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Lactogenic Hormone Activation of Stat5 and Transcription of the β-Casein Gene in Mammary Epithelial Cells Is Independent of p42 ERK2 Mitogen-activated Protein Kinase Activity*

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HC11 mammary epithelial cells have been used to characterize molecular events involved in the regulation of milk protein gene expression. Treatment of HC11 cells with the lactogenic hormones prolactin, insulin, and glucocorticoids results in transcription of the β-casein gene. Prolactin induces a signaling event which involves tyrosine phosphorylation of the mammary gland factor, Stat5, a member of the family of signal transducers and activators of transcription (Stat). Here we show that HC11 cells express two Stat5 proteins, Stat5a and Stat5b. Phosphopeptide and phosphoamino acid analysis of Stat5a and Stat5b immunoprecipitated from phosphate-labeled HC11 cells revealed that both proteins were constitutively phosphorylated on serine. Lactogenic hormone treatment resulted in the appearance of a tyrosine-phosphorylated peptide in both Stat5 proteins. Consistent with this observation, a Western blot analysis of Stat5a and Stat5b showed that lactogenic hormones induced a rapid, transient increase in phosphotyrosine which paralleled the binding of Stat5 to its cognate recognition sequence in the β-casein gene promoter. Lactogenic hormone treatment of the HC11 cells also led to a rapid activation of the mitogen-activated protein (MAP) kinase pathway. We examined the role of this pathway in β-casein transcription using a specific MAP kinase kinase inhibitor, PD98059. Concentrations of PD98059 which completely abrogated lactogen-induced MAP kinase activation did not affect the phosphorylation state of Stat5, its DNA binding activity, or transcriptional activation of a β-casein reporter construct. This indicates that the MAP kinase pathway does not contribute to lactogenic hormone induction of the β-casein gene.

Mammary gland differentiation requires the coordinated action of growth factors and hormones that promote morphological development and milk protein production in the lactating gland (1). In order to understand the molecular events contributing to mammary cell differentiation, *in vitro* cell culture systems have proven invaluable. The HC11 mouse mammary epithelial cell line, a clonal derivative of the COMMA-D line (2) is a useful model system for studying mammary cell differentiation. Treatment of HC11 cells with the lactogenic hormones glucocorticoids, insulin, and prolactin leads to rapid stimulation of β-casein gene transcription (3). The β-casein promoter binds numerous transcription factors (4–8). One of these factors, which is indispensable for hormonal induction of β-casein transcription, binds to a conserved sequence present in the promoter of casein genes from different species (9). This factor is the mammary gland factor or Stat5,[1] a member of the Stat (signal transducer and activator of transcription) family (10) (reviewed in Ref. 11). Stat family members are activated in response to cytokines whose receptors are associated with tyrosine kinases of the Janus kinase family. Following ligand binding and receptor aggregation, Stats are phosphorylated on tyrosine residues by the receptor-associated Janus kinases. Tyrosine phosphorylation mediates the specific binding of Stats to IFN-γ activated (GAS)-like sites, leading to activation of target genes (reviewed in Refs. 12 and 13).

Binding of prolactin to its receptor, a member of the cytokine receptor superfamily, leads to Janus kinase-2-mediated phosphorylation of Stat5 on tyrosine (14, 15). In COS cells transfected with mammary gland factor-Stat5 mutants, it has been shown that phosphorylation of Tyr-694 is essential for its DNA binding and transcriptional activation (16). In addition to tyrosine, Stats are also phosphorylated on serine (17–20). Serine phosphorylation is required for maximal transcriptional activity of Stat1α and Stat3 (20). It has been suggested that mitogen-activated protein (MAP) kinase is responsible for the cytokine induced serine phosphorylation of Stat1α (20, 21).

In order to investigate the mechanism of Stat5 activation in mammary epithelial cells, we have examined the phosphorylation status of Stat5a and Stat5b in resting and in lactogen-treated HC11 cells. Both Stat5 proteins were constitutively phosphorylated on serine. Treatment of HC11 cells with lactogenic hormones led to a rapid increase in their phosphotyrosine content. Lactogenic hormone treatment of the HC11 cells also led to a rapid activation of the MAP kinase pathway. We examined the role of this pathway in β-casein transcription using a specific MAP kinase kinase inhibitor, PD98059 (22). Pretreatment of HC11 cells with PD98059 led to a repression of lactogenic hormone-induced MAP kinase activity but had no effect on the phosphorylation status of Stat5, on its DNA binding activity, or on lactogenic hormone-induced transcriptional activation of the β-casein promoter luciferase construct. These

