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Activation of STAT Factors by Prolactin, Interferon-γ, Growth Hormones, and a Tyrosine Phosphatase Inhibitor in Rabbit Primary Mammary Epithelial Cells*

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In numerous studies on mammary epithelial cell lines, multiple factors, added to the medium or contained in the serum, were required for casein gene expression. It has been shown in these systems that the mammary gland factor (MGF) is implicated in the activation of the β-casein gene promoter. In the present study, we determined the relationship between known agents that affect casein gene expression and MGF activity using the properties of rabbit primary mammary epithelial cells to respond to PRL alone, when cultured in chemically defined medium. We demonstrate that MGF is rapidly activated by PRL alone or by human growth hormone, a natural ligand of many PRL receptors (PRL-Rs), in the cytoplasm and accumulated in the nucleus. The MGF activation by PRL occurred in the absence of endogenous extracellular matrix, a condition where casein synthesis is known to be markedly reduced. Different inhibitors of protein-tyrosine kinases, which have been shown to reduce casein mRNA synthesis, but not of protein kinase C, decrease the MGF activity. A tyrosine phosphatase inhibitor, sodium pervanadate, induced two GAS-binding complexes related to MGF and STAT1. Our data show that MGF is a latent cytoplasmic factor rapidly activated in mammary epithelial cells, by a mechanism involving a tyrosine kinase and a tyrosine phosphatase.

Prolactin (PRL) is a pleiotropic hormone that mediates a wide range of biological effects. PRL regulates diverse physiological mechanisms such as lactation, reproduction, cell growth, osmoregulation, steroidogenesis, and immune response (2–4). PRL and growth hormone (GH) are members of the GH family of polypeptide hormones produced in the anterior pituitary gland and share similarities in their primary structure (4, 5). Human growth hormone (hGH) is particular since it binds to both the GH receptor or PRL-R and is considered as a genuine lactogenic hormone.

PRL receptors are widely distributed in mammalian tissues and immune cells and they belong to the superfamily of genes encoding cytokines and hematopoietin class I receptors. This family includes receptors for GH, erythropoietin, interleukins 2–7 (IL-2–7), granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, leukemia inhibitory factor, oncostatin M, and ciliary neutrophilic factor. The molecules more distantly related class II family includes receptors for factor "in" (IFN), and interferon-γ (IFNγ) receptors (4, 6, 7). The cytokine/hematopoietic receptors do not contain intrinsic tyrosine kinase activity in their cytoplasmic domain. However, many studies suggest that members of the janus family of tyrosine kinases mediate this activity for these receptors, including the PRL-R (8–11). Studies of mutant cell lines have demonstrated that IFN-γ/β receptor signaling requires coexpression of Jak1 and Tyk2, whereas Jak1 and Jak2 were essential for IFNγ receptor signaling (12–14). Furthermore, these kinases have also been shown to be associated with the cytokine receptors (15–21). Both tyrosine-specific Jak2 and serine/threonine-specific Raf-1 kinases have recently been shown to be associated with PRL-R and to be activated by PRL in the PRL-dependent rat T-cell line Nb2 (22–24).

Recent progress has also been made in understanding the intracellular events which mediate receptor activated gene expression. For several cytokines it has been demonstrated that latent cytoplasmic transcription factors, termed signal transducing factors (STFs), are rapidly translocated to the nucleus after tyrosine phosphorylation. IFN-γ for example, stimulates the rapid tyrosine phosphorylation of STAT1 (p91). Activated p91 homodimerizes forming STF-IFNγ (STAT1:1), which is competent for nuclear translocation and binding to a GAS element (reviewed in Ref. 25). The DNA-binding sites for factors activated by epidermal growth factor (EGF), platelet-derived growth factor, IL-4, and GH are also closely related to the GAS consensus, and can in many cases bind STF-IFNγ (7, 26–30). The molecular cloning of transcription factors induced by interferons and other cytokines has revealed the existence of a growing family of signal transducing factors, STF/STATs. Recently cloned members of the family include STAT1α, STAT1β, STAT2, STAT3, STAT4, STAT5a, STAT5b, and IL-4 STAT (31–33, 46, 60, 75–77). It is well established in several species that casein genes are regulated at the transcriptional level by lactogenic hormones (PRL), glucocorticoids, insulin, and extracellular matrix. Only small amounts of caseins are detected in the resting mammary gland. The transcriptional activation of casein genes by PRL involves a highly conserved TTCnntGAA consensus element in

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the promoters of these genes. This sequence has been shown to bind MGF, whose activity is tightly coordinated with mammary gland differentiation. This factor is essential for the activity of the rat β-casein gene promoter (34, 35). As these studies were being completed, Wakao et al. (33) reported the cloning of MGF and demonstrated that tyrosine phosphorylation is required for the DNA binding activity of the recombiant factor. It has been suggested that MGF be renamed STAT5, based on its homology to other STAT factors (60). More recently, when our paper was ready for submission, tyrosine phosphorylation of STAT5 by prolactin in transfected COS cells and in vitro phosphorylation of STAT5 by tyrosine kinase jak2 was described (57).

Recent studies from our laboratory, on rabbit mammary cells, have shown that the induction of αS1- and β-casein genes by PRL is abrogated by protein kinase inhibitors, suggesting that the phosphorylation of transcription factors may be crucial for milk gene activation (1). In the present study, we have examined the events leading to MGF activation by PRL and GH, and compared it to the induction of STF by IFNγ in rabbit mammary epithelial cells (referred to throughout this report as mammary epithelial cells). The response to PRL of Nb2 cells, used before as a general model to evaluate lactogen properties of PRL and GH from different species, has also been examined. Our data support a model where receptor-associated tyrosine kinases rapidly mediate PRL signaling to the nucleus by the activation of a latent cytoplasmic transcription factor, MGF in mammary epithelial cells, but not in Nb2 cells, where a different STF-IFNγ-related factor could be involved. These findings support the idea that biological differences in hormonal action of PRL in different cell systems are supported at the molecular level by activation of different latent cytoplasmic factors.

