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Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription

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Mammary gland factor (MGF) is a transcription factor discovered initially in the mammary epithelial cells of lactating animals. It confers the lactogenic hormone response to the milk protein genes. We reported recently the isolation of the cDNA encoding MGF. MGF is a novel member of the cytokine-regulated transcription factor gene family. Members of this gene family mediate interferon α/β and interferon γ induction of gene transcription, as well as the response to epidermal growth factor and interleukin-6, and have been named signal transducers and activators of transcription (Stat). The name Stat5 has been assigned to MGF. We studied the mechanisms involved in the prolactin activation of Stat5 in COS cells co-transfected with cDNA encoding Stat5 and the prolactin receptor. Prolactin treatment of the transfected cells caused activation of Stat5 within 5–10 min. This activation does not require ongoing protein synthesis. Tyrosine kinase inhibitors prevent Stat5 activation in transfected COS cells. Treatment of recombinant Stat5 with a tyrosine-specific protein phosphatase *in vitro* abolishes its DNA binding activity. Prolactin stimulation of transfected cells induces Stat5 phosphorylation on tyrosine. Phosphorylation of *in vitro* transcribed and translated Stat5 with the Jak2 tyrosine kinase, but not with fyn, lyn or lck, confers DNA binding activity. The prolactin response of the β-casein milk protein gene promoter can be observed in COS cells transfected with cDNA vectors encoding Stat5 and the long form of the prolactin receptor. The short form of the prolactin receptor is unable to promote Stat5 phosphorylation and confer transcriptional induction in COS cells. Phosphorylation of the tyrosine residue at position 694 in the Stat5 sequence is essential for prolactin regulation. Replacement of Tyr694 by a phenylalanine residue prevents tyrosine phosphorylation, induction of DNA binding and transactivation by prolactin. Signal transduction via the prolactin receptor shares common features with other members of the cytokine/hematopoietin receptor gene family, i.e. the rapid activation of a factor through tyrosine phosphorylation which serves as a second messenger and an activator of transcription.

Key words: prolactin/signal transduction/transactivation/transcription factor/tyrosine phosphorylation

Introduction

The action of prolactin and the molecular mechanism of its signal transduction have proved difficult to study *in vitro*. Only a few lines of cultured cells are available which are prolactin-responsive. Mammary epithelial cells can be induced to synthesize casein proteins when cultured under special conditions and treated with lactogenic hormones (Danielson *et al.*, 1984; Reichmann *et al.*, 1989). HC11 cells (Ball *et al.*, 1988) have been extremely valuable in the elucidation of the sequential hormonal requirements for milk protein gene induction and have been used to define the role of the individual lactogenic hormones (glucocorticoids, insulin and prolactin) as well as epidermal growth factor (EGF) in the induction process (Hynes *et al.*, 1990; Taverna *et al.*, 1991). Positive and negative hormonal regulatory effects have been defined.

HC11 was also the first cell line used in studies of regulatory DNA elements conferring the lactogenic hormone response (Doppler *et al.*, 1989; Schmitt-Ney *et al.*, 1991; Doppler, 1994). Promoter–reporter fusions, containing regions of the rat β-casein gene promoter, led to the description of a minimal sequence required for the lactogenic hormone induction of a transfected gene construct (Schmitt-Ney *et al.*, 1991). This sequence is composed of multiple regulatory elements. Elements with positive and negative effects on transcription have been found (Altiok and Groner, 1993; Meier and Groner, 1994). The cells were also used for the identification of hormonally regulated nuclear DNA binding factors which bind specifically to the DNA sequences essential for the lactogenic hormone response. The factor studied in most detail is the mammary gland factor, MGF. It binds to the region between −80 and −100 in the β-casein gene promoter. The MGF binding site is indispensable for the hormonal induction of transcription, and MGF binding is regulated *in vitro* and *in vivo* (Schmitt-Ney *et al.*, 1992; Standke *et al.*, 1994; Welte *et al.*, 1994). MGF has been shown to interact with a prolactin response element (Standke *et al.*, 1994; Wakao *et al.*, 1994). The gene encoding MGF has been cloned recently, and sequence analysis has revealed that it belongs to the family of signal transducers and activators of transcription (Stat) transcription factors. These factors confer the response to interferon α/β (Stat1α and Stat2), interferon γ (Stat1α; Fu *et al.*, 1992; Schindler *et al.*, 1992a,b), interleukin (IL)-6 and EGF (Stat3; Akira *et al.*, 1994; Zhong *et al.*, 1994a). Stat4 has been cloned but its activating cytokine is not yet known (Zhong *et al.*, 1994b). MGF has been named Stat5 (Darnell *et al.*, 1994).

Nb-2 pre-T lymphoma cells are prolactin-dependent for their growth and have been used to study the prolactin receptor-associated kinases (Rui *et al.*, 1992). It has been found that tyrosine kinase activation plays a role in the prolactin receptor signaling pathway. A protein of 120 kDa becomes rapidly phosphorylated on tyrosine residues following receptor activation. This protein is receptor-associated and has been identified as the Jak2 kinase (Dusanter-Fourt *et al.*, 1994; Rui *et al.*, 1994). Jak2 belongs to a family of tyrosine kinases found initially in hematopoietic cells. These kinases are distinguished by two kinase domains and the lack of SH-2 or SH-3 domains (Ziemiecki *et al.*, 1994). Jak2 is involved in the signal transduction of additional cytokines, such as erythropoietin, IL-3, IL-6 and growth hormone (Argetsinger *et al.*, 1993; Silvennoinen *et al.*, 1993a; Witthuhn *et al.*, 1993; Narazaki *et al.*, 1994).

Both cell lines have been valuable (HC11 cells mainly for studies on the transcriptional regulation exerted by prolactin and Nb-2 cells for studies of the receptor-proximate signaling events), but they are limited in their usefulness. HC11 cells express low levels of prolactin receptor and attempts to derive stable lines with high expression through gene transfer procedures have been unsuccessful. HC11 cells require confluency, as well as EGF and glucocorticoid hormone priming, before a prolactin response can be observed. Transiently transfected cells are not responsive. The multi-hormonal synergisms probably partially reflect the complexity of regulation of milk protein synthesis *in vivo* and are interesting to study in their own right.

