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Lactogenic Hormones and Tenascin-C Regulate C/EBPα and β in Mammary Epithelial Cells

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Abstract Mammary epithelial cell differentiation depends on lactogenic hormones, growth factors, and cell-cell and cell-substrate interactions, all of which modulate transcription factors essential for milk protein gene expression. The CCAAT/enhancer binding protein (C/EBP) family and the signal transducer and activator of transcription 5 (Stat5) have been implicated in mammary epithelial cell growth and differentiation. We have investigated the effects of extracellular matrix components and lactogenic hormones on C/EBP and Stat5 activity. In the mammary gland, tenascin is expressed mainly during embryogenesis and carcinogenesis and in cell culture tenascin downregulates β-casein gene expression. In HC11 mammary cells, we found that tenascin, but not laminin or fibronectin, specifically downregulated C/EBPα levels but had no effect on Stat5 amount or DNA binding activity. Furthermore, we found that the lactogenic hormones, glucocorticoids, prolactin, and insulin, had no effect on C/EBPα and C/EBPβ protein levels but downregulated the DNA binding activity of the transcriptional repressor C/EBPβLIP. Thus, C/EBPα and β are regulated by tenascin and lactogenic hormones in mammary epithelial cells. J. Cell. Biochem. 76:394–403, 2000.

Key words: extracellular matrix; HC11 cells; Stat5; β-casein transcription

Lactogenic hormones, extracellular matrix (ECM), and intercellular interactions communicate signals to mammary epithelial cells that converge on defined transcription factors, which act in concert to modulate milk protein gene expression. Among these molecules, positive and negative regulators have been described [Schmitt-Ney et al., 1991; Meier et al., 1994; Altiok and Groner, 1994; Li et al., 1995]. The C/EBPs belong to the leucine-zipper family of transcription factors, which are involved in growth control and differentiation in various tissues including liver, fat, ovary, and hematopoietic cells. The C/EBPα isoform has two alternative translation products of 42 and 30 kDa that display different transactivation potentials [Ossipow et al., 1993] and regulate growth arrest and terminal differentiation in hepatocytes and adipocytes [Tanaka et al., 1997; Timchenko et al., 1997]. C/EBPβ has three variants that arise by leaky ribosome scanning [Descombes and Schibler, 1991]: two liver-enriched activator proteins (LAPs) of 39 and 36 kDa, and their inhibitor liver-enriched inhibitory protein (LIP), of 20 kDa. LIP has higher affinity for its DNA binding site and can therefore can inhibit LAPs in substoichiometric amounts. Hence, the ratio of LAP/LIP, rather than their absolute quantity, regulates gene transcription [Descombes and Schibler, 1991]. C/EBP expression is regulated during mammary gland development [Raught et al., 1995; Gigliotti and DeWille, 1998], and binding sites for C/EBP have been mapped in the hormone-responsive region of the β-casein gene promoter [Doppler et al., 1995]. These transcription factors are important in the mammary gland since C/EBPβ-deficient mice have deficiencies in normal development and functional differentiation [Robinson et al., 1998; Seagroves et al., 1998].

The extracellular signals that modulate C/EBP activity in mammary cells have not yet been explored. HC11 cells have been a useful model with which to study various aspects of mammary epithelial cell biology [Taverna et al., 1991; Cella et al., 1996, 1998; Merlo et al., 1997]. We have previously reported that the ECM protein tenascin has a negative effect on β-casein protein expression [Chammas et al.,

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1994]. In this article, we show that tenascin affects β-casein expression at the transcriptional level. In an attempt to identify the mechanism of tenascin action, we observed that tenascin downregulated C/EBPβ protein in HC11 cells. The effect of tenascin was selective since neither laminin nor fibronectin affected β-casein expression. The level of C/EBPβ was not altered by tenascin. In contrast to results obtained with laminin [Streuli et al., 1995], tenascin did not alter Stat5 DNA binding activity.

We have previously shown that lactogenic hormones stimulate Stat5 DNA binding activity [Cella et al., 1998]. In this study, we analyzed the effect of lactogenic hormones on C/EBP protein expression and DNA binding activity in HC11 cells. We found that C/EBPα and β were detected in undifferentiated cells. No significant change in their level was observed after lactogenic hormone treatment. In eletrophoretic mobility shift assays, we detected four protein-DNA complexes. Three of these complexes contained C/EBPβ isoforms. Lactogenic hormone induction downregulated LIP-containing complexes, indicating that hormone-dependent transcriptional regulation functions, at least in part, via repression of LIP. In summary, these data demonstrate that C/EBP proteins are targets in the signaling pathways of both tenascin and lactogenic hormones.