MATERIALS AND METHODS
Cell Culture—Primary mammary epithelial cells were isolated from mid-pregnant rabbits by enzymatic digestion of mammary gland essentially as described (1) with some modifications. Cells were maintained for 1 day in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 or 3 days in a serum- and a hormone-free synthetic medium (Dulbecco's modified Eagle's medium, 4% Ultroser SF; IBF, France). Oxine-prolactin (NIH PS13), bovine GH (recombinant bGH provided by G. Kann), human GH (recombinant hGH provided by SF; IBF, France). Ovine prolactin (NIH PS13), bovine GH (recombinant bGH provided by G. Kann), human GH (recombinant bGH provided by J. Djiane), porcine IFNγ (recombinant protein kindly provided by C. La Bonnardière), protein kinase inhibitors, protein phosphatase inhibitors, and cytoskeleton disrupting agents were added to the medium prior to collecting cells for cytoplasmic and nuclear extracts preparation. The Nb2-1IC cells (provided by A. Giordano) were grown in RPMI 1640 supplemented with 5% fetal calf serum. They were factor-starved for 20–24 h in the above medium containing 5% gelled horse serum (not containing PRL). All experimental procedures with U937 cells and M12.4.1 (M12) cells stimulated by IFNγ and HepG2 cells stimulated by IL-6 were as described previously (28).

Antibodies—Monoclonal anti-phosphotyrosine antibody PT 66 was from Sigma. Rabbit N1593 antisemum prepared from a nonimmunized rabbit was unexpectedly found to be able to recognize MGF. Polyclonal rabbit antiserum (Ab 1-4) were generated against various domains of STAT1 (p91) and used as described (28). Ab 1 recognizes p91 amino acids 607–716; Ab 2 recognizes p91 amino acids 715–750; Ab 3 recognizes p91 amino acids 2–66, and Ab 4 recognizes amino acids 515–607 of the denatured form of STAT1. Electrophoretic Mobility Shift Assay (EMSA) and Cell Extracts—Mammary tissue was frozen in liquid nitrogen and reduced to powder prior to making extract. Extracts from rabbit PME and Nb2 cells were prepared using the solutions containing MOPS instead HEPES and nucleotides separated from cytoplasmic fraction on the sucrose gradient as described previously (30). To avoid individual differences between rabbits which are not isogenic, each experiment described in this paper has been repeated generally several times. Cytosplasmic, nuclear, or whole cell extracts were prepared essentially as described previously (37, 38). Protein concentrations were estimated using the Bio-Rad assay kit. Standard shift reactions were carried out with 4 μg of crude extract incubated with 0.1 ng of labeled double-stranded probe for 20 min at 25°C. Oligonucleotides were labeled by filling in 5′-protruding ends with Klenow enzyme, using [α-32P]dCTP (3000 Ci/mmole). Rabbit β-CAS GAS probe was a double-stranded oligonucleotide representing the sequence –81 to –106 in the rabbit β-casein gene promoter (39): CATATGGGATCTGCTGGAATATTGACT. The sequence of the mutated probe was CATATGGGATCTGCTGGAATTTAAC. For the preparation of the double-stranded probes, the upper strand was hybridized to a primer (agttcataaat) and filled in with Klenow enzyme. Oligonucleotide NTAT was designed as palindrome agttcagattattaattcct, containing single-stranded 5′ overhangs at both ends and filled in with Klenow enzyme after annealing. Rabbit β-CAS GAS probe was used in EMSA analyzing the shifts in 1 × TAE, 5% acrylamide (39:1) gels. The following probes: the rat β-CAS GAS (gatccAGCTTCTTGAATT)T, the IRF-1 GAS (gatcGATTCTCCCGGAAT), the IFP GAS (gatcAGATTCTGAAATGA), and the Ly6 GAS (gatcAGATGCTGAAATGAGT) were prepared and used in EMSA in 0.2 × TBE, 4.5% acrylamide (29:1) gels as described previously (28). Phosphotyrosine phosphatase PTP 1B coupled to agarose beads (Upstate Biotechnology Inc.) was preincubated with extracts for 1 h at 37°C before adding labeled probe. When needed, the tyrosine phosphatase inhibitor sodium vanadate (1 mM) was added to the reaction mixture. To perform antibody supershift assay, antisera were added to the binding reaction in a final dilution of 1:200 unless otherwise indicated.