These features of the naturally prolactin-responsive cell lines complicate molecular biological investigations into the prolactin signaling mechanism. The cloning of crucial components of the prolactin signaling pathway, i.e. the isoforms of the prolactin receptor (Boutin *et al.*, 1988; Davis and Linzer, 1989; Edery *et al.*, 1989; Shirota *et al.*, 1990; Zhang *et al.*, 1990; Ali *et al.*, 1991) and the prolactin-regulated transcription factor Stat5 (Wakao *et al.*, 1994), provides the tools to study the molecular details of prolactin signaling in transfected COS cells. We report here that Stat5 can be expressed to a much higher level in transfected COS cells than it is normally expressed in HC11 cells. Prolactin induces the rapid activation of Stat5 without the requirement of ongoing protein synthesis. A tyrosine-specific protein kinase is essential in this rapid activation. Jak2 can activate Stat5 DNA binding *in vitro*. Phosphorylation on Tyr694 is necessary for Stat5 DNA binding activity and is induced by prolactin. Transactivation of a β-casein gene promoter construct by prolactin is mediated through Stat5 binding sites.

Results

Prolactin rapidly induces the activation of DNA binding activity of Stat5 in transfected COS cells

Transfection experiments with cDNA expression plasmids encoding Stat5 and the prolactin receptor were carried out. COS cells expressing both genes were induced with prolactin. Nuclear extracts were prepared and bandshift experiments with a radioactive DNA probe containing a Stat5 binding site were performed (Figure 1A). The induction of Stat5 DNA binding activity was observed

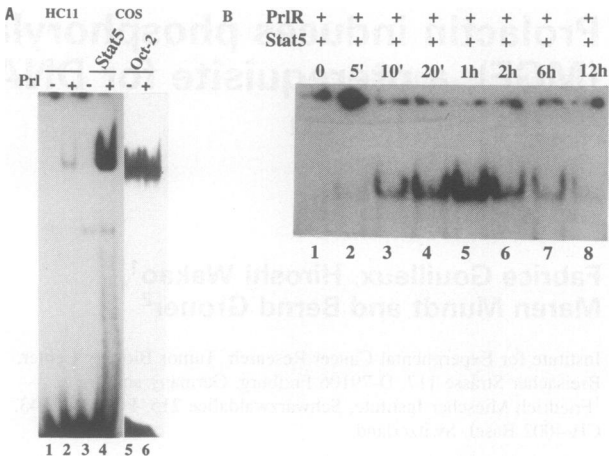

Fig. 1. Prolactin rapidly induces the activation of Stat5 DNA binding in transfected COS cells. (**A**) Nuclear extracts were prepared from HC11 (lanes 1 and 2), COS cells transfected with prolactin receptor cDNA and Stat5 cDNA (lanes 3 and 4) or Oct-2 cDNA (lanes 5 and 6). Cells were treated for 16 h with prolactin (lanes 2, 4 and 6) and nuclear extracts were analyzed in bandshift assays with a labeled DNA probe representing the bovine β-casein Stat5 binding site, as described in Materials and methods. (**B**) COS cells transfected with Stat5 cDNA and prolactin receptor cDNA were incubated with prolactin for the times indicated. Nuclear extracts were prepared and analyzed in bandshift assays with a Stat5-specific DNA probe.

(lane 4). The Stat5 DNA binding activity in transfected COS cells was compared with the endogenous Stat5 activity in HC11 mammary epithelial cells. Prolactin activates both endogenous (lane 2) and recombinant (lane 4) Stat5, and DNA–protein complexes of identical size were generated. However, the amount of activated MGF in the transfected COS cells is ~50-fold higher than in HC11 cells. No background of endogenous MGF was observed. This provides a favorable situation for the study of the molecular details of the activation process.

Another DNA binding protein, Oct-2, was used as a control and introduced into COS cells under the control of the same promoter element as the Stat5 cDNA. An Oct-2-specific DNA binding site was used as a probe to visualize the Oct-2 DNA complex (lanes 5 and 6). This complex is observed independently of the expression of the prolactin receptor and its activation. These experiments suggest that prolactin activation of Stat5 involves a post-translational event.

We determined the time course of Stat5 activation. Transfected COS cells were treated for increasing times with prolactin. Nuclear extracts were prepared and Stat5 activity was visualized by bandshift assays with a specific DNA probe (Figure 1B). Rapid induction was observed. Stat5 activity can be detected 5 min after prolactin addition (lane 2) and reaches a maximum after 1 h (lane 5). Longer exposure times result in a decrease of Stat5 DNA binding activity (lanes 6–8). These induction kinetics are similar to those observed with endogenous Stat5 in HC11 cells (Standke *et al.*, 1994).

Prolactin activation of Stat5 does not require ongoing protein synthesis and is specifically inhibited by the protein kinase inhibitors genistein and staurosporin

We analyzed the dependence of prolactin activation of Stat5 in transfected COS cells on ongoing protein synthesis

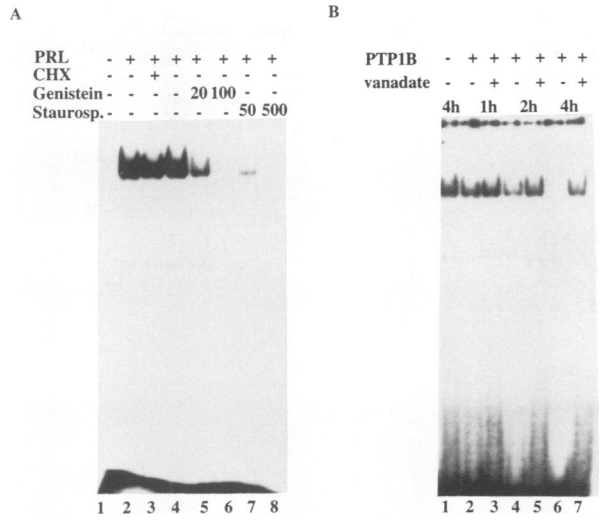

Fig. 2. (**A**) Prolactin activation of DNA binding of MGF does not require ongoing protein synthesis and involves tyrosine phosphorylation. COS cells transfected with MGF cDNA and prolactin receptor cDNA were induced with prolactin (lane 2). Cells were preincubated with cycloheximide for 1 h (lane 3) or with DMSO (lane 4), genistein (20 μg/ml, lane 5 and 100 μg/ml, lane 6) or staurosporine (50 nM, lane 7 and 500 nM, lane 8) for 30 min before prolactin addition. Nuclear extracts were analyzed in bandshift assays. (**B**) Tyrosine-specific protein phosphatase 1B (PTP1B) suppresses Stat5 cDNA binding activity. Nuclear extracts from COS cells transfected with Stat5 cDNA and prolactin receptor cDNA and treated for 1 h with prolactin were incubated with the PTP1B (lanes 2–7) in the absence (lanes 1, 2, 4 and 6) or presence (lanes 3, 5 and 7) of orthovanadate (100 μM) for the times indicated. Nuclear extracts were analyzed in bandshift assays.

