Coordinated Expression of Extracellular Matrix-degrading Proteinases and Their Inhibitors Regulates Mammary Epithelial Function during Involution

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Abstract. Extracellular matrix (ECM) plays an important role in the maintenance of mammary epithelial differentiation in culture. We asked whether changes in mouse mammary specific function in vivo correlate with changes in the ECM. We showed, using expression of β-casein as a marker, that the temporal expression of ECM-degrading proteinases and their inhibitors during lactation and involution are inversely related to functional differentiation. After a lactation period of 9 d, mammary epithelial cells maintained β-casein expression up to 5 d of involution. Two metalloproteinases, 72-kD gelatinase (and its 62-kD active form), and stromelysin, and a serine proteinase tissue plasminogen activator were detected by day four of involution, and maintained expression until at least day 10. The expression of their inhibitors, the tissue inhibitor of metalloproteinases (TIMP) and plasminogen activator inhibitor-1, preceded the onset of ECM-degrading proteinase expression and was detected by day two of involution, and showed a sharp peak of expression centered on days 4–6 of involution. When involution was accelerated by decreasing lactation to 2 d, there was an accelerated loss of β-casein expression evident by day four and a shift in expression of ECM-remodeling proteinases and inhibitors to a focus at 2–4 d of involution. To further extend the correlation between mammary-specific function and ECM remodeling we initiated involution by sealing just one gland in an otherwise hormonally sufficient lactating animal. Alveoli in the sealed gland contained casein for at least 7 d after sealing, and closely resembled those in a lactating gland. The relative expression of TIMP in the sealed gland increased, whereas the expression of stromelysin was much lower than that of a hormone-depleted involuting gland, indicating that the higher the ratio of TIMP to ECM-degrading proteinases the slower the process of involution. To test directly the functional role of ECM-degrading proteinases in the loss of tissue-specific function we artificially perturbed the ECM-degrading proteinase-inhibitor ratio in a normally involuting gland by maintaining high concentrations of TIMP protein with the use of surgically implanted slow-release pellets. In a concentration-dependent fashion, involuting mammary glands that received TIMP implants maintained high levels of casein and delayed alveolar regression. These data suggest that the balance of ECM-degrading proteinases and their inhibitors regulates the organization of the basement membrane and the tissue-specific function of the mammary gland. During involution an excess of active ECM-degrading proteinases and/or an absence of inhibitors disrupts cell–ECM interaction, leading to loss of differentiated lactational phenotype.

EXTENSIVE studies in culture have shown that the interaction of a cell with its extracellular matrix (ECM)[1] has a profound influence on the functional status of epithelia (reviewed by Talhouk et al., 1991*a*; Watt, 1991; Stoker et al., 1990). Evidence in support of an active role for ECM in vivo (Wicha et al., 1980) in the development and function of tissues is beginning to unfold (Darribe're et al., 1990; Silberstein et al., 1990). One way in which the cell regulates its interaction with ECM is through remodeling of its microenvironment by secretion of ECM-degrading proteinases and inhibitors (Matrisian, 1990; Alexander and Werb, 1991). ECM-degrading proteinases were previously shown to correlate with metastases and tumor growth presumably by facilitating invasion of cells through their basement membrane (Goldberg and Eisen, 1991). However, they are also believed to be involved in regulating development

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; PAI-1, plasminogen activator inhibitor-1; SL, stromelysin; TIMP, tissue inhibitor of metalloproteinases; tPA, tissue plasminogen activator.

of normal tissues (Behrendtsen et al., 1992; Talhouk et al., 1991*b*; Wilson et al., 1991; Brenner et al., 1989; Fukuda et al., 1988; Beers et al., 1975).

To explore further the role that ECM-degrading proteinases and their inhibitors play in regulating normal tissue development and tissue-specific function, we have used the mouse mammary gland as a model for our studies. We previously demonstrated that basement membrane remodeling activity in vivo is lowest during lactation, when the mammary gland is fully differentiated, and highest during involution when the gland ceases to produce milk (Talhouk et al., 1991*b*). Previous studies have shown that mammary epithelial cells can acquire function in culture only when they are provided with an exogenous basement membrane or when they deposit their own basement membrane (Streuli and Bissell, 1990; Li et al., 1987; Blum et al., 1987). That the differentiation acquired in culture is a result of direct cell–ECM interaction has been shown by disruption of function with the use of blocking antibodies against integrins (Streuli et al., 1991).

We now have asked whether the loss of mammary function during involution is due to ECM-remodeling events as initiated by ECM-degrading proteinases. We demonstrate that the loss of differentiated function of the mammary gland during involution is directly correlated with events that modulate cell–ECM interaction. Further, we provide evidence that despite hormonal modulation, involution can be delayed if the degradation of the basement membrane is inhibited after cessation of milking. The data presented here support the hypothesis that a critical and coordinated balance between ECM-degrading proteinases and their inhibitors regulates tissue development and function in vivo.

Materials and Methods

Mammary Tissue

Mammary tissue obtained from primigravidae female CD-1 mice (Charles River, Wilmington, MA) were used in all experiments. Tissue was collected from mice at various stages of lactation and during involution of the gland. The intervals of lactation or involution for tissue collections are indicated in the respective experiments. Tissue samples from at least two mice were collected at each stage of development. Each experiment was repeated twice except for cauterization experiments. All experiments were performed under protocols approved by the Animal Welfare and Research Committee, Lawrence Berkeley Laboratory (Berkeley, CA).

RNA Preparation and RNA Blot Analysis

Total RNA was prepared from mammary tissue according to the procedure of Chomczynski and Sacchi (1987). RNA samples were resolved on 1% agarose formaldehyde gels under denaturing conditions, and transferred to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, IL). The RNA was hybridized to mouse cDNA probes radiolabeled with ^{32}P-dCTP by random priming (Feinberg and Vogelstein, 1984). For β-casein (pBCL-1, subclone derived from a plasmid provided by J. Rosen, Baylor College of Medicine, Baylor, TX) blots with 2 μg total RNA were used. Blots with 20 μg total RNA were used for stromelysin (SL) (Ostrowski et al., 1988), full-length probe provided by L. Matrisian (Vanderbilt University, Nashville, TN), tissue plasminogen activator (tPA) (Rickles et al., 1988), probe provided by R. Rickles (State University of New York, Stony Brook, NY), tissue inhibitor of metalloproteinases (TIMP) (Gewert et al., 1987), probe provided by B. Coulombe (Universite de Montreal, Canada), and plasminogen activator inhibitor-1 (PAI-1) (Prendergast and Cole, 1989), probe provided by M. Cole (Princeton University, Princeton, NJ). Prehybridization, hybridization, and post-hybridization washes were carried out according to Streuli and Bissell (1990). The blots were exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) for fluorography at −80°C. Positions for RNA size standards (2 μg per lane, from Life Technologies Inc., Gaithersburg, MD) were determined after transfer by staining the marker lane with methylene blue.

Intensity of ^{32}P-cDNA/mRNA hybridization signals was quantified by scanning films of exposed blots with an LKB 2202 Ultroscan Laser Densitometer (LKB-Produkter AB, Bromma, Sweden). Intensities of bands from different blots were standardized to each other by assigning the most intense band on each gel an arbitrary value of 10, except for the β-casein blot, which was assigned an arbitrary value of 30. This was done to show the relationship of the various proteins during the critical interval of proteinase-inhibitor changes that take place concurrent with the drop in β-casein expression. The standardized intensity values were plotted against days of involution.