MATERIALS AND METHODS

Materials

[^{32}P]ATP was purchased from Amersham. The following antibodies were used: anti-C/EBPα (sc-061) and anti-C/EBPβ (sc-150) for Western blot (Santa Cruz). Anti-LAP and anti-LAP/LIP antisera, used for supershifts, were a generous gift from Dr. Ueli Schibler (University of Geneva, Switzerland).

Cell Culture and Lactogenic Induction

HC11 mammary epithelial cells were grown to confluency and maintained for 3 days in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10 ng/ml epidermal growth factor (EGF), and 5 μg/ml insulin. These competent cells [Taverna et. al., 1991] were washed and incubated for 18 h in serum-free medium (RPMI-1640 containing 1 mg/ml fetuin and 10 μg/ml transferrin) and then treated for the indicated times with serum-free medium supplemented with the lactogenic hormones 10^{-6} M dexamethasone, 5 μg/ml insulin, and 5 μg/ml ovine prolactin (luteotropic hormone; Sigma Chemical Co., St. Louis, MO) (DIP). Cells that were replated on matrix-coated dishes were left in medium supplemented with 2% serum plus insulin overnight so that they could attach; they were then placed into the indicated medium.

Whole Cell Extracts

Cells were washed with phosphate-buffered saline (PBS) and scraped into 700 μl of a buffer containing 10 mM $NaHPO_4$ pH 7.4, 1 mM EDTA, 1 mM DTT, 400 mM KCl, 10% glycerol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM PMSF, 5 μM NaF, 2 mM Na_2VO_4, 50 μM Na-glycerophosphate. After 3 cycles of freezing and thawing, extracts were centrifuged for 15 min at 17,000*g*, supernatants were recovered and stored at −70°C [Cella et al., 1998].

Subcellular Fractionation

Cells were fractionated as described [Schreiber et al., 1989]. In this investigation, 10–15 × 10^6 cells (1 confluent 10-cm dish) were washed in Tris-buffered saline (TBS) buffer and collected in this buffer. Cell were recovered by centrifugation (1,500*g* 5 min), resuspended in 400 μl of buffer containing 10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF, 5 μg/μl aprotinin, 5 μg/ml leupeptin, 2 mM Na_2VO_4 plus 50 μM Na-glycerophosphate and allowed to swell on ice for 15 min. 60 μl of a 10% Nonidet P-40 (NP-40) solution was added and the tube was vortexed vigorously for 10 s. The homogenate was centrifuged for 30 s in a microcentrifuge, the supernatant was removed, and the nuclear pellet was resuspended in 20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT plus the inhibitors described above. The tube was incubated under agitation for 15 min at 4°C , centrifuged for 5 min in a microcentrifuge, and supernatants were recovered and stored in aliquots at −70°C.

Electrophoretic Mobility Shift Assay

A total of 20 μg of nuclear extract was incubated with the C/EBP binding site (GATCCGTGTATTTATCAATGTTAC) [Tollet et al., 1995] (30,000 cpm, 0.5 ng) for 30 min at room temperature in 20 μl of electrophoretic mobility shift assay (EMSA) buffer: 10 mM Hepes pH 7.6, 2

mM $NaHPO_4$, 0.25 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 80 mM KCl, 2% glycerol, and 100 μg/ml poly (dI-dC). Specific binding was analyzed on a native 6% polyacrylamide gel, prerun for 2 h at 200 V, in 0.25 × TBE (22.5 mM Tris-borate, pH 8.0, 0.5 mM EDTA). The samples were loaded and eletrophoresed in the same buffer for 1 h at 200 V. The gels were dried and exposed to x-ray film. For supershifts and competition studies, antibodies (anti-LAP/LIP and anti-LIP), or nonlabeled competitor oligonucleotides were added to the binding reaction and incubated for 1 h before the labeled probe was added. EMSA for analysis of Stat5 binding activity was performed as follows: 6 μg of whole cell extract was incubated with the Stat5 binding site from the bovine β-casein promoter (5′-AGATTTCTAGGAATTCAATCC-3′) [Wakao et al., 1994] (30,000 cpm, 5 fmol) for 30 min on ice in 20 μl of the EMSA buffer described above. For competition, nonlabeled wild-type (Col 7) (5′-GTGGAC**TTCTTGGAA**TTAAGGAACTTTTG-3′) or mutated (Col 7.3) 5′-TGTGGACT**A**TT**TT**AATTAAGGAACTTTTG-3′ Stat5 binding site were used [Schmitt-Ney et al., 1992]. Complexes were resolved on a 4% native gel, using the same conditions as described above.