(Figure 2A). Cellular protein synthesis was inhibited with cycloheximide for 1 h before prolactin induction. Nuclear extracts were prepared and analyzed for Stat5 DNA binding activity in bandshift assays. DNA binding activity was not affected by the inhibitor (lane 3), indicating that ongoing protein synthesis is not required.

Phosphorylation of Stat5 has been shown to be involved in the regulation of Stat5 DNA binding activity (Schmitt-Ney *et al.*, 1992). We evaluated the action of the kinase inhibitors genistein and staurosporin on prolactin induction (Figure 2A). Staurosporin inhibits protein kinase C and tyrosine kinases at high concentrations. Genistein is a specific tyrosine kinase inhibitor. Transfected COS cells were treated with 20 (lane 5) and 100 μg/ml (lane 6) genistein or 50 (lane 7) and 500 nM (lane 8) staurosporin for 30 min before the addition of prolactin. Both inhibitors prevent the prolactin-induced Stat5 activation and the effect is concentration-dependent. We conclude that the signaling pathway of prolactin, which causes Stat5 activation, involves the action of a tyrosine-specific protein kinase.

The sensitivity of Stat5 DNA binding activity towards a tyrosine-specific protein phosphatase was investigated (Figure 2B). Nuclear extracts from transfected COS cells induced by prolactin were incubated with the purified recombinant tyrosine phosphatase PTP1B for increasing times in the absence (lanes 1, 2, 4 and 6) or presence of orthovanadate (lanes 3, 5 and 7), a tyrosine phosphatase inhibitor. Prolonged treatment abolished Stat5 DNA binding activity (lane 6). Vanadate prevented the loss of Stat5 binding, confirming the specificity of the phosphatase towards phosphotyrosine (lane 7).

Prolactin induces tyrosine phosphorylation of Stat5 in transfected COS cells

We examined if prolactin induction of COS cells leads to an increase in the phosphotyrosine content of recombinant Stat5. To analyze Stat5 we provided it with an epitope tag (Munro and Pelham, 1984; Field *et al.*, 1988). A short peptide sequence, corresponding to an antigenic epitope of an influenza virus protein, was added to the N-terminus of Stat5. This method has been used previously in the investigation of transcription factors (Fu and Zhang, 1993). The prolactin activations of this epitope–Stat5 fusion protein (E–Stat5) and non-modified Stat5 in transfected COS cells were compared (Figure 3A). Nuclear extracts were prepared from cells cultured in the absence or presence of prolactin. Stat5 and E–Stat5 were visualized by bandshift assays. Both were activated by prolactin. Incubation of the DNA–protein complexes with a monoclonal antibody (12CA5) specific for the added epitope resulted in a decreased electrophoretic mobility of the E–Stat5 DNA complexes (supershift, lanes 7 and 8), but not of the Stat5 DNA complexes (lanes 3 and 4).

To evaluate if E–Stat5 is tyrosine phosphorylated we used the monoclonal antibody 12CA5 in immunoprecipitation experiments. The prolactin receptor (long form) expression vector and E–Stat5 cDNA were transfected into COS cells. Nuclear extracts were prepared from cells treated with and without prolactin, and E–Stat5 was precipitated with antibody 12CA5. The immunoprecipitates were resolved on SDS–PAGE, blotted onto filters and probed with the monoclonal anti-phosphotyrosine antibody 4G10 (Figure 3B). A band of ~100 kDa was observed in the precipitate of extracts from prolactin-stimulated COS cells (lane 4). This corresponds to the size of the epitope-tagged Stat5 fusion protein. This band did not appear in the absence of prolactin induction (lane 3).

Two forms of prolactin receptor have been cloned from different tissues and cell lines. The short form and the long form differ in the length of their cytoplasmic domains (Boutin *et al.*, 1988; Davis and Linzer, 1989; Edery *et al.*, 1989; Ali *et al.*, 1991) and seem to be involved in different signaling pathways (Lesueur *et al.*, 1991). The short form of the prolactin receptor is not able to confer the activation of Stat5. We investigated if the failure of the short form of the prolactin receptor to activate Stat5 is due to its inability to stimulate tyrosine phosphorylation. We transfected the cDNA encoding the E–Stat5 construct with the short form of the prolactin receptor into COS cells. Immunoprecipitation with the monoclonal antibody 12CA5 and analysis of the phosphotyrosine content with the anti-phosphotyrosine antibody 4G10 revealed that E–Stat5 was not phosphorylated in cells expressing the short form of the prolactin receptor (lane 6). Expression of E–Stat5 is similar in the COS cells transfected with the long and short forms of the prolactin receptor (Figure 3B, lower panel).

The results indicate that prolactin induces the phosphorylation of Stat5 on tyrosine via the long form of the receptor in transfected COS cells. To establish a correlation with the *in vivo* situation, the presence of phosphorylated

