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Conditional deletion of the *bcl-x* gene from mouse mammary epithelium results in accelerated apoptosis during involution but does not compromise cell function during lactation

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Abstract

In the mammary gland Bcl-x is the most abundant cell survival factor from the Bcl-2 family. Since Bcl-x null mice die around day 12 of embryogenesis, the relevance of this protein in organ development and function is poorly understood. In erythroid cells *bcl-x* gene expression is controlled by cytokines and the transcription factor Stat5 (signal transducer and activator of transcription). However, we identified that *bcl-x* RNA levels in mammary tissue from prolactin receptor- and Stat5-null mice were indistinguishable from wild type mice. We have proposed that Bcl-x might control the survival of mammary epithelial cells throughout pregnancy, lactation, and the early stages of involution, and we have now tested this hypothesis through the conditional deletion of the *bcl-x* gene from mouse mammary epithelium. Conditional (floxed) *bcl-x* alleles were excised from alveolar cells during pregnancy using a Cre transgene under the control of the whey acidic protein gene promoter. Deletion of the *bcl-x* gene from the entire epithelial compartment (ducts and alveoli) was achieved by expressing Cre-recombinase under control of the mouse mammary tumor virus long terminal repeat. The absence of Bcl-x did not compromise proliferation and differentiation of mammary ductal and alveolar epithelial cells in virgin mice and during pregnancy and lactation. However, epithelial cell death and tissue remodeling were accelerated in the *bcl-x* conditional knockout mice during the first stage of involution. Concomitant deletion of the *bax* gene did not significantly modify the Bcl-x phenotype. Our results suggest that Bcl-x is not essential during mammopoiesis, but is critical for controlled apoptosis during the first phase of involution. Published by Elsevier Science Ireland Ltd.

Keywords: Conditional gene deletion; Mammary gland; Epithelium; Bcl-x; Bax; Apoptosis; Involution; Cre-recombinase

1. Introduction

The developmental cycle of mammary tissue encompasses the proliferation of alveolar epithelium during pregnancy, its functional differentiation at parturition, and a lactational period followed by death and remodeling of the epithelium during involution (for review see Hennighausen and Robinson, 1998). Involution of mouse mammary tissue occurs in two distinct phases, a reversible and a non-reversible phase (Li et al., 1997; Lund et al., 1996). While apoptotic cell death is characteristic for both phases, the irreversible phase is also characterized by protease-mediated remodeling of mammary epithelium (Lund et al., 1996). Upon weaning, the levels of lactogenic hormones decrease sharply, which results in the downregulation of the prolactin/Stat5 pathway (Liu et al., 1996) and a concomitant reduction in milk protein gene expression (Burdon et al., 1991). Apoptosis can be observed within 24–48 h, which is followed by tissue remodeling.

As demonstrated in nipple sealing experiments (Li et al., 1997), and in oxytocin deficient mice that lack the milk ejection reflex (Wagner et al., 1997b), epithelial cell death in mammary tissue is triggered, in part, by local stimuli and not by declining levels of prolactin. Experimental mouse genetics has demonstrated that apoptosis and involution are independent of p53 (Li et al., 1996). The precise control of involution is possibly achieved through a shift in the balance of molecules that mediate cell survival and cell death. While the activity of the transcription factor Stat5 is lost within 24 h

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of involution, Stat3 activity is induced (Liu et al., 1996). Deletion of the *Stat3* gene specifically from mammary epithelium during pregnancy demonstrated that Stat3 is partly responsible for cell death in mammary epithelium (Chapman et al., 1999). It has been proposed that the Bcl-2 family of cell survival and death proteins controls the initial phase of involution. There are more than 15 members of the Bcl-2 family that are divided into two groups, pro-apoptotic and pro-survival. The pro-survival factors include Bcl-2, Bcl-w, and Bcl-x_L, and the pro-apoptotic factors include Bax, Bad, Bcl-x_S, and Bak (Adams and Cory, 2001). Bcl-x and Bax are the two most prominent Bcl-2 family members expressed in mammary tissue (Heermeier et al., 1996). In tissue culture cells, transfected Bcl-x was shown to convey resistance to apoptosis induced by cytokine deprivation (Boise et al., 1993). Bax expression has been shown to be elevated in some tissues after apoptotic stimuli and to promote death when overexpressed in tissue culture cells (Heermeier et al., 1996; Oltvai et al., 1993). Bcl-x mRNA levels are low in virgin mammary tissue, but increase during pregnancy in parallel with Stat5 activation and cell differentiation. Stat5 can activate *bcl-x* gene transcription in erythroid cells (Socolovsky et al., 1999), but this pathway has not been investigated in mammary cells. Bcl-x mRNA levels decrease during lactation, but increase again sharply within 48 h of involution (Heermeier et al., 1996). Similarly, Bax levels increase with the onset of involution (Heermeier et al., 1996). Two isoforms of Bcl-x have been described. The long form (Bcl-x_L), consisting of 233 amino acids, is more abundant and promotes cell survival. The short form (Bcl-x_S) has been identified as a reverse transcriptase polymerase chain reaction (RT-PCR) product, which consists of 170 amino acids and induces cell death upon overexpression in tissue culture cells (Boise et al., 1993). However, its presence and relevance in vivo are unclear.

The abundance of Bcl-x in mammary epithelial cells and its developmental regulation suggest that it could serve two functions. First, it may promote the survival of mammary epithelial cells during pregnancy. Second, Bcl-x may provide survival cues at the transition of lactation and involution and thus balance the death-inducing function of Bax to bring about a controlled remodeling of the gland. Experimental mouse genetics has provided information on the roles of Bcl-2 (Murphy et al., 1999; Schorr et al., 1999), Bcl-w (Metcalfe et al., 1999; Print et al., 1998), and Bax (Brady and Gil-Gomez, 1998; Knudson et al., 1995; Naka et al., 1998; Schorr et al., 1999; Shindler et al., 1997) in organogenesis. No adverse effects on mammary development have been reported in these mice. A genetic evaluation of the role of Bcl-x in mammary gland development has not been feasible since Bcl-x null mice die at around day 12.5 of embryonic development (Motoyama et al., 1995).

Three main objectives have been addressed. First, we defined the role of Bcl-x in mammary gland development. Second, we examined the possibility that the prolactin/Stat5 pathway may control *bcl-x* gene transcription in the mammary epithelium. Third, we investigated the proposed balance of anti-apoptotic Bcl-x and pro-apoptotic Bax in the mammary gland. We studied the role of Bcl-x through the deletion of the gene specifically in mammary epithelium using a Cre-loxP recombination approach. We have generated mice that carry loxP sites in the *bcl-x* gene promoter region and the second intron (Rucker et al., 2000), and transgenic mice that express Cre-recombinase in mammary epithelium under control of the whey acidic protein (WAP) gene promoter and the MMTV-LTR (Wagner et al., 1997a). Through the generation of mice that carry two targeted *bcl-x* alleles and a Cre transgene, we were able to genetically define the roles of Bcl-x in mammary gland development and function. We also deleted the *bax* gene from these mice and investigated the possible balancing effect of Bcl-x and Bax. Finally, using PrlR- and Stat5-null mice, we tested whether prolactin and Stat5 regulate bcl-x gene expression.

2. Results

2.1. Expression of the bcl-x gene throughout mammary development

Although the profile of Bcl-x during mammary development has been published (Heermeier et al., 1996), a quantitative analysis of *bcl-x* RNA in direct comparison to other family members has not been reported. We analyzed *bcl-w*, *bfl-1*, *bcl-x*, *bak*, *bax*, *bcl-2*.and *bad* RNA levels using a quantitative RNase protection assay (Fig. 1A). RNA (7 μg) from the different stages of mammary development were analyzed and *L32* and *GAPDH* transcripts were used as loading controls. *Bcl-x*, *bak*, and *bax* transcripts were expressed most highly at all stages of development, but their respective levels did not change dramatically between day 10 of pregnancy and lactation (Fig. 1A, lanes 3–5). However, their expression increased sharply within the first 24 h after weaning (Fig. 1A, lanes 7–9), and subsequently declined around days 3–4 (Fig. 1A, lanes 10, 11). In particular, while *bcl-x* and *bax* RNA levels were comparable 24 h after weaning, the ratio shifted towards *bax* by day 4 of involution (compare Fig. 1A, lanes 9 and 11). The levels of *bfl-1* transcripts were low throughout development, but increased sharply at 4 days post-weaning (Fig. 1A, lane 11). Since remodeling of the epithelium is in full progress by day 4 of involution and lymphocytes have invaded the mammary gland, it is possible that the increased expression results from the presence of these cells. In contrast, the elevated levels of *bcl-x* and *bax* RNA are the result of increased gene expression in the epithelium as previously demonstrated by in situ hybridization (Heermeier et al., 1996). The level of *bcl-w* transcripts remained stable throughout development. *Bcl-2* transcripts were not detected at any developmental stage. The reason for the rather low expression levels of Bcl-2 family members (but not *L32* and *GAPDH*) at 6 h after weaning is not clear.

