorticoids delay STAT3 phosphorylation, the expression levels of *lif* mRNA was analyzed in mammary glands. Figure 4A shows qRT-PCR results for *lif* mRNA. As expected, 48 and 72 h involuting control glands showed higher *lif* mRNA levels than lactating mammary glands. Surprisingly, in DEX-treated mice, expression of this cytokine was even stronger than in postlactating control glands. These findings indicate that DEX effect on STAT3 activation is not due to the impairment of involution-associated *lif* up-regulation.

Because LIF receptor interacts with the Gp130 subunit to activate STAT3, the DEX treatment effect on *gp130* mRNA levels was also analyzed by qRT-PCR. Figure 4B shows that *gp130* expression was significantly up-regulated from lactation to involution and that DEX impaired this increment. Therefore, we propose that the impairment of *gp130* up-regulation might be a mechanism by which glucocorticoids delay STAT3 activation independently of *lif* expression levels.

DEX inhibits mammary epithelium apoptosis only if it is administered within 48 h after weaning

The histoarchitecture features of postlactating glands treated or not with DEX were analyzed in hematoxylin/eosin-stained sections in mammary glands. After 72 h of involution, untreated glands showed that alveolar structures started to collapse, apoptotic cells were extruding from the epithelial layer, and apoptotic bodies were localized in the ductal lumen (Fig. 5A and Supplemental Fig.

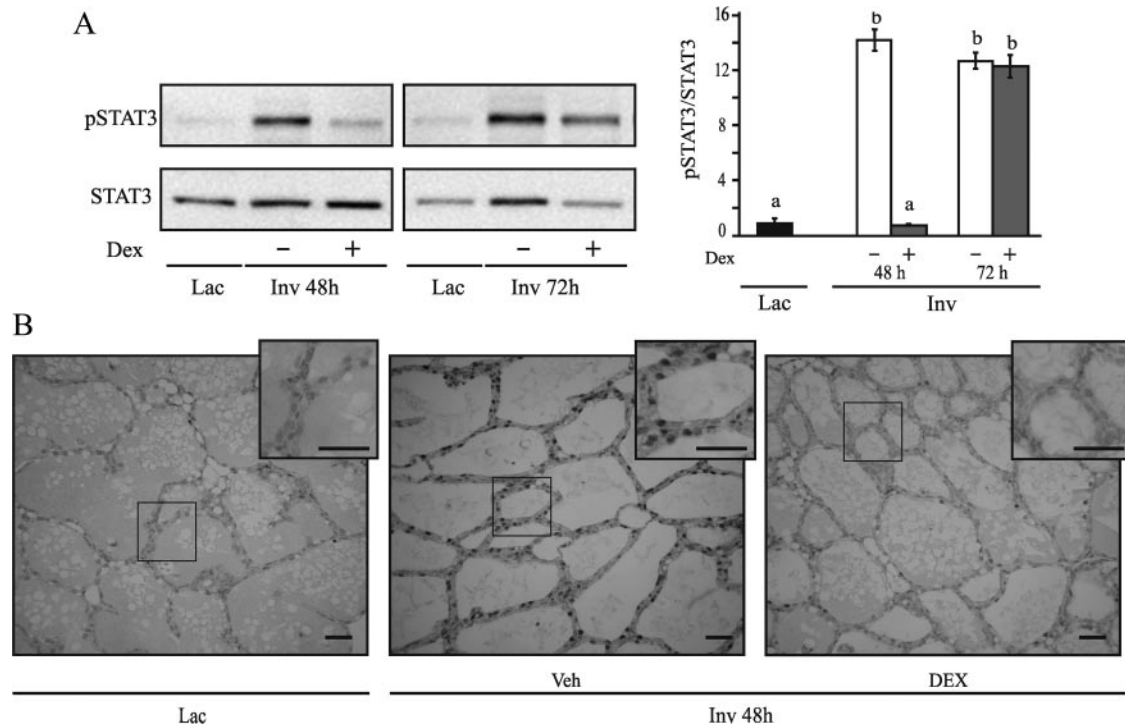


FIG. 3. DEX delays STAT3 activation. A, Protein extracts were prepared from lactating mammary glands (Lac) and from 48 (Inv 48 h) or 72 h (Inv 72 h) of mammary involution from mice treated (+) or not (–) with DEX, and Western blots were performed with antibodies against pSTAT3 and total STAT3 (left panel). Immunoblots were quantified using ImageJ software, and STAT3 activation was calculated as pSTAT3 to STAT3 ratio. Values are expressed as fold induction relative to lactation. The means \pm SE from three independent experiments are shown (column graphs). Bars with different superscript letters are significantly different from each other ($P < 0.05$). B, STAT3 activation analysis of lactating mammary glands (left panel) or 48 h of involution mice treated with DEX (right panel) or with vehicle (Veh) (middle panel). Tissue was fixed and immunohistochemical assays were performed by using a specific antibody against the STAT3 followed by DAB visualization. Bar (amplified section), 50 μ m. Images correspond to one representative experiment ($n = 3$).