RESULTS
MGF Is Rapidly Activated by PRL and Down-regulated during Casein Synthesis in Primary Mammary Epithelial Cells Independently of PRL Concentration—It has been shown previously that MGF binding activity can be detected in HC11 line of mouse mammary epithelial cells after several days of combined PRL and glucocorticoids treatment or 24 h after PRL treatment in COS cells cotransfected with cDNAs for MGF and PRL-R (33, 35). As our studies were being completed, our observation of a very rapid activation of MGF in HC11 cells was independently corroborated (40, 41). HC11 cells, derived from COMMA-1D cell line, have been used for studies on the transcriptional regulation of β-casein gene expression. However, they have some limitations in their use because of their relatively poor response to PRL. Furthermore, the induction of MGF by PRL must be accompanied by additional hormonal stimulation by glucocorticoids and insulin at confluence to activate β-casein gene expression (61, 63). Additionally, it was observed that the activation of MGF in HC11 cells is not sufficient to stimulate the transcription from the promoter composed of multimeric MGF sites, whereas the same promoter is activated by IL-6 in HepG2 cells (40). Moreover, all studies on this cell line were conducted in the presence of serum. Another cell model have been used by B. Gröner and co-workers. They used COS cells overexpressing the PRL-R and STAT5 (57). Here also, authors used a serum-containing medium, thus not avoiding possible contamination by factors and hormones interfering with milk genes expression. Thus it is not clear from all these studies if MGF DNA binding and its transactivation potential are regulated by PRL alone or require synergistic action with insulin, glucocorticoids, and seric factors. We have examined the time course of MGF activation by oPRL in rabbit mammary epithelial cells, where the induction of milk genes can be mediated by PRL alone in chemically defined medium. When cells were incubated in a hormone-free synthetic medium for 24 h, MGF was then not detectable (Fig. 1, lane 2). However, nuclear MGF was rapidly detected by EMSA in nuclear extracts (NE) prepared from PRL-treated mammary cells. This GAS binding activity peaked at 15 min and then began to decrease by 1 h, reaching a stable level between 4 and 24 h post-PRL treatment (Fig. 1, lanes 3–7). The MGF complex in NE from cultured cells has the same electrophoretic mobility as the MGF complex prepared from lactating mammary gland (lane 1). In all studies on the MGF activation published elsewhere, PRL was used at supraphysiological concentrations, e.g. 1–5 μg/ml. It has been shown previously in our laboratory that there exists a reciprocal correlation between the rate of casein synthesis, casein mRNA concentration, and the down-regula-
The Activation of MGF by PRL Occurs at the Post-translational Level and is Not Affected by Extracellular Matrix, Glucocorticoids, or EGF—The rapid activation of MGF by PRL is similar to the rapid activation of the other STAT transcription factors (17, 37, 42, 43) and suggests that a post-translational activation mechanism is involved. If PRL activates a preformed factor, its induction should not require de novo protein synthesis and should therefore be resistant to cycloheximide (CHX). We therefore examined the ability of PRL to activate MGF in the presence or absence of CHX in mammary epithelial cells. The inability of CHX to block MGF activation (Fig. 2A) is consistent with the fact that PRL stimulates the accumulation of casein mRNA in mammary epithelial cells for at least 2 h in the presence of CHX (44).

The down-regulation of MGF activity has previously been observed in murine mammary HC11 cells when they are cultured in EGF for 3 days (35) or most recently for several hours (41). No results are available on the early, within minutes, action of EGF on mammary cells. Since EGF is known to rapidly induce tyrosine phosphorylation of STAT proteins (27, 45, 46), we wanted to determine whether EGF treatment might affect the activation of MGF in our system or also activate different GAS-binding factor. Mammary epithelial cells were incubated in synthetic medium with EGF and PRL for 15 min, or pretreated with EGF for 3 h prior to PRL stimulation. EGF was neither able to induce MGF binding activity, nor interfere with the appearance of MGF activated by PRL (Fig. 2A). This suggests that EGF does not control the initial activation of MGF and is not directly involved in the slow down-regulation of MGF activity.

ECM proteins act synergistically with PRL in the induction of genes by PRL and moreover, only limited casein mRNA synthesis occurs in the absence of ECM. Furthermore, ECM has been shown to regulate gene expression at the transcriptional level of several genes including the β-casein in mammary epithelial cells (reviewed in Ref. 47). An enhancer element, possibly responding to the ECM and PRL, has been localized in

![Fig. 1. Kinetics of MGF induction by prolactin in the nucleus.](image)

Rabbit mammary cells were incubated for 24 h in a hormone-free synthetic medium and then submitted to prolactin treatment. Nuclear extracts were prepared at different times after PRL addition to rabbit mammary epithelial cells and examined by mobility shift assay with a labeled rabbit β-CAS GAS probe in a 5.0% polyacrylamide gel. PRL-stimulated nuclear extracts were prepared from mammary epithelial cells, untreated (lane 2) or treated with oPRL added at concentrations of 2 μg/ml (lanes 3–7) and 50 ng/ml (lanes 8–11) for indicated time. MGF, nuclear extract from lactating rabbit mammary gland (lane 1). MGF complex is indicated with an arrow.

![Fig. 2. Effect of EGF, cycloheximide, dexamethasone, and extracellular matrix on the MGF induction by PRL in rabbit mammary epithelial cells.](image)

A, deinduced mammary epithelial cells were pretreated for 3 h or 15 min with EGF (10 ng/ml) (lanes 4 and 5 and lanes 6 and 7, respectively) or with CHX (5 μg/ml) for 1 h (lane 3). oPRL (500 ng/ml) was then not added (lanes 1, 4, and 6) or added to the culture medium for 15 min (lanes 2, 3, 5, and 7). B, mammary epithelial cells were cultured directly on plastic dishes (lanes 1–5). Cells from several dishes were trypsinized and reseeded on plastic (PL) support (lane 6) or on Engelbreth-Holm-Swarm (EHS) exogenous extracellular matrix (lane 7). Cells were treated with oPRL (500 ng/ml) for indicated periods of time. C, mammary epithelial cells were not treated (lanes 1 and 2) or treated with dexamethasone (Dex) for 24 h (lanes 3 and 4). PRL was then added as described in A to nontreated cells (lane 2) or to dexamethasone-treated cells (lane 4).
distal region of bovine β-casein gene promoter. Although ECM and PRL can activate the enhancer independently, the ECM response has not been differentiated from the PRL response in terms of sequence requirements (48, 49). Curiously, this DNA fragment contains MGF consensus sequence TTC TCA GAA, raising the possibility of the involvement of MGF in ECM response. To determine if ECM proteins play a role in MGF activation, mammary cells were trypsinized and reseeded on plastic support to eliminate the endogenous ECM present in primary cultures. In these conditions caseins are not detected in the culture medium of primary cells reseeded on plastic and stimulated by PRL as we have previously demonstrated (1). Nuclear extracts were prepared from both cells replated in the absence and presence of exogenous matrix and no difference was observed in MGF binding activity (Fig. 2B).