Immunocytochemistry

Immunofluorescence staining for type IV collagen, laminin A-chain, and casein was performed on frozen sections. Mammary tissues were fixed in 2% paraformaldehyde, infused with 18 and 30% sucrose before embedding in Tissue-Tek OCT compound (Miles Sc. Div., Elkhart, IN), and frozen in an ethanol/dry ice bath. Sections (5 μm) were cut with a Leitz cryotome onto gelatin-coated slides and stained by immunofluorescence (Harlow and Lane, 1988). Briefly, nonspecific binding sites were blocked with wash solution. All washes were done for at least 1 h at ambient temperature or overnight at 4°C in PBS (130 mM NaCl, 7 mM $Na_2HPO_4.7H_2O$, 3 mM $NaH_2PO_4.H_2O$) containing 0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20, and 0.05% NaN_3. Sections were incubated for 1 h, at ambient temperature with either polyclonal rabbit antiserum to mouse laminin A-chain (gift of E. Engvall, La Jolla Cancer Res. Inst., La Jolla, CA) at 1:100 dilution, or polyclonal rabbit antiserum to mouse type IV collagen (E.Y. Labs, San Mateo, CA) at 1:300 dilution, or with FITC-conjugated (Sambrook et al., 1989) mouse mAb to rat β-casein (gift of C. Kaetzel, Case Western Reserve University, Cleveland, OH). In the case of type IV collagen and laminin A-chain, biotinylated anti-rabbit IgG (Amersham Corp.) followed by Texas red streptavidin were used at 1:50 dilution and 1:2,000 dilution, respectively, for 30 min each at ambient temperature. In all sections, nuclei were counterstained with 0.5 μg/ml DAPI (4,6-diamino-2-phenylindole) (Sigma Chemical Co., St. Louis, MO) for 5 min before the last wash. Zeiss epifluorescence optics (Oberkochen, Germany) were used, and photography was with TX-400 film (Eastman Kodak Co.).

For SL staining, paraffin-embedded sections were deparaffinized and rehydrated. Nonspecific protein binding sites were blocked by incubation of sections with ovalbumin (1 mg/ml) in PBS for 30 min, followed by incubation with a 20% solution of milk in PBS for 30 min. Sections were further blocked with 5% rabbit antiserum in PBS. Monoclonal mouse anti-human SL antibody (clone 2, 5 μg/ml in blocking buffer, gift of S. M. Wilhehm, Miles Research Center, West Haven, CT) was added to the sections and incubated for 1 h at ambient temperature and rinsed several times with PBS. Sections were then incubated in 5% PBS for 30 min, rinsed with PBS, and incubated with biotinylated secondary anti-mouse antibody (1:100) (Sigma Chemical Co.) for 30 min at ambient temperature. After washing as before, alkaline phosphatase-conjugated streptavidin (1:100) was added for another 30 min. Localization of SL was detected by alkaline phosphatase substrate (ABC kit; Vector Laboratories Inc., Burlingame, CA). Endogenous alkaline phosphatase activity was blocked by levamisole (Sigma Chemical Co.).

Protein Blotting

Mammary gland tissue extracts prepared as described previously (Talhouk et al., 1991*b*), were resolved on 12% polyacrylamide gels under denaturing conditions. After electrophoresis, resolved proteins were transferred to Immobilin-P membranes (Millipore Continental Water Systems, Bedford, MA) using a dry blot apparatus (American Bionetics, Hayward, CA). Membranes were blocked overnight in a wash buffer (100 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.3% Tween 20) with 2% fatty acid free BSA (Sigma Chemical Co.). The membranes were then incubated for 1 h in polyclonal rabbit anti-mouse milk antiserum (diluted 1:1,000 in blocking buffer) and washed 3 times, for 20 min each time, to remove unbound antiserum. Bound antibody was detected by addition of alkaline phosphatase-conjugated anti-rabbit IgG (Caltag Laboratories, South San Francisco, CA), followed by BCIP/NBT (bromo-chloro-indolyl phosphate/nitroblue tetrazolium) (Sigma Chemical Co.). All washings and incubations were done at ambient temperature. Biotinylated size markers were detected with alkaline phosphatase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA).

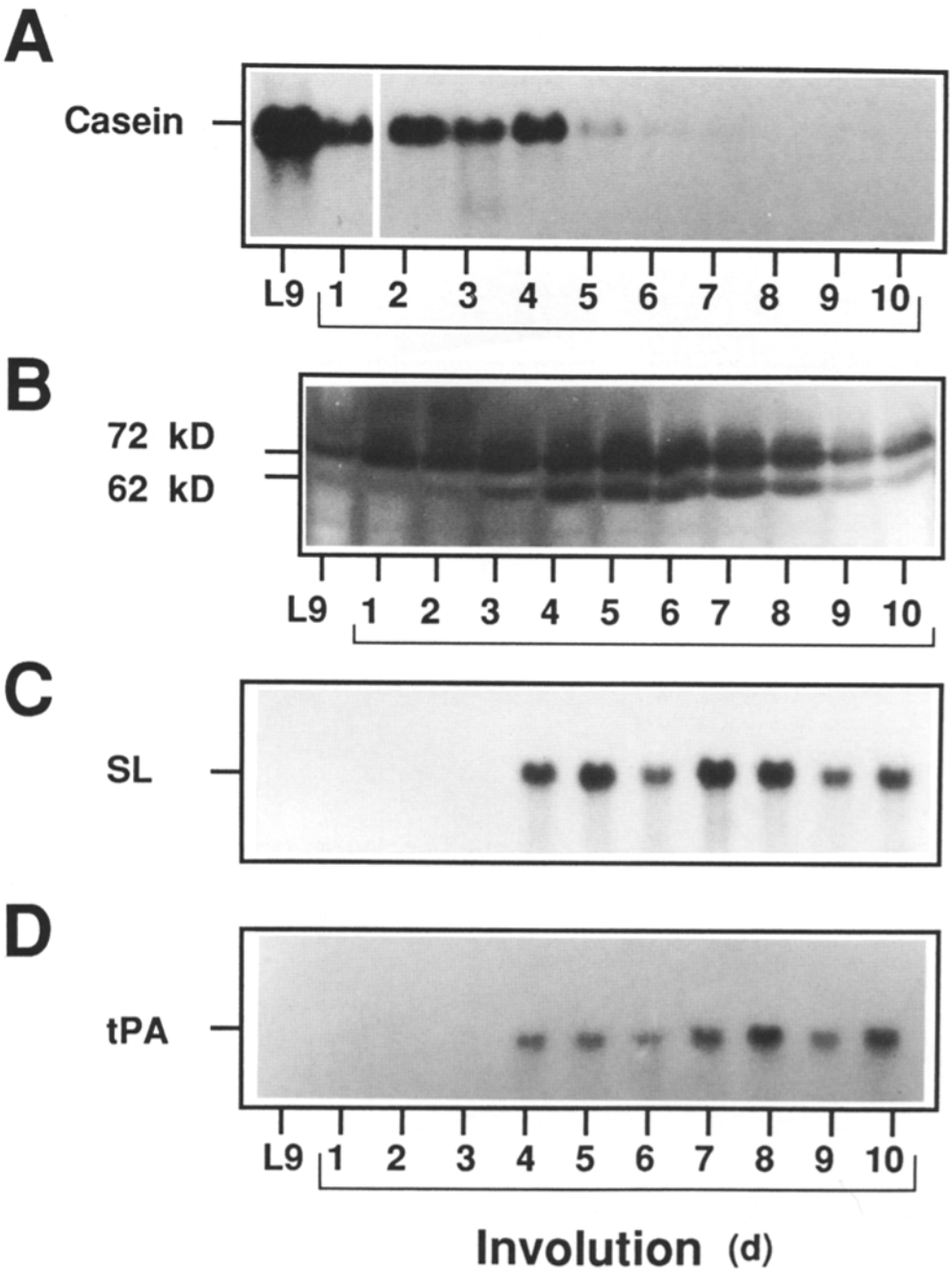

Figure 1. Reciprocal expression of milk proteins and ECM-degrading proteinases during involution. RNA blots of (*A*) β-casein and (*C* and *D*) ECM-degrading proteinases are shown. Expression of β-casein mRNA was maintained at high levels up to 4 d after involution and declined rapidly after that. A zymogram (*B*) shows that as early as 1 d after involution, the 72-kD gelatinase increased markedly over that observed in lactating mammary tissue (*L9*). The active form of 72-kD gelatinase (*62 kD*) began to appear on day three of involution, and remained prominent from days 4–10. mRNA expression of ECM-degrading proteinases SL (*C*) and tPA (*D*) was noted only after day three of involution.