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[1] The abbreviations used are: Stat, signal transducer and activator of transcription; GAS, interferon-γ activated site; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; ECM, extracellular matrix; IFN, interferon.

data demonstrate that MAP kinase activation is not involved in the transcriptional induction of the β-casein gene mediated by the lactogenic hormones.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]Orthophosphate and [γ-^{32}P]ATP were purchased from Amersham Corp. Purified kinase-inactive extracellular signal-regulated kinase-2 (ERK2) (K52R) protein was generously provided by Dr. N. Ahn (University of Colorado, Boulder). The following antisera were employed: rabbit anti-MEK-1 (2880), obtained from Dr. R. Davis (23); rabbit anti-MAP kinase (ERK1 and ERK2) (24); and a monoclonal antibody for phosphotyrosine (25). The Stat5 sera were produced in rabbits against specific COOH-terminal peptides (26). The peptides were -LDARLSPPAGLFTSARSSLS- for Stat5a and -MDSQWIPHAQS- for Stat5b. The MEK inhibitor PD98059 was kindly provided by Dr. Alan Saltiel (Parke-Davis, Research Division, Warner Lambert Co., Ann Arbor, MI).

Cell Culture and Lactogenic Hormone Induction—All experiments were carried out with HC11 mammary epithelial cells stably transfected with a β-casein promoter luciferase construct (p-344/-1βc-Lux) (27). The cells, referred to as HC11-Lux, were grown to confluency and maintained 3 days in medium containing RPMI 1640, 10% fetal calf serum, 10 ng/ml epidermal growth factor, and 10 μg/ml insulin (growth medium). The competent cells were then washed and incubated for 18 h in serum-free medium (RPMI 1640 supplemented with 1 mg/ml fetuin and 10 μg/ml transferrin), 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).

Luciferase Assays—HC11-Lux cells were harvested after 4 h of treatment with the lactogenic hormones, and luciferase activity was determined on triplicate samples using the luciferase assay system (Promega) as described by the manufacturer. Total light emission was measured during the first 3 s of the reaction using a luminometer (Berthold Microlumat LB96). The results are expressed in light units.

In Vitro Protein Kinase Assays—Cells were harvested at 4 °C in lysis buffer (TLB) (20 mM Tris, pH 8.0, 137 mM NaCl, 2 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1% Triton X-100, 10% glycerol, 25 mM β-glycerophosphate). MEK activity was measured in an immune complex kinase assay using recombinant kinase-inactive MAP kinase as an exogenous substrate. MEK-1 was immunoprecipitated for 1 h at 4 °C with 5 μl of anti-MEK-1 serum (23) immobilized on 20 μl of protein A-Sepharose beads. The immunoprecipitates were washed three times with TLB and twice with kinase buffer (25 mM Hepes, pH 7.5, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 5 mM $MnCl_2$, 15 mM $MgCl_2$). The washed MEK-1 immunoprecipitates were incubated with 1 μg of kinase-inactive ERK2 (K52R) (29) and 50 μM [γ-^{32}P]ATP (10 Ci/mmol). The reaction was terminated by addition of sample buffer, boiled, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (7%). The gel was dried, and the phosphorylation of ERK2 was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics). The activity of ERK1 and ERK2 MAP kinase was measured *in vitro* using myelin basic protein as a substrate after specific immunoprecipitation of each kinase from 200 μg of protein from total cell lysates as described previously (24). Substrate phosphorylation was analyzed as described above.

Electrophoretic Mobility Shift Assay—Competent, serum-starved HC11 cells were pretreated for 60 min with 30 μM PD98059 or with vehicle (1% dimethyl sulfoxide, v/v) and then treated with lactogenic hormones for 5 or 60 min. Whole cell extracts were prepared in extraction buffer: 400 mM KCl, 10 mM NaHPO4, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 5 μM NaF, 50 μM β-glycerophosphate, and 2 mM sodium vanadate. For a Stat5 specific band shift, 6 μg of whole cell extract was incubated with the Stat5 DNA binding site from the bovine β-casein promoter (5′-AGATTTCTAG-GAATTCAATCC-3′) (10) (50,000 cpm, 5 fmol) for 30 min on ice 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 dithiothreitol, 5 mM $MgCl_2$, 80 mM KCl, 2% glycerol, and 50 μg/ml poly(dI-dC). Specific binding was analyzed on a 4% 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 electrophoresed for 1 h at 200 V, the gels were dried and exposed to film, and the specific band was quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics).