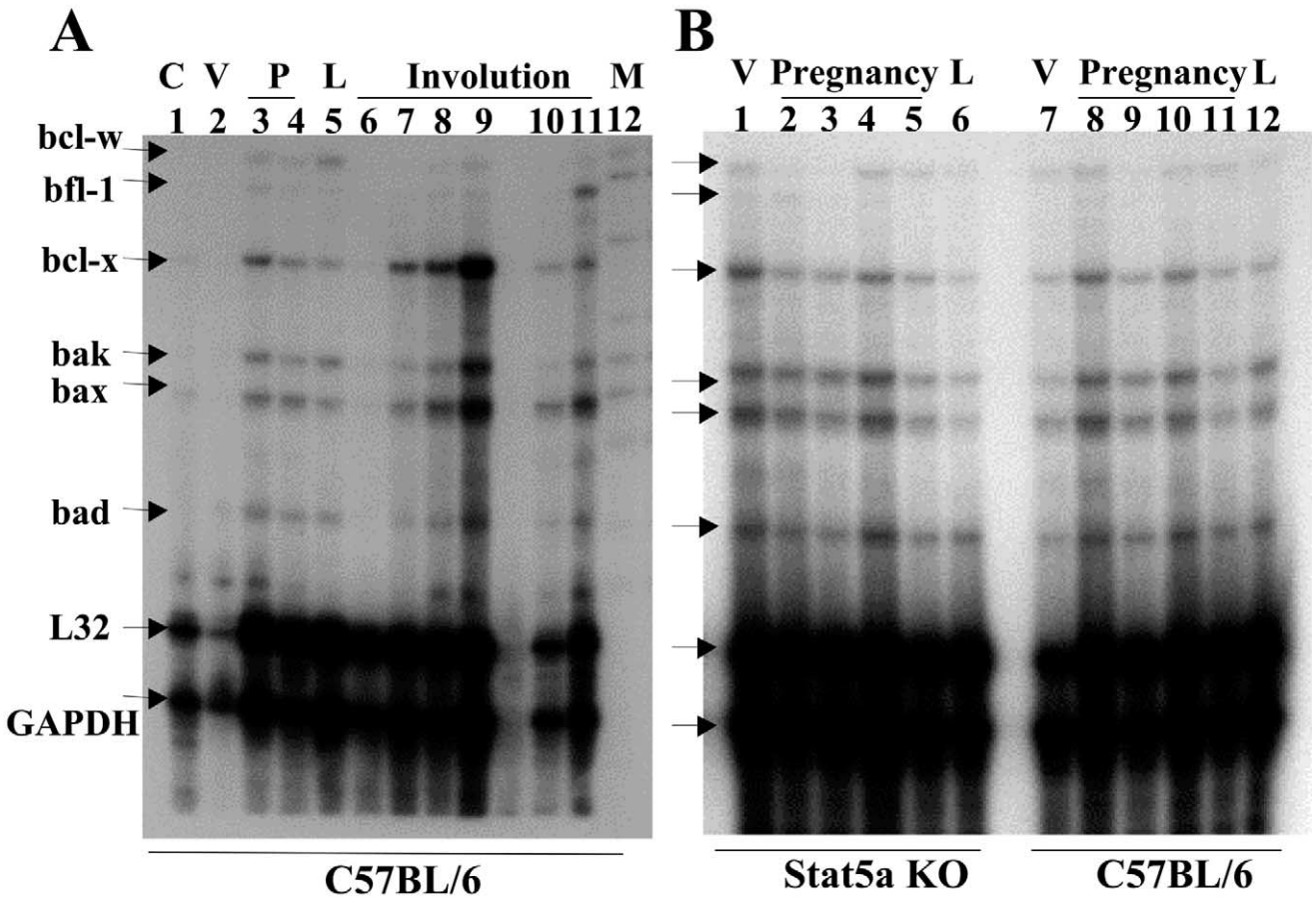

Fig. 1. RNase protection assays of mammary gland RNA with bcl-2 family member probes (bcl-w, bfl-1, bcl-x, bak, bax, and bad) and analysis of bcl-x RNA from PRLR knockout and Stat5a/5b double knockout mice. (A) Various developmental stages of wild type (C57BL/6) mammary tissue (7 μg of RNA were analyzed for each time point). Lanes 1–12 in order are: kit control (1), virgin (2), pregnancy day 10 (3), pregnancy day 13 (4), lactation day 10 (5), involution 6 h (6), involution 12 h (7), involution 18 h (8), involution 24 h (9), involution 3 days (10), involution 4 days (11), and molecular weight marker (12). The band observed between bad and L32 may be the result of non-specific nicks. (B) Various developmental stages of mammary tissue from Stat5a-null (lanes 1–6) and wild type (lanes 7–12) mice. Lanes 1 and 7, virgin; lanes 2 and 8, pregnancy day 9; lanes 3 and 9, pregnancy day 13; lanes 4 and 10, pregnancy day 16; lanes 5 and 11, pregnancy day 18; and lanes 6 and 12, lactation day 1.

2.2. Expression of the bcl-x gene in mammary epithelium is not under control of the prolactin receptor or Stat5

In erythroid cells *bcl-x* gene activity is induced by the transcription factor Stat5 (Kieslinger et al., 2000; Socolovsky et al., 1999). Since Stat5 conveys cell survival signals in both erythroid (Socolovsky et al., 1999) and mammary alveolar cells (Humphreys and Hennighausen, 1999), we asked whether Stat5 also controls *bcl-x* gene expression in mammary epithelium. RNA was isolated from mammary tissue of wild type (Fig. 1A,B) and Stat5a-null (Fig. 1B) mice at different stages of development. Analysis of the Bcl-2 family members was performed using RNase protection assays as discussed above. The expression pattern of all genes, including *bcl-x*, was similar in wild type and Stat5a-null mice (Fig. 1B).

Since PrlR- and Stat5-null (lack Stat5a and 5b) mice are infertile we could not evaluate directly Bcl-x levels after a full pregnancy. We therefore transplanted wild type, PrlR- and Stat5-null epithelium into cleared fat pads of wild type mice, and analyzed mammary protein at parturition (lactation day 1) after one pregnancy (Fig. 2). Bcl-x levels in the PrlR- and Stat5-null tissues were even higher than in wild type tissue. Expression of Stat1 and Stat3 was similar in PrlR-null, Stat5-null, and wild type littermates. The weak Stat5a and 5b signals in the Stat5-null transplants were derived from the stroma of the wild type hosts. Bcl-x, Stats 1 and 5 and SGP-2 were found in wild type stroma. From these experiments we conclude that *bcl-x* gene transcription in mammary tissue, unlike in the erythroid system, is not controlled by Stat5.