3A, middle panel). On the other hand, in DEX-treated glands, the alveoli were not broken down, only a few apoptotic cells appeared, and accumulation of secreted proteins was observed. Interestingly, some adipocytes, usually found in involuting but not lactating glands, were seen in these glands (Fig. 5A and Supplemental Fig. 3A, right panel). This observation suggests that glucocorticoid administration inhibited but did not completely block mammary gland involution.

The antiapoptotic effect of DEX on epithelial cells was also analyzed by immunohistochemical assays against activated cysteine-dependent aspartate-specific proteases 3 and 9 (caspase-3 and caspase-9). Figure 5B (see also Supplemental Fig. 3B) shows that these caspases were activated in 48-h involuting glands (middle panels), while they seemed to be completely inactive in 48 h DEX-treated mice (right panels).

The consequences of applying glucocorticoid treatment not immediately but either 24 or 48 h after weaning were then determined. In each case, apoptosis induction in the mammary gland was evaluated by DNA fragmentation. Figure 5C shows that the ladder pattern observed in DNA samples from 72-h involuting glands (Fig. 5C, lane 3) was prevented by DEX when it was administrated either im-

mediately or 24 h after suckling cessation (Fig. 5C, lanes 4 and 5). However, apoptosis prevention was not observed when DEX treatment had been initiated 48 h after weaning (Fig. 5C, lane 6). These results suggest that DEX is able to inhibit involution-associated events only if it is applied within a specific temporal window that corresponds to early involution.

DEX delays GR down-regulation during involution

It has been shown that mammary regression is triggered by local tissue factors (32, 33). In fact, involution occurs, even in the presence of high levels of circulating lactogenic hormones, which reportedly preserve mammary cell survival (33). It has been observed that the abundance of GR molecules may determine the intensity of cell response to glucocorticoids (48). Hence, the hypothesis that GR down-regulation during involution (49) might be responsible for desensitizing mammary epithelial cells after weaning was evaluated. If this were true, DEX treatment should inhibit involution-associated GR down-regulation. To test this proposition, GR expression levels were determined. The results shown in Fig. 6 indicate that DEX treatment inhibited GR protein decrease after weaning.

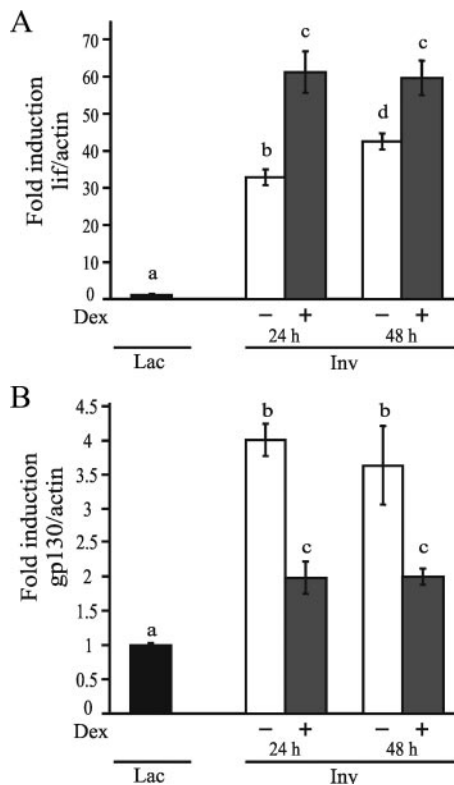


FIG. 4. DEX prevents *gp130* up-regulation independently of *lif* expression. Total RNA was extracted from lactating mammary glands (Lac) and from 24 h (Inv 24 h) or 48 h (Inv 48 h) of mammary involution from mice treated (+) or not (-) with DEX. Then qRT-PCR for *lif* (A) and *gp-130* (B) were performed with specific primers as described in *Materials and Methods*. Gene expression was normalized against *actin* expression, and values are expressed as fold induction relative to lactation. The means \pm SE from three independent experiments are shown. Bars with different superscript letters are significantly different from each other ($P < 0.05$).