Luciferase Assay

Competent HC11-Lux cells [Wartmann et al., 1996] were plated onto dishes coated with purified tenascin (50 μg/ml) or bovine serum albumin (BSA) and induction was carried out in RPMI 10% FCS plus the lactogenic hormones prolactin, dexamethasone, and insulin. Cell were harvested and luciferase activity was measured and expressed in arbitary units/μg of protein. As a control, HC11 cells were stably transfected with an SV40 promoter-luciferase construct and the same procedure was repeated.

Western Blot Analysis

A total of 80 μg of whole cell extracts was resolved by 12–14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF filters, and immunoblotted in TTBS solution (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20, and 20% normal horse serum) with specific antisera against C/EBPα and β. Proteins were visualized with peroxidase-coupled second antibody using the ECL detection system (Amersham). For reprobing, membranes were stripped in 62.5 mM Tris pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol at 40°C for 40 min.

RESULTS

Effect of Lactogenic Hormones on C/EBPα and β Protein Expression

C/EBP binding sites have been mapped to the lactogenic hormone-responsive region of the β-casein gene promoter, suggesting that hormones might influence C/EBP activity [Doppler et al., 1995]. To address this question, C/EBPα and β were analyzed by Western blot in HC11 cells after lactogenic hormone treatment. The 42-kDa variant of C/EBPα was present in noninduced cells, and its expression did not change significantly after hormone treatment (Fig. 1, left, top arrow). (The apparent decrease in lanes 4–5 is a blotting artifact of this particular experiment.) Low constant levels of the 30-kDa variant were detected throughout the time course (Fig. 1, left, bottom arrow). The three C/EBPβ isoforms, LAPs and LIP, shown at the right side of Figure 1, were present in noninduced cells and as seen for the α form, no alterations were observed after lactogenic hormone treatment (Fig. 1, right, three arrows, lanes 7–12).

Effect of Lactogenic Hormones on C/EBP DNA Binding Activity

Although the levels of C/EBP proteins remained constant throughout lactogenic hormone treatment, we postulated that their DNA binding activity might be regulated. In order to test this possibility, nuclear protein extracts were prepared from serum-starved HC11 cells or from cells induced with lactogenic hormones for 1 h, 3 h, 1 day, or 2 days (Fig. 2A, lanes 1–5). Binding activity was analyzed in an EMSA, using a C/EBP-specific binding oligonucleotide [Tollet et al., 1995]. Four protein-DNA complexes were identified (a, b, c, and d, as indicated). The specificity of the protein-DNA complexes was verified by using 50- or 100-fold excess of nonlabeled probe as competitors. A 100-fold excess completely abolished the DNA binding activity of the four complexes (Fig. 2B, lanes 1–3). Binding activity was detected both in non-induced and hormone-treated cells (Fig. 2A, lanes 1 and 2–5, respectively). Although the same complexes were present throughout, the level of complexes b, c, and d, but not a, was decreased after 1 or 2 days of lactogenic hormone treatment.

