Stat5 as a Target for Regulation by Extracellular Matrix*

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Transcription of tissue-specific genes in mammary gland requires signals from both prolactin and basement membrane. Here we address the mechanism by which this specialized extracellular matrix regulates transcription. Using mammary cell cultures derived from transgenic mice harboring the ovine $\beta$-lactoglobulin gene, we show that either a basement membrane extract, or purified laminin-1, induced high levels of $\beta$-lactoglobulin synthesis. It is known that prolactin signals through Stat5 (signal transducer and activator of transcription). This transcription factor interacts with $\gamma$-interferon activation site-related motifs within the $\beta$-lactoglobulin promoter, which we show are required for matrix dependence of $\beta$-lactoglobulin expression. The DNA binding activity of Stat5 was present only in extracts of mammary cells cultured on basement membrane, indicating that the activation state of Stat5 is regulated by the type of substratum the cell encounters. Thus, basement membrane controls transcription of milk protein genes through the Stat5-mediated prolactin signaling pathway, providing a molecular explanation for previous studies implicating extracellular matrix in the control of mammary differentiation.

Cell-matrix interactions are critical for regulating the phenotype of many cells. In mammary gland, basement membrane is necessary for the prolactin-mediated control of lactation (1-3). However, the mechanism by which extracellular matrix (ECM)* influences differentiation in mammary epithelial cells has not been elucidated. We have shown previously that functional $\beta_1$ integrins are required for mammmary differentiation (4) and that the basement membrane component laminin-1 directs milk protein gene transcription coordinately with prolactin (5).

The prolactin pathway is driven through the protein tyrosine kinase Jak2 (6-10) and one of its substrates, the transcription factor Stat5 (11, 12). Stat5 factors associate with cytokine receptors following ligand binding and subsequently become phosphorylated by receptor-associated Jak s. They then dimerize and translocate to the nucleus where they bind specific DNA sequence motifs, thus contributing to transcriptional activation (13-15).

In this paper we examine whether an element of the prolactin signaling pathway is modulated directly by cell-matrix interactions, thereby mediating the ECM control of transcription. Using primary and first passage cultures of mammary epithelial cells, we demonstrate that the activity of the promoter for the milk protein $\beta$-lactoglobulin (BLG) is dependent on basement membrane and that Stat5 recognition sites within this promoter are required for transcription. Moreover, we show that the ability of Stat5 to bind its cognate DNA sequence within the BLG promoter requires cell interactions with both basement membrane and prolactin. Thus, matrix and cytokine signals converge on a single pathway.

Our data demonstrate, for the first time, that the activity of a Stat transcription factor is a target for regulation by ECM. This establishes a novel signaling route that is subject to control by cell-matrix interactions, in addition to those already described involving mitogen-activated protein kinases (16, 17), insulin receptor substrate-1 (18), and Ras (17).

MATERIALS AND METHODS

Substrata—Collagen I-coated dishes were prepared by incubating plates overnight at 4 °C with rat tail collagen in PBS to give a coating density of 8 $\mu$g/cm². The plates were washed twice with cold PBS and once with medium before use. EHS matrix was prepared from the Engelbreth-Holm-Swarm tumor and used as a substratum as described (2, 4, 5). In some experiments, cells were seeded onto glass coverslips precoated with 3 $\mu$g/cm² vitronectin (Sigma) before being overlaid with 200 $\mu$g/ml of either complete EHS matrix or purified laminin-1 (19) diluted into differentiation medium (5).

Cell Culture—Mammary epithelial cells were isolated from 14.5-18.5-day pregnant transgenic mice (BLG.SX/45, BLG.Dp/39, BLG.Dp/46 (20), Bc.Dp/188, and BLG.SAA (22) (Fig. 1)) or normal ICR mice and established in culture as described (2, 4, 5, 23). Most experiments were done with primary mammary epithelial cells. Cells were plated on tissue culture plastic or on dishes coated with either a thin layer of collagen I or with EHS matrix at a density of 2.5-5 $\times$ 10³ cells/cm² for 48-72 h in growth medium, F12 (Sigma) containing 10% heat-inactivated fetal calf serum (Advanced Protein Products, Brierley Hill, UK), 10 ng/ml epidermal growth factor (Promega Corp., Southampton, UK), 1 mg/ml fetuin, 5 $\mu$g/ml insulin, and 1 $\mu$g/ml hydrocortisone (Sigma). The cultures were washed extensively, and the medium was changed to differentiation medium (DMEM/F12 (Life Technologies Ltd., Paisley, Scotland) containing 5 $\mu$g/ml insulin, 1 $\mu$g/ml hydrocortisone, and 3 $\mu$g/ml prolactin (Sigma). In some experiments, first passage mammary epithelial cells were used. In this case, primary cells were grown on dishes coated with collagen I in growth medium for 48-72 h as described above and then trypsinized to single cell suspensions and plated at 1 $\times$ 10⁵ cells/cm² on appropriate substrata.

