TGFβ Suppresses Casein Synthesis in Mouse Mammary Explants and May Play a Role in Controlling Milk Levels during Pregnancy

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Abstract. Mammary explants from 14–15-d-pregnant mice synthesize and secrete milk proteins in culture in response to insulin, hydrocortisone, and prolactin. Here we demonstrate that transforming growth factor β (TGFβ) treatment suppresses, in a dose dependent and reversible manner, the ability of explants to synthesize and secrete milk caseins. TGFβ does not affect the level of casein mRNA within explants but inhibits casein synthesis posttranscriptionally. We also show increased expression of TGFβ2 and TGFβ3 in intact mammary gland as pregnancy progresses, with reduced expression of all three TGFβs at the onset of lactation. These findings suggest that endogenously produced TGFβ may limit the accumulation of milk caseins that are produced in the mammary gland during pregnancy.

THIS paper describes a potential role for locally produced transforming growth factor beta (TGFβ)[1] in limiting the accumulation of milk proteins in the mouse mammary gland during pregnancy. Previous results from our laboratory demonstrated abundant expression of TGFβ2 and TGFβ3 transcripts in the mammary glands of pregnant mice, but very low levels of expression of all three mammalian TGFβs in the glands of lactating mice. We also showed that none of the TGFβs alter normal alveolar growth and morphogenesis when administered to the mammary glands of pregnant mice using miniature slow-release plastic implants (Daniel et al., 1989; Robinson et al., 1991). These findings suggested that TGFβs might play a role in preparing the gland for lactation. We investigated this possibility by treating cultured mammary explants from 14–15-d-pregnant mice with TGFβ and lactational hormones.

Milk proteins are classified into the acid-precipitable caseins and the whey proteins. The caseins are a family of milk phosphoproteins whose biological function is to provide supersaturating concentrations of calcium, phosphates, and essential amino acids to the neonate (Vandahaar and Ziska, 1989). α- and β-caseins, the most abundant of the milk caseins, are described as calcium sensitive because they precipitate in the presence of low concentrations of calcium (Waugh, 1971). They are maintained in stable suspension in milk because of their interaction with κ-casein (Mackinlay and Wake, 1971). Their relative abundance in milk makes caseins ideal markers of differentiated mammary function.

The regulation of casein synthesis and secretion is a complex process regulated by multiple interactions between peptide and steroid hormones (Topper, 1970; Topper and Freeman, 1980). Whole mammary organs or mammary explants synthesize and secrete milk proteins in the presence of the lactogenic hormones insulin, prolactin, and hydrocortisone (Elias, 1957; Jeurgens et al., 1965; Forsyth, 1971; Rivera, 1971). Expression of caseins in culture is also influenced by cell–cell and cell–matrix interactions (Levine and Stockdale, 1985; Bissell and Ram, 1989), and there is now increasing evidence that growth factors, such as EGF, TGF, fibroblast growth factors, and insulin-like growth factor play important roles in mammary growth and differentiation (Oka et al., 1991). TGFβ1 inhibits the expression of β-casein in lactogenic hormone-induced HC11 mouse mammary epithelial cells (Mieth et al., 1990). Here, we show that all three TGFβs regulate mammary explant production of caseins by suppressing their synthesis and subsequent secretion.

We also expand our previous analysis of TGFβ mammary mRNA expression patterns during pregnancy and lactation. TGFβ2 and TGFβ3 levels increase as pregnancy continues, with greatly reduced expression of all three TGFβs after birth. These findings suggest that TGFβ may help limit the accumulation of milk caseins during pregnancy and that its own expression may be regulated by the hormones of pregnancy and lactation.

Materials and Methods

Explant Cultures

14–15-d-pregnant, primiparous, Balb/C mice were anesthetized with Nem-

1. *Abbreviation used in this paper*: TGF, transforming growth factor.

butal (60 μg/g body weight) and the number three thoracic and number four inguinal mammary glands were removed aseptically and rinsed in DME/F12 medium containing 5 μg/ml gentamycin sulfate. The tissue was removed to a sterile surface and minced with sterile stainless steel razor blades. A number of explants, weighing a total of 30–100 mg, were transferred to a 15-ml conical culture tube containing the appropriate medium. Cultures were gassed briefly with 5% CO_2:95% air, sealed tightly, then placed at 37°C on a rotary shaker at 300 rpm. Medium was replaced every 48 h (Western blot analysis and characterization studies) or every 24 h (all other experiments). The glands from two or more animals were used in all studies. Lactogenic hormones were 5 μg/ml each of insulin (Sigma Chemical Co., St. Louis, MO), hydrocortisone (Sigma Chemical Co.), and prolactin (National Institutes of Health). Aldosterone (Sigma Chemical Co.) at 5 μg/ml was also included in the culture medium (see Ganguly et al., 1981).