Detection of Stat5 Tyrosine Phosphorylation and Phosphopeptide Mapping—Stat5a and Stat5b were immunoprecipitated from 200 μg of whole cell extracts made in extraction buffer with the specific antisera described above. Immunoprecipitates were washed three times with TLB and then subjected to SDS-PAGE (7.5%). Proteins were transferred to polyvinylidene difluoride membranes, and a Western blot analysis was performed with an anti-phosphotyrosine monoclonal antibody. The levels of immunoprecipitated Stat5 proteins were monitored by reprobing the membranes with specific antisera.

Phosphopeptide mapping was performed as follows. 10-cm dishes of competent HC11-Lux cells were incubated for 12 h in phosphate-free, serum-free Dulbecco's modified Eagle's medium, complemented with transferrin and fetuin, in the presence of 0.5 mCi/ml [^{32}P]orthophosphate (2 mCi/4 ml medium). At the end of the labeling period the cells were pretreated for 1 h with 30 μM PD98059 or with vehicle and then treated with lactogenic hormones for 0, 5, or 60 min. Lysates were made in 500 μl of TLB, clarified by centrifugation at 14,000 rpm for 10 min at 4 °C, then precleared by incubation for 30 min with protein A-Sepharose. The Stat5a and Stat5b specific antisera described above were prebound to protein A-Sepharose for 60 min before they were used to precipitate the Stat5 proteins from the clarified lysates. Immunoprecipitates were washed once with TLB, once with 0.5 M LiCl, 100 mM Tris, pH 7.4, 25 mM β-glycerophosphate, 1 μM sodium vanadate, and once with 10 mM Tris, pH 7.4, 25 mM β-glycerophosphate, 1 μM sodium vanadate. Under these conditions there was no coimmunoprecipitation of the two Stat5 proteins. Proteins were resolved by SDS-PAGE (7.5%), and phosphorylated Stat5 proteins, detected after 15 h of autoradiography, were excised from the gel. Tryptic digestion and two-dimensional phosphopeptide analysis were performed as described previously (30). Briefly, dried gel slices were rehydrated in 1 ml of 50% acetonitrile for 4 h, then air-dried. The dried slice was soaked in 200 μl of 100 mM $NaHCO_3$ (pH 8.3) containing 5 μg of tosylphenylalanyl chloromethyl ketone-treated trypsin (Worthington) and subjected to tryptic digestion for a total of 24 h at 30 °C. A second aliquot of trypsin (5 μg) was added after the first 5 h. The buffer containing the tryptic peptides was removed, and the gel slice was again extracted by two consecutive additions of 1 ml of 50% acetonitrile. The pooled peptides were dried in a SpeedVac concentrator, resuspended in 10 μl of pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid), and spotted onto cellulose thin layer chromatography plates. The first dimension was electrophoresis for 1 h at 1000 V in pH 1.9 buffer. After drying, the plates were placed in a chromatography tank containing phosphochromatography buffer (37.5% 1-butanol, 25% pyridine, and 7.5% acetic acid) for 12 h for separation in the second dimension. Plates were dried and subjected to autoradiography and PhosphorImager analysis. Individual peptides were recovered and extracted with 30% acetonitrile followed by SpeedVac concentration. Dried peptides were then resuspended in 50 μl of 6 N HCl, and partial acid hydrolysis was performed by incubation at 110 °C for 60 min. The dried sample was resuspended in 10 μl of pH 1.9 buffer containing 2 μg of each phosphoamino acid as internal standards and then subjected to electrophoresis for 90 min at 1000 V in pH 3.5 buffer. Phosphoamino acids were detected by autoradiography and PhosphorImager analysis.

RESULTS

Treatment of HC11 Cells with Lactogenic Hormones Activates the MAP Kinase Pathway—HC11 cells are immortalized, nontransformed mammary epithelial cells (3) which have been used to examine the requirements for transcriptional induction of the milk protein gene β-casein. The HC11 cells must be grown to confluency in medium containing epidermal growth factor and insulin (31). This results in competent cultures, which, when treated with the lactogenic hormones glucocorticoids, insulin, and prolactin, produce the milk protein β-casein. All three hormones are required for transcriptional activation of the β-casein gene (32), suggesting that multiple intracellular signaling pathways converge to activate the β-casein gene promoter.