Since glucocorticoids are also known to enhance milk production and are generally used in cultures of mammary cells, we tested the effect of glucocorticoids in the same series of experiments in serum-free medium (Fig. 2C). Glucocorticoids appeared to have no effect on PRL-stimulated MGF activation even after 24 h (Fig. 2C, lanes 3 and 4). The data reported in this section thus demonstrate that EGF, ECM and glucocorticoids do not directly affect the activation of MGF.

MGF Activation by PRL Is Affected by the Protein-Tyrosine Kinase Inhibitors Involved in the Down-regulation of Casein mRNA—It has been shown that tyrosine phosphorylation event is essential for DNA binding activity of MGF purified from mammary gland of lactating sheep (33) or overexpressed in COS cells (57). The situation is not clear for mammary epithelial cell line HC11. While Welte et al. (41) shown that the treatment of HC11 cells with genistein, or incubation of NE from 15-min PRL-stimulated cells with anti-phosphotyrosine antibody reduce the DNA binding activity of MGF, Marte et al. (69) described that a staurosporine derivative CGP 41251, a potential inhibitor of protein kinase C (PKC), reduced the DNA binding of MGF induced by 24 h of combined PRL/insulin/glucocorticoid treatment of HC11 cells. Thus it is not still clear if the MGF activity depend essentially on tyrosine phosphorylation event or serine/threonine phosphorylations could also be required. Therefore, we used several protein-tyrosine or serine/threonine kinase inhibitors, which have been shown recently to abrogate or not to abrogate the induction of rabbit α51- and β-casein genes in cultures of mammary epithelial cells (7, 71). MGF activity was evaluated in cells cultured in synthetic medium and induced by PRL for 20 h, followed by treatment with inhibitors (Fig. 3A). Similar results were obtained when cells were pretreated with inhibitors for 4 h, prior to a 15-min stimulation with PRL (Fig. 3B). This suggests that the same kinases are involved in both the rapid and prolonged activation of MGF. Staurosporine, 6-dimethylaminopurine, which are broad spectrum kinase inhibitors, and genistein, a more specific tyrosine kinase inhibitor (see Ref. 1, and references therein), prevented the PRL-induced activation of MGF (Fig. 3, panel A, lanes 4–6, and panel B, lanes 3–6), while a potent EGF-R tyrosine kinase inhibitor, tyrphostin 25, was ineffective (Fig. 3B, lane 7). Interestingly, the inhibitory effect of staurosporine on the MGF activity was dose-dependent, analogous to its dose-dependent inhibition of casein gene expression. Furthermore, a significant inhibitory effect on the MGF binding activity was observed in extracts prepared from cells pre-treated with staurosporin for either 15 min or 1 h, followed by a 15-min PRL stimulation. To address the involvement of PKC, we used a well known PKC inhibitor, H7, and an additional very selective inhibitor of most of PKC isoforms, bisindolylmaleimide GF 109203X (50). This compound has been shown to inhibit completely PKC in HC11 cells and in primary rabbit mammary cells without altering the induction of casein gene expression by prolactin (72).2 GF 109203X only marginally decreased the intensity of MGF shift (Fig. 3B, lane 8), and H7 did not affect the MGF activity (Fig. 3A, lane 7). The fact that serine/threonine kinase inhibitors neither reduce STAT5 activity over 15 min or 24 h of incubation with PRL nor abolish the casein mRNA synthesis suggests the essential role of tyrosine phosphorylations in MGF activity. To further confirm the role of tyrosine kinases in STAT5 activation in mammary cells, nuclear extracts prepared from PRL-stimulated cells were treated with human PTP 1B, a tyrosine-specific phosphatase (Fig. 3C, lanes 2–5) or with monoclonal anti-phosphotyrosine antibody (pY-pY) PT66 (lanes 6 and 7) and control (C) ascites (lanes 8 and 9). PV (1 μM) were added to inhibit the action of PTP1B in the reaction mixture (lanes 3 and 5). Labeled β-CAS GAS probe was then added for an additional 15-min incubation and analyzed in gel retardation assay.

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Fig. 3. Tyrosine phosphorylation of MGF is necessary for its DNA binding activity. Nuclear extracts from mammary epithelial cells were analyzed as described in Fig. 1. A, mammary epithelial cells were noninduced (lane 1) or induced with 2 μg/ml PRL for 20 h (lanes 2–7). Protein kinase inhibitors: staurosporine (50 nM, lane 3; 20 nM, lane 4), genistein (25 μg/ml, lane 5), 6-dimethylaminopurine (2.5 μM, lane 6), and H7 (10 μM, lane 7) were then added for 4 h. Four micromgs of nuclear protein extracts from these cells was analyzed in a gel retardation assay using the rabbit β-CAS GAS as probe B, mammary epithelial cells were incubated for 4 h in the presence of protein kinase inhibitors staurosporine (20 nM, lane 3; 200 nM, lane 4), 6-dimethylaminopurine (2.5 μM, lane 5), genistein (25 μg/ml, lane 6), tyrphostin 25 (TP, 10 μM, lane 7), and GF 109203X (GF, 10 μM, lane 8), and then induced by PRL (500 ng/ml) for 15 min. C, effect of protein-tyrosine phosphatase and monoclonal anti-phosphotyrosine antibody on the MGF DNA binding activity. Nuclear extracts from PRL-induced mammary epithelial cells (at 500 ng/ml for 15 min) were preincubated for 1 h at 37 °C with increasing amounts of recombinant PTP 1B (Upstate Biotechnology Inc.) (lanes 2–5) or with monoclonal anti-phosphotyrosine antibody (pY-pY) PT66 (lanes 6 and 7) and control (C) ascites (lanes 8 and 9). PV (1 μM) were added to inhibit the action of PTP1B in the reaction mixture (lanes 3 and 5). Labeled β-CAS GAS probe was then added for an additional 15-min incubation and analyzed in gel retardation assay.
(lanes 8 and 9) further confirms that a phosphotyrosine residue is critical for STAT5 binding. These observations, along with the kinase inhibitor results, are consistent with a model in which PRL activation of MGF, like other STFs, requires a tyrosine phosphorylation event.