We are defining secreted proteins as those that can be detected in culture medium. The bulk of secretion in explants is directed toward the lumens which have been cut by dissection of the explants. Shaking the explants during the culture period, which served to prevent the explant lumens from becoming clogged with secreted proteins, was critical for the continuous release of proteins into the medium.

RNA Preparation

Tissues or explants were frozen in liquid nitrogen immediately after removal from the animal or from culture media and total RNA was prepared as previously described (Robinson et al., 1991). For tissues, total RNA was isolated from 2–6 animals for each stage examined.

Northern Hybridization

RNA was electrophoresed in 1.0% agarose containing 2.3 M formaldehyde in MOPS buffer (0.2 M morpholinopropane sulphonic acid, 50 mM sodium acetate, 5.0 mM EDTA. pH 7.0). RNA was transferred to Magna NT (MSI, Inc., Westboro, MA) by the established procedure of Maniatis et al. (1982). The RNA was hybridized, under high stringency conditions, to mouse cDNA probes radiolabeled with ^{32}P by random priming. Washes after the hybridizations were in 0.1× SSPE/0.1% SDS at 65°C. 18 S rRNA was detected with a complementary 18-base oligonucleotide end-labeled with ^{32}P. Here, washes were in 2× SSPE/0.1% SDS at 57°C.

TGFβ clones were provided by P. Kondaiah (Laboratory of Chemoprevention, Bethesda, MD). Casein clones, provided by M. Bissell (Lawrence Berkeley Laboratory, Berkeley, CA), were originally isolated by J. Rosen (Baylor College of Medicine, Houston, TX). The 18 S rRNA oligonucleotide was provided by H. Noller (University of California, Santa Cruz, CA).

Immunoblot Analysis

To detect caseins in culture medium, 1-ml samples were precipitated with a final concentration of 10% TCA. Protein pellets were resuspended in electrophoresis sample buffer (2% SDS, 10% glycerol, 0.08 M Tris-HCl, pH 6.8, 2 mM EDTA, 0.1 M DTT, 0.01% bromophenol blue) and denatured at 95°C for 10 min before loading onto 13% denaturing acrylamide gels. Sample loading was standardized on the basis of total TCA-precipitable counts present in the original sample. After electrophoresis, proteins were transferred to Immobilon P membrane (Millipore Co., Medford, MA) in 25 mM Tris-base, 192 mM glycine, 15% (vol/vol) methanol. Membranes were blocked for 2 h at 37°C with 5% BSA (Sigma Chemical Co.) in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl (TBS). The blots were then washed 3 × 5 min in 0.1% BSA in TBS. The membranes were incubated for 1 h in polyclonal rabbit anti-mouse milk casein antiserum (diluted 1:3,000 in 1% BSA, 0.05% Tween-20 in TBS) and washed as above. Bound antibody was detected by addition of HRP-conjugated anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA), followed by 4-chloro-1-napthol and H_2O_2. All washes and antiserum incubations were at ambient temperature. Biotinylated size markers were detected with HRP-conjugated streptavidin (Bio-Rad Laboratories). The rabbit anti-mouse casein antiserum was a gift from Victor Rocha (University of California, Santa Cruz, CA).

Metabolic ^{35}S-labeling

For Western blot, dose-dependence, and characterization studies, explants were incubated during the final 24 h of culture in 4.5 ml of DME/F12 medium to which 100 μCi of [^{35}S]methionine from translabel (ICN, 1200 Ci/mmol) was added. The medium was harvested and stored at −70°C until immunoprecipitation. For pulse–chase studies explants were cultured for 4 d. During the final hours of culture, medium was replaced with 1 ml of methionine-free DME/F12 and 250 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) (1200 Ci/mmol) was added. For pulse only studies, labeling was stopped by quick freezing, and explants were stored at −70°C until further analysis. For pulse–chase studies, 1 h of labeling was followed by rinsing in ice-cold DME/F12 containing an excess of unlabeled methionine (4 mg/ml). The explants were then incubated in 1.5 ml of the same methionine-rich medium for varying amounts of time and processed for further analysis as described above.

Immunoprecipitation

Media fractions were adjusted in 1.0 ml to radioimmunoprecipitation (RIPA) buffer conditions (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM Na_2EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.1% SDS, 0.2% NP-40, and 0.5% sodium deoxycholate). The explants were homogenized in 1.5 ml of RIPA buffer without detergents and immunoprecipitation was conducted by adjusting these samples in 0.5 ml to RIPA detergent concentrations. The total incorporation of radioactivity was calculated from the TCA-precipitable counts contained in the medium and explant homogenate samples and aliquots representing equal numbers of total TCA-precipitable cpms were used for immunoprecipitation. RIPA-adjusted samples were incubated for 45 min at 4°C with anti-casein antiserum and subsequently for 1 h with 0.1 vol of 10% (wt/vol) protein A-sepharose beads (Pharmacia, Milwaukee, WI). The protein A-Sepharose beads were collected by centrifugation, and washed (3× RIPA buffer; 1× 50 mM Tris-HCl, pH 7.4) for 20 min with end-over-end rotation at 4°C. The precipitated proteins were eluted in electrophoresis sample buffer by heating at 95°C for 10 min and then separated by electrophoresis in denaturing 13% acrylamide gels. For fluorography, gels were treated for 30 min with Amplify (Amersham Corp.), dried, and exposed at −70°C to x-ray film.