SDS-Substrate Gels

Zymograms and tissue extracts were prepared as described previously (Talhouk et al., 1991*b*). Zymogram gels consisted of 7% polyacrylamide impregnated with gelatin at 1 mg/ml. After electrophoresis the gels were washed twice, for 30 min each time in 2.5% Triton X-100 solution at ambient temperature, then incubated for 24 h in substrate buffer (50 mM Tris-HCl buffer, pH 8.0, 5 mM $CaCl_2$, 0.02% NaN_3) at 37°C. The gels were then stained in Coomassie blue R250 for 1 h and destained in water overnight. Gelatin-degrading enzymes were visualized as clear bands, indicating proteolysis of the substrate protein. Metalloproteinases are secreted in a latent form and require cleavage of a peptide from their NH_2 terminus for activation. However, exposure of proenzymes of the tissue extracts to SDS during gel separation procedures leads to activation without proteolytic cleavage (Talhouk et al., 1991*b*). Zymograms are shown as negative images for clarity.

For preparation of samples, freshly isolated mammary tissue was immediately frozen in liquid nitrogen. The tissue was then pulverized to a fine powder, weighed, and either stored at −70°C or suspended 1:5 (wt/vol) in extraction buffer (1% Triton X-100 in 500 mM Tris-HCl buffer, pH 7.6, containing 200 mM NaCl and 10 mM $CaCl_2$). The suspension was frozen on dry ice, thawed four times, and microfuged (12,000 *g* for 30 min at 4°C). The supernatant was removed and stored at −70°C until needed for zymography. Analysis of proteinase activity was based on equal amounts of protein (15 μg) in tissue extracts.

Sealing of Primary Duct of Mammary Gland

The mammary glands from two groups of mice were sealed by cauterization of the teat opening and of the surgically exposed primary duct after 2 d of lactation. The first group consisted of normal lactating mice; the second group had their pups removed immediately after cauterization, leading to involution. Mammary tissues from lactating, sealed, and involuting glands were collected at 6 and 7 d after cauterization and processed for immunocytochemistry or RNA blot analysis as described above. Mammary tissues from two mice from each group were sampled for each stage of involution.

Preparation of Slow-release Pellets

Ethylene/vinyl acetate copolymer (EVAc; Du Pont Co., Boston, MA) slow-

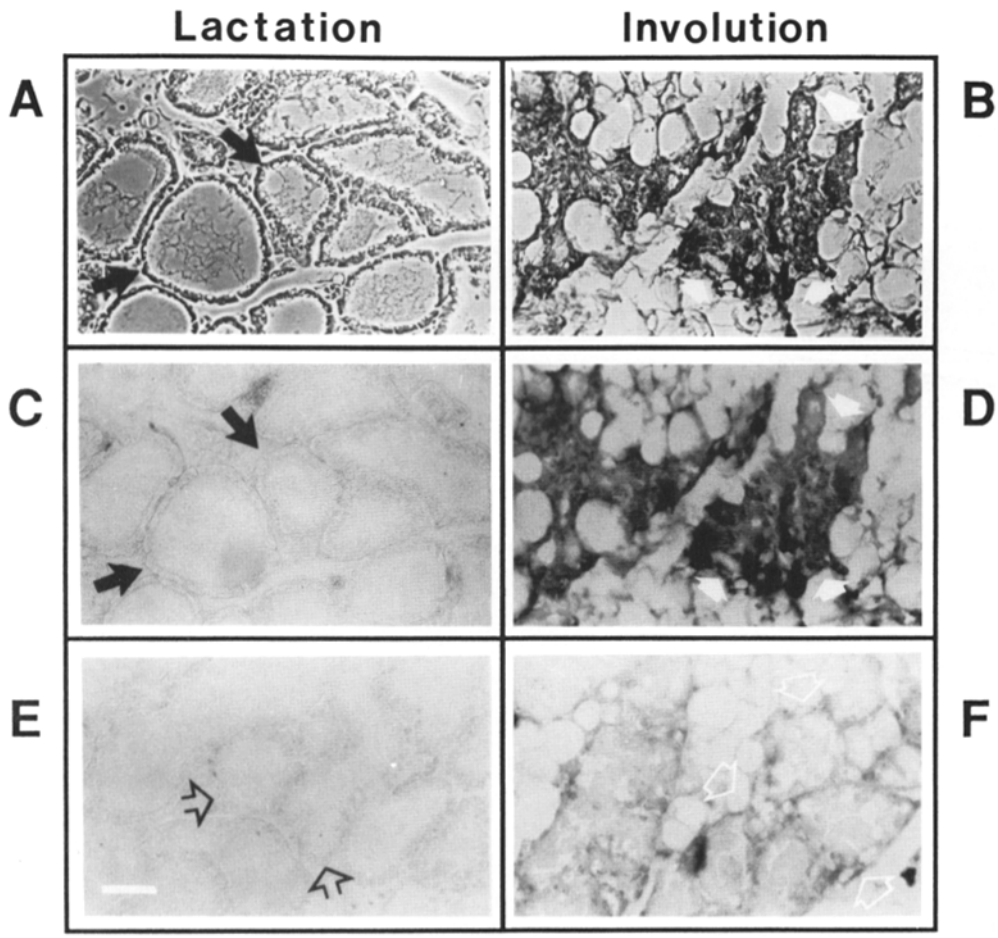

Figure 2. Immunolocalization of SL in involuting mammary gland. Transverse sections of paraffin-embedded tissue from (*A*, *C*, and *E*) a 9-d lactating mammary gland, and (*B*, *D*, and *F*) 4-d involuting mammary gland after a 9-d lactation were stained with antibody to SL (*A–D*) or control (*E* and *F*) are shown. *A* and *B* are phase-contrast micrographs and *C* and *D* are bright field micrographs, of the same sections as in *A* and *B*, respectively, of mammary tissue incubated with SL antibody. SL was observed around regressed alveoli (*solid white arrow heads* point to a group of regressed alveoli) of an involuting gland, but not around alveoli (*solid black arrow heads* point to two alveoli) of a lactating gland. *E* and *F* are bright field micrographs of control transverse sections incubated without the SL antibody (*open black arrow heads* point to alveoli in a lactating gland, and *open white arrow heads* point to a group of regressed alveoli). Note the dark staining observed around the alveoli in *D* but not in *F*. Bar, 50 μm.

release implants were prepared according to Silberstein and Daniel (1987). A lyophilized mixture of BSA (25 mg) and various concentrations of recombinant human TIMP (expressed in *Escherichia coli*, a gift of D. Carmichael, Synergen Corp., Boulder, CO) was dispersed with 0.125 ml of EVAc dissolved in dichloromethane (Sigma Chemical Co.). This mixture was immediately frozen, dried, and cut into 1–2-mg pieces. The pellets were then surgically implanted into the inguinal mammary gland (one mouse for each TIMP concentration, and two implants per gland) after 2 d of involution which was initiated by cessation of suckling after 2 d of parturition. Mammary glands with implants and the contralateral inguinal gland in the same mouse were sampled at 1, 2, and 3 d after implantation (equivalent to 3, 4, and 5 d of involution, respectively). Each gland was cut in two, fixed in 2% paraformaldehyde, and then either embedded in paraffin for hematoxylin and eosin staining or frozen in OCT compound as described earlier for immunofluorescence.