MAP kinase cascades are conserved intracellular signaling pathways by which cells respond to a variety of external stimuli including hormones, cytokines, and growth factors (33). In mammalian cells the 44- and 42-kDa ERKs are members of the archetype growth factor-regulated MAP kinase cascade. Generally, ERK1 and ERK2 are activated by the sequential stimulation of Ras, a small GTP-binding protein, Raf-1, a 74-kDa

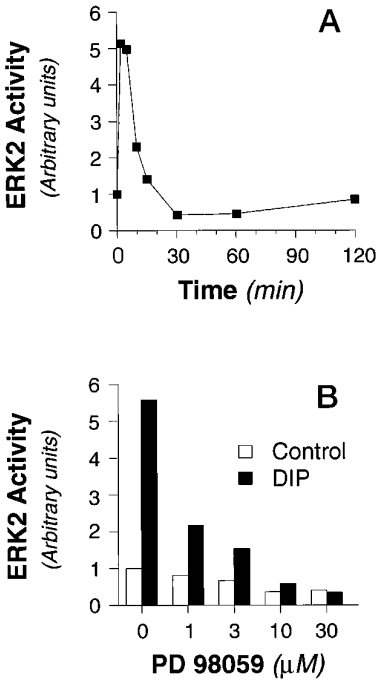

FIG. 1. **Lactogenic hormone activation of MAP kinase is inhibited by PD98059.** *A*, competent HC11-Lux cells were incubated for 18 h in serum-free medium and then treated for the indicated times with serum-free medium supplemented with lactogenic hormones and lysates were prepared in TLB buffer. p42ERK2 MAP kinase activity was measured following immunoprecipitation with a specific antiserum with myelin basic protein as a substrate. Reaction products were resolved by SDS-PAGE and quantified using a PhosphorImager and ImageQuant software. *B*, HC11-Lux cells were pretreated with the indicated concentration of PD98059 or with vehicle for 60 min, then incubated with lactogenic hormones for 5 min. p42ERK2 MAP kinase activity was determined and quantified as in *A*. *DIP* refers to medium containing the lactogenic hormones dexamethasone, insulin, and prolactin.

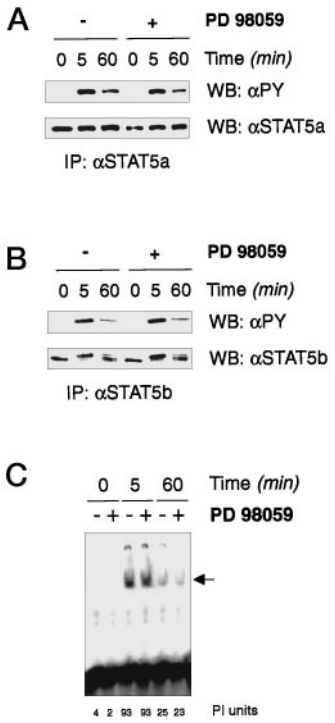

FIG. 2. **Lactogenic hormone-induced tyrosine phosphorylation and activation of Stat5 proteins.** Competent, serum-starved HC11-Lux cells were pretreated for 60 min with 30 μM PD98059 or with vehicle and treated with lactogenic hormones for 5 or 60 min, and whole cell extracts were prepared. Stat5a (*A*) and Stat5b (*B*) were immunoprecipitated with specific antisera, subjected to SDS-PAGE (7.5%), and transferred to polyvinylidene difluoride membranes, and Western blot analyses were performed with anti-phosphotyrosine monoclonal antibody (*αPY*) (*upper panel*). The blots were reprobed with Stat5-specific sera (*lower panel*). *C*, whole cell extracts were incubated with the Stat5 DNA binding site from the β-casein promoter, and specific binding was analyzed by EMSA. Quantification was performed with a PhosphorImager and ImageQuant Software. The numbers below (*C*) refer to the arbitrary PhosphorImager units (*PI*).