Results

TGFβ mRNA Levels during Pregnancy and Lactation

We previously showed (Robinson et al., 1991) that steady-state mRNA levels for TGFβ2 and TGFβ3 are high in the

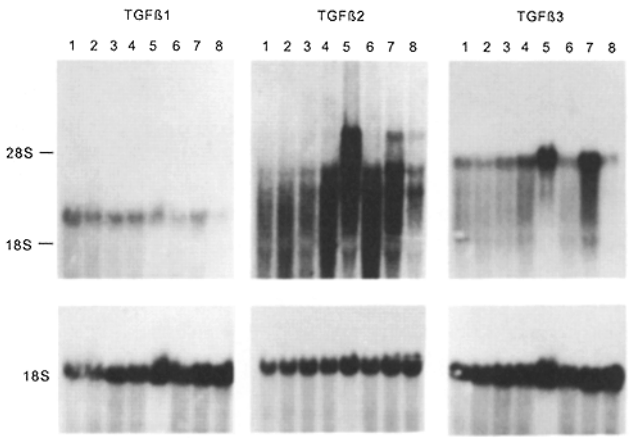

Figure 1. Expression of TGFβ mRNAs during pregnancy and early lactation. Blots containing total RNA (15 μg per lane) isolated from mammary glands of virgin, pregnant, and lactating animals were hybridized to radiolabeled, isoform-specific TGFβ cDNA probes and washed under conditions of high stringency (0.1 × SSPE, 0.1% SDS, 65°C). To control for equivalent loading of the mRNA, rRNA was examined by UV shadowing after electrophoresis (the positions of 28 S and 18 S ribosomal RNAs are indicated for reference). As a control for equivalent transfer and integrity of the samples, the blots were later probed for the expression of 18 S ribosomal RNA (*lower panels*). Lanes: (*1*) virgin; (*2*) 6 d pregnant; (*3*) 9 d pregnant; (*4*) 12 d pregnant; (*5*) 15 d pregnant; (*6*) 17 d pregnant; (*7*) day of birth; (*8*) 3 d after birth.

mammary gland during pregnancy but are low during lactation. Here, we performed a more detailed analysis of the mammary gland expression patterns of TGFβ1, TGFβ2, and TGFβ3 mRNAs during pregnancy and early lactation. RNA was isolated from mammary glands of virgin, staged pregnant, and early lactating animals and RNA blots were probed with radiolabeled TGFβ cDNAs (Fig. 1).

TGFβ1 transcript levels did not change significantly during pregnancy and lactation, with the exception of slight decreases at 17 d of pregnancy and 3 d after birth. TGFβ2 and TGFβ3 mRNA levels, on the other hand, increased during pregnancy, with peak expression at 15 d. The levels decreased transiently at 17 d of pregnancy and decreased again 3 d after birth. We previously showed that the levels of all three TGFβ transcripts are low after seven days of lactation (Robinson et al., 1991).

The temporal variation of TGFβ mRNA levels between pregnancy and lactation suggested that the loss of TGFβ expression is necessary for the onset of lactation.

TGFβ Suppresses Casein Secretion

Mammary explants were isolated from mouse mammary glands at 14 d of pregnancy as described in Materials and Methods. The nature of secreted milk caseins was determined by Western blot analysis of the medium from explants which were cultured for four days in the presence of lactogenic hormones alone, or in lactogenic hormones plus 5 ng/ml of TGFβ1, TGFβ2, or TGFβ3.

The positions of α_1-casein (43 kD), α_2-casein (39 kD), β-caseins (26 kD), and γ-casein (23.7 kD) in mouse milk are indicated in lane *6* of Fig. 2 *A* and are consistent with those reported by Hennighausen and Sippel (1982). γ-casein is not as abundant as the other caseins and was not readily detected in most of our assays. Medium from explants cultured in the absence of lactogenic hormones was analyzed as a control for antiserum specificity and is shown in lane *1* of Fig. 2 *A*. The levels of secreted caseins were high in the medium from explants cultured in lactogenic hormones (lane *2*). With the addition of TGFβ, casein secretion was dramatically suppressed (lanes *3*, *4*, and *5*). As shown in lane *5*, suppression of casein secretion was always greatest with TGFβ3. For this reason, and because the response was similar with all three TGFβs, further discussion will focus on the results we obtained with TGFβ3.