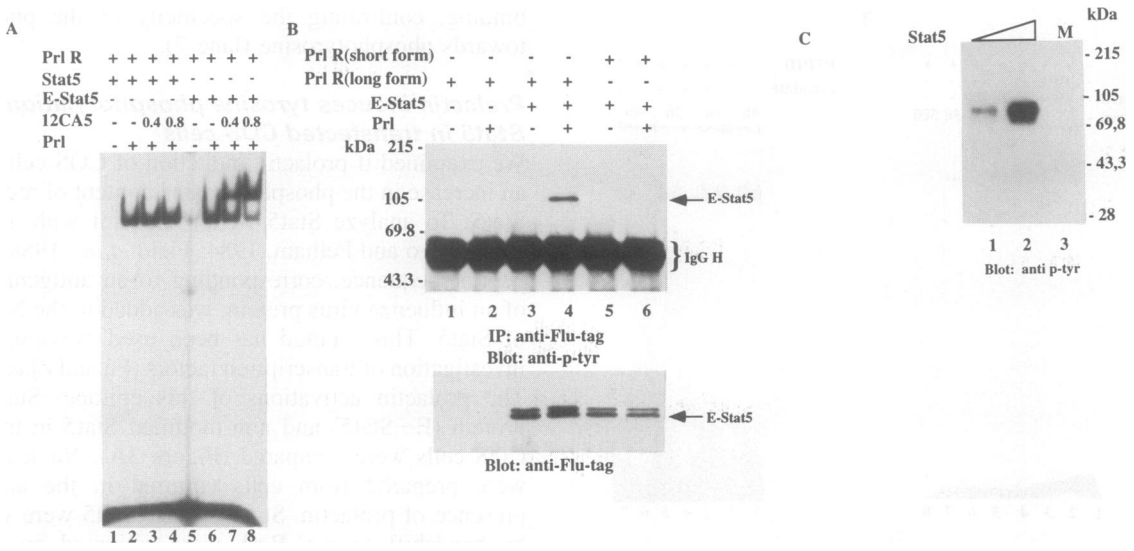

Fig. 3. Induction of tyrosine phosphorylation of recombinant Stat5 by prolactin in transfected COS cells. (**A**) Specificity of antibody recognition. COS cells were transfected with Stat5 cDNA and prolactin receptor cDNA (lanes 1–4) or an epitope-tagged Stat5 (E−Stat5) cDNA and prolactin receptor cDNA (lanes 5–8) and treated with prolactin (lanes 2–4 and 6–8). Nuclear extracts were prepared and analyzed in bandshift assays. Monoclonal antibody 12CA5, which recognizes the influenza virus epitope, was added to the binding reactions (0.4 μg, lanes 3 and 7; 0.8 μg, lanes 4 and 8). (**B**) COS cells were transfected with the vector expressing the long form (lanes 1–4) or the short form of the prolactin receptor (lanes 5 and 6) and Stat5 cDNA (lanes 3–6). Nuclear extracts were prepared before (lanes 1, 3 and 5) or after (lanes 2, 4 and 6) treatment with prolactin for 1 h. Immunoprecipitations were performed with the monoclonal antibody 12CA5 (anti-Flu-tag, E epitope). Immunoprecipitates were analyzed by Western blotting with the anti-phosphotyrosine antibody 4G10. IgG H, heavy chain of immunoglobulin G. (**C**) Stat5 from mammary gland tissue of lactating animals is tyrosine phosphorylated. Two concentrations of purified Stat5 (lanes 1 and 2) were immunoblotted with anti-phosphotyrosine antibody 4G10. Markers proteins were loaded in lane 3.

tyrosine residues in Stat5 purified from mammary tissue of lactating sheep by oligonucleotide affinity chromatography was analyzed. The monoclonal antibody directed against phosphotyrosine (4G10) strongly recognized Stat5 protein (Figure 3C, lanes 1 and 2).

In vitro phosphorylation of Stat5 with the protein tyrosine kinase Jak2 confers specific DNA binding activity

The cytoplasmic region of the prolactin receptor does not comprise a tyrosine kinase domain, but it is associated in the unliganded and prolactin-bound forms with the cytoplasmic tyrosine kinase Jak2 (Rui *et al.*, 1992). Ligand binding probably induces receptor homodimerization and tyrosine phosphorylation of Jak2 (Rui *et al.*, 1994). Because of the close association of Jak2 with the prolactin receptor, we tested if Stat5 can serve as a substrate for Jak2 *in vitro*. For this purpose Stat5 cDNA was transcribed *in vitro* from a promoter specific for the T7 RNA polymerase, and the mRNA was translated in a cell-free reticulocyte lysate. The resulting Stat5 protein has no specific DNA binding activity (Figure 4, lane 1). Specific DNA binding activity was observed when the *in vitro* transcribed and translated Stat5 was incubated with purified Jak2 in the presence of ATP (lane 2). Incubation with the tyrosine-specific protein kinases fyn, lyn or lck did not result in the activation of the DNA binding activity of Stat5 (lanes 4–6). This result suggests that the tyrosine-specific phosphorylation observed after activation of the long form of the prolactin receptor and required for the ability of Stat5 to bind to its DNA response element is effected by Jak2.

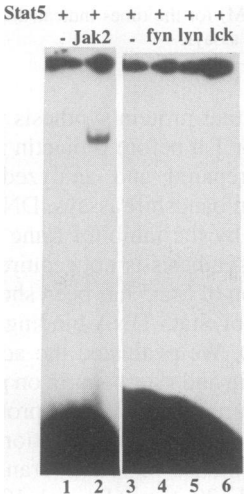

Fig. 4. *In vitro* phosphorylation with Jak2 results in Stat5 DNA binding activity. *In vitro*-translated Stat5 was incubated with the kinases Jak2, fyn, lyn and lck (lanes 2 and 4–6) in the presence of 10 μM ATP. Stat5 DNA binding activity was measured in bandshift experiments.

Transcriptional activation of the β-casein gene promoter by prolactin in COS cells is mediated by Stat5

The transcription of the β-casein gene promoter is under the control of the lactogenic hormones in HC11 mammary epithelial cells. The β-casein gene promoter, truncated at position −344, is sufficient to confer lactogenic hormone induction to a linked reporter gene in these cells (Doppler