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† The abbreviations used are: ECM, extracellular matrix; BLG, $\beta$-lactoglobulin; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; EHS, Engelbreth-Holm-Swarm; GAS, $\gamma$-interferon activation site; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); bp, base pair(s).

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strata in DMEM/F12 medium containing 10% heat-inactivated fetal calf serum, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, and 1 μg/ml hydrocortisone for 24–48 h before changing the medium to differentiation medium (5). Depending on the experiment, cells were harvested up to 4 days later. Under these conditions, both primary and first passage cells cultured on EHS matrix form lactational alveoli that undergo functional (1, 2, 4, 5) differentiation.

Protein Analysis— Cultures were washed 3 times with methionine-free DMEM/F12 medium containing appropriate hormones and, after 30–60 min this was replaced with similar medium containing 0.25 mCi/ml [35S]methionine (Trans³⁵S-label, ICN Biomedicals Ltd., High Wycombe, UK) for 25–240 min. To harvest cell proteins, cultures were washed 3 times with warm medium, chilled on ice, and extracted with radioimmunoprecipitation buffer containing proteinase inhibitors, and the detergent-insoluble proteins were cleared by centrifugation, all as described (5, 23). Metabolic incorporation into newly made protein was measured by precipitation with trichloroacetic acid, and aliquots representing equal numbers of precipitable counts were separated on 15% polyacrylamide gels under reducing conditions. Mouse milk proteins were precipitated with a rabbit anti-mouse milk antiserum followed by Protein A-Sepharose (Zymed Laboratories Inc., South San Francisco, CA) before separation by SDS-PAGE (5, 23). Either gels were analyzed by fluorography or digitized images were obtained from dried gels using storage phosphor imaging plates and a Fujix Bas 2000 bioimaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). The levels of β-casein and BLG in whole cell lysates were quantitated with Fuji software and normalized to the total number of counts on each lane of the polyacrylamide gel. No proteins corresponding to BLG were detected in cultures from non-transgenic mice or from BCΔDp188 transgenic mice. To confirm the identity of the protein designated BLG in cultures from BLG.SX/45, BLGΔDp39, BLGΔDp46, and BCΔDp188 transgenic mice, immune precipitations were performed with a rabbit anti-ovine BLG antiserum.

CAT Assays—To measure chloramphenicol acetyltransferase (CAT) enzyme levels as an indicator of BLG promoter activity, primary or first passage cells derived from BCΔDp39 transgenic mice were cultured in the extracts normalized, and the CAT activity measured, all as described (24, 25). Cells cultured on EHS matrix were washed twice with Puck’s saline, harvested by treatment with 1% dispase (Boehringer Mannheim Ltd., Lewes, UK) for 30 min at 37°C, washed to remove dispase, and suspended in 0.25 M Tris-Cl, pH 7.8. Cells cultured on plastic were detached with trypsin-EDTA, washed three times with PBS, and suspended in 0.25 M Tris-Cl, pH 7.8. A cell extract was prepared by three cycles of freeze-thawing followed by centrifugation at 14,000 × g for 5 min, and the total protein concentration was estimated using a micro-Bradford assay (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). To confirm that equal quantities of cell protein were used for CAT assays, 1 μg of total cell protein was separated by SDS-PAGE, and the gel was stained with silver. The CAT reaction was done with 10 μg of total cell protein and carried out for 1 h at 37°C in a 150-μl reaction mixture containing 2 μl of [14C]chloramphenicol (DuPont NEN Products, Stevenage, UK), 0.53 μM acetyl coenzyme A, 0.25 μM Tris-Cl, pH 7.8. After [14C]chloramphenicol extraction with ethyl acetate, acetylated [14C]chloramphenicol was separated from the nonacylated form by thin-layer chromatography. CAT activity was determined by measuring the level of the acetylated chloramphenicol, using storage phosphor imaging plates and a Fuji Bas 2000 bioimaging analyzer.