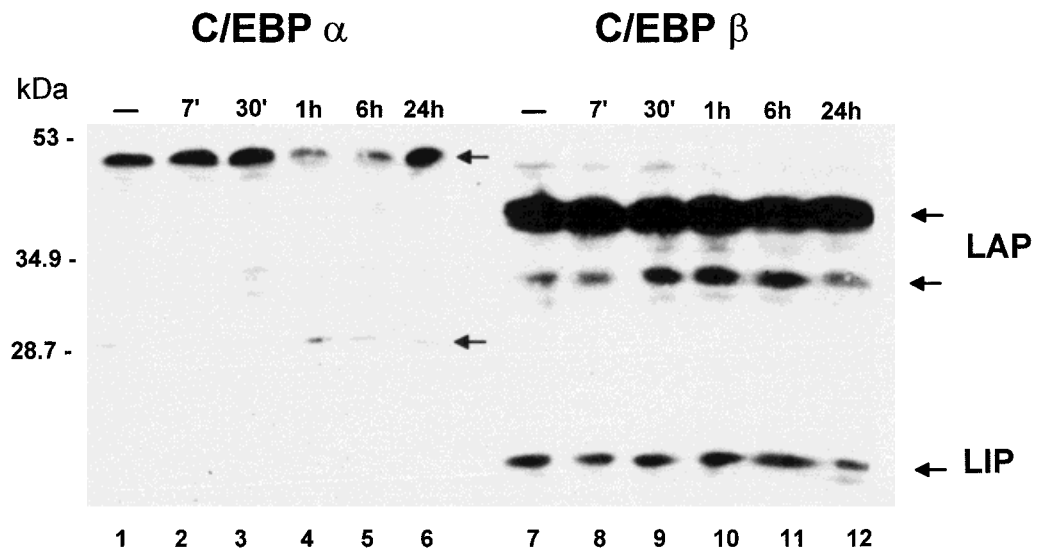

Fig. 1. Lactogenic hormones do not change C/EBPα and β protein levels. Competent serum-starved HC11 cells were induced with lactogenic hormones in RPMI serum-free medium for the indicated times. Whole cell extracts were prepared, resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF filter. Samples were loaded in duplicate. The left and the right sides were probed for C/EBPα and C/EBPβ, respectively. Arrows on the right side of each blot indicate the C/EBP isoforms. Molecular-weight markers (kDa) are indicated on the left.

In order to identify the C/EBPβ proteins detected in the EMSA, nonimmune serum, or antisera raised against LAP or against both LAP and LIP, were added to the binding reaction before the labeled probe (Fig. 2C). The addition of anti-LAP/LIP serum completely abolished complex d, (Fig. 2C, lane 2). The addition of anti-LAP specific serum resulted in a strong decrease in the intensity of complex c and a moderate decrease in complex b, compared with nonimmune serum (Fig. 2C, cf. lane 1 with lane 3). Complex a was not affected by the addition of the antisera. These results show that complex a does not contain C/EBPβ isoforms, complexes b and c contain LAPs, and complex d is composed exclusively of LIP, since anti-LAP serum did not alter its level. In conclusion, C/EBPβ-LIP DNA binding activity is partially downregulated by lactogenic hormones. Since complexes b and c are at least partially composed of LAP proteins, it is possible that LAP DNA binding activity is also downregulated by lactogenic hormones.

Tenascin Effect on β-Casein Gene Expression

In the mammary gland, tenascin is expressed by the mesenchyme in close proximity to the growing epithelial bud [Chiquet-Ehrismann et al., 1986; Sakakura, 1991], in the involuting gland [Jones et al., 1995], in neoplasia [Mackie et al., 1987; Yoshida et al., 1997], and in milk [Kalembey et al., 1997]. In HC11 cells, tenascin deposition and assembly correlates negatively with β-casein protein production. In addition, cells plated on tenascin produce less β-casein compared with control cells [Chammas et al., 1994]. To analyze whether tenascin exerts its negative effect at the transcriptional level, HC11 cells were stably transfected with the β-casein gene promoter (-344/-1) construct linked to the luciferase reporter gene [Wartmann et al., 1996]. This segment contains the *cis*-acting sequences required for minimal and hormone induced expression of the β-casein gene [Doppler et al., 1995]. These cells were plated on either tenascin or BSA-coated culture dishes and induced with DIP or insulin for the indicated periods of time (Fig. 3A and B, respectively). As a control, HC11 cells were stably transfected with the SV40 promoter-luciferase construct and the experiment was repeated as described above (Fig. 3A,B, small graphics). Cell extracts were prepared and luciferase activity was determined. We observed that β-casein promoter-induced luciferase activity was decreased by 52% in cells plated on tenascin. No significant differences in SV40-induced luciferase activity were observed between cells plated on tenascin or on BSA, sug-