serine/threonine kinase and MEK, a dual specificity kinase. In order to investigate the role of the ERKs in lactogenic hormone-induced β-casein gene transcription, HC11 cells stably transfected with a rat β-casein promoter (-344/-1)-luciferase construct, HC11-Lux cells (27) were utilized. The kinetics of activation of the 44-kDa ERK1 and 42-kDa ERK2 MAP kinases was examined in HC11-Lux cells incubated over a 2-h time period with lactogenic hormones. p42 ERK2 MAP kinase (Fig. 1*A*) was rapidly activated with maximal stimulation between 2 and 5 min, whereas the p44 ERK1 isoform of MAP kinase was not stimulated by this treatment.[2] Treatment of HC11 cells with potent mitogens such as epidermal growth factor or *neu* differentiation factor does stimulate ERK1 activity (24, 32). MEK1 was rapidly activated, approximately 2-fold, by lactogenic hormones, whereas Raf-1 was minimally stimulated.[2] Although the amplification capacity of the MAP kinase cascade is well known (22), we cannot exclude that a MEK kinase distinct from Raf-1 might contribute to the hormone induction of MEK-1 and ERK2 in these cells (34).

To examine the relevance of MAP kinase activation by lactogens, PD98059, a compound which prevents the activation and phosphorylation of MEK1 and MEK2 *in vitro* and *in vivo* (22), was employed. The IC_{50} for inhibition of ERK2 activity in HC11-Lux cells was 3 μM (Fig. 1*B*). At 30 μM PD98059 lactogenic hormone-induced ERK2 activation was completely inhibited over a 2-h time course.[2]

Inhibition of the ERK2 Does Not Alter Stat-5 Phosphorylation or DNA Binding—The transcriptional activation of the β-casein gene following lactogenic hormone stimulation is mediated at least in part by Stat5. Phosphorylation on tyrosine is essential for Stat5 binding and transcriptional activation (16). We examined the phosphorylation status and DNA binding activity of Stat5 following lactogenic hormone treatment of HC11-Lux cells.

The mouse mammary gland expresses two Stat5 proteins, Stat5a and Stat5b, the products of two closely related genes (26). Peptide antisera were generated against the specific COOH terminus of each Stat5 protein. The sera only recognize their cognate antigen.[3] The phosphotyrosine content of both Stat5 proteins was examined by Western blotting following their immunoprecipitation from lactogenic hormone treated HC11-Lux cells. In unstimulated cells no tyrosine phosphorylation of either Stat5 protein was detected. The maximal amount of phosphotyrosine was observed after 5 min of hormone treatment, and its level decreased thereafter (Fig. 2, *A* and *B*, *left side*). Interestingly, hormone treatment resulted in an apparent decrease in the electrophoretic mobility of Stat5b, but not Stat5a. Alignment of the two Western blots revealed that only the slower migrating Stat5b band contained phosphotyrosine (Fig. 2*B*).

To follow the activation of Stat5 we investigated the formation of Stat5-DNA complexes by EMSA using lactogenic hormone-treated whole cell extracts and a Stat5 binding site as a

[2] M. Wartmann, unpublished results.

[3] N. Cella, unpublished results.

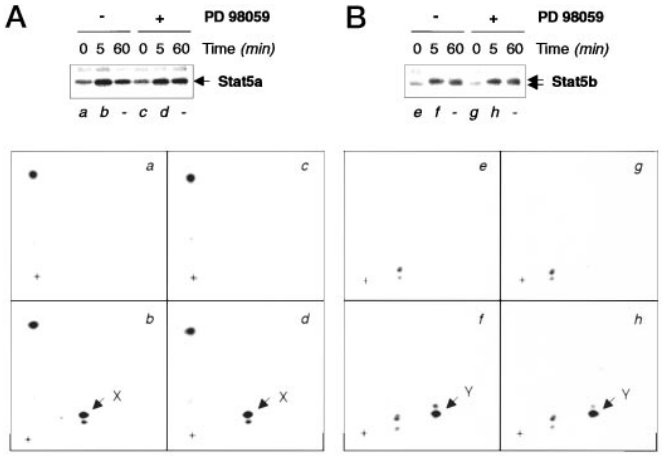

FIG. 3. **Phosphopeptide mapping of trypsin digests of ^{32}P-labeled Stat5 proteins.** Stat5a (*A* and *maps a–d*) and Stat5b (*B* and *maps e–h*) were immunoprecipitated from lysates of ^{32}P-labeled HC11-Lux cells that had been pretreated with 30 μM PD98059 or with vehicle for 60 min and then treated with lactogenic hormones for 0, 5, or 60 min. Phosphorylated Stat5a (*A*) and Stat5b (*B*) were resolved by 7.5% SDS-PAGE and detected by autoradiography. The Stat5 proteins were further analyzed by tryptic phosophopeptide mapping (*lower panels*). *Panels c*, *d*, *g*, and *h* were from samples treated for 5 min with hormones. *X* and *Y* indicate the phosphotyrosine-containing phosphopeptides in Stat5a (*X*) and Stat5b (*Y*). The samples from the 60-min treatment gave essentially the same results with the exception of the reduced amount of radioactivity in phosphopeptides X and Y. The application origin is indicated by +. First dimension was electrophoresis (cathode on the right) and second dimension was ascending chromatography. Plates were dried and subjected to PhosphorImager analysis.