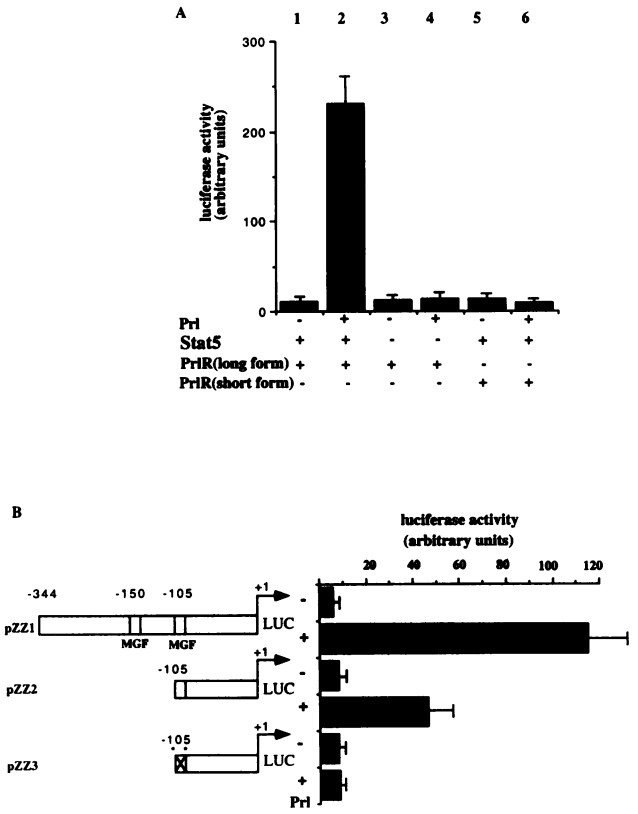

Fig. 5. Prolactin activation of β-casein gene transcription is mediated by Stat5. (**A**) COS cells were transfected with the (−344/−1) β-casein promoter luciferase construct (pZZ1), the long form (lanes 1–4) or the short form (lanes 5 and 6) of the prolactin receptor cDNA and Stat5 cDNA (lanes 1, 2, 5 and 6). One day after transfection, COS cells were incubated overnight with prolactin and cell extracts were prepared. Luciferase activities were determined. All transfections included plasmid pCH110. β-Galactosidase assays were used as a control for transfection efficiency. The values shown represent mean values of four independent experiments. (**B**) A single Stat5 binding site is sufficient for prolactin induction. COS cells were transfected with the (−344/−1) β-casein promoter construct (pZZ1), the (−105/−1) β-casein promoter construct (pZZ2) or the (−105/−1) β-casein promoter construct containing a point mutation in the Stat5 box (pZZ3). This mutation prevents the binding of Stat5. All transfections included cDNA vectors encoding prolactin receptor, Stat5 and pCH110. After overnight treatment with prolactin, luciferase activities were determined. The values represent the mean values of four independent experiments.

et al., 1989). We determined if Stat5 activated by prolactin in COS cells is able to transactivate the β-casein promoter. We used a construct comprising the β-casein promoter fragment (−344/−1) and the luciferase gene as a reporter (Figure 5B). This construct was transfected with a prolactin receptor expression vector into COS cells. Extracts were prepared from cells grown in the absence or presence of prolactin, and luciferase activity was determined (Figure 5A). In prolactin-treated cells (lane 2), luciferase activity was 20 times higher than in non-treated cells (lane 1). Transcriptional activation was not observed in the absence of Stat5 (lanes 3 and 4). We conclude from this experiment that the prolactin activation of Stat5 is essential for transcriptional induction of the β-casein gene promoter in COS cells. The short form of the prolactin receptor is not able to cause transactivation (lanes 5 and 6).

We tested additional β-casein promoter constructs (shown in Figure 5B) in transfection experiments in COS cells. These constructs contain a truncated β-casein promoter (−105 to −1, pZZ2) and a truncated promoter with a point mutation in the Stat5 binding site (pZZ3). Mutations in the Stat5 binding site, located between positions −105 and −85, have been shown previously to abolish the lactogenic hormone response of the β-casein promoter in HC11 cells (Schmitt-Ney *et al.*, 1992). The constructs were transfected in COS cells together with the prolactin receptor cDNA and Stat5 cDNA. Cell extracts were prepared from cells cultured in the absence or presence of prolactin. Deletion mutant pZZ2 (−105 to −1) was still prolactin-inducible, but only to 60% of the luciferase activity observed with the pZZ1 (−344 to −1) construct (Figure 5B). The point mutation in the Stat5 binding site of construct pZZ3 abolished the response of the promoter to prolactin. These results indicate that Stat5 binding to its recognition sequence in the β-casein promoter is essential for prolactin regulation.

Phosphorylation on Tyr694 is required for prolactin activation of Stat5

Five tyrosine residues are present in the C-terminal region of Stat5. Two tyrosines (Tyr665 and Tyr668) are part of the SH-2 domain of the molecule. Tyr682, 683 and 694 are present in a similar sequence context in Stat1α and Stat2 (H.Wakao, unpublished results). We investigated if these tyrosine residues are important for the prolactin response. Site-directed mutagenesis was employed. Single amino acid changes were introduced into E−Stat5. Tyr665 was changed into a glycine, and tyrosines 668, 682, 683 and 694 were changed into phenylalanines. The functional properties of these mutants were investigated.

Wild-type E−Stat5 and mutant E−Stat5 variants were transfected into COS cells with the prolactin receptor cDNA. Extracts of cells cultured in the absence or presence of prolactin were prepared. Bandshift assays were carried out (Figure 6A, upper panel). The prolactin activations of the DNA binding activity of the mutants 665, 668, 682 and 683 (lanes 3–10) were indistinguishable from the wild-type protein (lanes 1 and 2). No induction of DNA binding was observed with mutant 694 (lanes 11 and 12). Immunoprecipitations and blotting experiments with the antibody recognizing the influenza virus epitope (12CA5) were carried out on the same extracts to control the possible effect of the mutations on the level of protein expression (Figure 6A, middle panel). The results show that all mutants are expressed at the same level as the wild-type protein. Immunoblotting with the anti-phosphotyrosine antibody 4G10 was also carried out. No signal was detected for the mutant Tyr694 in COS cell extracts from prolactin-stimulated cells (Figure 6A, lower panel).

Mutant 694 failed to produce a prolactin-responsive DNA binding protein. The functional insufficiency of this mutant protein was confirmed when the transcriptional activation mediated by the mutants 668, 682 and 694 was investigated. Transfection of COS cells with the β-casein promoter construct, the prolactin receptor and the indi-