Discussion

The present work focuses on the role that glucocorticoids exert on STAT5A and STAT3 signaling during mammary gland involution. STAT5A and STAT3 have reciprocal patterns of activation throughout a mammary developmental cycle: STAT5 is strongly activated toward the end of pregnancy, persists in an activated state during lactation, and is rapidly inactivated after cessation of suckling, whereas STAT3 activation is hardly detectable during lactation but is strongly induced at the onset of involution.

First, our results show that DEX administration delays STAT5A inactivation and degradation preserving *b-cas* and *pip* expression. It is well known that activated STAT5A plays a vital role in PRL-induced mammary cell differentiation. The activation of this transcription factor and the consequent expression of milk protein genes are often used as molecular markers for determining the complete differentiation of mammary epithelial cells (50). In fact, it has been demonstrated that JAK2 and STAT5A activation are able to induce three-dimensional organoid

differentiation accompanied by a shift from mesenchymal to epithelial cell phenotype in breast cells (51). Therefore, it can be proposed that inhibition of STAT5A inactivation helps to maintain the differentiation of postlactating glands in DEX-treated animals.

Second, our results show that DEX-treatment also delays STAT3 activation and translocation into epithelial cells nuclei, despite *lif* expression induction. Because the LIF receptor interacts with the Gp130 subunit to activate STAT3, it is plausible that DEX dependent down-regulation of STAT3 activation would be due to the retardation of *gp130* up-regulation. Our results show that DEX impaired the increment in *gp130* expression, which is normally significantly up-regulated from lactation to involution.

Third, the results presented in this work also indicate that DEX administration right after weaning inhibited apoptosis of mammary epithelial cells, in agreement with Feng *et al.* (44). Interestingly, this effect occurred only when DEX was administered within the first 48 h, when complete STAT5A inactivation and the highest levels of STAT3 activation were reached in control mammary glands. Therefore, these results suggest that glucocorticoid treatment maintains mammary differentiation and impairs epithelial cell apoptosis by modulating early involution associated events as STAT5A and STAT3 signaling pathways.

In the mammary gland and mammary epithelial cell lines, it has been found that STAT5 and GR proteins interact physically and functionality in the cell nucleus (9, 14) in which they synergize the gene expression regulation (15). In fact, Wyszomierski *et al.* (9) found that GR/STAT5 interaction increases STAT5 tyrosine phosphorylation levels, enhancing and prolonging STAT5/DNA binding activity by protecting this factor from inactivation by tyrosine phosphatases. Therefore, it is plausible that in the experiments described herein, DEX treatment delayed STAT5A inactivation by inducing or maintaining GR/STAT5 physical interaction. However, the possibility that DEX might also sustain STAT5A tyrosine phosphorylation by modulating prolactin receptor expression or activity and/or by modulating JAK2 kinase pathway cannot be ruled out. In this sense, further studies should be done to find out the specific mechanism by which DEX modulates STAT5A activation *in vivo*.

In mammary cells, no physical interaction has been previously detected between activated GR and STAT3 (11); therefore, direct binding between these two factors would not be the cause of the DEX-induced delay in STAT3 activation observed in this work. On the other hand, the observed DEX-induced *lif* up-regulation discards the possibility that this glucocorticoid inhibits STAT3 phosphorylation by lowering the expression of its natural inducer.