Electrophoretic Mobility Shift Assays—Primary cultures of mammary epithelial cells from pregnant non-transgenic ICR mice were harvested by trypsinization either at the end of the initial plating or at the end of the initial plating medium containing appropriate hormones and, after 30–60 min this was replaced with similar medium containing 0.25 mCi/ml [35S]methionine (Trans³⁵S-label, ICN Biomedicals Ltd., High Wycombe, UK) for 25–240 min. To harvest cell proteins, cultures were washed 3 times with warm medium, chilled on ice, and extracted with radioimmunoprecipitation buffer containing proteinase inhibitors, and the detergent-insoluble proteins were cleared by centrifugation, all as described (5, 23). Metabolic incorporation into newly made protein was measured by precipitation with trichloroacetic acid, and aliquots representing equal numbers of precipitable counts were separated on 15% polyacrylamide gels under reducing conditions. Mouse milk proteins were precipitated with a rabbit anti-mouse milk antiserum followed by Protein A-Sepharose (Zymed Laboratories Inc., South San Francisco, CA) before separation by SDS-PAGE (5, 23). Either gels were analyzed by fluorography or digitized images were obtained from dried gels using storage phosphor imaging plates and a Fujix Bas 2000 bioimaging analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan). The levels of β-casein and BLG in whole cell lysates were quantitated with Fuji software and normalized to the total number of counts on each lane of the polyacrylamide gel. No proteins corresponding to BLG were detected in cultures from non-transgenic mice or from BCΔDp188 transgenic mice. To confirm the identity of the protein designated BLG in cultures from BLG.SX/45, BLGΔDp39, BLGΔDp46, and BCΔDp188 transgenic mice, immune precipitations were performed with a rabbit anti-ovine BLG antiserum.

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Electrophoretic Mobility Shift Assays—Primary cultures of mammary epithelial cells from pregnant non-transgenic ICR mice were harvested by trypsinization either at the end of the initial plating period in growth medium or following a switch for 1, 2, or 3 days to differentiation medium with or without prolactin. Cell pellets were snap frozen in liquid N2 and nuclear extracts prepared as described (26). The total protein concentration was estimated by the Pierce BCA protein assay (Pierce and Warriner(UK)Ltd.,Chester,UK),andequal quantiles of protein were added to end-labeled double-stranded oligodeoxyribonucleotides for electrophoretic mobility shift assays. The oligodeoxyribonucleotides used were the recognition motifs for Stat5 STM site (5'-GATTCCGGGAGACCGCGT), NF-1 (5'-GATCTTTGCCTGGAAGCCAAAT), and Sp-1 (5'-ATTCTAGCAGGCGGGCGGCGAG). In some assays, gel retardation reactions were performed overnight at 4°C with antibodies to Stat5 (27), Stat1 (E23, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or phosphoryro sine (PY20, Santa Cruz) before addition of DNA.

**RESULTS**

In order to dissect the mechanism of basement membrane control of milk protein expression, we used primary and first passage cultures of mammary epithelial cells. This enabled us to examine the signaling requirements for cells in culture that were as closely related as possible to those for cells in vivo. Initially we studied the activity of a transgenic ovine BLG promoter using a well established culture model for mammary differentiation (1, 2, 4, 5).

In transgenic mice, the BLG promoter sequence is strongly active in mammary gland during pregnancy and lactation (20). To assess whether the activity of this promoter is regulated by cell-matrix interactions, mammary epithelial cells from a series of transgenic mice expressing BLG constructs (Fig. 1) were plated on plastic dishes or on a reconstituted basement membrane extract from the EHS tumor and cultured under differentiation conditions with lactogenic hormones. After 3 days, the cultures were pulse-labeled with [35S]methionine, and cell lysates were prepared by detergent extraction (5, 23). Total lysates of mammary epithelial cells isolated from pregnant BLGΔDp39 mice showed that although BLG was synthesized by cells cultured both on plastic and EHS matrix, its expression was up-regulated dramatically by basement membrane (Fig. 2A). Quantitative measurements indicated a 3.7-fold increase of BLG expression on EHS matrix over plastic. Immune precipitation experiments showed that endogenous mouse milk protein synthesis was also up-regulated by this matrix (Fig. 2B), as expected from earlier studies (1, 2, 4, 5). Very similar results were obtained from cultures of BLG.SX/45 mice, which contain 4.3 kb of the BLG promoter sequence rather than just the 406 bp of BLGΔDp39. Thus, the expression of a transgenic ovine BLG gene in mammary epithelial cells is under the control of ECM.