Results

ECM-Degrading Proteinases and Their Inhibitors Are Temporally Expressed during Involution

We determined the expression of ECM-degrading proteinases during transition of the mammary gland from a differentiated lactational phenotype to a nonlactating phenotype during involution. As involution of the gland was initiated after 9 d of lactation, three general events occurred. First, expression of β-casein mRNA, a marker of lactational phenotype, dropped to one third immediately after involution, remained there for 4 d after cessation of suckling, and then declined dramatically (Fig. 1 *A*). Second, expression of a number of ECM-degrading proteinases rose dramatically: the most prominent ECM-degrading proteinase detected in involuting tissue by substrate gel zymography was the 72-kD gelatinase (Fig. 1 *B*), as shown previously (Talhouk et al., 1991*b*). The 72-kD gelatinase, while low during lactation, was abundant as early as 1 d into involution and remained high throughout. The active form of the 72-kD gelatinase (the 62-kD band) began to be detected on day three of involution. SL, known for its wide range of ECM substrates (Talhouk et al., 1992; Alexander and Werb, 1991), and tPA, which is implicated indirectly in degradation of several ECM components (Alexander and Werb, 1991) were not expressed during lactation or the first 3 d of involution (Fig. 1, *C* and *D*). After 4 d of involution, and at least up to day 10 both SL mRNA and tPA mRNA were expressed at high levels. We have previously shown that the 72-kD gelatinase is secreted in the direction of the basement membrane (Talhouk et al., 1991*b*). Using a monoclonal anti-SL antibody that recognizes mouse SL in protein blots (not shown) and immunolocalization analysis (Fig. 2), we observed SL in 4-d involuting, but not lactating, tissue. Secreted SL localized proximally to the basement membrane underlying the mammary epithelial cells in alveoli (Fig. 2) and around ducts (not shown), but not around fat cells, which are surrounded by their own basement membrane. The third event that took place early in involution was the expression of TIMP, an inhibitor of SL, 72-kD gelatinase, and other metalloprotein-

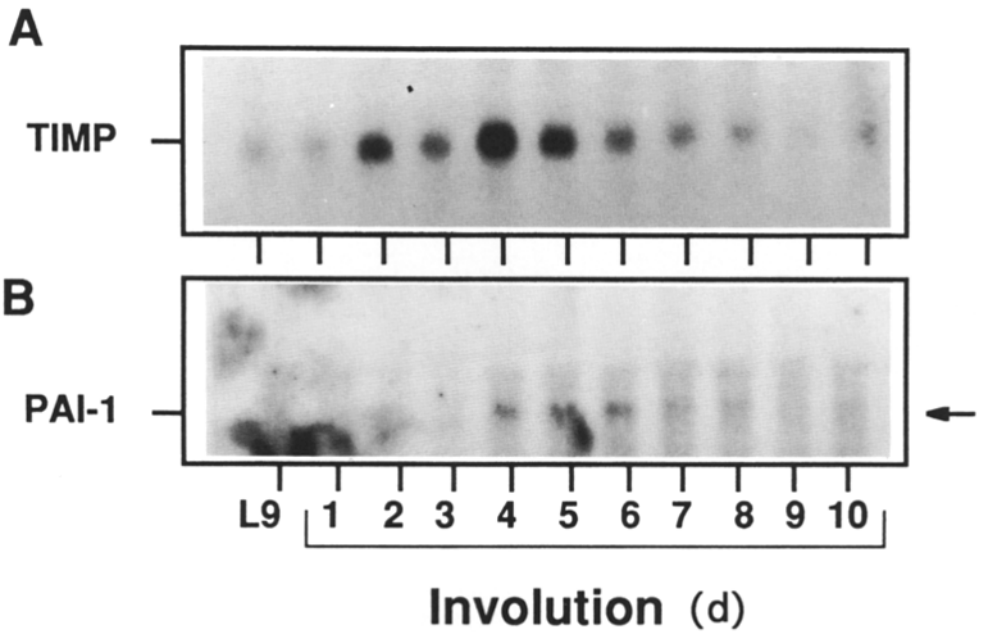

Figure 3. Regulation of TIMP and PAI-1 mRNA expression during involution. Total RNA prepared from lactating and involuting mammary tissue was analyzed by RNA blotting for expression of (*A*) TIMP and (*B*) PAI-1 (*arrow head*). Note that expression of these inhibitors peaked when 72-kD gelatinase, SL and tPA were first expressed during involution (see Fig. 1, *B–D*). Expression of inhibitors declined with that of β-casein mRNA (see Fig. 1 *A*). The first three lanes of the RNA blot for PAI-1 mRNA (*B*) are obscured by nonspecific background. This particular RNA blot was used because the RNA samples shown are from the same experiment as those in Fig. 1, *A–D* and those in Fig. 3 *A*. In another experiment with RNA samples isolated from comparable stages of mammary development as those that are obscured above, we did not detect PAI-1 mRNA expression (not shown).

ases, and PAI-1, an inhibitor of plasminogen activators. Peak levels were reached at days 4–6, and subsequently declined (Fig. 3). On the basis of these data, we hypothesize that these events in involution are linked and that a high ratio of inhibitors to ECM-degrading proteinases prevents degradation of the basement membrane for up to 4–5 d, maintaining accurate cell–ECM interactions and thus preserving functional epithelia that express β-casein. A diagram summarizing the change in expression of these gene products with time of involution is shown in Fig. 4. The loss of β-casein expression correlated closely with the onset of SL expression and the decline of TIMP expression more than it did with the onset of tPA expression and decline of PAI-1 expression. Thus subsequent studies focused on the interrelation of metalloproteinase levels and their inhibitor, TIMP.

Modification of Involution Is Correlated with Altered Expression of ECM-degrading Proteinases and TIMP

To test the first part of the above hypothesis, i.e., that the pattern of expression of ECM-degrading proteinases and inhibitors and the loss of mammary gland function are linked, it was necessary to modify involution and determine if temporal expression of these three events would change. We decreased the interval of lactation to 2 d, resulting in a more rapid involution than that observed after 9 d of lactation. While milk proteins were readily detectable by immunoblot analysis in gland extracts obtained after 9 d of lactation and persisted up to 2–3 d into involution (Fig. 5 *A*), when the lactation period was reduced to 2 d they had diminished dramatically by 2 d into involution (Fig. 5 *B*). After 9 d of lactation, the expression of β-casein mRNA was still abundant on day four of involution (Fig. 1 *A*), whereas after 2 d of lactation, β-casein mRNA disappeared by day four of involution (Fig. 6 *A*). After 2 d of lactation, expression of SL mRNA (Fig. 6 *B*) and tPA mRNA (Fig. 6 *C*) was low at day two but became high by day four and remained high at day seven of involution. Expression of TIMP mRNA (Fig. 6 *D*) peaked at 2 d of involution but had markedly decreased by day four. This temporal shift of critical events from 4–6 d after a 9-d lactation to 2–4 d after a 2-d lactation confirms the hypothesis that the three events discussed are interrelated.

Presence of Lactogenic Hormones Modifies Metalloproteinases and Their Inhibitors

To further examine the interrelation of metalloproteinase expression and loss of casein gene expression, we next took advantage of reports that involution can be retarded in a nonsuckled, hormonally stimulated gland (Schmidt, 1971). We asked if this retardation of involution could be due to a change in the ratio of inhibitors to ECM-degrading proteinases. To ensure that suckling did not occur, the primary duct of one of the mammary glands was sealed by cauterization. The sealed gland maintains its ability to make milk for prolonged periods if suckling of other nonsealed glands continues owing to continued lactational stimulus. 6 d after cauterization, the sealed gland expressed high levels of TIMP mRNA and low levels of SL mRNA, whereas its normal lactating counterpart in the same animal expressed neither (Fig. 7 *A*). β-Casein expression was maintained for at least 7 d after cauterization, with levels comparable with those of the lactating counterpart. On the other hand, in involuting tissue from completely weaned mice, β-casein had declined substantially by days 6–7 as shown above, and, as expected, these involuting glands expressed low levels of TIMP and high levels of SL. Therefore, β-Casein expression was maintained only when the ratio of SL to TIMP was low (Fig. 7 *B*). Immunohistochemical analysis (Fig. 7 *C*) in lactating, sealed, and involuting mammary glands showed that casein and alveolar structures were still abundant in the sealed gland comparable with those in lactating tissue. Alveolar structures in involuting gland were regressed, and casein was