2.3. Targeted deletion of the bcl-x gene from mammary epithelium

Based on our expression studies and in situ hybridization experiments (Heermeier et al., 1996), we hypothesized that Bcl-x could mediate cell survival during pregnancy and the first phase of involution. To test this hypothesis in the mouse, we deleted the *bcl-x* gene specifically from

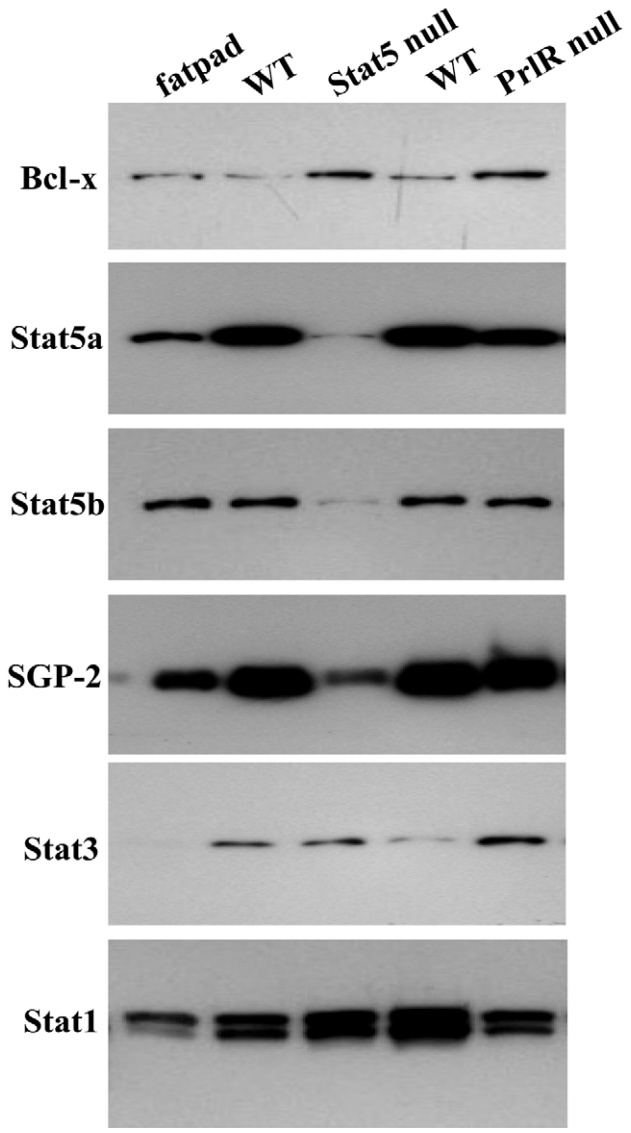

Fig. 2. Western blots of protein extracts from the mammary glands of transplanted mice at lactation day 1 probed with anti-Bcl-x, anti-Stat5a, anti-Stat5b, anti-SGP-2, anti-Stat3, and anti-Stat1 (p84-91). Lanes are wild type cleared fat pad, wild type (sibling of Stat5-null) transplant, Stat5-null transplant, wild type (sibling of PRLR-null) transplant, and PRLR-null transplant.

mammary epithelium using the Cre-loxP recombination system. LoxP sites had been introduced into the promoter and second intron of the mouse *bcl-x* gene (Rucker et al., 2000) and transgenic mice had been generated that carry the Cre gene under control of the MMTV-LTR and the mammary-specific WAP gene promoter (Wagner et al., 1997a). While the *MMTV-Cre* mice expressed Cre-recombinase in ductal epithelium of virgin mice and in alveolar epithelium throughout pregnancy (Wagner et al., 2001), the *WAP-Cre* gene was active in differentiating alveolar cells at late pregnancy and lactation (Fig. 6A and data not shown). We utilized these characteristics in expression kinetics to delete the *bcl-x* gene in specific cell types and during different developmental stages. Mice were generated that carried two conditional *bcl-x* alleles (*fl/fl*) and the *MMTV-Cre* (*fl/fl*; *MC* mice) or *WAP-Cre* (*fl/fl*; *WC* mice) transgene. Deletion of the *bcl-x* gene was evaluated on the level of DNA and protein. To avoid potential problems with the preferential death and therefore loss of Bcl-x-null cells, we performed Southern blot analyses on mice that contained one wild type and one conditional *bcl-x* allele (*fl/+*) and the *WAP-Cre* transgene (*fl/+*; *WC* mice). The 6 kb fragment represented the *bcl-x* null allele, whereas the 7 kb fragment indicated the wild type and the non-recombined floxed alleles (Fig. 3A). Since the Cre transgene was only active in mammary epithelium and not in the stroma, the fragment containing the wild type and floxed alleles was more intense (Fig. 3A). In contrast, deletion of the *bcl-x* gene from hematopoietic cells (Wagner et al., 2000) resulted in the selective amplification of Bcl-x null cells in spleen, and thus a complete deletion was apparent (Fig. 3A). Western blot analyses established the reduction of Bcl-x in mammary tissue of *bcl-x fl/fl*; *WC* mice (Fig. 3B). Similarly, Bcl-x was absent from Bcl-x null hematopoietic cells (Fig. 3B). Quality of loading was monitored through Coomassie Blue staining (Fig. 3B, lower panel). Loss of Bcl-x was also seen in mice whose *bcl-x* alleles were conditionally deleted with MMTV-Cre (Fig. 6B).

2.4. Bcl-x is not required for functional mammary gland development

Bcl-x is the most abundant cell survival factor of the Bcl-2 family (as assessed by RNase protection assays) during pregnancy and the first phase of involution. To investigate its role in mammopoiesis we monitored ductal and alveolar development and function in mice from which the *bcl-x* gene had been deleted using MMTV-Cre or WAP-Cre transgenes. In the absence of Bcl-x, mammary ducts exhibited normal elongation and branching during puberty, and alveoli formed and differentiated during pregnancy (data not shown). Furthermore, Bcl-x-null mammary tissue was fully functional and dams could support their litters (data not shown). During lactation, the mammary fat pads of *fl/fl*; *WC* (Fig. 4C) and *fl/fl*; *MC* (Fig. 5C) were filled with secretory lobulo-alveolar structures. Even after several pregnancies these mice were able to raise their litters (data not shown).

2.5. Bcl-x promotes cell survival in the first stage of involution

Bcl-x gene expression is highly induced in the first stage of involution. We addressed the question as to whether the presence of Bcl-x at the transition of lactation to involution participates in the controlled death of mammary epithelium. Pups were removed from *fl/fl* and *fl/fl*; *WC* dams at peak lactation and the number of alveoli containing dead cells and the numbers of shed cells were determined at 24 and 48 h of involution (Figs. 4 and 8A). No significant differences

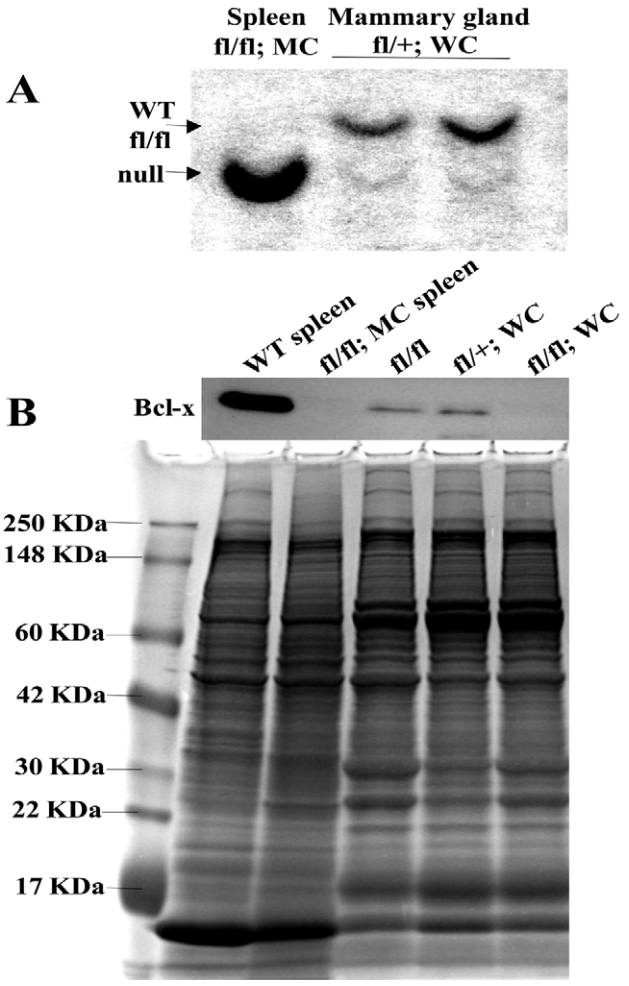

Fig. 3. DNA and protein analyses of mammary tissue from control and conditionally deleted *bcl-x* mice. (A) Southern blot of genomic DNA digested with EcoRI and probed for the *bcl-x* gene. Lanes are: spleen DNA from a *bcl-x* fl/fl; MMTV-CreD mouse (Wagner et al., 2000) (note that *bcl-x* null erythroid cells are preferentially amplified so that a complete deletion was observed), and DNA from lactating mammary tissue of two mice carrying the *WAP-Cre* transgene, one *floxed bcl-x* allele, and one wild type *bcl-x* allele; the wild type and *floxed* alleles appear as the upper band (7 kb) and the null allele as the lower band (6 kb). Note that the WAP gene promoter targets Cre expression only to mammary epithelial cells, and that the underlying stroma will carry only non-recombined *bcl-x* alleles. (B) Western blot of total protein lysate using anti-Bcl-x antibodies (top panel) with corresponding Coomassie Blue staining (bottom panel). Lanes are: molecular marker ladder (seen in Comassie Blue staining only), protein from the spleen of a mouse with two wild type *bcl-x* alleles, protein from the spleen of a mouse with the *MMTV-CreD* transgene, and two *floxed bcl-x* alleles (Wagner et al., 2000), mammary protein from a *bcl-x* fl/fl mouse during lactation, mammary protein from a *bcl-x fl/+*; *WAP-Cre* mouse during lactation, mammary protein from a *bcl-x fl/fl*; *WAP-Cre* mouse during lactation. The nature of the lower molecular band seen in the *bcl-x fl/+*; *WC* lane is not clear but it could represent Bcl-x_S.