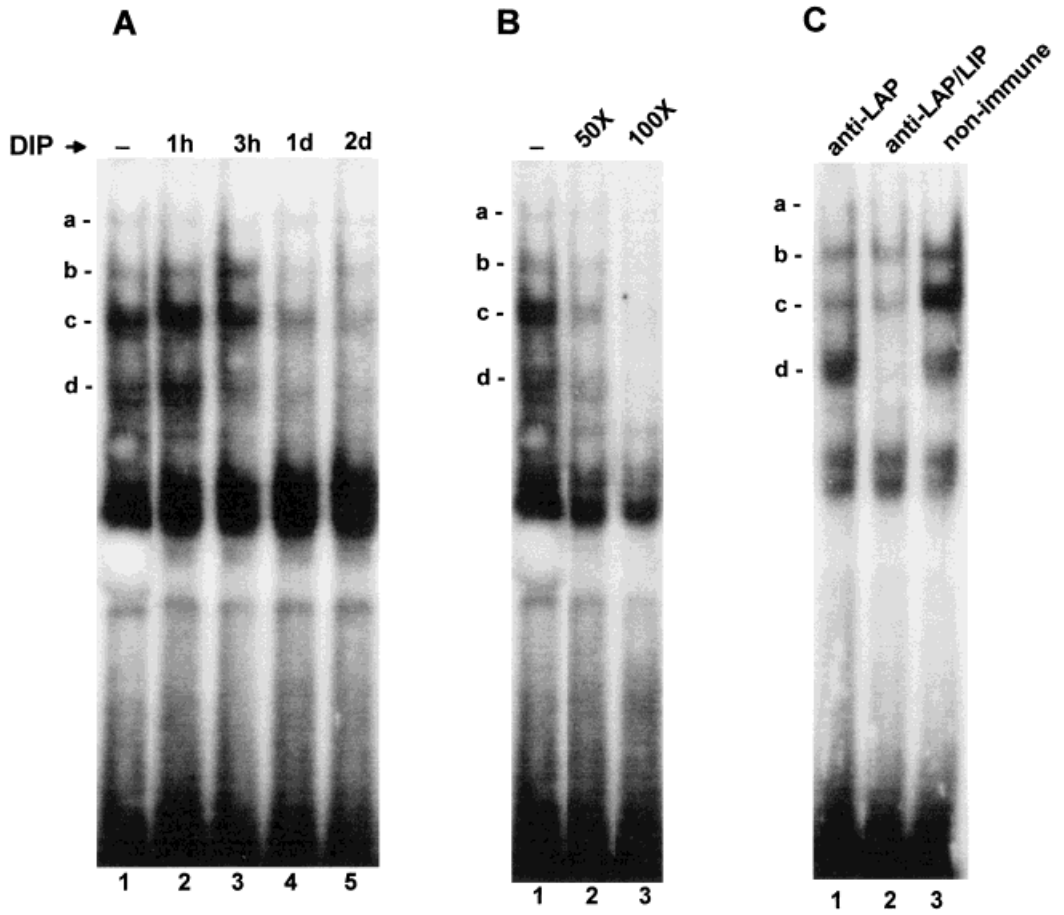

Fig. 2. Lactogenic hormones modulate C/EBP protein-DNA binding activity. **A:** Competent, serum-starved HC11 cells were treated with lactogenic hormones (DIP) in RPMI serum-free medium for the indicated times. Nuclear protein extracts were incubated with radiolabeled probe containing the C/EBP binding site and resolved on a 6% native polyacrylamide gel. **B:** The binding reaction was carried out with no additional probe (**lane 1**) or with 50- and 100-fold excess of nonlabeled probe as competitors (**lanes 2**, and **3**, respectively). **C:** Nuclear extracts were incubated with anti-LAP (**lane 1**), anti-LAP/LIP (**lane 2**), or nonimmune serum (**lane 3**) before addition of the radiolabeled-C/EBP binding oligonucleotide. Specific C/EBP-DNA complexes are referred to as a, b, c, and d, indicated on the left side of each panel. This result is representative of three independent experiments.

gesting that tenascin acts specifically on the β-casein gene promoter. The decrease in β-casein gene promoter activity was detected in both DIP- and insulin-treated cells, suggesting that tenascin might inhibit basic transcription. In addition, inhibition of transcription was first detected 12 h after hormone addition, showing that the cellular response to tenascin is slow.

Effect of Tenascin on Stat5 Binding Activity

Stat5 plays an essential role in mammary gland growth and differentiation and mediates the prolactin response [Groner and Gouilleux, 1995]. Stat5 DNA binding activity is also regulated by extracellular matrix [Streuli et al., 1995], suggesting that it could be a target for regulation by tenascin. To address this question, HC11 cells were plated either on tenascin or on plastic and treated with lactogenic hormones for 1 h, 1 day, or 2 days. Whole cell extracts were prepared, and binding activity was analyzed in an EMSA using the specific Stat5 DNA binding site as a probe (Fig. 4A). The specificity of the protein-DNA complex was accessed by using nonlabeled wild-type or mutated Stat5 binding site as competitors (Fig. 4B). No differences in the level of specific complex were observed in cells plated on tenascin compared with those plated on plastic. Western blot analysis demonstrated no differences in Stat5 levels in cells plated on tenascin (data not