Explants were labeled with ^{35}S-translabel during the final 24 h of culture and for gel loading, individual medium samples were standardized on the basis of total TCA-precipitable counts. To determine whether TGFβ selectively suppressed casein secretion the transfer membrane was exposed to x-ray film following Western blot analysis (Fig. 2 *B*). The medium pattern of total, ^{35}S-labeled proteins was identical in the presence or absence of TGFβ. Determination of the total counts incorporated into TCA-precipitable proteins in both explants and medium indicated that the cells of the explants secreted ~10% of total protein synthesized. This ratio was not changed as a result of TGFβ treatment, providing further support for the selectivity of the response.

Dose Dependence of the TGFβ Response

The dose dependence of TGFβ-induced suppression of casein secretion was examined in explants which were cultured for 4 d in lactogenic hormones plus varying concentrations of TGFβ3. Cultures were labeled with ^{35}S-translabel during the final 24 h of culture and the levels of secreted caseins were detected in media samples by immunoprecipitation. Fig. 3 *A* shows a representative example of the increased suppression of casein secretion that occurred with increasing concentrations of TGFβ3.

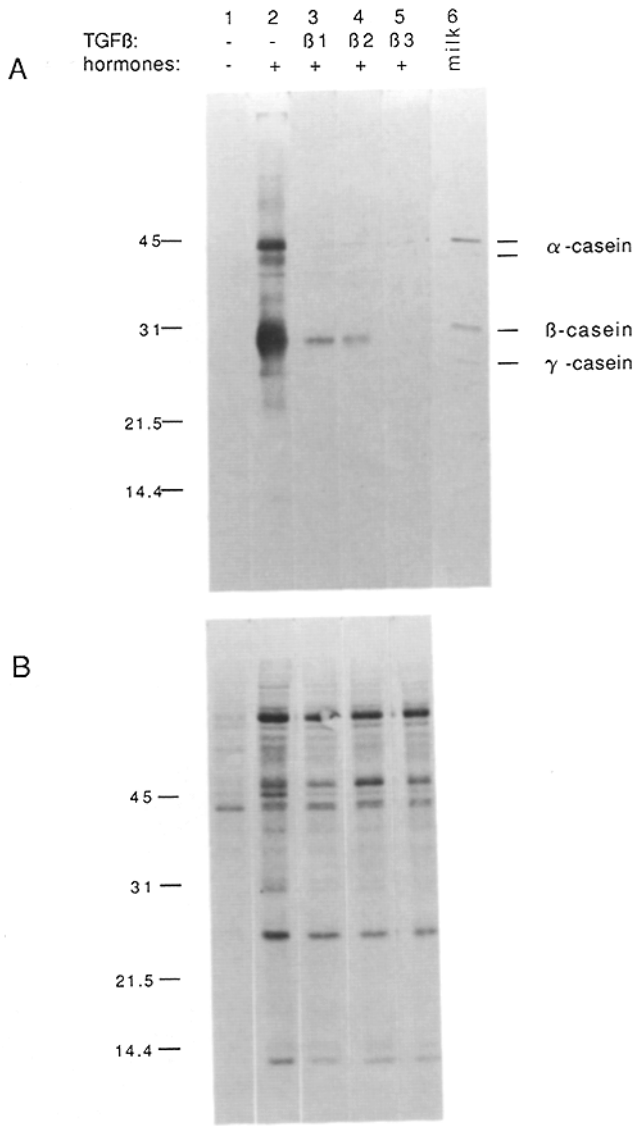

Figure 2. Western blot analysis of caseins in the medium of explant cultures. Explants were cultured in the presence (+) or absence (−) of lactogenic hormones for 4 d. TGFβ1 (lane *3*), TGFβ2 (lane *4*), or TGFβ3 (lane *5*) was included in the indicated cultures at a concentration of 5 ng/ml. Cultures were labeled with ^{35}S-translabel during the final 24 h. (*A*) A blot containing TCA-precipitable proteins from media samples incubated with a rabbit polyclonal antiserum directed against milk caseins. Loading was standardized on the basis of total TCA-precipitable counts present in the media samples. Mouse milk (lane *6*) was used as a control (20 μl of a 1:30,000 dilution in electrophoresis sample buffer). Molecular weight standards (kD) are indicated to the left of the figure. (*B*) Autoradiograph of the same blot to illustrate total ^{35}S-labeled proteins present in the media samples.