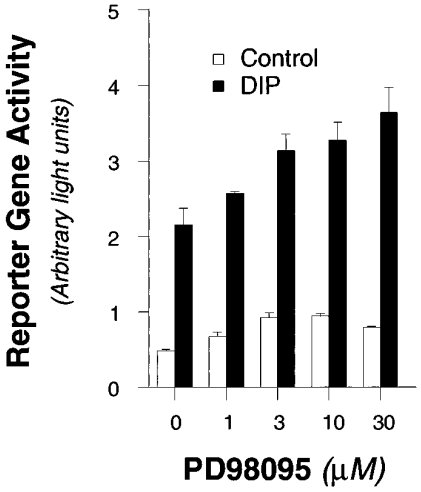

FIG. 4. **The MEK kinase inhibitor PD98059 does not affect lactogenic hormone induced β-casein promoter activity.** Competent, serum-starved HC11-Lux cells were pretreated for 60 min with the indicated concentration of PD98059 or with vehicle. HC11-Lux cells were then treated for 4 h with lactogenic hormones, extracts were prepared, and luciferase activity was determined. *DIP* refers to medium containing the lactogenic hormones dexamethasone, insulin, and prolactin.

probe (10). The kinetics of appearance of the protein DNA complex paralleled the lactogenic hormone-induced phosphorylation of the Stat5 proteins on tyrosine (Fig. 2*C*). Binding was maximal within 5 min and declined by 1 h. The complex contains both Stat5a and Stat5b as determined by a shift Western analysis.[3] Pretreatment of the HC11-Lux cells with 30 μM PD98059, a concentration which completely blocks ERK2 activity (Fig. 1*B*), had no effect upon the lactogen-stimulated phosphotyrosine content of the two Stat5 proteins (Fig. 2, *A* and *B*), nor did it affect the ability of activated Stat5 to bind DNA (Fig. 2*C*).

It has been shown that serine phosphorylation is important for cytokine-mediated stimulation of transcriptional activity of Stat1α, Stat3, and Stat5 (20, 35). Thus, we examined the effect of PD98059 on the phosphorylation state of Stat5a and Stat5b immunoprecipitated from [^{32}P]orthophosphate-labeled HC11 cells stimulated with lactogenic hormones. Under the conditions employed, the two Stat5 proteins did not coimmunoprecipitate.[2] The total phosphate content of Stat5a and Stat5b increased upon hormone addition (Fig. 3, *A* and *B*, *upper panels*). Phosphoamino acid analysis revealed that basal phosphorylation occurred on serine. Lactogen treatment resulted in the additional appearance of phosphotyrosine.[2] The proteins were further analyzed by two-dimensional tryptic phosphopeptide mapping. Autoradiographs of the separated peptides in the samples from control cells revealed one major phosphopeptide for Stat5a (Fig. 3, *panel a*) and a set of minor phosphopeptides for Stat5b (Fig. 3, *panel e*). Inferring from the phosphoamino acid analysis, these peptides are likely to be phosphorylated on serine. While lactogenic hormone treatment did not alter the phosphorylation state of these peptides, it did result in the appearance of an additional phosphopeptide in both Stat5a and Stat5b (Fig. 3, *panels b* and *f*, *X* and *Y*). As expected, peptide X and Y contain phosphotyrosine. These peptides do not contain phosphoserine.[2] Since a mutant lacking the mammary gland factor-Stat5 Tyr-694 exhibits no tyrosine phosphorylation in response to prolactin (16), it is likely that these phosphopeptides contain the amino acids surrounding Tyr-694 and Tyr-699, in Stat5a and Stat5b, respectively (see Liu *et al.* (26) for numbering). Importantly, neither the total phosphate content nor the phosphopeptide maps for Stat5a and Stat5b were affected by prior incubation of the cells with PD98059 (Fig. 3, *panels c*, *d*, *g*, and *h*). This suggests that ERK2 is not involved in the regulation of basal or lactogen-induced Stat5 phosphorylation.