were observed between *fl/fl* and *fl/fl*; *WC* mice at 24 h (Fig. 4B,D). In contrast, the number of alveoli with shed cells and the number of shed cells per alveolus at 48 h was three-fold greater in the Bcl-x-null tissue as compared to control tissue (Figs. 4F,H and 8A,B). Similar increases were seen in the Bcl-x null tissue deleted with MMTV-Cre as compared to the control tissue (Figs. 5B,D and 8A,B). To determine the cells from which the *bcl-x* gene had been deleted we generated *fl/fl*; *WC* and *fl/fl*; *MC* mice that also carried the *ROSA26* reporter gene. After parturition, more than 80% of the alveolar cells stained blue (Fig. 6A,B), suggesting that the majority of cells had undergone Cre-mediated recombination. In addition, at 48 h after weaning most cells shed into the lumina stained blue (data not shown). Deletion of the *bcl-x* gene using the MMTV-Cre transgenic mice also resulted in increased cell death at 48 h of involution (Fig. 5D). We further investigated the integrity of mammary tissue after 5 days of involution and did not observe a difference between *fl/fl*; *WC* and *fl/fl* mice (data not shown).

We measured alveolar cell death using ApopTag assays on tissue during pregnancy, at day 10 of lactation and 24 and 48 h of involution. No significant differences were observed during pregnancy, at day 10 of lactation and 24 h of involution (data not shown). However, at 48 h of involution the apoptotic rate was 4% in *fl/fl* mice, 10% in *fl/fl*; *WC* mice, and 9% in *fl/fl*; *MC* mice (Fig. 8C).

2.6. Alveolar differentiation in the absence of Bcl-x

We investigated whether the loss of Bcl-x altered the expression of genes that define alveolar differentiation and apoptosis. Specifically, the expression of Stats 1, 3, and 5 and SGP-2 was analyzed during lactation and involution. Bcl-x levels were sharply reduced in *bcl-x fl/fl* mice carrying either the *WAP-Cre* (Fig. 7A) or the *MMTV-Cre* transgene (Fig. 7B). As controls we used spleen from wild type and *bcl-x fl/fl*; *MC* mice (Wagner et al., 2000). Bcl-x levels were greatly reduced in the mutant spleens (Fig. 7A,B). In wild type mice Stat5a and 5b levels were highest during lactation and decreased during involution (Fig. 7). A similar pattern was observed in *fl/fl*; *WC* mice (Fig. 7A). In contrast, the decrease of Stat5 was more profound during involution in *bcl-x fl/fl*; *MC* mice (Fig. 7B). The levels of Stat3, a protein that has been linked to the initiation/progression of involution (Chapman et al., 1999), increased slightly during involution in wild type mice (Fig. 7). Increased Stat3 levels were also observed in *bcl-x fl/fl*; *WC* mice (Fig. 7A), but not in *fl/fl*; *MC* mice (Fig. 7B). The presence of a shorter form of Stat3 in spleens from *bcl-x fl/fl*; *MC* mice may reflect the altered cell type in these mice (Wagner et al., 2000). SGP-2 (Strange et al., 1992) is highly induced in mammary epithelium from wild type mice during the early phase of involution (Fig. 7). A similar induction was observed during involution of the *bcl-x fl/fl* mice in the presence of either Cre transgene (Fig. 7). The expression profiles during lactation and involution were similar in *fl/fl*; *WC* mice that also lacked the two *bax* alleles (data not shown).

3. Discussion

Bcl-x is the most abundant member of the Bcl-2 family of proteins in a wide variety of cell types, including hemato-

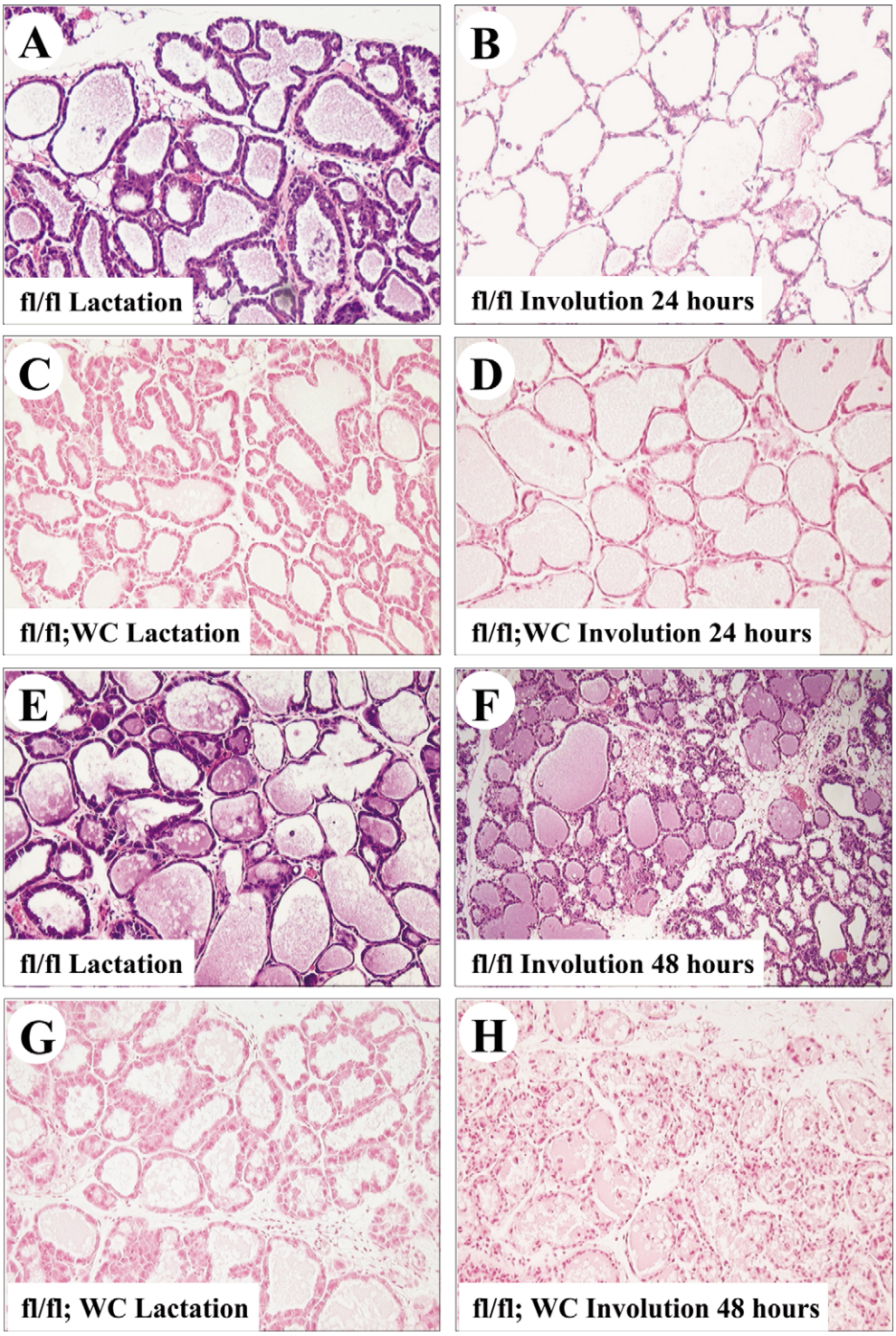

Fig. 4. Accelerated apoptosis of mammary epithelial cells upon deletion of the *bcl-x* gene by WAP-Cre. Mammary tissue from *bcl-x fl/fl* (panels A, B, E, F) and *bcl-x fl/fl*; *WC* (panels C, D, G, H) mice day 10 of lactation (panels A, C, E, G), 24 h of involution (panels B, D), and 48 h of involution (panels F, H). (A) Lactating (L10) fourth mammary gland from a *fl/fl* mouse. (B) Opposite side fourth mammary gland taken from the same mouse at 24 h of involution. (C) Lactating fourth mammary gland from a *bcl-x fl/fl*; *WC* mouse. (D) Opposite side fourth mammary gland taken from the same mouse as (C) at 24 h of involution. (E) Lactating (L10) fourth mammary gland from a *fl/fl* mouse. (F) Opposite side fourth mammary gland taken from the same mouse as in (E) at 2 days of involution. (G) Lactating fourth mammary gland from a *bcl-x fl/fl*; *WC* mouse. (H) Opposite side fourth mammary gland taken from the same mouse as (G) at 2 days of involution. Panels A–H, 100× magnification.