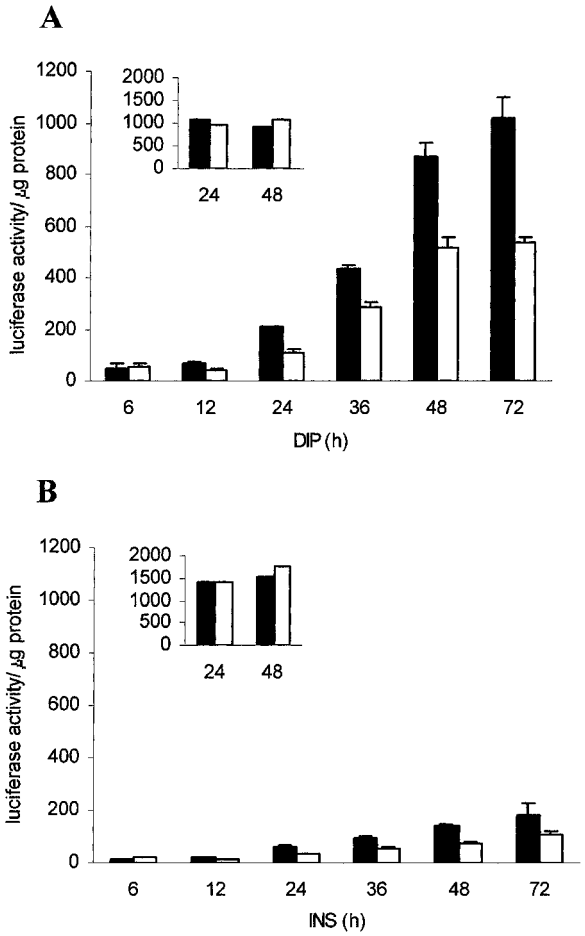

Fig. 3. Tenascin downregulates β-casein gene expression. Competent HC11-Lux cells or HC11 cells transfected with the SV40 promoter-luciferase construct (insert) were plated on tenascin (white bars) or bovine serum albumin (BSA)-coated dishes (black bars) and treated either with dexamethasone + insulin + prolactin (DIP) (**A**) or insulin (INS) (**B**) for the indicated times. Cell extracts were prepared and luciferase activity determined and expressed in luciferase arbitrary units/µg protein. Standard deviation between independent experiments is shown.

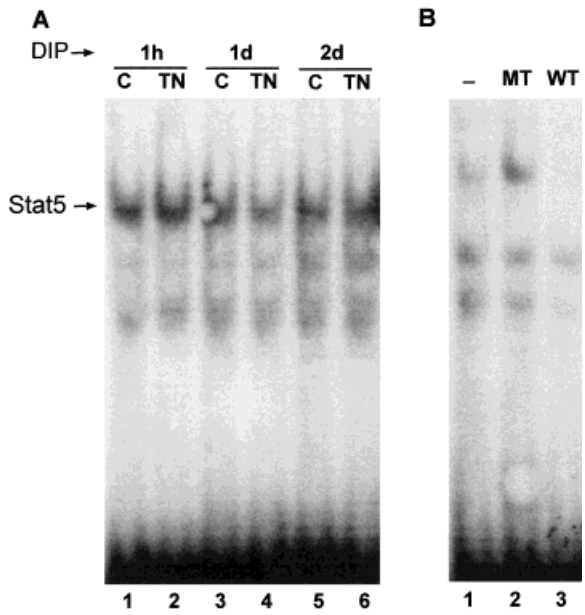

Fig. 4. Tenascin does not alter Stat5 DNA-binding activity. **A:** Competent HC11 cells were plated either on plastic culture dishes (C) or on tenascin (TN) coated dishes and treated with RPMI serum-free medium containing dexamethasone + insulin + prolactin (DIP) for 1 h, 1 day, or 2 days. Nuclear protein extracts were prepared and binding activity analyzed in an EMSA, using a specific Stat5 binding site. **B:** The same binding reaction shown in A was repeated adding 100-fold excess of nonlabeled mutated (MT) or wild type (WT) Stat5 binding site as competitors.

shown), indicating that tenascin does not alter Stat5 protein amount and binding activity.

Effect of ECM on C/EBPα and β Expression

C/EBPβ have complex effects on transcription, reflected by the interactions between the activator LAPs, and the repressor, LIP [Descombes et al., 1991]. In hepatocytes, C/EBPα is regulated by the ECM [Rana et al., 1994; Tollet et al., 1995, Runge et al., 1997]. It has been suggested that cell confluency, which is important for optimal β-casein gene expression in HC11 cells [Chammas et al., 1994], upregulates C/EBPα protein levels [Raught et al., 1995]. These data prompted us to analyze the effect of tenascin on the expression of the C/EBP isoforms. HC11 cells were plated on plastic or tenascin-coated dishes and induced with lactogenic hormones for 2 days. Whole cell extracts were prepared and analyzed by Western blot using C/EBPα-specific antiserum (Fig. 5A, top). Tenascin strongly downregulated the 42-kDa variant of C/EPBα protein in a dose-dependent manner (cf. lanes 2 and 3). The membrane was stripped and reprobed with anti-C/EBPβ. Tenascin had no effect on C/EBPβ protein expression (the 39-kDa LAP variant is shown in Figure 5A, bottom). In order to check whether other ECM molecules might affect C/EBPα expression, HC11 cells were plated on plastic, fibronectin, or laminin (Fig. 5B, lanes 1–2, 3–4, and 5–6, respectively) and were induced with lactogenic hormones for 2 days. Western blot analysis showed that neither laminin nor fibronectin had an effect on the level of C/EBPα protein. In summary, tenascin selectively downregulates the C/EBPα isoform, but not the β isoform.