MAP Kinase Does Not Contribute to Lactogenic Hormone-induced β-Casein Promoter Activity—In order to examine the effect of MAP kinase inhibition upon the induction of the β-casein gene promoter, HC11-Lux cells were treated 4 h with lactogenic hormones in the presence or absence of PD98059 and transcriptional activation of the luciferase reporter construct was measured. MAP kinase is inhibited throughout the time course of the experiment in PD98059-treated cells.[2] Compared to the control, lactogenic hormones induced β-casein luciferase activity approximately 4-fold irrespective of the dose of PD98059 employed (Fig. 4). Interestingly, the basal as well as the hormone-induced luciferase activity was higher in the presence of PD98059.

DISCUSSION

Stat5 is activated by numerous cytokines and growth factors in diverse cell types (36–38) and was first detected as a prolactin-inducible transcription factor in the mammary gland (4). In the present study we investigated the role of the MAP kinase pathway in lactogen-induced β-casein promoter function and Stat5 phosphorylation. We also present here the first phosphopeptide analysis of Stat5 proteins in lactogenic hormone-treated mammary epithelial cells. The results can be summarized as follows: 1) in competent HC11 cells before lactogen treatment, Stat5a and Stat5b are constitutively phosphorylated on serine; 2) in response to lactogenic hormones, Stat5a and Stat5b yield one phosphotyrosine-containing tryptic peptide; 3) ERK2 MAP kinase is activated in response to lactogenic hormones, but its inhibition with PD 98059 has no effect upon Stat5 phosphorylation, DNA binding, or lactogenic hormone-stimulated β-casein transcription.

The intracellular signaling molecules activated by prolactin

have been examined most extensively in mammary cells and in Nb2 lymphoma cells, which are dependent upon prolactin for growth (39). Treatment of Nb2 cells with prolactin leads to an elevation in Ras-GTP levels and Raf-1 kinase activation (40, 41). There is also a rapid appearance of ERK1 in the nucleus of these cells (42). In Nb2 cells inhibition of the MAP kinase signaling pathway with broad range kinase inhibitors leads to an inhibition of cell growth (43, 44), suggesting that the mitogenic effect of prolactin depends on MAP kinase activity. Here we show that prolactin, which is a mitogen as well as a lactogen for mammary cells (1), activates the MAP kinase pathway. However, the lactogenic effect of prolactin, *i.e.* its ability to induce β-casein transcription, does not require MAP kinase activity. Interestingly, we observed that basal as well as lactogen-induced β-casein promoter activity was elevated in PD98059-treated cells (Fig. 4), suggesting that MAP kinase activity might have a negative effect upon β-casein gene transcription. We have previously reported that HC11 cells transformed by the Ha-*ras* and the v-*raf* oncogenes, potent activators of the MAP kinase pathway, no longer synthesize β-casein in response to lactogenic hormones (45, 46). Furthermore, prolactin-induced Stat5 DNA binding activity was also impaired in the Ha-*ras* and v-*raf* transformed HC11 cells (46). We are currently investigating the molecular mechanisms involved in the negative modulation of β-casein transcription by the MAP kinase pathway.

While it is clear that phosphorylation on one specific tyrosine residue is necessary for Stat DNA binding, whether or not this tyrosine phosphorylation is sufficient to promote transcriptional activation is not yet clear. Due to the diversity of signals which lead to Stat activation as well as the numerous genes whose transcription is stimulated in response to Stat activation, it is unlikely that there will be a simple answer. Experiments examining the IFN-γ-responsive Stat1α were the first to explore the role of multiple signaling pathways and transcriptional activation of Stat target genes. The COOH terminus of Stat1α, as well as Stat3 and Stat4, has a consensus recognition motif for phosphorylation by MAP kinase -P*X*S/TP- which, for Stat1α and Stat3, is phosphorylated in response to IFN treatment (20). A mutant Stat1α in which the serine in the consensus MAP kinase site was changed to alanine binds DNA as well as wild type Stat1α. However, transcriptional activation of an IFN-γ-responsive gene was reduced in cells expressing the mutant Stat1α (20). These results show that serine phosphorylation is important for full transcriptional activation of Stat1α. Indirect evidence suggests that MAP kinase might be responsible for this phosphorylation, since it has been shown that ERK2 associates with the α chain of the IFN-α/β receptor and that ERK2 and Stat1 are coimmunoprecipitated after IFN-β treatment (21).