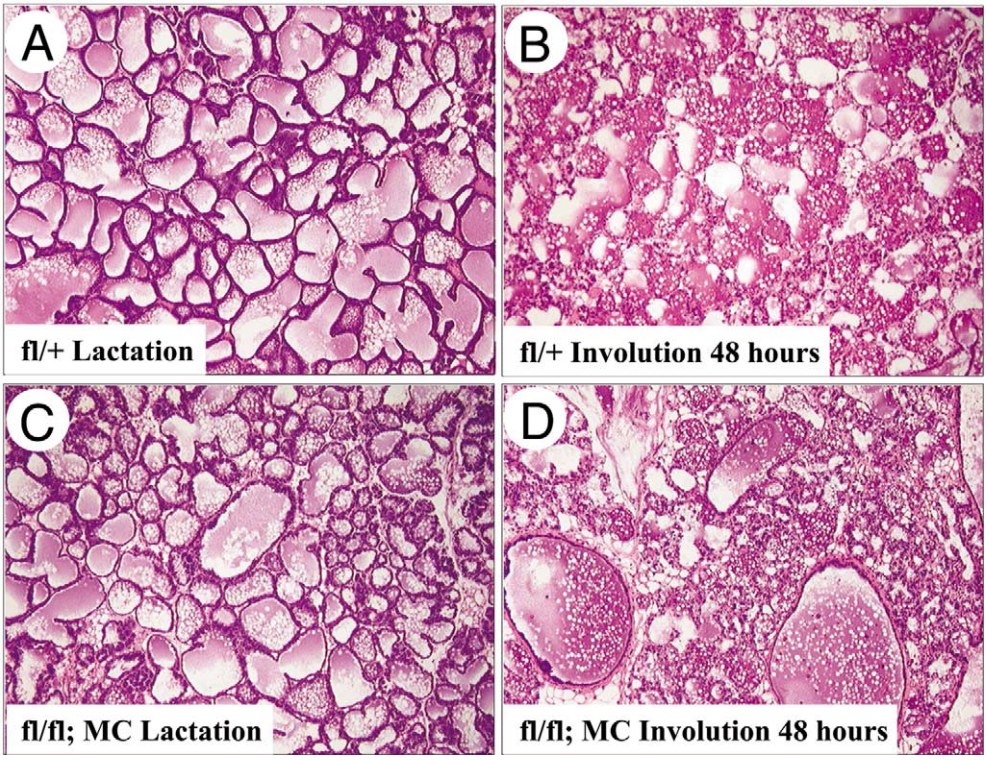

Fig. 5. Accelerated apoptosis of mammary epithelial cells upon deletion of the *bcl-x* gene by MMTV-CreD. Mammary tissue from *bcl-x fl/+* (panels A, B) and *bcl-x fl/fl*; *MC* mice (panels C, D) at day 10 of lactation (panels A, C), and at 48 h of involution (panels B, D). (A) Lactating mammary gland from a fourth mammary gland from a fl/+ mouse. (B) Opposite side fourth mammary gland taken from the same *fl/+* mouse at 48 h of involution. (C) Lactating fourth mammary gland from a *bcl-x fl/fl*; *MC* mouse. (D) Opposite side fourth mammary gland taken from the same mouse as (C) at 48 h of involution. Magnification for A–D is 100×.

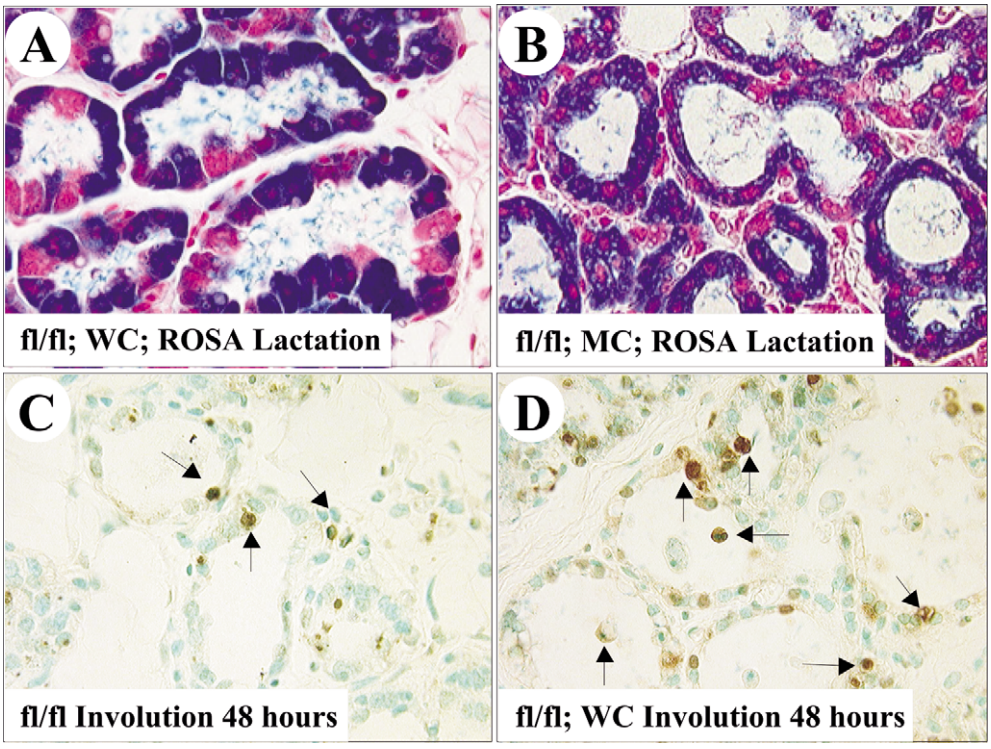

Fig. 6. LacZ and ApopTag staining of control and *bcl-x* conditionally deleted mammary glands. (A) LacZ stained mammary tissue from a *bcl-x fl/fl*; *WC*; *ROSA26* mouse at day 10 of lactation. (B) LacZ stained mammary tissue from a *bcl-x fl/+*; *MC*; *ROSA26* mouse at day 10 of lactation. (C, D) ApopTag staining of *bcl-x fl/fl* (C) and *bcl-x fl/fl*; *WC* (D) mammary tissue at 48 h of involution. Panels A–G, 630× magnification.

poietic cells, germ cells and mammary epithelial cells. Its vital role in mouse development and physiology has been confirmed through gene inactivation experiments (Motoyama et al., 1995; Rucker et al., 2000; Shindler et al., 1997; Wagner et al., 2000). Based on our work and that of others it is now clear that the physiological role of Bcl-x in promoting cell survival is not only dependent on the cell type, but also linked to defined developmental stages. Here we show that the physiological role of Bcl-x in mammary epithelium is confined to the first stage of involution, a phase characterized by increased apoptosis. Although Bcl-x is the most abundant cell survival factor throughout pregnancy and lactation, its presence is not required for the development and function of mammary epithelial cells. At this point it is not clear whether any of the Bcl-2 survival factors are critical by themselves during