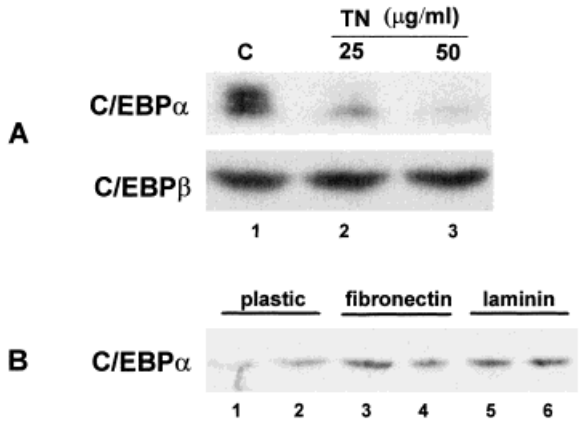

Fig. 5. Tenascin, but not laminin or fibronectin downregulates C/EBPα levels. **A:** Competent HC11 cells were plated on plastic culture dishes (C) or tenascin-coated dishes and treated with dexamethasone + insulin + prolactin in RPMI serum-free medium for 2 days. Whole cell extracts were prepared, resolved on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a PVDF membrane. The filter was successively probed for C/EBPα (**top**) and C/EBPβ (**bottom**). **B:** The same procedure was repeated, plating cells on either plastic, fibronectin, or laminin-coated dishes. **Lanes 1–2**, **3–4**, and **5–6** are duplicates. C/EBPα analysis was carried out as described in A.

DISCUSSION

We report that C/EBPα and β proteins are differentially regulated by tenascin and by lactogenic hormones in HC11 mammary epithelial cells. We have identified four C/EBP-DNA complexes that are present in both noninduced and hormone-treated HC11 cells. Lactogenic hormones partially downregulated the DNA binding activity of C/EBPβ-LAP and C/EBPβ-LIP. Tenascin is known to downregulate β-casein gene expression [Jones et al., 1995; Wirl et al., 1995; this report). We show here that tenascin affects the basal transcriptional activity of the β-casein promoter, since insulin-treated cells also displayed lower promoter activity when plated on tenascin-coated dishes. Tenascin did not alter Stat5 DNA binding activity, and its effects on transcription were only observed after 24 h, suggesting that it acts indirectly. Finally, tenascin, but not laminin or fibronectin, selectively downregulated C/EBPα protein levels.

ECM and C/EBPs

The ECM can interfere with gene expression directly, evoking an intracellular signal that affects transcription, or indirectly, regulating cell shape and cytoskeleton organization, which can also modulate transcription. Although HC11 cells adhere and spread equally well on tenascin and on control dishes, after 1 day in culture, cells were more loosely attached and could be detached by gentle rinsing (data not shown). Similar observations were made with uterine epithelial cells plated on tenascin [Julian et al., 1994]. In addition, tenascin had a negative effect on β-casein gene expression without causing changes in cell morphology or three-dimensional structure [Jones et al., 1995]. These data suggest that tenascin modulates transcription by triggering an intracellular signal independent of cell shape and cell-cell interaction. The tenascin domain TNfnA-D, which inhibits β-casein gene transcription [Jones et al., 1995], leads to a loss of focal adhesion integrity [Murphy-Ullrich et al., 1991; Chung et al., 1996]. Appropriate signals provided by cell-substrate interaction are essential for mammary epithelial cell growth, differentiation, and milk protein gene expression [Roskelley et al., 1995]. In this context, loss of proper cell-substrate interaction in cells on tenascin could be responsible for inhibition of β-casein gene expression. It has been shown that the expression and deposition of ECM components by mammary epithelial cells are regulated by the substratum on which cells are initially plated [Streuli and Bissell, 1990]. Tenascin could act by inducing the cells to secrete soluble factors, which might in turn act negatively on β-casein gene expression. Alternatively, tenascin might inhibit deposition of an appropriate matrix, which normally occurs in HC11 cells plated on plastic [Chammas et al., 1994]. Absence of this matrix might interfere with β-casein gene expression and might also explain the weak adhesion of the cells after 1 day in culture. This proposed mechanism correlates well with the fact that the inhibitory effect of tenascin on β-casein gene transcription was detected only after a day in culture.