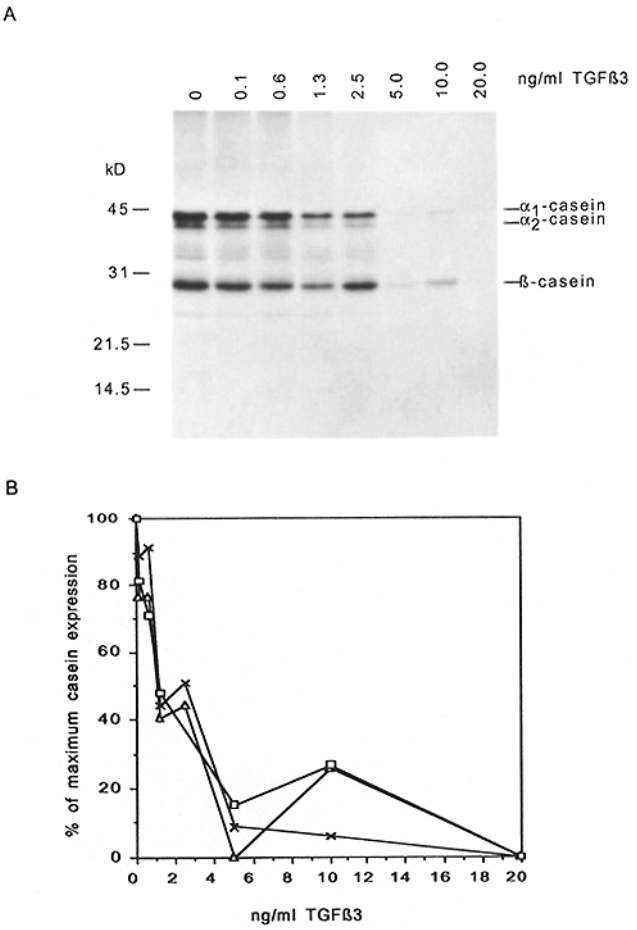

Figure 3. Dose dependence of the TGFβ response. Explants were cultured in the presence of lactogenic hormones and increasing amounts of TGFβ3 for 4 d. Cultures were labeled with ^{35}S-translabel during the final 24 h. (*A*) Media samples were immunoprecipitated with anti-casein antiserum and electrophoresed on a 13% acrylamide gel. The fluorogram was obtained after 7 d of exposure to x-ray film. Molecular weight markers (kD) are indicated to the left of the figure. (*B*) A graphical representation of the data in *A*. The fluorogram was quantitated using a scanning densitometer and values obtained for the bands representing α_1-casein (X), α_2-casein (△), and β-casein (□) at each dose of TGFβ3 and plotted as a percentage of the level of casein present in the absence of any TGFβ3.

Fig. 3 *B* is a graphical representation of the data in Fig. 3 *A*. The exposed fluorogram shown in Fig. 3 *A* was quantified using a scanning densitometer (Hoeffer, San Francisco, CA). Values obtained for the bands representing α_1-casein, α_2-casein, and β-casein at each dose of TGFβ3 were plotted as percentages against the respective values in the absence of TGFβ3. Doses of TGFβ3 as low as 0.1 ng/ml led to the suppression of casein secretion; 5 ng/ml and higher concentrations of TGFβ3 gave nearly complete suppression. The graph indicates similar dose response curves for all three of the caseins examined.

Characterization of the TGFβ Effect

The response of explants to TGFβ was examined in greater detail by culturing explants for eight days in various combinations of lactogenic hormones and TGFβ3. ^{35}S-translabel was added during the final 24 h of culture and secreted caseins were analyzed by immunoprecipitation.

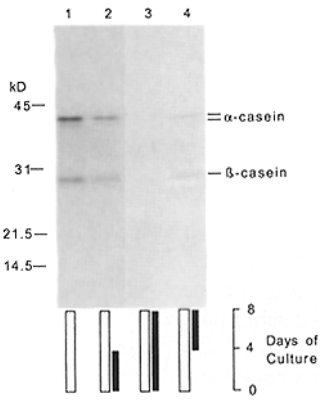

Figure 4. Characterization of TGFβ-induced suppression of casein secretion. Explants were cultured for 8 d and ^{35}S-translabel was added to each culture during the final 24 h. Media samples were immunoprecipitated with anti-casein antiserum. Lanes (also represented diagrammatically at the bottom of the figure): (*1*) lactogenic hormones alone throughout the 8-d culture; (*2*) lactogenic hormones plus TGFβ3 (5 ng/ml) for the first 4 d of culture, followed by lactogenic hormones alone for the remaining 4 d of culture; (*3*) lactogenic hormones plus TGFβ3 (5 ng/ml) throughout the 8 d of culture; and (*4*) lactogenic hormones alone for the first 4 d of culture followed by lactogenic hormones plus TGFβ3 (5 ng/ml) for the remaining 4 d of culture. The fluorogram was obtained after 5 d of exposure to x-ray film. Molecular weight standards (kD) are indicated to the left of the figure. (□) Lactogenic hormones present; (■) TGF-β present.