The Tyrosine Phosphatase Inhibitor Sodium Pervanadate Activates Two DNA-binding Complexes in Primary Mammary Epithelial Cells—Signaling through PRL receptor is promoted by the activation of one or more phosphotyrosine kinases and presumably down-regulated by one or more phosphotyrosine phosphatases. No data are known on the involvement of phosphatases in the regulation of PRL signaling. To assess the possible role of endogenous tyrosine phosphatases in STAT5 activation, we treated mammary epithelial cells with sodium pervanadate (PV), a known protein-tyrosine phosphatase inhibitor (51). In these studies we employed the peroxysome proliferator-activated receptor-γ agonist, which penetrates intact cells more readily. A 15-min incubation of mammary epithelial cells with pervanadate resulted in the appearance of two GAS-binding complexes, one of which comigrated with MGF induced by PRL. The maximal stimulation was observed after 1 h of treatment, comparable to the intensity of MGF complex after 1 h of PRL induction (Fig. 4A, lanes 3, 4, and 8). The formation of both complexes was inhibited by staurosporine, added 1 h before PRL treatment (lane 5) but not by the serine/threonine inhibitor GF 109203X (lane 7), suggesting that the inhibitory effect of staurosporine is tyrosine-specific. Additionally, PRL was not able to restore MGF activity in cells treated by staurosporine and then induced by PV (Fig. 4A, lane 6). The induction of two GAS-binding complexes by PV is an intriguing result. Recently the existence of two APRF (STAT3) forms, which differ in electrophoretic mobility (67), has been described. Most recently, the slower migrating form of APRF, associated with the slower migrating form of APRF, migrates faster, is a different factor but not a converted form of APRF. This raises a question about their identity. Several lines of evidence demonstrate that one complex is STAT5 and the second complex, migrating faster, is a different factor but not a converted form of MGF as a result of post-transcriptional modifications induced by PV.

First, the experiments with cytoskeletal disrupting agents (e.g. cytochalasin D and colchicine) demonstrate that these two complexes respond differently to this treatment (Fig. 4B). While cytochalasin D and colchicine inhibit the accumulation of casein mRNA in several species (52, 53), they did not affect the formation of the PV-induced MGF-comigrating band (lanes 3–5) and MGF (lanes 7–9). On the contrary, we observed that these agents both preferentially affected formation of the faster migrating complex induced by PV (lanes 3–5).

It has been shown recently that STAT5 is induced in HC11 cells and STAT1 from mouse macrophage cell line J774A.1 have different mobility in EMSA experiments (41). To further characterize the nature of the faster migrating complex, we compared it to GAF, activated in rabbit mammary epithelial cells, using unique properties of our cell system to respond to PRL as well to IFNγ. Cells were treated with oPRL and porcine IFNγ (pIFNγ) for 15 min, or with PV for 1 h. As shown in Fig. 4C, the pIFNγ-induced complex (referred to as PME-GAF) comigrates with the faster PV complex. PME-GAF was induced in a dose-dependent manner, with a detectable signal at 50 ng/ml pIFNγ, and maximum signal between 0.5 and 5 μg/ml (not shown). Furthermore, the two DNA binding activities induced by PV were inhibited by the staurosporine pretreatment in the same manner as MGF and PME-GAF (pIFNγ treatment, lanes 3, 5, and 7). Interestingly, IFNγ enhanced both MGF and PME-GAF complexes when added to PV-treated cells (lane 8), suggesting that common protein kinases may be involved in MGF and PME-GAF activation. Additionally, another tyrosine phosphatase inhibitor, phenylarsine oxide, failed to induce MGF DNA-binding factors and even blocked PRL induction of MGF complex (data not shown). These data demonstrate that PV is able to stimulate the activation of two DNA-binding complexes that appear to be MGF and GAF-related, and suggest that tyrosine phosphorylation in addition to a tyrosine kinase is involved in the activation of casein gene expression.

MGF is Rapidly Activated in the Cytoplasm by PRL and GH prior to Nuclear Translocation—While rapid activation of nuclear STAT5 was described elsewhere and confirmed in the present work, nothing is known about the possible cytoplasmic activation of this factor. To address this question, cytoplasmic

FIG. 4. Induction of two different β-CAS GAS-binding complexes by a phosphatase inhibitor, sodium pervanadate. Gel retardation assay was performed with 4 μg of nuclear extracts incubated with the labeled rabbit β-CAS GAS probe as described in Fig. 1. A, mammary epithelial cells were not treated with inhibitors (lanes 1–4 and 8) or pretreated for 1 h with the protein kinase inhibitor staurosporine (200 nm, lanes 5 and 6) and with GF 109203X (10 μM, lane 7) and then nonstimulated (lane 1) or stimulated with 500 ng/ml oPRL for 15 min (lane 2) or 1 h (lanes 6 and 8) or with 10 μM PV for 15 min (lane 3) or 1 h (lanes 4–7). B, effect of cytoskeleton-disrupting agents on MGF and PV-induced factors. Mammary epithelial cells were submitted to colchicine (1 μM, lanes 3 and 7) or cytochalasin D (2 μM, lanes 4, 5, 8, and 9) treatment before induction with 10 μM PV for 1 h (lanes 2–5) or with 500 ng/ml oPRL for 15 min (lanes 6–9), C, comparison of electrophoretic mobility of PV-, PRL-, and IFNγ-induced β-CAS GAS binding activities. Mammary epithelial cells were not treated (lanes 1, 2, 4, 6, and 8) or pretreated with 200 nm staurosporine for 15 min (lanes 3, 5, and 7) and then induced with 500 ng/ml oPRL for 15 min (lanes 2 and 3), with PV at 10 μM for 1 h (lanes 4 and 5), with pIFNγ at 500 ng/ml for 15 min, or with combination action of PV for 45 min followed by pIFNγ treatment for additional 15 min (lane 8).
and nuclear extracts were prepared and tested by EMSA using the rabbit β-CAS-MGF probe. To further characterize complex hormonal regulation of mammary epithelial cells, we examined the ability of other lactogenic hormones, human and bovine GH, to stimulate MGF in mammary epithelial cells. Human GH and oPRL bind the rabbit PRL-R with high affinity and exhibit a potent lactogenic activity in these cells. However, bovine GH is considered as totally devoid of PRL-like activity in explants from bovine mammary gland and has a very low affinity for recombinant rabbit PRL-R (54, 55).3 Cells were explants from bovine mammary gland and has a very low affinity for recombinant rabbit PRL-R (54, 55).3 Cells were treated by oPRL, hGH, bGH, and bGH for 15 min. MGF activity was detected in the cytoplasm within 5 min after PRL stimulation, with comparable intensity at the 15-min time point (data not shown). As expected, hGH markedly induced a DNA binding activity that comigrated with MGF both in cytoplasm and nuclear extracts (Fig. 5, lanes 2, 4, 7, and 9). bGH was only able to induce a weak complex that comigrated with MGF (Fig. 5, lanes 5 and 10). Interestingly, pIFNγ induced two complexes in the cytoplasm, but only one of them was translocated to nuclei. The nature of the slower complex, which comigrates with MGF, but fails to translocate to the nucleus is unclear.