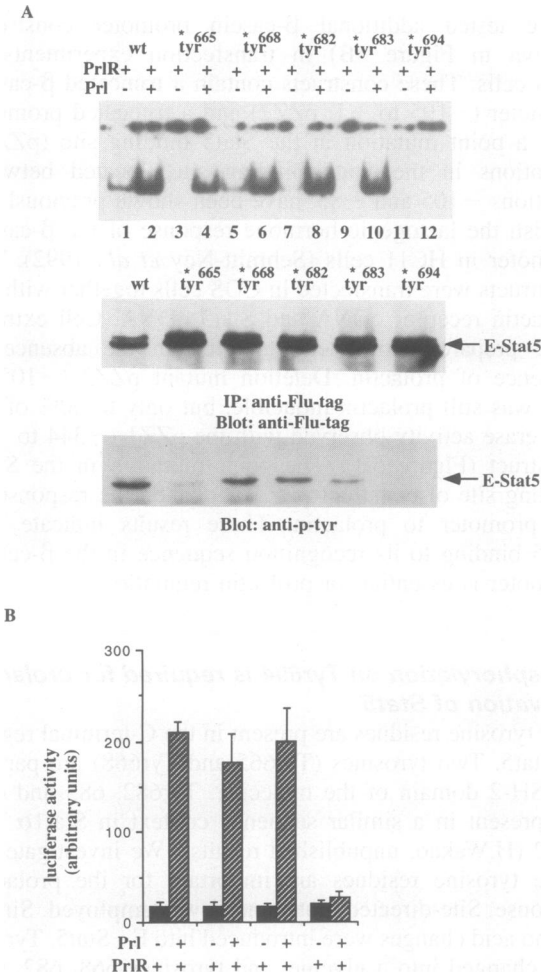

Fig. 6. Tyr694 is required for prolactin-dependent Stat5 activation. (**A**) COS cells were transfected with a cDNA vector encoding wild-type E−Stat5 (lanes 1 and 2) and mutants of E−Stat5 in which the tyrosines at the indicated positions have been replaced (lanes 3–12) together with the prolactin receptor. Cell extracts were prepared from cells not treated (lanes 1, 3, 5, 7, 9 and 11) or treated (lanes 2, 4, 6, 8, 10 and 12) with prolactin. Cell extracts were used in bandshift assays (upper panel) and for immunoprecipitation and immunoblotting experiments with the 12CA5 antibody (anti-Flu-tag, middle panel) or the anti-phosphotyrosine antibody 4G10 (lower panel). (**B**) Wild-type and mutant Stat5 constructs 668, 683 and 694 were transfected into COS cells together with the prolactin receptor and the β-casein promoter construct (pZZ1). Cell extracts of prolactin-treated and non-treated cells were prepared. Luciferase and β-galactosidase activities were determined. The values represent the mean values of four experiments.

vidual mutant genes showed that the mutant 694 is not able to mediate transcriptional activation of the β-casein promoter by prolactin (Figure 6B). The transactivation potential of mutants 668 and 682 is similar to the potential of the wild-type protein. These experiments show that phosphorylation on Tyr694 is absolutely required for prolactin-dependent activation of Stat5 DNA binding and induction of transcription.

Discussion

We used transfections of Stat5 and prolactin receptor cDNAs into COS cells to elucidate the mechanisms involved in prolactin activation of Stat5. Recombinant Stat5 in COS cells shares properties with endogenous Stat5 expressed in HC11 cells. Because Stat5 and the prolactin receptor can be expressed to much higher levels in COS cells, this experimental approach presents a favorable setting for the study of the molecular details of prolactin signaling.

Prolactin activation of Stat5 is rapid and requires the action of a protein tyrosine kinase

Stat5 is rapidly activated by prolactin and this activation is independent of protein synthesis inhibition by cycloheximide. Rapid activation has been also observed in HC11 cells with endogenous Stat5 upon prolactin stimulation (Standke *et al.*, 1994). A protein tyrosine kinase is involved in this activation since genistein, a protein tyrosine kinase inhibitor, prevents activation of Stat5 by prolactin. Prolactin receptor activation induces tyrosine phosphorylation of several cellular proteins, e.g. p120, p97 and p40, in rat T lymphoma cells, Nb-2 (Rui *et al.*, 1992). The protein tyrosine kinase Jak2 has been implicated recently in the signaling pathway of the prolactin receptor in Nb-2 cells and found to be associated with the prolactin receptor (Rui *et al.*, 1994). A similar time course of activation was observed for Stat1α upon interferon γ induction of Swiss 3T3 cells. Maximal levels of Jak2-mediated phosphorylation of Stat1α were found after 1 h (Silvennoinen *et al.*, 1993b).

Other protein kinases, like PKC, also seem to be important. Staurosporine, an inhibitor of PKC at low concentration (10 nM) and of other serine−threonine and tyrosine kinases at high concentration (500 nM), seems to be more potent than genistein in preventing Stat5 activation by prolactin. This implies that other kinases might be involved either directly or indirectly in Stat5 activation. Stat5 activity is increased after *in vitro* treatment of nuclear extracts with PKC or suppressed *in vivo* by a selective inhibitor of PKC in HC11 cells (Marte *et al.*, 1994; Marti *et al.*, 1994). Stat5 binding activity is sensitive to treatment with tyrosine phosphatase, but not to serine−threonine phosphatase (data not shown). These data suggest that PKC acts in an indirect manner, perhaps by inhibiting a tyrosine phosphatase. The role of a tyrosine phosphatase has been suggested recently as a negative regulator of GAF and ISGF3, factors activated by interferon γ and interferon α/β (David *et al.*, 1993; Igarashi *et al.*, 1993). Alternatively, PKC could activate the protein tyrosine kinase involved in Stat5 activation.

Tyrosine phosphorylation required for Stat5 DNA binding activity is induced by prolactin and effected by Jak2

Prolactin activation of Stat5 DNA binding is the result of the prolactin-dependent tyrosine phosphorylation of Stat5. Tyrosine phosphorylation of transcription factors has been described in the signaling pathways of different cytokines: interferon γ, interferon α/β, IL-6, IL-4, IL-3, IL-10 and GM-CSF (Schindler *et al.*, 1992a,b; Igarashi *et al.*, 1993; Kotanides and Reich, 1993; Larner *et al.*, 1993; Wegenka *et al.*, 1993). Our observation that Jak2 can phosphorylate Stat5 *in vitro* and thus confer specific DNA binding activity makes it very likely that this kinase also mediates Stat5 activation *in vivo*. Cell-free *in vitro* activation of DNA binding proteins has been reported for ISGF3 by interferon α (David *et al.*, 1993) and a protein which

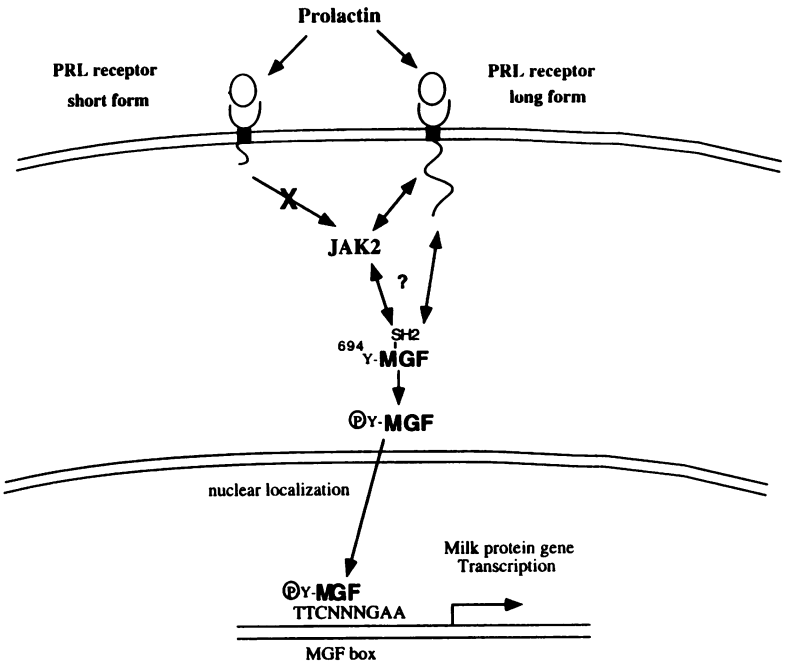

Fig. 7. Model for the activation of Stat5 (MGF) by the prolactin receptor. Binding of prolactin to its plasma membrane receptor (long form) activates the tyrosine-specific protein kinase Jak2. This kinase catalyzes the tyrosine phosphorylation of Stat5. Stat5 then translocates into the nucleus where it binds to the DNA sequence of the Stat5 box and activates transcription of milk protein genes. Binding of prolactin to the short form of the receptor does not activate Jak2. Double arrows represent possible interactions between Stat5 through its SH2 or SH3 domains with Jak2 and the prolactin receptor.

binds to the SIE (c-sis inducible element) in the c-*fos* promoter and which is tyrosine phosphorylated through the activity of the EGFR (Sadowski and Gilman, 1993).