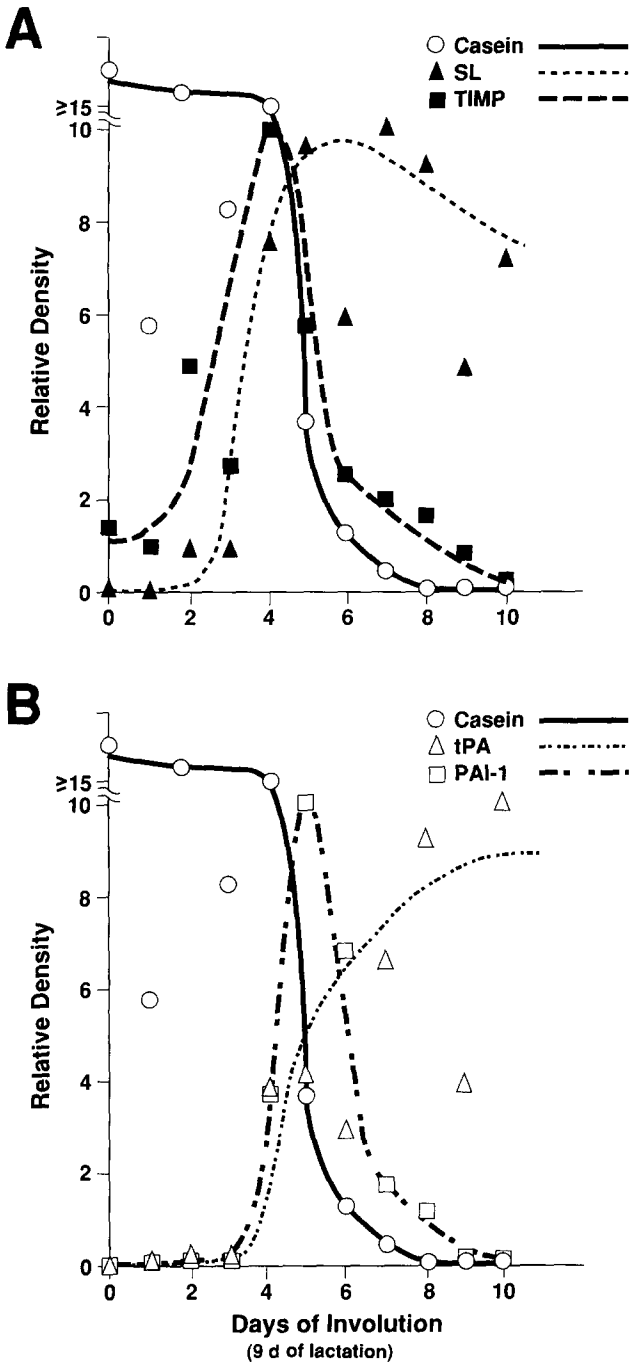

Figure 4. Summary of expression of ECM-degrading proteinases, inhibitors, and casein genes during involution after 9 d of lactation. RNA blots were quantified by densitometry and normalized to arbitrary units relative to the highest hybridization signal of mRNA/cDNA for each transcript, as described in the text. Average values from duplicate readings were plotted against days of involution. Data plotted show the temporal regulation of β-casein mRNA expression in relation to that of (*A*) SL and TIMP, and (*B*) tPA and PAI-1. Critical events after a 9-d lactation occurred at 4–6 d of involution.

hardly evident in the remaining alveoli. Involuting glands sealed before weaning were indistinguishable from normal involuting glands (data not shown). These results show that expression of TIMP and, to a lesser extent, SL, is induced by local rather than systemic factors. Furthermore, they suggest that the differentiated status of the mammary gland is dependent on a coordinated and critical balance between ECM-degrading proteinases and inhibitors: the lower the ratio of ECM-degrading proteinases to TIMP in an involuting gland, the slower the process of involution.

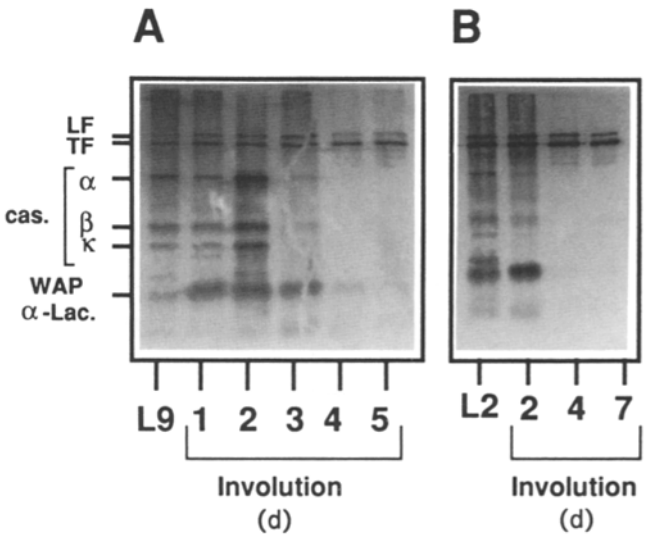

Figure 5. Effect of lactation interval on accumulation of milk proteins in the mammary gland during involution. Immunoblots of mammary tissue extracts from (*A*) a 9-d lactating gland (*L9*) and subsequent days of involution and (*B*) a 2-d lactating gland (*L2*) and subsequent days of involution are shown. Tissue extracts were subjected to electrophoresis on 12% SDS-polyacrylamide gels, transferred onto a membrane, and analyzed with a broad-spectrum milk antibody for milk proteins, lactoferrin (*LF*), transferrin (*TF*), casein (*cas*), whey acidic protein (*WAP*), and α-lactalbumin (*α-lac*). Both TF and LF are found in milk and in circulating blood.

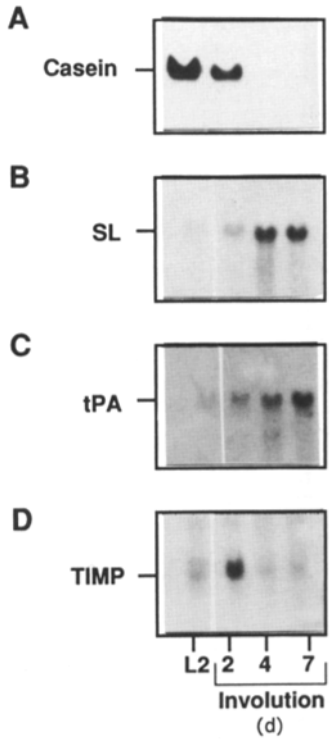

Figure 6. Effect of lactation interval on expression of β-casein and the ECM-degrading proteinase-inhibitor balance during involution. RNA blots for (*A*) β-casein, (*B*) SL, (*C*) tPA, and (*D*) TIMP in tissue from lactating and involuting mammary glands after a short (2 d) lactation (*L2*) interval are shown.