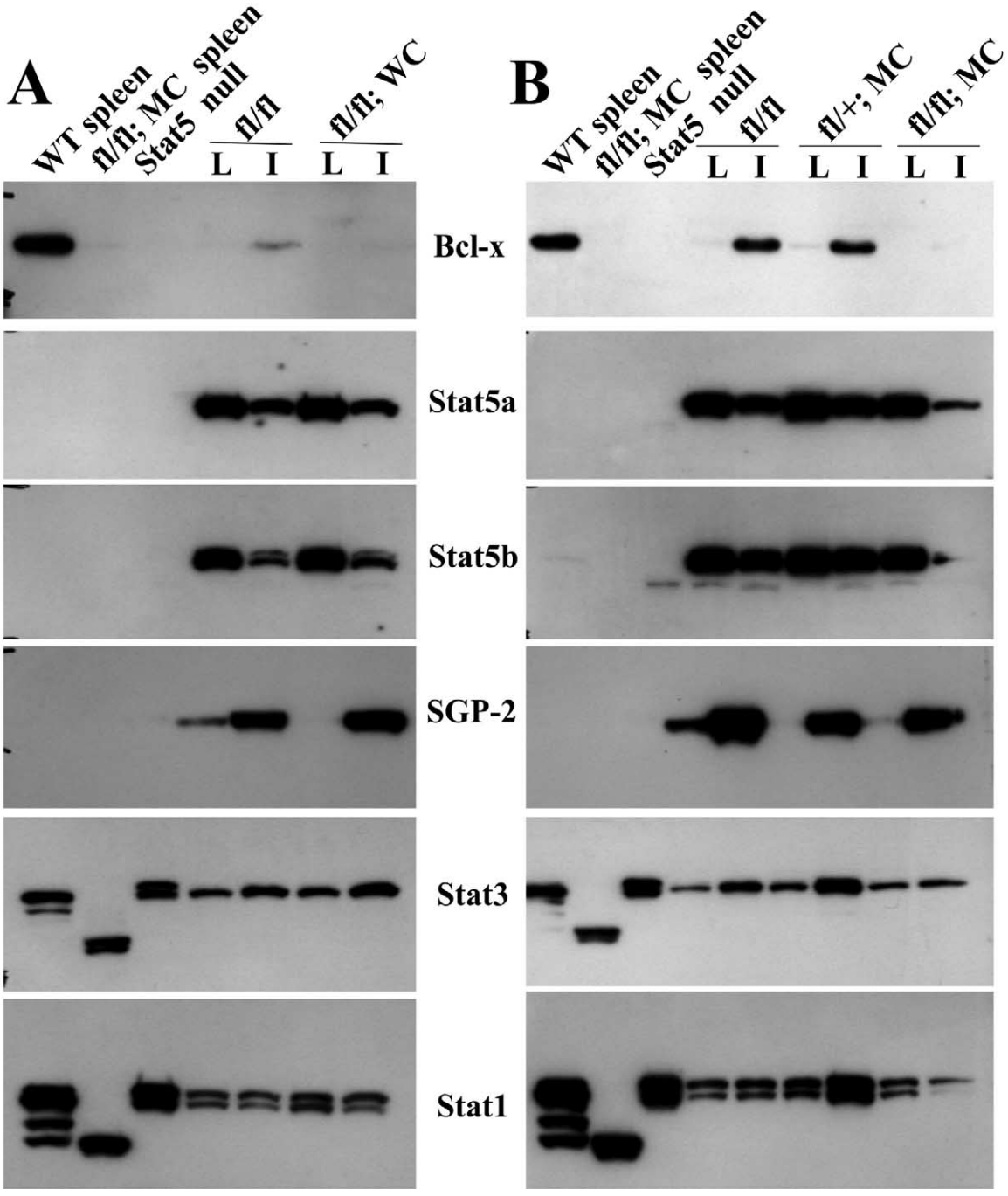

Fig. 7. Western blot analyses of differentiation and death markers upon deletion of the *bcl-x* gene using WAP-Cre (A) and MMTV-Cre (B) transgenic mice. Western blots of mammary tissue were probed for Bcl-x, Stat5a, Stat5b, SGP-2, Stat3, and Stat1. Spleen extract from wild type and *fl/fl*; *MC* mice was described earlier (Wagner et al., 2000). L, mammary tissue from mice at day 10 of lactation; I, mammary tissue from mice at 48 h of involution.

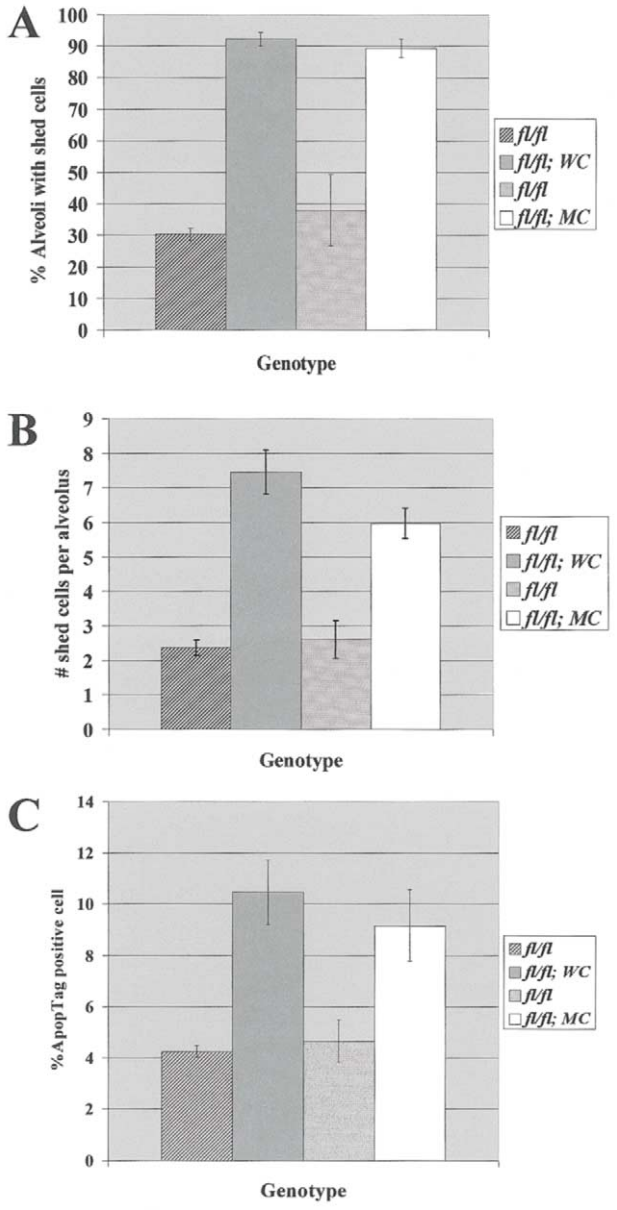

Fig. 8. Number of mammary alveoli during involution that contain shed (apoptotic) cells (A), number of shed cells per alveolus (B), percent of ApopTag positive alveolar cells (C). Three *fl/fl* and three *fl/fl*; *WC* littermates were analyzed, and three *fl/fl* and three *fl/fl*; *MC* littermates were analyzed. The hatched bar represents *fl/fl* mice, the solid gray bar represents the *fl/fl*; *WC* mice, the spotted bar represents *fl/fl* mice, and the solid white bar represents *fl/fl*; *MC* mice. Details of counting are in Section 4.4.

pregnancy and lactation. No mammary phenotype has been reported in Bcl-w-null (Print et al., 1998) and Bcl-2-null mice (Veis et al., 1993). Overexpression of Bcl-2 in mammary epithelium results in increased proliferation within the terminal end bud (Humphreys et al., 1996) and increased resistance to apoptosis (Schorr et al., 1999). It has been suggested that members of the Bcl-2 family promote cell survival during pregnancy and that a shift towards cell death factors induces apoptosis during involution (Metcalfe et al., 1999). These investigators hypothesized that the decline of Bcl-w during involution is a key initiating event of cell death. We now show that Bcl-x constitutes a mandatory cell survival signal after weaning and its loss results in an accelerated epithelial cell death. Thus, the presence of Bcl-w by itself cannot maintain cell survival.

The physiological role of Bcl-x in the first phase of mammary involution is subject to speculation. It is clear that the absence of Bcl-x does not adversely affect development and function of the gland, and that accelerated involution does not interfere with the gland's ability to function after subsequent pregnancies. It is possible that the presence of Bcl-x prevents premature involution of a gland under more natural conditions. In addition to the survival role that Bcl-x plays in involution, it also seems to have an effect on the survival of transformed cells and tumor cells. Ectopic overexpression of Bcl-x or Stat5-mediated activation of the *bcl-x* gene exists in transformed cell types and may contribute to the survival properties of tumor cells. For example, in lymphoid cells Bcr/Abl can induce *bcl-x* gene expression through the activation of Stat5 (Gesbert and Griffin, 2000; Horita et al., 2000). In addition, overexpression of Bcl-x can promote chemotherapy resistance of mammary tumors in a syngeneic mouse model (Liu et al., 1999) and malignant conversion of skin papillomas (Pena et al., 1998).