To investigate further these DNA-binding complexes, competition with several different GAS oligonucleotides was carried out. MGF and MGF-comigrating rabbit β-CAS GAS binding activities all competed well with the rabbit β-CAS GAS (MGFW) and IFP GAS (whose consensus is identical to those of β-CAS GAS), less effectively with the Ly6 GAS (whose sequence lacks G residue, critical for MGF binding), and not at all by a mutated β-CAS GAS (MGFM) or a nonspecific heterologous oligonucleotide, NTAT (Fig. 6). The competition profile of MGF from lactating mammary gland was identical to MGF from primary mammary cells (data not shown). PME-GAF DNA binding was also well competed by MGFW IFP and Ly6 GAS but not with MGFM or heterologous NTAT. Competition experiments with PV-induced complexes indicate that they had the same profile as MGF and PME-GAF. These studies further support the notion that PRL, hGH, and bGH activate the same MGF complex. In addition, PV appears to induce simultaneously MGF and another factor different from MGF.

Treatment of Nb2 Cells by Lactogenic Hormones Induces GAF-related Factor—We were interested in comparing the above described PRL actions to those that have been extensively characterized in Nb2 cells, which were used as a model to study the prolactin-like activity of many hormones. These cells express PRL-R and are PRL-dependent for their growth. They also proliferate in response to hGH, but not of bGH. Cytoplastic and nuclear extracts were prepared from Nb2 cells after 15 min of oPRL, hGH, bGH, or pIFNγ stimulation and compared with the complexes stimulated in mammary epithelial cells. No complexes were detected after bGH or pIFNγ treatment (Fig. 7A and data not shown). Two GAS-binding complexes were detected in cytoplasmic and nuclear fractions of PRL- and hGH-treated cells (Fig. 7A). None of the two complexes comigrated with MGF. The faster complex comigrated with PME-GAF and other GAFs, and the slower migrating complex had the similar mobility to APRF/STAT3 complex from IL-6-stimulated HepG2 cells (Fig. 7A and B).

Our other results (not shown) demonstrated that the faster but not the slower migrating complex effectively competed with the Ly6 GAS, and partially with the IFP GAS. Moreover, one antiserum raised against STAT1 (Ab 1) recognized faster migrating complex induced by PRL in Nb2 cells (not shown). These data suggest that lactogenic hormones PRL and hGH, but not bGH or IFNγ, induce a STAT1-related factor. This is consistent with reports indicating that STAT1 is activated in Nb2 cells (24).

PRL- and GH-activated DNA-binding Proteins Are Antigenically Similar and Recognized by MGF-specific Antiserum; Anti-p91 Antibodies Recognize PME-GAF but Not MGF—We wanted to further confirm whether MGF complexes induced by PRL, GH, and PV are identical, by testing each DNA-binding complex for reactivity to a panel of antisera generated against various conserved domains of STAT1, as well as naturally occurring MGF-specific antisera (NIS93). To confirm the antibody nature of supershifted complexes, the IgG fraction from NIS93 and from a control nonimmune rabbit (SCL) was pre pared by ammonium sulfate precipitation (56). Nuclear or cytoplasmic extracts were incubated with antibodies and then analyzed by EMSA. These experiments revealed a dose-de-
Fig. 7. PRL and hGH, not bGH or pIFNγ, activate rat β-CAS GAS-binding factors in Nb2 cells. A, PRL and hGH rapidly activate rat β-CAS GAS-binding factor in Nb2 cells to translocate from the cytoplasm to the nucleus. Cytoplasmic (lanes 7–10) or nuclear (lanes 11–14) extracts prepared from Nb2 cells treated with oPRL (lanes 8 and 12), bGH (lanes 9 and 13), or hGH (lanes 10 and 14) for 15 min at 500 ng/ml each were assayed by EMSA with rat β-CAS GAS probe in 4.5% polyacrylamide gel. Lanes 7 and 11, untreated Nb2 cells; lanes 1–5, nuclear extracts from mammary epithelial cells (PME) untreated (lane 1) or treated with oPRL (lane 2), pIFNγ (lane 3), hGH (lane 4), or bGH (lane 5). Lane 6, whole cellular extracts from IL-6-treated HepG2 cells. The position of migration is shown for MGF from mammary epithelial cells extract and for faster migrating complex (designated as FMC), for Nb2 extracts. B, analysis of the electrophoretic mobility of STF-PRL from Nb2 cells with different GAFs by EMSA. Nuclear extracts from human U937 cells treated by IFNγ (lane 1), rat Nb2 cells treated by oPRL (lane 2), rabbit mammary epithelial cells treated by pIFNγ (lane 3), murine M12 cells treated by IFNγ (lane 4), and IL-6-treated HepG2 cells were prepared as described under “Materials and Methods” and assayed by EMSA in 5% polyacrylamide gel using the rat IRF-1 GAS probe.