Tyrosine phosphorylation of GAF or Stat1α is sufficient for nuclear translocation, DNA binding and gene activation. This involves a single tyrosine residue, Tyr701 (Shuai *et al.*, 1993a,b). Tyr694 of Stat5 is required for its DNA binding activation by prolactin. Stat5 shares homologies with Stat1α and Stat2, the components of the ISGF3 complex. There is an overall sequence identity of ~30% (H.Wakao, unpublished results). Sequence alignments show that Tyr694 of Stat5 occurs in a similar sequence context to Tyr701 of Stat1α. This suggests that Tyr694 and 701 could be the sites of phosphorylation by a common protein tyrosine kinase, most likely Jak2.

Transcriptional activation of the β-casein promoter by prolactin is mediated by Stat5 and requires the long form of the prolactin receptor

Transcriptional activation by interferon γ and IL-6 of responsive genes involves the tyrosine phosphorylation of the GAF and APRF transcription factors (Wegenka *et al.*, 1993; Akira *et al.*, 1994; Pine *et al.*, 1994). Transcriptional activation of the β-casein promoter in COS cells by prolactin is mediated by Stat5. Deletion or mutation of the MGF binding sites abolishes prolactin induction of the promoter. Only 105 bp of the promoter sequence are required to observe the prolactin response. This sequence contains a single Stat5 binding site. A delimitation analysis of the β-casein gene promoter in HC11 cells has shown previously that at least 180 nucleotides of the promoter region are required for lactogenic hormone inducibility in mammary epithelial cells (Altiok and Groner, 1993). This 180 nucleotide promoter fragment comprises two MGF binding sites in addition to further positively acting promoter elements. HC11 cells express very low amounts of active Stat5 (Schmitt-Ney *et al.*, 1991; Standke *et al.*, 1994). It is therefore possible that high expression of Stat5 and the prolactin receptor can render cells more sensitive to prolactin and override subtle regulatory features observed in the HC11 cells (Altiok and Groner, 1993; Meier and Groner, 1994).

The long form but not the short form of the prolactin receptor is able to confer the prolactin response to the β-casein gene promoter in COS cells. Similar results were obtained with the β-lactoglobulin gene promoter in CHO cells (Lesueur *et al.*, 1991). A Stat5 binding site is also present in this promoter. Our results clearly show that prolactin failed to activate Stat5 via the short form of the prolactin receptor. The short and long forms of the prolactin receptor bind prolactin with equal affinity. However, they differ in the length of their cytoplasmic domains. The cytoplasmic domains of cytokine receptors are crucial for signal transduction upon ligand binding (Murakami *et al.*, 1991; Harada *et al.*, 1992; Miura *et al.*, 1993). Different reports have shown that the cytoplasmic domain of the EPO receptor, gp130 and the GH receptor interact with and/or activate protein tyrosine kinases after ligand binding (Argetsinger *et al.*, 1993; Witthuhn *et al.*, 1993; Stahl *et al.*, 1994). The critical region required for both signal transduction and tyrosine kinase interaction has been identified in the cytoplasmic domain of EPO receptor (Witthuhn *et al.*, 1993). Since Stat5 is not tyrosine phosphorylated after prolactin stimulation via the short form of the prolactin receptor, it is reasonable to assume that the short form lacks the critical region needed for the interaction with Jak2.

A model for prolactin signaling and Stat5 activation

Prolactin shares features of transcription factor activation with interferon γ, interferon α/β, IL-6, IL-4, IL-3 and GM-CSF which has been reviewed recently by Darnell *et al.* (1994). In this review a nomenclature for the transcription factors of the Jak−Stat pathways has been suggested. Upon prolactin addition, Stat5 is rapidly phosphorylated on tyrosine. It translocates into the nucleus where it binds to specific DNA sequence and activates transcription of specific genes. This is shown schematically in Figure 7. It is possible that the tyrosine phosphorylation results in the ability of the protein to form homodimers via the SH2 phosphotyrosyl interactions, as has been suggested for Stat1α (Shuai *et al.*, 1994), and that only the dimers are able to bind to DNA. Early events in Stat5 activation have yet to be determined. The protein tyrosine kinases involved in EPO, GH, interferon γ, interferon α/β, IL-6 and IL-3 signaling belong to the Jak−Tyk family of protein tyrosine kinases (Velazquez *et al.*, 1992; Argetsinger *et al.*, 1993; Müller *et al.*, 1993; Silvennoinen *et al.*, 1993a,b; Watling *et al.*, 1993; Witthuhn *et al.*, 1993; Stahl *et al.*, 1994). Jak2 is involved in the prolactin signaling pathway and is most likely responsible for Stat5 activation *in vivo*. Stat5 contains SH2 and SH3 domains like Stat1α and Stat2 (Fu, 1992; H.Wakao *et al.*, manuscript submitted). It is possible that Stat5 associates with other proteins and possibly the protein tyrosine kinase Jak2 or the prolactin receptor via its SH2 and SH3 domains. It has been shown recently that APRF associates with gp130, the signal-transducing chain of the IL-6 receptor (Lütticken *et al.*, 1994). Mutagenesis of the SH2 domain will allow us to elucidate the exact mechanisms involved in the early steps of prolactin signal transduction.