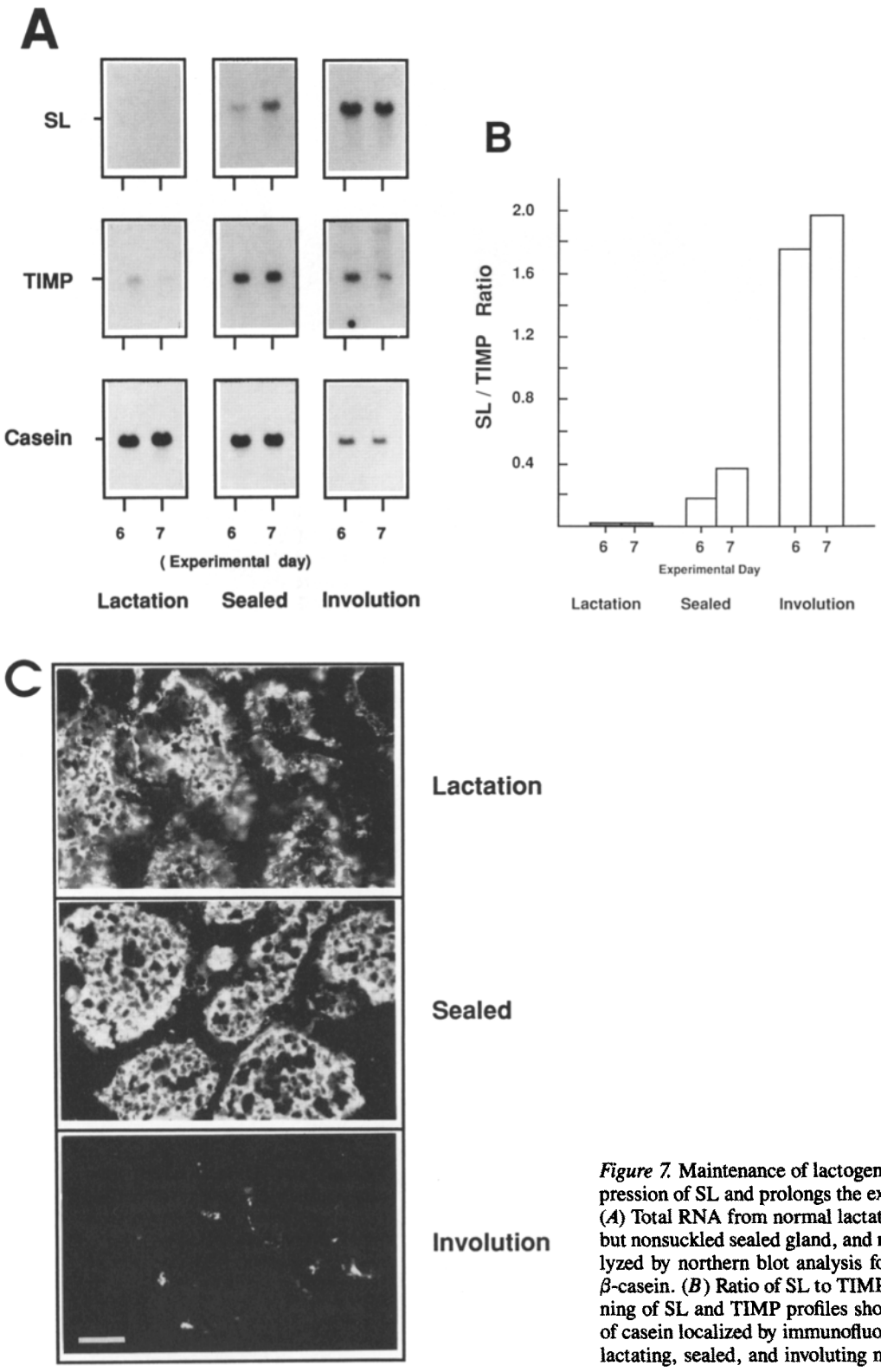

Figure 7. Maintenance of lactogenic stimuli delays the onset of expression of SL and prolongs the expression of TIMP and β-casein. (*A*) Total RNA from normal lactating gland, hormonally sufficient but nonsuckled sealed gland, and normal involuting gland was analyzed by northern blot analysis for expression of SL, TIMP, and β-casein. (*B*) Ratio of SL to TIMP obtained by densitometer scanning of SL and TIMP profiles shown in *A*. (*C*) Photomicrographs of casein localized by immunofluorescence in frozen sections from lactating, sealed, and involuting mammary glands. Bar, 50 μm.

The Ratio of TIMP to ECM-degrading Proteinases Regulates Involution

The experiments described so far showed a strong correlation between an increased ratio of ECM-degrading proteinases to inhibitors and the loss of lactational differentiation. However, maintenance of the functional status of the sealed gland, although accompanied by a shift in the ratio of SL to TIMP, could have been a result of maintenance of lactational stimulus and independent of ECM remodeling events. To test directly the role of ECM remodeling in loss of tissue-specific function, we artificially disrupted the proteinase-inhibitor ratio during normal involution, a milieu devoid of lactational stimuli. If the loss of function of mam-

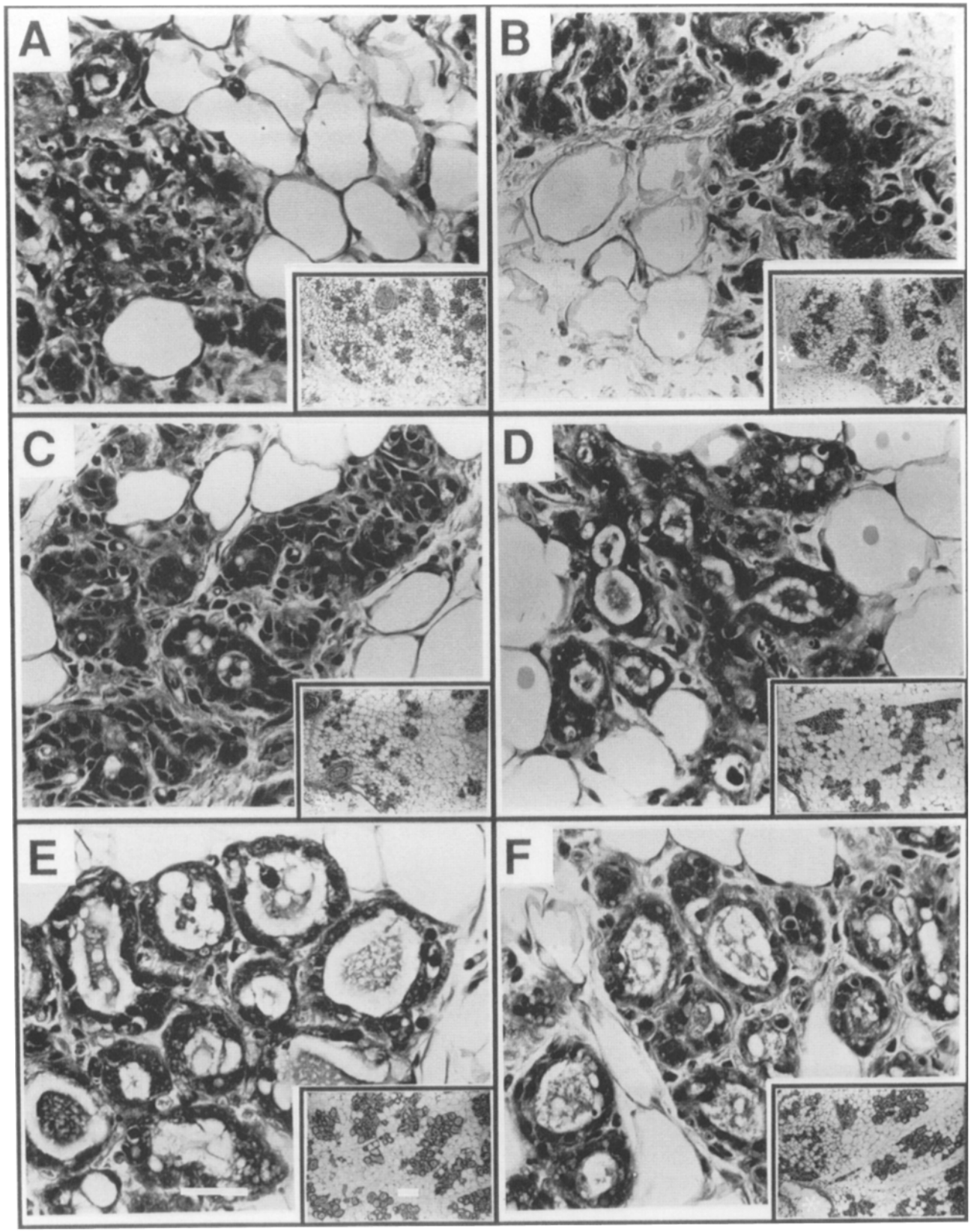

Figure 8. Exogenous TIMP inhibits involution of the mammary gland. (*A*) Transverse section of a typical day four involuting mammary gland without an implant, stained with hematoxylin and eosin, with a predominant fat stroma and regressed alveolar structures. Slow-release EVAc pellets surgically implanted in a 2-d involuting mammary gland (after 2 d of lactation) with (*B*) no TIMP, (*C*) 0.1, (*D*) 1, (*E*) 5, and (*F*) 10 μg TIMP show a concentration-dependent effect evident by a delay in the regression of the alveoli 2 d after implantation of the pellet (equivalent to 4 d of involution). Note the large size of lumina and evidence of secretory activity in mammary glands receiving 5 or 10 μg of TIMP (*E* and *F*). Insets show the same tissue sections at lower magnification. The asterisk indicates the location of the EVAc pellet. Bars: (*A–E*) 50 μm; (insets) 500 μm.