Fig. 8. Analysis of cross-reactivity of MGF with STAT1- and MGF-specific antibodies by Ab interference mobility shift assay. A, the increasing amounts of the IgG fraction of NIS93 antisera (lanes 3–6) were incubated for 10 min with 4 μl of nuclear extract prepared from mammary epithelial cells induced in the synthetic medium for 15 min by oPRL. Labeled rabbit β-CAS GAS probe was then added for additional 15 min prior to the electrophoresis in nondenaturing 5.0% polyacrylamide gel. The volume (in μl) of antisera added to the reactions is indicated on the top of the panel. SCL: control rabbit serum (lane 2). B, NE from PRL-induced mammary epithelial cells were incubated for 15 min with different antibodies: STAT1-specific Ab 3 (lane 2) and Ab 1 (lane 3), MGF-recognizing IgG fraction of NIS93 (0.5 μl) (lanes 4–6), ovine antisera raised against rabbit IgG, SMAL (lane 7), and rabbit control serum, SCL (lane 8). Additionally, SMAL and SCL antisera were also added in the two reactions with NIS93 antisera 7 min later (lanes 5 and 6). Gel retardation analysis was performed after additional incubation for 15 min with the labeled rabbit β-CAS GAS probe followed by the electrophoresis in nondenaturing 5.0% polyacrylamide gel. C, MGF binds to the IRF-1 GAS element but does not cross-react with anti-p91 antibodies. Supershift reactions were performed with anti-p91 Ab 1–4 (lanes 3–6), NIS93 (lane 2), and nonimmune serum (lane 7) preincubated with nuclear extract of PRL-treated mammary epithelial cells (PME/oPRL) before adding IRF-1 GAS probe and analyzed in the 5% polyacrylamide gel.
by a 15-min incubation with the labeled probe before submitting the epithelial cells treated with PV (lanes 3, STAT1 (lane 5) and analyzed by supershift antibody assay with the with NIS93 (lanes 4).

While these hormones have been shown to activate MGF in HC11 cells, required the presence of glucocorticoids along with activation. Previous studies on MGF activation, carried out in systems, which require the addition of several hormones for casein induction. This has enabled us to study casein gene induction in response to a single ligand, PRL. Our results demonstrate that dexamethasone does not affect MGF activation and that PRL alone is sufficient for MGF activation. Previous studies on MGF activation, carried out in HC11 cells, required the presence of glucocorticoids along with PRL. While these hormones have been shown to activate MGF in HC11 cells, EGF has been shown to have an inhibitory effect (35). As our studies were being completed, rapid activation of MGF by PRL in murine HC11 cells was reported (40). However, the cells were cultured in medium containing 10% fetal calf serum. Thus, it is not clear from this work whether other factors may have contributed to PRL activation of MGF. Additionally, the rapid effect of EGF on MGF activity was not observed in our system (i.e. mammary epithelial cells). This is consistent with recent data indicating that the inhibitory action of EGF was only observed after several hours of incubation of cells with this growth factor and was half-maximal after 10 h (41). This suggests that the inhibitory effect observed in HC11 cells is therefore most likely indirect and does not interfere with the mechanism leading to the rapid activation of MGF by prolactin. Furthermore, present studies suggest that the effect of glucocorticoids on milk gene expression over 24 h of action on cells is not exerted at the level of MGF activation. This result is consistent with recent data describing the activation of MGF by PRL, observed in HC11 cells, cultured for 5 days at confluence in the absence of glucocorticoids (41). Although it is not clear why a very long culture conditions were used to induce MGF activity in the absence of glucocorticoids in HC11 cells in contrast with our cell system, these observations indicate that other mechanisms mediate transcriptional effect of glucocorticoids on casein gene expression.

A promoter element, BCE-1, which is thought to respond to ECM, has been described in the distal region of bovine β-casein gene (49). This enhancer is able to respond to PRL in the absence of ECM. The presence of the MGF consensus sequence within the BCE-1 enhancer suggests that MGF is involved in its activation by PRL, but its contribution to ECM response is not clear. However, our results, demonstrating the activation of MGF in secondary cultures where ECM proteins are not detectable (under conditions where only limited casein synthesis occurs), suggest that ECM exerts its effect on casein genes expression independently of MGF activation. Furthermore, our results suggest that ECM signaling is distinct from that of PRL (71).

Data presented here provide evidence that the activation of endogenous MGF involves tyrosine phosphorylation. The activation is blocked by tyrosine kinase inhibitors, and GAS binding activity is sensitive to treatment with a tyrosine-specific phosphatase or with anti-phosphotyrosine antibody. This is consistent with recent work demonstrating that tyrosine phosphorylation of MGF is necessary for its DNA binding and recent reports indicating a role for the Jak2 kinase in activation of STAT5 in vitro (33, 57). Moreover, the kinetics of MGF activation described here correlate well with the kinetics of Jak2 activation reported by others (10). Thus, the modulation of receptor-associated protein-kinase Jak2 activity may determine the MGF activity in response to PRL. Other recent data indicate that Jak1 and Jak3 can also be involved in STAT5 activation in response to IL-2 in CTLL-2 cells (78). As our manuscript was being revised, another group reported that, although STAT3 DNA binding is not blocked by H7, transcriptional activation is blocked. In contrast, our results with PKC inhibitors H7 and GF 109203X indicate that PKC does not play a role in β-casein expression (1, 71, 72). These observations are consistent with very low PKC activity in mammary cells during late pregnancy and lactation, periods closely associated with the expression of milk protein genes (58).