Lane *2* of Fig. 4 shows the levels of secreted caseins from explants cultured for 4 d in lactogenic hormones plus 5 ng/ml TGFβ3, followed by 4 d of culture in lactogenic hormones alone. The secreted casein levels approached those seen from explants cultured in lactogenic hormones alone for the entire 8 d (lane *1*), demonstrating that the suppression of casein secretion was reversible upon removal of TGFβ from the system. When TGFβ3 was present throughout the 8-d culture period, secreted casein levels were very low (lane *3*). Since secretion is fully suppressed after 4 d in culture (Figs. 2 and 3), continued suppression over four additional days of treatment indicated that the explants did not become refractory to extended TGFβ treatment. If cultures were first treated for 4 d with lactogenic hormones alone, followed by 4 d of treatment with lactogenic hormones plus TGFβ3, casein secretion was still suppressed (lane *4*), illustrating TGFβ's ability to suppress already-established casein secretion.

Casein Transcript Levels in TGFβ-treated Explants

The effects of TGFβ isoforms on casein gene expression were investigated by isolating total RNA from explants cultured for 48 h in the presence of lactogenic hormones, either alone or with the addition of 5 ng/ml TGFβ1, TGFβ2, or TGFβ3. In COMMA-D cells (Eisenstein and Rosen, 1988) and in rat mammary organ cultures (Guyette et al., 1979), β-casein mRNA accumulates for up to 48 h in the presence of lactogenic hormones. For this reason, and because casein secretion in our explants is fully suppressed after 48 h of TGFβ treatment (not shown), we chose this time of treatment feeling it would offer the best opportunity of detecting differences in casein transcript accumulation.

RNA blots containing 5 μg of total RNA per lane were probed with a radiolabeled α-casein or β-casein cDNA clone. TGFβ1, TGFβ3, or TGFβ2 treatment (Fig. 5, lanes *5*, *6*, and *7*, respectively) had no effect on the accumulation of

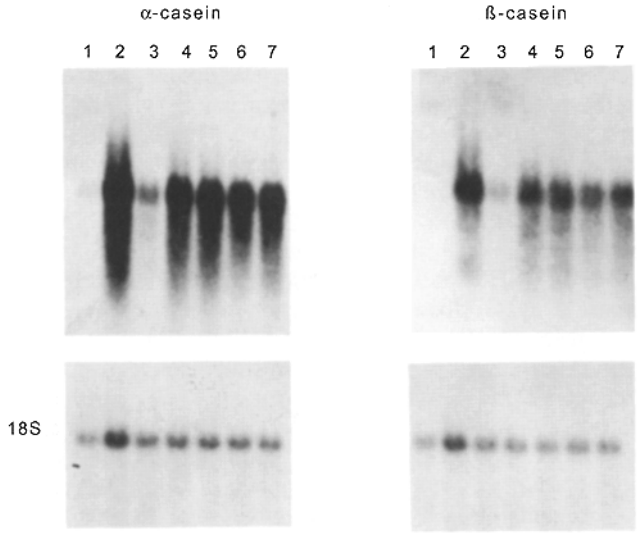

Figure 5. Expression of casein mRNAs in mammary explants. Blots containing total RNA (5 μg per lane) isolated from either explants or mammary glands were hybridized to radiolabeled α-casein cDNA or β-casein cDNA and washed under conditions of high stringency (0.1 × SSPE, 0.1% SDS, 65°C). To control for equivalent loading of the mRNA, rRNA was examined by UV shadowing following electrophoresis. As a control for equivalent transfer and integrity of the samples, the blots were later probed for the expression of 18 S ribosomal RNA (lower panels). Lanes: (*1*) virgin mammary gland; (*2*) 3-d lactating mammary gland; (*3*) explants cultured in the absence of lactogenic hormones; (*4*) explants cultured in lactogenic hormones alone; (*5*) explants cultured in lactogenic hormones plus 5 ng/ml TGFβ1; (*6*) explants cultured in lactogenic hormones plus 5 ng/ml TGFβ3; and (*7*) explants cultured in lactogenic hormones plus 5 ng/ml TGFβ2.

α-casein or β-casein transcripts in explants; transcript levels were identical to those seen in explants treated with lactogenic hormones alone (lane *4*). The low levels of casein transcripts present in the absence of lactogenic hormones (lane *3*) or in mammary tissue from virgin mice (lane *1*) and the high levels seen in lactating tissue (lane *2*) are shown for reference. α_1- and α_2-caseins are produced from transcripts of identical size (Hennighausen and Sippel, 1982).

Kinetics of Casein Synthesis and Secretion in Explants

The kinetics of casein synthesis and secretion within cultured explants were analyzed by pulse–chase labeling (Fig. 6). Casein levels within explant homogenates and media samples were evaluated by immunoprecipitation.