Stat3 is phosphorylated on tyrosine and serine following treatment of cells with both INF-α and interleukin-6 (17, 18). The interleukin-6-induced serine phosphorylation, which is sensitive to the broad spectrum serine/threonine kinase inhibitor H7, has been reported to influence the formation of stable Stat3 homodimers on some GAS-like sites (18). The H7-sensitive kinase responsible for the phosphorylation is apparently not on the MAP kinase pathway (47) and may be protein kinase C (48). IFN-α-induced phosphorylation of Stat3 occurs on the serine residue in the consensus MAP kinase site. Mutation of this serine had no effect on the DNA binding activity; however, there was a 2-fold reduction in IFN-α induced transcription (20). The reason for the discrepancy in the results concerning the DNA binding of Stat3 is not clear but could reflect, *e.g.*, a difference in the serine residue that is phosphorylated in response to IFN-γ and interleukin-6.

Stat5 is phosphorylated on tyrosine and serine in interleukin-2-stimulated T cells (35) and in liver cells from growth hormone-treated rats (49). In T cells, serine phosphorylation leads to an H7-sensitive reduction in the electrophoretic mobility of Stat5. The DNA binding of Stat5 was not affected in H7 treated cells, although its ability to function as a transcriptional activator was decreased. The H7-sensitive kinase, although not yet identified, is evidently neither MAP kinase nor S6 kinase (35). Using a more specific inhibitor, the data presented here corroborate those findings. However, β-casein transcription is not reduced in H7-treated HC11 cells[2] (50). The difference in transcriptional sensitivity of the target promoters to H7 treatment could reflect the different cytokines used to activate Stat5, differences in the site of serine phosphorylation as well as the promoter examined.

Here we demonstrate that Stat5a and Stat5b are phosphorylated on serine in competent, serum-starved HC11 cells and that this phosphorylation is not influenced by lactogenic hormones. The kinase responsible for this serine phosphorylation is currently unknown. It is unlikely to be MAP kinase for two reasons: serum-starved HC11 cells have very low basal MAP kinase activity (24, 32) (this report), and treatment of the cells with PD98059 further reduces basal activity (Fig. 1*B*). We have previously reported that treatment of the HC11 cells with lactogenic hormones, in the presence of the protein kinase C inhibitor CGP41256, negatively affected Stat5 DNA binding and transcriptional activation of the β-casein gene promoter (27). *In vitro* treatment of nuclear extracts from lactating mammary glands with protein kinase C (51) or casein kinase II (9) resulted in enhanced Stat5 DNA binding making it possible that both protein kinase C and casein kinase II directly phosphorylate Stat5. Interestingly, growth hormone-induced serine phosphorylation also modulates the DNA binding of Stat5 (49). These results suggest that serine phosphorylation of Stat5, which in our experiments is present before lactogenic treatment, could, together with tyrosine phosphorylation, promote optimal DNA binding activity of Stat5.

The induction of milk protein gene expression in the mammary gland requires multiple signals including peptide and steroid hormones as well as those emanating from the extracellular matrix (52, 53). In primary cultures of mammary gland cells, prolactin-dependent Stat5 DNA binding and transcriptional activation of milk protein genes is only observed in cells plated on a laminin-containing extracellular matrix (ECM) (54). This implies that there is a hierarchy of signaling in mammary cells and that the cell contact with the ECM disposes them to respond to lactogenic hormones. The HC11 cells appear to have retained this characteristic of primary mammary cells. Growing cultures of HC11 cells deposit an ECM which influences their ability to produce β-casein in response to lactogenic hormones (55). Interestingly, despite the fact that growing cultures of HC11 cells contain Stat5,[4] its binding activity cannot be stimulated by lactogenic hormones[4] (7). This may in part be due to the lack of an appropriate matrix and may reflect the results seen with the primary mammary cells. We find it noteworthy that in the competent cultures, that is, HC11 cells that have deposited the appropriate ECM and are primed to respond to lactogenic hormones, the Stat5 proteins are phosphorylated on serine. It will be interesting to see if ECM influences the serine phosphorylation status of Stat5 in the HC11 cells.

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[4] N. Cella and I. Beuvink, unpublished results.

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