Although termination of protein-tyrosine kinase signals most likely involves a tyrosine dephosphorylation, it is not known if protein-tyrosine phosphatases regulate PRL trans-

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duction. A number of studies have demonstrated that activation of STAT proteins occurs after the addition of phosphoryrosine phosphatase inhibitors to cells (73, 74). Our studies demonstrate for the first time that two different GAS-binding complexes are simultaneously detected in the NE of cells treated with sodium pervanadate. We used a unique properties of rabbit mammary cells which respond to PRL as well to IFN γ to characterize these complexes. PME-GAF, the factor induced by IFN γ in mammary epithelial cells, is different from MGF, based on their distinct mobilities in EMSA and their different reactivity to a panel of antisera. PME-GAF interacts with the MGF site of the rabbit β-casein promoter. However, IFN γ alone is not able to induce caseins. Furthermore, our preliminary results indicate that treatment of mammary epithelial cells with pIFN γ shows a dose-dependent inhibitory effect on PRL induced casein synthesis (results not shown). This suggests that PME-GAF might have an inhibitory effect on casein gene expression. The mechanism that would allow the β-casein promoter to bind yet discriminate between STF-IFN γ and STF-PRL is unknown but could be related to the presence of a YY1 site, which is juxtaposed to the β-CAS GAS. It has been proposed that YY1 is involved in the repression of casein gene transcription (61, 62).

We present data which suggest that PV-induced complexes correspond to MGF and IFN γ-inducible factor. Their activation is prevented by the protein kinase inhibitor staurosporine, indicating that tyrosine kinases are involved in the PV mediated activation of GAS-binding complexes. Although the exact mechanism of activation by PV remains unclear, studies on the receptors for IL-3, granulocyte-macrophage colony-stimulating factor, erythropoietin, and ciliary neurotrophic factor-IL-6 suggest that a receptor-associated tyrosine phosphatases SH-PTP1 may play an essential role in signal down-regulation (see Ref. 59, and references therein).

Our results demonstrate that PV alone is unable to induce casein mRNA in mammary explants after 8 h of treatment and even show some inhibitory effect on prolactin action in this system. Interestingly, some enhancement of existing basal level of casein gene transcription, generally observed in mammary explants, is detected after 24 h of PV treatment (71). These data strongly imply other factors involved in PV activity, such as positive or negative regulators. For instance, the relief of transcriptional repression by another factorsregulated by lactogenic hormones, single-stranded DNA-binding transcriptional regulators, may be necessary to activate casein gene expression (63). Thus, PV treatment that activates MGF may not be sufficient to relieve the repression by single-stranded DNA-binding transcriptional regulator, or activate another essential factor. We cannot exclude the hypothesis that, additionally, PME-GAF could exert its inhibitory effect on mRNA casein synthesis by competing with MGF when both factors are induced by PV. Furthermore, it is not clear if possible post-translational modifications, other than tyrosine phosphorylations, are necessary to confer the transactivation potential to STAT5 by PRL action. If it is the case, then PV treatment could only induce MGF DNA binding but not its transcriptional activity.

Our results demonstrate the activation of different STFs in mammary epithelial cells after a number of stimuli. Interestingly, hGH and, to a much lower extent, bGH appear to induce MGF in mammary epithelial cells. These factors show the same competition pattern, are recognized by the same antisem NIS93, and comigrate with MGF. Furthermore, hGH is known to be a ligand for PRL-R and to induce lactation in the rabbit, whereas bGH shows only a low affinity for rabbit PRL-R with little lactogenic activity (54, 55). Thus, we suggest that hGH, and to a much lesser degree bGH, interact with the rabbit PRL-R to activate MGF. This low activation of MGF by bGH may explain why this hormone is not able to induce casein gene expression. We cannot exclude, however, the possibility that GH may also activate MGF through interactions with the GH receptor. We compared the PRL and other cytokines signaling in highly differentiated mammary epithelial cells and Nb2 cells whose proliferation is PRL- or hGH-dependent. PRL-dependent tyrosine phosphorylation of several proteins in these cells has been reported (8, 9). Our results demonstrate that two GAS-binding complexes were detected in cytoplasmic and nuclear fractions of PRL- and hGH-treated cells. The faster complex comigrated with IFN γ-induced factor in rabbit mammary cells and other GAFs, and is antigenically related to STF-IFN γ. This indicates that in these cells, PRL signaling could use a STAT1-related factor. The characterization of a second complex will indicate if rat STAT5 is also induced with PRL and hGH. Data from another group indicate that PRL can activate factor immunologically related to STAT5 in Nb2 cells. Our observations together with data from other groups raise the question of weather some STAT proteins may be involved in the proliferative and growth factors, while another set of specific STFs are involved in the functioning of nonproliferating highly differentiated mammary epithelial cells. The molecular mechanisms of such specificity of response to the cytokines by cognate receptors and transcription factors activation remains an area of intense investigation. Recent publications of Heim et al. (64) and Stahl et al. (65) give insight in the understanding of molecular mechanisms of ligand-dependent specificity activation of STFs. They show that tyrosine-containing motifs in cytokine receptors and STAT SH2 groups may play a crucial role in determining the specificity of their interactions. Proposed models involve ligand-driven receptor dimerization and activation of associated kinases, which in turn activate STATs. The use of chemically defined medium clearly demonstrate in the present work that pervanadate alone is able to induce the formation of two distinct DNA-binding complexes. This first example of simultaneous activation of different STFs in the absence of ligands for IFN γ-R and PRL-R, which suppose the presence of only monomeric forms of cognate receptors, raises the question how per- vanadate could activate signal transducing factors with different specificity. Moreover, our data indicate that the binding activity of MGF does not strictly reflect its ability to induce casein gene expression. These data and our other results (71) suggest that a tyrosine phosphorylation is also involved in triggering the induction of casein gene expression.

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