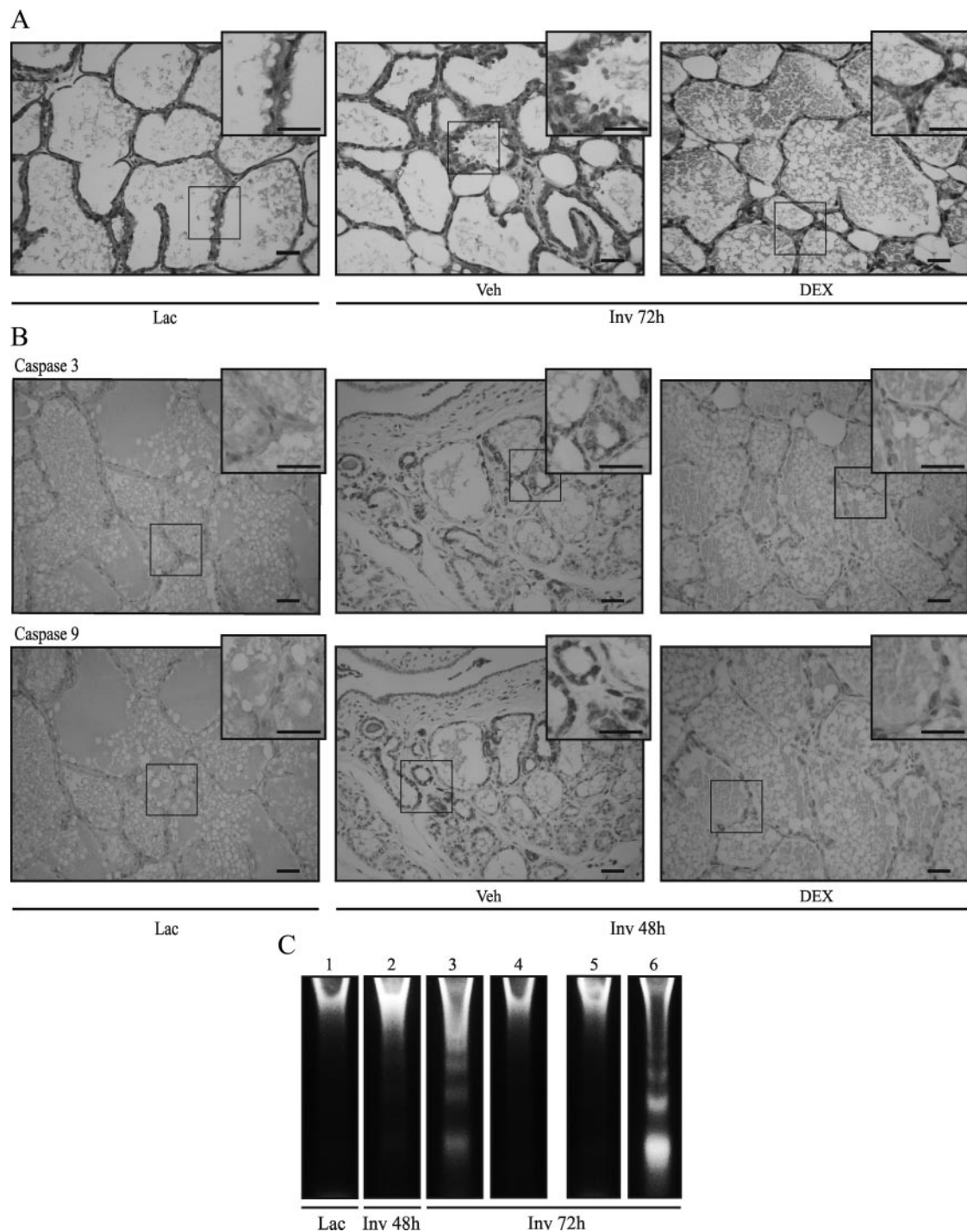


FIG. 5. DEX prevents apoptosis when it is administered immediately upon weaning. **A**, Hematoxylin and eosin-stained sections of lactating mammary glands (Lac) (left panel) and 72 h (Inv 72 h) of mammary involution from mice treated with DEX (right panel) or with vehicle (Veh) (middle panel). Bar (amplified section), 50 μ m. **B**, Analysis of activated caspase-3 (upper panels) and caspase-9 (lower panels) of lactating mammary glands (Lac) (left panels) and 48 h (Inv 48 h) of mammary involution from mice treated with DEX (right panels) or with vehicle (Veh) (middle panels). Tissue was fixed, and immunohistochemical assays were performed by using specific antibodies against active caspase-3 and caspase-9 followed by DAB visualization. Bar (amplified section), 50 μ m. **C**, DNA fragmentation ladder from lactating mammary glands (lane 1) and 48 h (lane 2) and 72 h (lanes 3–6) of involution, treated with DEX immediately upon weaning (lane 4), after 24 h (lane 5), or 48 h (lane 6). DNA was analyzed by electrophoresis on 2% agarose gel and evaluated by dyeing with ethidium bromide. Gels correspond to one representative experiment ($n = 3$).