The rate of casein synthesis in explants was analyzed by terminating the incorporation of label into explants at 15-min intervals during a 1-h pulse (Fig. 6, *left panels*). TGFβ-treated explants synthesized caseins at a lower rate than untreated, as indicated by lower casein levels in TGFβ-treated explants at all time points examined.

For chase studies, explants were labeled with [^{35}S]methionine for 1 h (over which time they secrete little labeled protein [not shown]), washed with ice-cold buffer, and then incubated in label-free medium. In untreated explants, incorporation of label into caseins continued during the first hour of chase and over the next 4 h the amount of label decreased

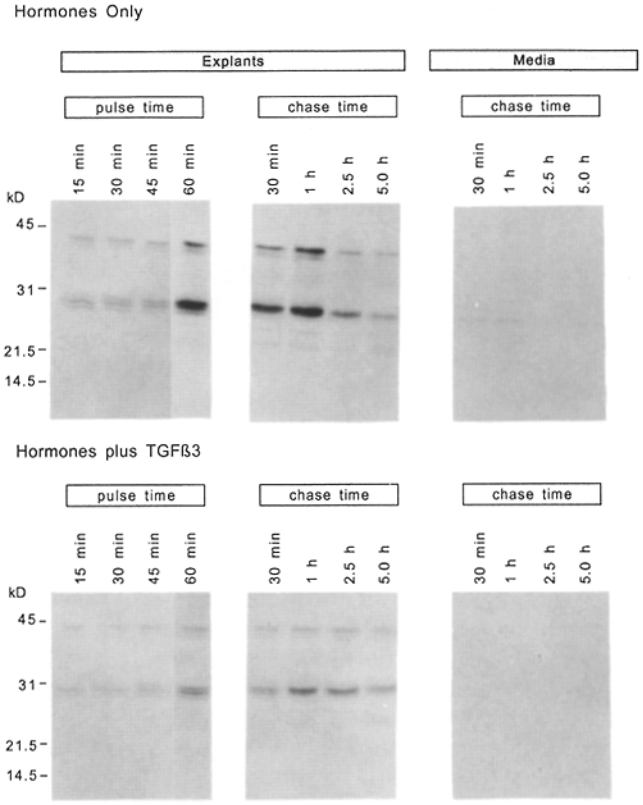

Figure 6. Pulse–chase analysis of casein processing in TGFβ treated and untreated explants. Explants were cultured for a total of 4 d in the presence of lactogenic hormones or lactogenic hormones plus 10 ng/ml TGFβ3 and pulse-chased during the final 6 h of culture. Medium and explant homogenates were collected at the indicated times. Samples were normalized on the basis of TCA-precipitable counts and immunoprecipitated with anti-casein antiserum. Panels: (*upper left*) casein levels in homogenates of untreated explants after indicated pulse times; (*lower left*) casein levels in homogenates of TGFβ3-treated explants after indicated pulse times; (*upper middle*) casein levels in homogenates of untreated explants after a 1-h pulse and indicated chase times; (*lower middle*) casein levels in homogenates of TGFβ3-treated explants after a 1-h pulse and indicated chase times; (*upper right*) casein levels in medium samples of untreated explants after a 1-h pulse and indicated chase times; (*lower right*) casein levels in medium samples of TGFβ3-treated explants after a 1-h pulse and indicated chase times. Fluorograms were obtained after 3 d of exposure to x-ray film. The positions of molecular weight markers (kD) are shown to the left of the top and bottom panels.

(Fig. 6, *upper middle panel*). A similar pattern of label incorporation and turnover was seen in TGFβ-treated explants (Fig. 6, *lower middle panel*). This pattern of label incorporation and intracellular casein turnover also occurs in rabbit mammary explants that have been grown in culture for 48 h (Al-Sarraj et al., 1979; Razooki Hasan et al., 1982; O'Hare et al., 1986). By 5 h of chase, the intracellular casein levels were identical in TGFβ-treated and untreated explants, a finding that is consistent with the overall level of caseins present in explants if they are labeled continuously for 24 h (data not shown).

After pulse labeling, untreated explants secreted ^{35}S-labeled caseins within 1 h (Fig. 6, *upper right panel*); no fur-

ther release was detected at later time points. Caseins were only barely detectable in the medium of TGFβ-treated explants (Fig. 6, *lower right panel*). Prolonged radiographic exposure, however, indicated a pattern of casein secretion identical to that in untreated explants (not shown).

Discussion

The regulation of casein gene expression during pregnancy and lactation is a complex process involving a coordinated response at several levels. A variety of hormones are known to play roles in lactogenesis during pregnancy, and growth factors are now being implicated as local mediators of hormonal function (reviewed in Oka et al., 1991). Here, we show that TGFβ may be one of these local mediators. All three TGFβ isoforms suppress the synthesis and secretion of caseins in mammary explants cultured in the presence of lactation-inducing hormones.