We have shown that the basement membrane ligand responsible for triggering β-casein transcription in mammary epithelial cells is laminin-1 (5). We therefore examined whether laminin-1 could also activate BLG transcription in mammary cultures derived from BLGΔDp39 transgenic mice. Using an
Dependent on the type of substratum that the cells encounter. After 27 min, the medium was collected, and the cells were cultured for 4 days with the lactogenic hormones, prolactin, hydrocortisone, and insulin. At the end of the culture period, the cellswere pulse-labeled with [35S]methionine for 75 min and extracted, and equal aliquots of newly synthesized total cell protein were separated by SDS-PAGE. Shown is a lysate representing equal amounts of newly synthesized protein were separated by 15% reducing SDS-PAGE and immunoprecipitated using an antibody specific for mouse milk proteins, but not ovine BLG, that purified laminin-1 induced expression of BLG. For this assay, first passage BLG-dp/39 cells were plated on plastic (P) or on a reconstituted basement membrane from the EHS tumor (E) and cultured for 3 days with the lactogenic hormones, prolactin, hydrocortisone, and insulin. At the end of the culture period, the cells were pulse-labeled with [35S]methionine for 27 min, the medium was collected, and the cells were extracted with radiimmune precipitation buffer. Alliquots of cell lysate representing equal amounts of newly synthesized protein were (A) separated by 15% reducing SDS-PAGE and (B) immune precipitated using an antibody specific for mouse milk proteins, but not ovine BLG, before separation by SDS-PAGE. The band designated BLG was confirmed by immune precipitation with a BLG-specific antibody (not shown). No proteins of this size were detected in radiolabeled whole cell extracts from non-transgenic mouse cultures. Similar results to those shown were obtained from BLG-dp/46 and BLG-SX/45 mice. C shows that purified laminin-1 induced expression of BLG. For this assay, first passage BLG-dp/39 cells were plated on coverslips and then treated with 200 μg/ml soluble ECM proteins diluted into the culture medium for 4 days in the presence of lactogenic hormones (5). Controls received hormones but no ECM protein. Alternatively, cells were plated on top of EHS gels. At the end of the culture period, cells were pulse-labeled with [35S]methionine for 75 min and extracted, and equal aliquots of newly synthesized total cell protein were separated by SDS-PAGE. Shown is the region of the gel containing BLG. D shows that the 406-bp promoter of the BLG gene itself contains matrix-dependent control elements. Mammary epithelial cells from pregnant BC3Dp/188 mice were either plated directly on plastic or EHS matrix (P and E; left panel) or were cultured on collagen I, or EHS matrix (P, C, E; right panel). After 4 days in the presence of lactogenic hormones, cells were lysed and the activity of CAT enzyme was measured. CAT enzyme activity in the EHS cultures was increased 3.4-fold over plastic in primary cells and 2.9-fold over plastic in first passage cells.