Alternatively, our results suggest that inhibition of *gp130* up-regulation might be a potential mechanism by which DEX treatment retards STAT3 activation. In agreement

with this, Zhao *et al.* (52) have shown that in mammary epithelium *gp130*-deficient mice, there was no induction of STAT3 phosphorylation upon weaning. In those ani-

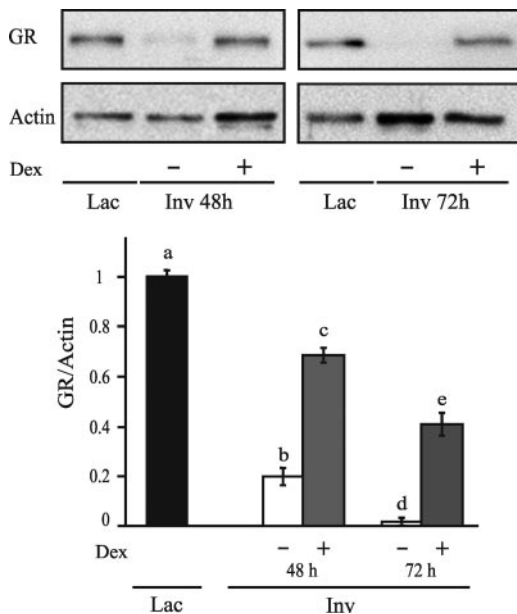


FIG. 6. DEX delays GR down-regulation. Protein extracts were prepared from lactating mammary glands (Lac) and from 48 (Inv 48 h) or 72 h (Inv 72 h) of mammary involution from mice treated (+) or not (–) with DEX, and Western blots were performed with an antibody against GR. Immunoblots were quantified using ImageJ software, and GR levels were normalized against actin expression. Values are expressed as fold induction relative to lactation. The means \pm SE from three independent experiments are shown (column graphs). Bars with different superscript letters are significantly different from each other ($P < 0.05$).

mals, postlactational involution and tissue remodeling was greatly impaired, pSTAT5 decline was delayed, and glands showed a secretory appearance, even 2 wk after weaning. Therefore, the experiments shown in this work suggest that impairment of *gp130* up-regulation and/or delay in STAT3 activation might be involved in inhibition of STAT5A deactivation. Alternatively, it has been shown that STAT5 activation protected cells from STAT3 mediated death signals (40). Thus, DEX-induced pSTAT5A maintenance could also be involved in the impairment of STAT3 activation. Hence, from these results, one could wonder whether there is any hierarchy in the glucocorticoid capacity of regulating the STAT5 and STAT3 pathways.

As indicated above, DEX treatment caused the enhancement of *lif* expression after weaning. To our knowledge, no previous reports have shown that GR activation induces the enhancement of this cytokine in mammary epithelial cells. However, glucocorticoid direct action might not be responsible for *lif* expression induction. It is possible that milk accumulation in the mammary alveoli, due to involution inhibition, generated high levels of mechanical stress and that triggered such an effect, as recently reported in mammary cultured cells (53).

Different cell types regulate their responsiveness to glucocorticoids, controlling the receptor levels by transcrip-

tion modulation and/or protein stability (54–63). Despite the fact that in most tissues and cell types, glucocorticoids down-regulate GR expression, positive feedback was demonstrated in several cases (61–63). Similarly, in the mammary gland, our results indicate that DEX treatment delayed weaning-triggered GR down-regulation. We believe that this effect might be a key event in STAT5A and STAT3 activity modulation exerted by glucocorticoids. Moreover, the lack of antiapoptotic effect when DEX was administered 48 h after weaning might be due to the low GR expression levels observed at that time.

GR decrease during mammary gland involution has been already reported (49). Although the mechanisms involved are still unknown, Kariagina *et al.* (64) demonstrated that LIF administration reduced GR mRNA and protein expression in the hypothalamus-pituitary-adrenal axis and in mouse AtT20 corticotroph cells, through the STAT3 signaling pathway. Therefore, it is possible that during mammary gland involution, induction of the LIF-STAT3 signaling pathway would cause GR down-regulation. Thus, the results presented here suggest that the DEX inhibitory effect on STAT3 activation might result in GR maintenance, enabling glucocorticoid activity in postlactating mammary glands.

It has been demonstrated that mammary local factors are responsible for triggering the whole involution process (33). However, the data shown herein suggest that circulating hormones and their specific receptors might be relevant in setting up the action thresholds for those factors. We believe that DEX induces the raising-the-bar effect by keeping high STAT5A and GR expression and activation levels and inhibiting STAT3 phosphorylation. During DEX treatment, the intensity of mechanical stress and local factor concentration might keep building up until reaching the new threshold established by hormone treatment. In this sense, it is not possible to assert whether the retardation in relevant early events would result in a long-term inhibition of tissue regression. For instance, Lund *et al.* (31) have found that systemic daily treatment with hydrocortisone delayed epithelial apoptosis and regression of the gland for up to 10 d in a dose-dependent manner. In summary, our results suggest that glucocorticoid-induced shifts in the kinetics of pivotal involution-associated pathways would be revealing how circulating hormones can act as physiological (and/or pathological) controllers and determinants of mammary regression after weaning.

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