mary epithelium during involution is regulated by a decrease in the ratio of TIMP to SL, then the introduction of exogenous TIMP at the regulatory point should minimize ECM remodeling and alter the course of involution. To accomplish this, we prepared slow-release pellets (Silberstein and Daniel, 1982; and Coleman and Daniel, 1990) containing recombinant human TIMP, which has previously been shown to inhibit mammary metalloproteinases in zymogram assays (Talhouk et al., 1991*b*). In mammary glands weaned for 2 d after a 2-d lactation exposure to exogenous TIMP released from the implants delayed the onset of involution and prolonged the differentiated status of the gland. By day four of weaning, mammary glands containing TIMP implants showed a concentration-dependent delay of involution (Fig. 8). The alveoli in involuting mammary glands containing TIMP implants maintained a secretory morphology resembling that of lactating alveoli. The majority of alveoli were well rounded, rather than convoluted, and many still surrounded a lumen that contained secretory products and fat-like droplets. In contrast, alveoli in the involuting glands in the same animal that did not contain implants, or that contained implants with no TIMP or only low concentrations of TIMP, were smaller, convoluted, and had small lumina evident in only a few of the alveoli. This concentration-dependent effect was noted in mice at days three and four of involution (1–2 d after implantation of the pellet). 3 d after implantation, no differences were noted between the glands that contained the TIMP implant and the glands that did not. This suggests that as the release of TIMP is lessened, the ECM-degrading proteinases and/or other soluble mammary effectors were able to override the inhibitory effect of exogenous TIMP.

We next determined whether the implants containing TIMP could indeed retard the dissolution of the mammary alveolar basement membranes. In 4-d involuting gland, after a 2-d lactation, the basement membranes of both epithelial alveoli and fat cells stained with type IV collagen (Fig. 9 *A*), making it difficult to distinguish whether the basement membrane surrounded the alveoli or fat cells. We exploited the observation that the basement membrane of fat cells does not contain the laminin A-chain isoform (Aratani and Kitagawa, 1988) and used an antibody to laminin A-chain to examine the basement membrane of the mammary epithelial alveoli. In a 4-d involuting mammary gland with no implant, laminin A-chain antibody stained the small alveoli with an apparently continuous basement membrane (Fig. 9 *B*). Very little staining for casein was observed in this gland. The contralateral gland with a 10 μg TIMP pellet implanted on day two of involution, on the other hand, showed much larger alveoli with continuous basement membranes, and greater numbers of alveoli and lumina. These alveoli contained abundant casein (Fig. 9 *C*). Therefore, only alveoli in the gland with the TIMP implant, but not the contralateral one, maintained a lactational status.

Discussion

Few reports in the literature describe an active role for ECM-degrading proteinases and their inhibitors in the regulation and maintenance of tissue-specific function (for review see Alexander and Werb, 1991; and Matrisian, 1990). The transition of the mammary gland from a differentiated lactational phenotype to a nonlactating phenotype during involution, and the concomitant dissolution of the basement membrane, is an ideal model for studying the effect of ECM on the function of secretory mammary epithelial cells in vivo. There is now compelling evidence that basement membrane instructs mouse mammary epithelial cells to acquire and maintain the tissue-specific lactational phenotype in culture (Streuli et al., 1991; Streuli and Bissell, 1990; Chen and Bissell, 1989; Barcellos-Hoff et al., 1989; Blum et al., 1987; Li et al., 1987). An earlier study by Wicha et al. (1980) demonstrated that inhibition of basement membrane deposition in vivo in mammary glands of rats disrupted ductular branching and alveolar development. We have shown recently that loss of mammary gland function during involution (Lascelles and Lee, 1978) correlates with an increase in expression of ECM-degrading proteinases (Talhouk et al., 1991*b*). The present study was designed to test the hypothesis that proteolysis of basement membrane is responsible for loss of function of mammary gland during involution, and thus to provide direct critical evidence for the role of basement membrane in regulating functional differentiation in vivo.

To dissect the mechanisms involved in loss of differentiated functions of mammary gland, we used three different ways to induce involution: (*a*) slow involution after 9 d of lactation; (*b*) rapid involution after 2 d of lactation; and (*c*) "involution" in a hormone-sufficient mammary gland in which the duct leading to one nipple was cauterized while other glands were still being suckled. Our data indicate that, while the patterns of regulation of ECM-degrading proteinases and inhibitors were distinct in each case, increased expression of ECM-degrading proteinases relative to inhibitors correlated with the onset of loss of tissue-specific function, measured by β-casein expression and morphology of the mammary gland.

Loss of lactational hormonal stimulus during involution (Forsyth, 1982) resulted in an immediate decrease in β-casein expression on day one, but expression could be maintained at intermediate levels for up to 5 d of involution. The initial drop probably correlates with cessation of suckling and secretion and hence feed back inhibition by intracellular accumulated milk (Wilde et al., 1990). The subsequent loss of β-casein expression in involution correlated with expression of ECM-degrading proteinases that could alter cell–ECM interaction. Early in involution, when resumption of lactation and expression of milk proteins is still a possibility, the gland is engorged with milk for at least 2 d into involution (Hurley, 1989), as indicated by immunoblot analysis. At this point, ECM-degrading proteinases and their inhibitors become critical players in regulating mammary gland function. During the early stage of involution (days 1–4), the gland could still maintain milk expression because the activity of ECM-degrading proteinases relative to their respective inhibitors was low. As the ratio of ECM-degrading proteinases to inhibitors rose, there was an abrupt decrease in expression of milk proteins at 4 d. We believe this to be solely the function of the proteinase to inhibitor ratio rather than additional hormonal or other stimuli, because when we kept other parameters constant but changed this ratio, we could modulate lactation. For example, a shorter interval of lactation altered the kinetics of involution by precipitating an earlier decline in expression of milk proteins.