Activation of *bcl-x* and *bax* gene expression is rapidly induced upon weaning, and gamma interferon activated sequence (GAS) sites (binding sites for Stat proteins) have been identified in the promoter and body of the *bcl-x* gene. The induction of *bcl-x* gene expression during pregnancy and involution can be correlated with the activation of Stat5 and Stat3, respectively. However, no reduction of Bcl-x levels were observed in mammary tissue isolated from Stat5-null mice (this study) or mammary-specific Stat3 null mice (Chapman et al., 1999). Although there are reports based on tissue culture cells and gene knockout mice (Socolovsky et al., 1999) that Stat5 can induce transcription of the *bcl-x* gene in erythroid cells through direct binding to GAS sites in the promoter of the *bcl-x* gene, our data demonstrate that this is not the case in mammary epithelium. Clearly, *bcl-x* gene expression is independent of the prolactin receptor and Stat5. We have also demonstrated that *bcl-x* gene expression is independent of Stat5 in the ovary (unpublished). It is possible that activation of the *bcl-x* gene in mammary epithelium is controlled by glucocorticoids (Schorr and Furth, 2000).

While it is generally recognized that members of the Bcl-2 family can control the balance between cell survival and death (Vaux and Korsmeyer, 1999), research from our laboratory and that of others also point to an underlying cell- and developmental-specificity (Table 1). Cells from the erythroid, mammary, neuronal, and ovarian compartments exemplify the cell- and stage-specificity of Bcl-x function. While Bcl-x is mandatory for the survival of mature erythrocytes, it is dispensable for immature erythrocytes (Wagner et al., 2000). Bcl-x is also required for the survival of primordial germ cells (Rucker et al., 2000), but is not necessary in follicles and mature oocytes (unpublished).

Table 1
Contributions of Bcl-x and Bax in mouse development

Genotype	Mammary gland	Erythroid	Germ cells	Neurons
Conditional bcl-x deletion (mouse)	Normal development	Severe hemolytic anemia	Loss of primordial germ cells	Not determined
	Loss of resistance to apoptosis	Hyperplasia of immature erythroid cells[a]	Males are sterile, females have decreased fertility[b]	
bcl-x null (mouse and ES cell)	Lethal by E12.5	Lack adult definitive erythrocytes[c]	Lethal by E12.5	Increased apoptosis in the brain stem and spinal cord[c]
	Not determined		Not determined	
bax null (mouse)	Reduced apoptosis only during first stage of involution[d]	Normal erythroid development[e]	Higher ovarian reserve[f]	Increased number and survival of various types of neurons[h]
			Males sterile, accumulate premeiotic germ cells[e,g]	
bcl-x null and bax null (mouse or cell line)	Normal development	Same phenotype as tissue specific bcl-x deletion, function not restored[a]	Functional germ cells, loss of bax prevents bcl-x deletion phenotype[b]	Greatly reduced levels of apoptosis in vivo and in vitro when compared to mice lacking just bcl-x[g]
	Loss of resistance to apoptosis alleviated			

[a] (Wagner et al., 2000).
[b] (Rucker et al., 2000).
[c] (Motoyama et al., 1995).
[d] (Schorr et al., 1999).
[e] (Knudson et al., 1995).
[f] (Perez et al., 1999).
[h] (Deckwerth et al., 1996).
[g] (Shindler et al., 1997).

It has been shown in vitro that Bcl-x and Bax constitute a rheostat that controls cell survival and death (reviewed in Vaux and Korsmeyer, 1999). In vivo data now suggest that the presence of such a rheostat is cell-specific (Rucker et al., 2000; Wagner et al., 2000) (Table 1). To investigate whether the loss of Bax could rescue the accelerated apoptosis seen in *bcl-x fl/fl*; *WC* mice, we generated *fl/fl*; *WC* mice that were also null for the *bax* gene. Pregnancy-induced mammopoiesis appeared normal and these mice were able to nurse their pups (unpublished). Involution of mammary tissue in these mice was similar to that seen in the presence of Bax (unpublished). Despite extensive efforts, we were unable to obtain the expected number of *fl/fl*; *MMTV-Cre*; *bax null* mice (one out of 37). Since the MMTV-LTR is expressed in many cell types, it is possible that the combined deletion of Bcl-x and Bax resulted in embryonic death. Similar to the mammary gland, erythrocytes from which both Bcl-x and Bax were absent still exhibited a Bcl-x null phenotype (loss of mature erythrocytes) (Wagner et al., 2000). In contrast, the deletion of the *bax* gene from Bcl-x hypomorphic mice resulted in the rescue of the defect (restoration of germ cells) (Rucker et al., 2000). Likewise, the loss of immature neurons in the developing central nervous system (CNS) in the absence of Bcl-x was partially reversed upon the deletion of the *bax* gene (Shindler et al., 1997). Thus Bcl-x and Bax constitute a critical survival–death balance in specific cell types during defined developmental periods.

4. Experimental procedures

4.1. Transgenic mice

Conditional *bcl-x* mice had been generated by inserting loxP sites into the upstream region of the *bcl-x* gene and into the second intron (Rucker et al., 2000). These mice were mated with *WAP-Cre* and *MMTV-Cre* transgenic mice (Wagner et al., 1997a) and with *ROSA26* reporter mice (Soriano, 1999). The mice were of mixed background (129 and C57/B6).

The genotype of the mice was determined by PCR analyses. Primers for the *bcl-x* gene were 5′-CTG-CTC-GCC-ACC-TCA-TCA-GTC-3′ and 5′-GTC-TCA-GAA-GCC-GCA-ATA-TCC-3′ which yielded a 175 base pair (bp) product from the floxed allele and a 135 bp product from the wild type allele after 30 cycles at 65°C annealing. Primers for the Cre transgenes were 5′-TAG-AGC-TGT-GCC-AGC-CTC-TTC-C-3′ which binds in the WAP gene promoter, 5′-GGT-TCT-GAT-CTG-AGC-TCT-GAG-TG-3′ which binds in the MMTV-LTR, and 5′-CAT-CAC-TCG-TTG-CAT-CGA-CCG-G-3′ which binds in the Cre sequence. Using the respective promoter primers with the Cre primer, the *WAP-Cre* transgene produced a 240 bp fragment and the *MMTV-Cre* transgene produced a 280 bp fragment after 30 cycles at 65°C annealing. The *ROSA26* transgene produced a 425 bp product with primers 5′-

GAT-CCG-CGC-TGG-CTA-CCG-GC-3′ and 5′-GGA-TAC-TGA-CGA-AAC-GCC-TGC-C-3′ annealing at 65°C for 30 cycles. All products were separated in 2.5% agarose TAE gels.

For all mice studied, the right side fourth mammary gland was biopsied after lactating for 7–10 days. The pups were removed at the time of the biopsy, and the left side fourth mammary gland was harvested at 24 h, 2-, 3-, or 4 days post-weaning. Lymph nodes were also surgically excised at the time of mammary gland biopsies. More than 30 *fl/fl*; *WAP-Cre* mice and more than 30 control littermates, more than 20 *fl/fl*; *MMTV-Cre* mice and more than 20 control littermates, and five *fl/fl*; *WAP-Cre*; *bax*-null and 15 control littermates were analyzed. Only one out of 37 mice genotyped was *fl/fl*; *MMTV-Cre*; *bax-null* (20 of these mice were from matings of *fl/fl*; *MMTV-Cre*; *bax* +/−).

Stat5a-null (Liu et al., 1997), Stat5-null (lack both Stat5a and 5b) (Teglund et al., 1998) and PrlR-null (Teglund et al., 1998) mice were used to examine if bcl-x transcription is under the control of the Prl/Jak2/Stat5 pathway. Protein from virgin mammary tissue was analyzed. Since Stat5-null and PrlR-null mice are infertile, we transplanted mammary epithelium (Robinson et al., 1998) from these mice into wild type hosts and analyzed the tissue after one pregnancy.