The BLG promoter contains three DNA recognition sequences for the transcription factor, Stat5 (also known as mammalian gld factor (11, 12) or milk protein binding factor (26)). Stat5 is expressed in pregnant and lactating mammary gland (28) and binds γ-interferon activation site (GAS)-related DNA sequence elements (29). Since the GAS motifs bound by Stat5 are required for maximum BLG promoter function in vivo (22), we asked whether they were also required for its activity in culture. Dinucleotide sequences within the three GAS motifs in the proximal 406 bp of the BLG promoter were mutated to abolish Stat5 binding activity, and mice that contained multiple copies of this transgene were produced. In mammary cultures prepared from the resulting BLG-SAA transgenic mice (Fig. 1), low levels of BLG were transcribed from the mutant promoter, but there was no increased synthesis of BLG in cells cultured on EHS matrix (Fig. 3, A and B). This result contrasted with that for endogenous β-casein, which was induced to a similar extent by the EHS matrix, both in cultures from BLG-dp/39 and BLG-SAA transgenic mice harboring the wild type or mutant BLG promoter and in cultures from BC3Dp/188 mice expressing the CAT reporter gene (Fig. 3, A and C). These results confirm previous conclusions that Stat5 binding sites within the BLG promoter are required for a high level of activity (22, 29). Our observation that BLG expression was not completely abrogated in the BLG-SAA cultures indicates that additional matrix-independent factors have a role in transcription from this promoter. However, since there was no increased expression of BLG in cells cultured on the basement membrane matrix, our results suggested strongly that Stat5 itself was necessary for the matrix-dependent control of expression.
We therefore examined the activity of Stat5 directly, in cells cultured on the different substrata in the presence or absence of prolactin. Cells from non-transgenic mice were cultured on plastic, collagen I, or EHS matrix and then harvested, and nuclear extracts were prepared (26). DNA electrophoretic mobility shift assays were performed using the oligodeoxynucleotides used as the recognition motifs for Stat5, NF-I, and SP1. In the control lanes (Co), an additional extract from mammary gland was included for the Stat5 assay, and partially purified NF-I protein was included for the NF-I assay. B, equal quantities of cell protein from nuclear extracts of mammary gland or from the EHS sample prepared above (2 days in differentiation medium) were used for electromobility shift assays with the Stat5 probe. Antibodies, as indicated, were incubated with nuclear extract before addition of the probe. PY, phosphotyrosine.

**FIG. 4.** Matrix dependence of transcription factor activity. A, primary mammary epithelial cells from non-transgenic midpregnant mice were cultured on plastic (P), collagen I (C), or EHS matrix (E) for 2 days in serum-containing medium. The cultures were washed, and the medium was changed to differentiation medium containing lactogenic hormones with or without prolactin for 0, 2, or 3 days. The cells were then harvested and equal quantities of cell protein were used for electrophoretic mobility shift assays. The DNA oligodeoxynucleotides used were the recognition motifs for Stat5, NF-I, and SP1. In the control lanes (Co), an additional extract from mammary gland was included for the Stat5 assay, and partially purified NF-I protein was included for the NF-I assay. B, equal quantities of cell protein from nuclear extracts of mammary gland or from the EHS sample prepared above (2 days in differentiation medium) were used for electromobility shift assays with the Stat5 probe. Antibodies, as indicated, were incubated with nuclear extract before addition of the probe. PY, phosphotyrosine.

**DISCUSSION**

It has been known for some time that expression of tissue-specific milk protein genes in mouse mammary gland requires cell interactions with basement membrane (1–3). The milk-producing cells of alveoli interact with a laminin-rich basement membrane in vivo. Culture studies have demonstrated that this association with basement membrane provides signals for milk protein synthesis that are mediated through integrins (4) and derived from the ECM component laminin (5). These locally acting signals operate together with lactogenic hormones, resulting in transcriptional activation of milk protein promoters (24, 30) via a mechanism that has not yet been elucidated.

In this work we have taken the problem one step back from the level of the promoter and shown that the DNA binding activity of a Stat transcription factor essential for milk protein transcription is controlled by cell-matrix interactions. This con-
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Several milk protein gene control regions, including the 406-bp BLG promoter (26), the β-casein proximal promoter and its enhancer element, BCE1 (30), and the whey acidic protein promoter (31), contain potential recognition sequences for Stat5. Since all of these genes are controlled by ECM, we propose that the matrix-dependent activation of Stat5 provides an essential contribution to the mammary differentiation program.

In conclusion, this study suggests one signaling mechanism to explain previous observations in the mammary system that implicate ECM in the control of differentiation (1–5). We have demonstrated that transcription from the BLG promoter requires mammary cells to interact with a basement membrane and that ECM and lactogenic hormones cooperate in the regulation of Stat5 transcription factor activity. To our knowledge, this is the first demonstration that the function of a Stat family member is dependent on signals other than cytokines/growth factors. In the light of recent data showing that cell-matrix interactions also control mitogen-activated protein kinase and insulin receptor substrate-1 activity, our findings now reveal a related mechanism by which extracellular matrix regulates cell phenotype. It is known that proliferation of normal mammary cells requires not just growth factors but also ECM proteins (40, 41), and recent studies have suggested that other fundamental processes such as cell survival are dependent on matrix (21, 42). Thus, together these studies point to a general control principle involving cross-talk between cytokine- and matrix-mediated signaling pathways.

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