In experiments where we prevented lactation in one set of

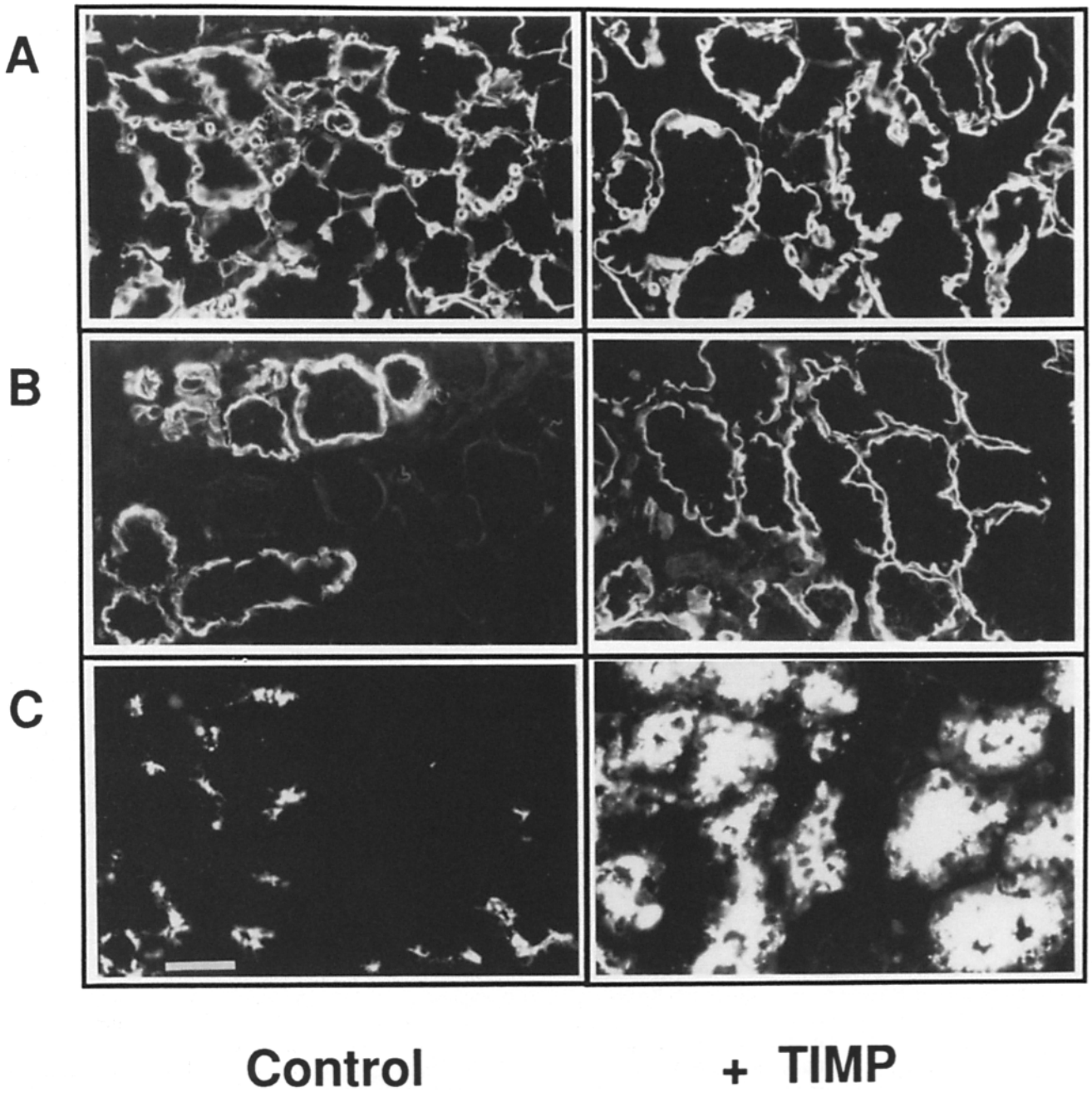

Figure 9. Immunohistochemical localization of (*A*) type IV collagen, (*B*) laminin A-chain, and (*C*) β-casein in 4-d involuting glands with no EVAc pellets (*Control*) and those with 10 μg TIMP pellets 2 d after surgical implantation (*+ TIMP*). As in Fig. 8, the size and morphology of alveoli are markedly different in mammary gland receiving TIMP. Bar, 50 μm.

mammary glands while other glands in the animal continued to be suckled, we could draw an additional conclusion: that expression of TIMP, in particular, and SL is independent of hormonal stimuli but related to loss of suckling, because the other glands in the same animal had no discernable enzyme expression. This observation perhaps explains why prevention of suckling delays involution but does not prevent it (Wellings and DeOme, 1963). The nonsuckled gland cannot maintain its functional status indefinitely in the presence of lysosomal enzymes (Hurley, 1987) and ECM-degrading proteinases.

In experiments where we introduced exogenous TIMP to the involuting gland, thus reversing involution, we provided definitive evidence for the overriding effect of TIMP. The higher the TIMP to metalloproteinase ratio, the larger the alveoli and the longer they could maintain a lactational phenotype, as shown by accumulation of casein in the lumen. The detailed mechanism by which TIMP inhibits the action of ECM-degrading proteinases is not well understood. However, the inhibitors may bind to the basement membrane and inactivate secreted ECM-degrading proteinases, as suggested by Saksela and Rifkin (1988) and Alexander and Werb (1991).

The literature on the fate of basement membrane surrounding the alveoli during involution is confusing. Earlier studies showed that the basement membrane is not lost dur-

ing involution but rather is thicker and more convoluted than it is in lactating tissue (Warburton et al., 1982; Richards and Benson, 1971; and Wellings and DeOme, 1963). Others have suggested that the basement membrane is hydrolysed during involution (Martinez-Hernandez et al., 1976). However, these studies may have been misinterpreted owing to the difficulty in differentiating between basement membrane of fat cells and that of epithelial cells. In this study, we used an antibody to laminin A-chain that clearly distinguishes an intact basement membrane underlying mammary epithelial cells during involution. We saw a greatly diminished number of alveoli and, hence, less basement membrane in involution. The remaining alveoli retained a stainable continuous basement membrane, which did appear to have thicker staining, perhaps due to internal folding as some cell death occurs. Because exogenous TIMP released from implants not only prolonged the functional status of the alveoli, but also led to maintenance of larger alveoli with more secretory cells, we speculate that there is a role for the local effect of ECM-degrading proteinases in the absence of their respective inhibitors. ECM-degrading proteinases secreted either by the epithelial cells themselves (Talhouk et al., 1991*b*; Monteagudo et al., 1990) or by fibroblasts (Basset et al., 1990) would alter the interaction of certain cells within an alveolus with ECM and eventually lead to detachment of cells from the basement membrane and from neighboring cells in an alveolus. The basement membrane underlying the detaching cells is degraded. Adjacent cells could be protected from ECM-degrading proteinases by local concentrations of inhibitors such as PAI-1 or TIMP bound to their basement membrane (Alexander and Werb, 1991; Saksela and Rifkin, 1988). The net result of this process would be smaller alveoli, as postulated by Wicha et al. (1980), as involution proceeds.

In the lactating gland, functional differentiation is maintained with a minimal remodeling of ECM. A corollary and critical test of our hypothesis that basement membrane plays an active role in regulating differentiated function in vivo would be to induce expression of ECM-degrading proteinases during lactation. In studies to be reported separately (Talhouk, R. S., C. Sympson, C. M. Alexander, S. Clift, M. J. Bissell, and Z. Werb, manuscript in preparation), we generated transgenic mice that inappropriately express autoactivatable SL (Sanchez-Lopez et al., 1988) under the control of the whey acidic protein promotor during lactation. In effect, a higher ratio of SL to inhibitor was artificially induced during lactation. Alveoli in the mammary glands of these transgenic mice were smaller than alveoli from nontransgenic, normal lactating mice and contained a discontinuous basement membrane. This loss-of-function phenotype is opposite to the gain-of-function phenotype in involuting mammary glands of mice with TIMP implants.

Given our results in this and previous studies on mammary gland differentiation (reviewed by Howlett and Bissell, 1992; Talhouk et al., 1992), we hypothesize that there are six distinct steps by which a mammary epithelial cell maintains its differentiated status. (*a*) Induction of lactogenic hormones. (*b*) Formation of a functional basement membrane in contact with mammary epithelial cells. What regulates formation of this basement membrane, and whether or not the composition is different during different stages of development, is not known. (*c*) Expression of various integrins (and other ECM receptors) to modulate interactions of the cell with its microenvironment. While a few mammary-specific integrins have been identified, lack of rodent-specific antibodies has hampered a complete analysis of the integrins. (*d*) Interaction of the cell with its ECM. This interaction is possibly mediated via integrins. We have shown previously that a broad-spectrum β_1-integrin antibody disrupts β-casein expression in mammary cells in culture (Streuli et al., 1991). (*e*) Interaction of integrins with second and third messengers for intracellular signalling. (*f*) Expression of differentiated functions. We now know that milk protein genes, as exemplified by β-casein, contain ECM response elements (Schmidhauser et al., 1990, 1992).

In this study we have dealt only with the fourth step. Not only have we shown the importance of ECM to mammary function in vivo, but we also have proven the corollary to the culture studies; i.e., if basement membrane is necessary for functional differentiation, then loss of basement membrane should lead to loss of function.

In summary, we now have evidence that functional epithelial tissue in vivo maintains its differentiated status when minimal remodeling of ECM is occurring. Cell–ECM interaction can be altered in the presence of increased levels of ECM-degrading proteinases and/or in the absence of their inhibitors. Because inhibitors play a role in minimizing ECM remodeling, thus maintaining a status quo of cell–ECM interaction, they are critical players in modulating cell morphology and function. What signals regulate the ECM-degrading proteinases and their inhibitors remains to be determined.

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