Materials and methods

Cell culture

COS7 cells were maintained in DMEM containing 10% FCS, 2 mM glutamine and 50 μg/ml gentamycin. HC11 cells were grown in RPMI-1640 medium containing 10% FCS, 5 μg/ml bovine insulin, 10 μg/ml murine EGF, 50 μg/ml gentamycin and 2 mM glutamine. HC11 cells were maintained for 3 days at confluency in this medium before prolactin addition. COS and HC11 cells were induced with 5 μg/ml ovine prolactin. In the protein synthesis inhibition experiments, COS cells were cultured with 10 μg/ml of cycloheximide (Sigma) for 1 h prior to the addition of prolactin. In the kinase inhibition experiments, COS cells were cultured with 50 and 500 nM of staurosporine (UBI) and 20 and 100 μg/ml of genistein (UBI) for 30 min prior to the addition of prolactin.

Plasmids and DNA transfections

pXM-Stat5, pXM-Oct2 and the two forms of the prolactin receptor-expressing constructs have been described (Wakao *et al.*, 1994). pE-Stat5 is a plasmid into which an influenza virus epitope which is recognized by the monoclonal antibody 12CA5 has been inserted. A double-stranded oligonucleotide containing the epitope sequence from the influenza virus, a Kozak consensus sequence and the cloning sites *Sal*I and *Xho*I (5′-TCGACGAATTCCACCATGTACCCCTACGACGT-CCCCGACTACGCCTCGA-3′), was inserted into the *Sal*I site of plasmid p10.3.D, a 5′ deletion construct of Stat5. The resulting construct contained the entire Stat5 coding sequence except the first Met initiation codon.

pZZ1 was constructed by inserting a *Nde*I−*Xho*I DNA fragment containing the β-casein promoter from −344 to −1 in front of the luciferase gene of the pLucDSS vector (Gouilleux *et al.*, 1991). pZZ2 contains the *Nde*I−*Xho*I fragment of the β-casein promoter truncated at −105. The mutated β-casein promoter fragment (−105/−1) was obtained by PCR. The 5′ primer served to add a *Bam*HI site and to create the mutation in the MGF box, and the 3′ primer served to add a *Xho*I site to the 3′ end. The *Bam*HI−*Xho*I fragment of pZZ2 has been replaced by the mutated one to create pZZ3.

Mutants of Stat5 in tyrosine residues were made by using the *in vitro*-directed mutagenesis kit from Promega. All mutations were verified by DNA sequencing. Tyr665 was changed into a glycine by alteration of the codon TAT into GGT; Tyr668, 682, 683 and 694 were changed to phenylalanine by alteration of the codons TAC into TTC.

Transfection experiments were performed by the calcium phosphate precipitation technique. Five micrograms of pXM-MGF, pXME-MGF or pXM-Oct-2 and 5 μg of the prolactin receptor expression vector were used for the transfections. In addition, 0.5 μg of the plasmid pCH110, encoding the β-galactosidase gene under the control of the SV40 promoter, were included in each transfection as an internal control for the transfection efficiency. In the case of the reporter gene assays, 2 μg of the β-casein promoter constructs were used. The DNA was adjusted to 20 μg with salmon sperm DNA.

Luciferase and β-galactosidase assays

Luciferase assays were carried out as described (Gouilleux *et al.*, 1991). β-Galactosidase assays were performed with the kit from Tropix Co. Cells were harvested, washed twice in PBS and lysed in 25 mM glycylglycine pH 7.8, 1 mM DTT, 15% glycerol, 8 mM $MgSO_4$, 1 mM EDTA and 1% Triton for 5 min at 4°C and centrifuged for 5 min at 14 000 r.p.m. Supernatants were used for the β-galactosidase assays. Two hundred microlitres of reaction buffer containing 100 mM K-phosphate pH 8.0, 1 mM $MgCl_2$ and 0.035 mM AMPGD were added to the extracts (5–20 μl) for 30 min at room temperature to keep the signals within the linear range. Measurements were made by injecting 300 μl of accelerator solution (10% Emerald luminescent amplifier and 0.2 N NaOH) and then counting for 10 s in the Luminometer 953 (Berthold). Luciferase assays were performed in the same apparatus. Luciferase activities and the amounts of nuclear extract used in bandshift experiments were correcting for the β-galactosidase values obtained in each experiment.

Preparation of nuclear extracts and electrophoretic mobility shift assays

Nuclear and cytosolic extracts were prepared as described (Standke *et al.*, 1994) with the following modifications. Nuclei were extracted with a hypertonic buffer containing 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 0.1% Triton, 20% glycerol, 1 mM DTT, 0.2 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin for 20 min at 4°C. The extracts were centrifuged at 14 000 r.p.m. for 5 min. Extracts were frozen in liquid nitrogen and stored at −70°C. Cytosolic and nuclear extracts were used for bandshift experiments or immunoprecipitations. The protocol for electrophoretic mobility shift assays has been described (Wakao *et al.*, 1992). In all bandshift experiments the Stat5 binding site of the bovine β-casein promoter was used as a probe (5′-AGATTTCTA-GGAATTCAAATC-3′).

This oligonucleotide was end-labeled with polynucleotide kinase to a specific activity of 8000 c.p.m./fmol.

Phosphatase treatment of Stat5

Purified Stat5 (Wakao *et al.*, 1994) or nuclear extracts from transfected COS cells was incubated at room temperature with the recombinant glutathione-*S*-transferase−PTP-1B fusion protein containing the entire 76 kDa human PTP-1B (UBI). 0.2 U of PTP-1B−agarose bead conjugate were used per reaction. After a brief centrifugation, supernatants containing Stat5 were used in bandshift experiments.

In vitro kinase reactions

Stat5 was synthesized in an *in vitro* transcription and translation reaction as described by Wakao *et al.* (1994). The *in vitro*-translated product of Stat5 was used for the *in vitro* kinase assay. Purified Jak2, fyn, lyn and lck were purchased from UBI. *In vitro* kinase assays were performed following the recommendations of the supplier. Reactions were performed for 1 h at room temperature in a buffer containing 10 mM HEPES, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 100 μM Na_3VO_4 and 10 μM ATP. After the reaction a bandshift assay was carried out.

Immunoprecipitation, antibodies and Western blotting analysis

For immunoprecipitations, cell extracts were incubated with 3 μl of ascites fluid containing the monoclonal antibody 12CA5 raised against the influenza virus epitope (Babco) for 3 h at 4°C. Immunoprecipitates were isolated with protein A−Sepharose with careful washes. Proteins in the immunoprecipitates were resolved on 8% SDS−polyacrylamide

gels, transferred to polyvinylidene difluoride membrane (Amicon) and incubated with the monoclonal antibody directed against phosphotyrosine (4G10, UBI). Immunoreactive bands were visualized using an epichemiluminescence (ECL) Western blotting system (Amersham) according to the manufacturer's protocol.

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