4.2. Southern analysis

Genomic DNA was isolated from mammary gland tissue by digesting in a solution consisting of Proteinase K (20 ng/μl) in 1% sodium dodecyl sulphate (SDS), 50 mM Tris–HCl at pH 8.0, 20 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA), extracted with phenol/chloroform, precipitated in isopropanol, air-dried, and resuspended in TE. Genomic DNA (30 μg) was cut with EcoRI and separated in a 0.7% TAE gel, blotted onto a nylon membrane and hybridized with the ^{32}P-labeled probe (described below) for 4 h at 65°C with QuikHyb (Stratagene) and sheared salmon sperm DNA. Membranes were washed with 2 × SSC, 0.1% SDS for 20 min and then twice with 2 × SSC, 0.01% SDS for 20 min, and exposed on autoradiography film.

Template DNA to be used as a probe in the Southern analysis was PCR amplified from C57BL/6 mouse genomic DNA using the following primers: 5′-TAG-TAG-CTG-TCG-TGG-TAG-GCG-3′ and 5′-GTT-CAT-TTA-CAG-ATT-AGC-TTT-CAA-G-3′, which bind in exon 2 of the *bcl-x* gene. The PCR product was run in a 1% TAE gel and purified using a gel extraction kit (Qiagen). Two hundred nanogram of template was used to transcribe the probe with the Stratagene PrimeIt II kit with ^{32}P-radiolabeled dCTP. Probes were purified through G-50 Sephadex columns and their incorporation of radioactivity was determined by Cherenkov counting. The probe was denatured and hybridized to the DNA on the membrane as described above.

4.3. Western analysis

Protein was extracted from various tissues by homogenization in lysis buffer (0.04 M Tris–HCl, 0.28 M NaCl, 20% (v/v) glycerol, 2%(v/v) NP-40, 4 mM EDTA, 20 mM NaF with the addition of 100 μl of 10 mg/ml PMSF, 40 μl of 10 mg/ml aprotinin, 40 μl of 10 mg/ml of leupeptin, and 200 μl of 0.1 M Na-vanadate to each 10 ml) and rotated for 1 h at 4°C. Protein concentration was determined by bicinchoninic acid (BCA) colorimetric detection kit (Pierce). Sixty micrograms of lysate were run in a 14% Tris–glycine SDS gel to separate the proteins. Blocking was performed for 2 h at room temperature in 3%(w/v) dried non-fat milk in 1 × TBST. Primary antibodies were diluted in 3% dried non-fat milk in 1 × TBST and incubated with the membrane at room temperature for 1–2 h. Primary antibodies tested were: anti-Bcl-x (Santa Cruz, sc-634) diluted 1:500, anti-Stat5a antibody (Liu et al., 1997) (1:20,000 dilution), anti-Stat5b (Liu et al., 1997) (1:10,000 dilution), anti-SGP-2 (Santa Cruz sc-6420) diluted 1:500, anti-Stat3 (Santa Cruz, sc-7179) diluted 1:1000, and anti-Stat1 (Santa Cruz, sc-346) diluted 1:1000. Secondary antibody, rabbit IgG-HRP, was diluted 1:5000 and incubated in 3%(w/v) milk in 1 × TBST for 1 h at room temperature. Excitation of the HRP was performed with the Super Signal kit from Pierce for 5 min. ECL films were exposed for 30 s, 1, 5, and 10 min.

4.4. Histology

Tissues were fixed in 2% paraformaldehyde for 2 h and stained for β-galactosidase followed by a 1 h post-fix in formaldehyde. Alternatively, they were kept for 4 h in Tellyesniczky's fixative. Following fixation, tissues were dehydrated, paraffin embedded, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin, or with nuclear fast red if they had been stained for β-galactosidase. For the β-galactosidase assay samples were pre-stained in 2 mM $MgCl_2$, 0.01%(w/v) Na-deoxycholate, and 0.02%(w/v) Igepal (NP-40) in 1 × PBS. Following pre-stain, the samples were stained in 30 mM ($K_4Fe(CN)_6$, 30 mM $K_3Fe(CN)$, 2 mM $MgCl_2$, 0.01%(w/v) Na-deoxycholate, and 0.02%(v/v) NP-40 in 1 × PBS with 1 mg/ml of X-gal. Samples were washed in 1 × PBS, dehydrated through alcohol, post-fixed for 1 h in formaldehyde, and processed as described above. A detailed description of the β-galactosidase staining can be found at http://mammary.nih.gov/tools/histological/Histology/index.html#a2. Counting of histological sections was performed on three mice of each genotype (*fl/fl*, *fl/fl*; *WC*, *fl/fl*, *fl/fl*; *MC*).

The number of alveoli with shed cells was determined for each sample by counting the alveoli with shed cells as a percentage of the total alveoli. The numbers counted were: 661/2064 (32%), 195/691 (28.2%), 579/1874 (30.9%) for *fl/fl* mice, 2213/2437 (90.8%), 2485/2628 (94.6%), 2648/2905 (91.2%) for *fl/fl*; *WC* mice, 185/528

(35%), 241/850 (28.4%), 754/1488 (50.6%) for *fl/fl* mice, and 805/885 (90.9%), 432/474 (91.1%), 799/930 (85.9%) for *fl/fl*; *MC* mice. These numbers gave averages of 30.37% (SD = 1.9553), 92.2% (SD = 2.0881), 38% (SD = 11.4000), and 89.3% (SD = 2.93), respectively. The *P* value for the *t*-test of *fl/fl* versus *fl/fl*; *WC* mice was 0.00141, and for *fl/fl* versus *fl/fl*; *MC* mice the *P* value was 0.02474.

The number of shed cells per alveolus was determined for each sample by counting the number of shed cells and the number of alveoli and calculating the average. The numbers counted were: 229/499 (2.26), 134/353 (2.63), 143/321 (2.24) for *fl/fl* mice, 155/1070 (6.9), 159/1160 (7.3), 160/1304 (8.15) for *fl/fl*; *WC* mice, 406/882 (2.17), 855/2177 (2.55), 975/3182 (3.26) for *fl/fl* mice, 550/3157 (5.74), 492/2800 (5.69), 532/3452 (6.49) for *fl/fl*; *MC* mice. These numbers gave averages of 2.373 (SD = 0.2223), 7.45 (SD = 0.6384), 2.6567 (SD = 0.5543), and 5.9733 (SD = 0.4392), respectively. The *P* value for the *t*-test of *fl/fl*; *WC* mice was 0.0002, and for *fl/fl* versus *fl/fl*; *MC* mice the *P* value was 0.0015.

4.5. ApopTag assay

Tissues were agitated in Tellyesniczky's fixative for 4 h and then dehydrated, paraffin embedded, and sectioned at 5 μm. ApopTag assay was performed using the Intergen ApopTag In Situ Apoptosis Detection Kit. Methyl green (0.01%) was used for counter staining. Counting of ApopTag positive cells was performed at 200× magnification and then confirmed by morphological appearance at 630×. The numbers counted were: 25/554 (4.5), 26/617 (4.2), 26/639 (4.1) for *fl/fl* mice, 67/578 (11.6), 58/543 (10.7), 51/555 (9.2) for *fl/fl*; *WC* mice, 30/750 (4), 37/651 (5.7), 37/831 (4.4) for *fl/fl* mice, 47/506 (9.3), 56/531 (10.5), 41/534 (7.7) for *fl/fl*; *MC* mice. These numbers gave averages of 4.25 (SD = 0.2248), 10.46 (SD = 1.2644), 4.667 (SD = 0.8327), and 9.1667 (SD = 1.405), respectively. The *P* value for the *t*-test of *fl/fl*; *WC* mice was 0.0095, and for *fl/fl* versus *fl/fl*; *MC* mice the *P* value was 0.0078.

4.6. RNA analysis

Total RNA was isolated from tissue samples using the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). All RNA samples were pooled from three or more mice. The mouse Apo-2 template set (Pharmingen; consists of templates for *bcl-w*, *bfl1*, *bcl-x*, *bak*, *bax*, *bcl-2*, *bad*, *L32*, *GAPDH*) was labeled with a ^{32}P-labeled UTP according to the Riboquant Multi-Probe RNase Protection Assay System Instruction Manual. Total RNA (7 μg)were hybridized with 2.4×10^5 cpm/μl of the Apo-2 labeled templates according to the manufacturer's specifications. Following RNase treatment and chloroform:isoamyl alcohol extraction, the protected products were run in a NOVEX QuickPoint precast sequencing gel and exposed on autoradiography film.

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