TGFβ inhibits the growth of many epithelial cells grown in culture (Roberts and Sporn, 1990), including mammary epithelial cells derived from reduction mammoplasties (Stampfer and Bartley, 1988; Valverius et al., 1989) and several transformed mammary epithelial cell lines (Dickson and Lippman, 1987). In situ, TGFβs reversibly inhibit ductal growth in the virgin mouse mammary gland (Silberstein and Daniel, 1987; Robinson et al., 1991). However, in situ administration of TGFβ to the mammary glands from pregnant mice does not influence alveolar morphogenesis or DNA synthesis in alveolar cells (Daniel et al., 1989; Robinson et al., 1991). Nor does TGFβ treatment influence the growth or morphology of explants in culture (not shown). For these reasons, we believe it unlikely that TGFβ's effect on casein expression is a result of growth inhibition.

We show the effect of TGFβ to be selective for caseins (Fig. 2), to be dose dependent (Fig. 3), and to be reversible (Fig. 4). In addition, TGFβ can suppress the secretion of caseins after the processes of milk production and secretion have been fully established (Fig. 4, lane *4*) and explants in culture do not become refractory to prolonged TGFβ treatment (Fig. 4, lane *3*). These data demonstrate that TGFβ exhibits many of the requirements expected of a pregnancy-associated, natural inhibitor of casein expression.

To ensure that explants maintained their full differentiated functional potential throughout the culture period, we initially examined the effects of TGFβ on the hormonal induction of explants by measuring secreted caseins. However, the influence of TGFβ on casein secretion appears to be linked to reduced intracellular production of these milk proteins. Pulse-chase analysis shows a dramatic reduction in the amount of caseins that are produced in the presence of TGFβ (Fig. 6). These studies also show that the patterns of casein processing and turnover as well as the steady-state intracellular accumulation of caseins are not altered by TGFβ treatment. Therefore, TGFβ does not affect the ability of hormonally treated explants to synthesize and store casein proteins; rather, it appears that TGFβ limits the rate of casein synthesis which results in reduced secretion. We have not, however, formally ruled out the possibility that TGFβ also influences the process of casein secretion.

We have not determined the exact point at which TGFβ suppresses casein synthesis. In contrast to the effects of EGF or TGFα on casein expression in HC11 cells (Hynes et al., 1990), TGFβ-mediated suppression is not a result of reduced casein transcript accumulation. Fig. 5 shows that there is no detectable difference in the overall levels of α- and β-casein transcripts in hormonally treated explants after TGFβ treatment. TGFβ could, however, influence casein synthesis by reducing the efficiency of casein translation from these transcripts.

We have also shown that TGFβ2 and TGFβ3 transcript levels increase as pregnancy continues, but that their expression drops off shortly after birth. The change in expression is not a result of TGFβ transcript dilution at lactation; we have demonstrated, immunohistochemically, the same expression pattern at the level of TGFβ protein (Robinson et al., 1991). Endogenous expression of TGFβ during pregnancy may, therefore, limit casein synthesis and secretion. We envision a mechanism whereby milk production is initiated by the hormones of pregnancy, but is kept in check by local growth factor mediators, such as TGFβ, whose expression rises in response to the hormones of pregnancy. Once lactation begins, efficient secretion of milk components can occur, casein synthesis need no longer be limited, and TGFβ expression decreases. TGFβ has been detected in milk (D. Danielpour, Laboratory of Chemoprevention, Bethesda, MD, personal communication), suggesting a possible autocrine type of feedback regulation on milk production during pregnancy.

The following questions now arise: (*a*) What TGFβ isoforms are important to this proposed mechanism in the animal? While TGFβ1 affects casein synthesis within explants, its effects in the animal may not be as significant as those of TGFβ2 and TGFβ3. Unlike TGFβ2 and TGFβ3, TGFβ1 transcript levels do not significantly change during pregnancy. (*b*) At what level within the cell is casein accumulation suppressed? The controls exerted on casein gene expression in explants and cell culture occur at both the transcriptional and posttranscriptional levels (Guyette et al., 1979; Eisenstein and Rosen, 1988). Secretion-coupled degradation has also been described as a posttranslational mechanism of casein gene regulation in mammary explants (O'Hare et al., 1986). However, we see no evidence for decreased casein transcript accumulation (Fig. 5) or increased intracellular casein turnover in response to TGFβ treatment (Fig. 6). (*c*) What factors regulate TGFβ expression during pregnancy and lactation? Progesterone is a likely candidate because of its known role in limiting lactogenesis and secretion (Davis et al., 1972; Assairi et al., 1974; Terada et al., 1988). (*d*) Are other milk proteins, such as whey acidic protein and α-lactalbumin, regulated in the same fashion as caseins? Studies to address these questions are currently in progress in our laboratory.

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