We show that the C/EBPα protein is selectively downregulated in HC11 cells plated on tenascin. A requirement of ECM-matrigel for maintenance of C/EBPα protein expression has also been observed in hepatocytes [Rana et al., 1994; Tollet et al., 1995; Runge et al., 1997]. This effect was only detected after 3 days, in agreement with the kinetics of C/EBPα downregulation in HC11 cells plated on tenascin. We have not been able to directly correlate the decrease in C/EBPα protein with the decrease

in β-casein gene transcription induced by tenascin. Bandshift analysis using nuclear extracts from HC11 cells plated on tenascin did not show any differences compared with control extracts (data not shown). Thus, whether the inhibitory effect of tenascin on β-casein gene expression is causally linked to its effect on C/EBPα remains to be established.

An ECM- and prolactin-responsive element (BCE-1) has been mapped to the distal site of the bovine β-casein gene promoter [Schmidhauser et al., 1992]. Binding sites for Stat5 and C/EBP have been identified in this region, suggesting that both proteins are potential targets for ECM regulation. In primary cells Stat5 tyrosine phosphorylation and DNA binding activity was only detected in cells plated on matrigel [Streuli et al., 1995]. In other cell lines, however, ECM had no influence on Stat5 or C/EBP DNA binding [Myers et al., 1998]. This discrepancy could be related to differences between these cell models.

C/EBPβ Is Regulated by Lactogenic Hormones in HC11 Cells

C/EBP binding sites have been mapped in the hormone-responsive region of the β-casein gene promoter, suggesting that these transcription factors could respond to lactogenic hormones. The LAP/LIP ratio, rather than their absolute levels, regulates transcriptional activation. LIP interferes with LAP by binding more avidly to the their common recognition site [Descombes et al., 1991]. We observed no significant change in the level of C/EBPα and β proteins in noninduced compared to hormone-induced HC11 cells. Considering the four C/EBP-DNA complexes we identified, C/EBPβ LAP was found in complexes b and c , and C/EBPβ LIP in complex d. We tried unsuccessfully to identify C/EBPα with different antibodies. Since the anti-C/EBPβ sera did not completely abolish all the complexes, C/EBPα could still be in one of them. We were unable to identify the protein(s) in complex a. Since this band is weaker than the others, protein(s) present in this complex must be less abundant in the nucleus, or its affinity to the DNA is lower. Protein-DNA complexes were detected in noninduced cells; lactogenic hormones induced a major decrease in the DNA binding activity of complex d-LIP. LAP-containing complexes b and c also exhibited decreased DNA binding after lactogenic hormone treatment; thus, LAP might also be a target for hormone regulation. These observations are interesting in light of previous reports on HC11 cells, in which it was shown that lactogenic hormones induced a decrease in the DNA binding activity of a complex [Schmitt-Ney et al., 1991], which was later shown to be a C/EBP consensus site in the β-casein gene promoter [Doppler et al., 1995]. These results suggest that relief of transcriptional repression of the β-casein gene involves hormone-induced post-translational modification of C/EBPβ isoforms, leading to downregulation of their DNA binding activity. C/EBPβ activity has been shown to be regulated by phosphorylation in other cell systems [Trautwein et al., 1993; Osada et al., 1996]. Furthermore, glucocorticoids decreased the levels of C/EBP-β-LIP in HC11 cells [Raught et al., 1995], suggesting that C/EBPβs are targets for glucocorticoids. In mammary cells, the synergism between glucocorticoids and prolactin is mediated by the physical association between the glucocorticoid receptor and Stat5 [Cella et al., 1998]. In addition, glucocorticoids have a rather slow effect on β-casein gene expression [Doppler et al., 1990], which might be mediated by C/EBPs. Similar situations have been reported during chick neural development and in hepatoma cells, where in both cases glucocorticoid responsiveness depends on C/EBP [Bem-Or and Okret, 1993; Gotoh et al., 1997]. Synergism between Stat and C/EBP has been reported previously [Kordula and Travis, 1996], and in HC11 cells mutations in the C/EBP sites abolish glucocorticoid and prolactin induction of the β-casein gene promoter [Doppler et al., 1995]. These data suggest that Stat5, glucocorticoid receptor, and C/EBPs may form a functional unit that regulates transcription through specific DNA-protein and protein-protein interactions. The LIP isoform has been correlated with mammary cell proliferation and neoplastic transformation [Raught et al., 1996]. The decrease in C/EBPβ-LIP DNA binding activity induced by lactogenic hormones may be part of the switch from proliferation to differentiation.

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