Growth Hormone Promotes the Association of Transcription Factor STAT5 with the Growth Hormone Receptor*

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Members of the cytokine/growth hormone (GH)/prolactin receptor superfamily transduce signals by association and activation of Jak tyrosine kinases. For GH receptor (GHR), both Jak2 and the GHR undergo tyrosine phosphorylation upon GH stimulation. Also, GH has recently been shown to activate the transcription factor STAT5 by tyrosine phosphorylation. In the present study, we demonstrate that GH induces rapid tyrosine phosphorylation of different isoforms of STAT5 in mouse L cells by transiently transfected with a cdna encoding porcine GHR (pGHR). In this cell system, STAT5 directly interacts with the GHR in a GH-dependent manner. Additionally, GH-induced tyrosine phosphorylation of STAT5 and the interaction of STAT5 with GHR can be observed in mouse 3T3-F442A cells which express endogenous mouse GHR. Interestingly, when cdnas encoding the two mouse STAT5 homologs (STAT5A and STAT5B) were individually transfected into mouse L cells expressing pGHR, only STAT5A demonstrated the ability to interact with the pGHR and subsequently underwent GH-dependent tyrosine phosphorylation. STAT5B did not. Therefore, the GH-dependent interaction of a particular STAT5 with tyrosine-phosphorylated GHR may play an important role in GH-mediated signal transduction.

The GH receptor (GHR) is a member of the cytokine/GH/prolactin receptor superfamily whose members share homology in the extracellular ligand binding domain (1–5). Members of the superfamily also share a proline-rich motif (Box-1) in the cytoplasmic domain (6, 7). GH binding to GHR has been shown to induce tyrosine phosphorylation and activation of Jak2 tyrosine kinase and tyrosine phosphorylation of GHR itself (8–13). GH also induces tyrosine phosphorylation of a number of cellular proteins which are known as signal transducers and activators of transcription (STATs), including STAT1, STAT3, and STAT5 (14–17). These findings suggest that activation of Jak-STAT pathways in response to GH plays an important role in the GH signaling system which leads to GH-induced biological responses.

STAT5 was originally purified and cloned from mammary epithelial cells in sheep and identified as a signal transducer that confers the specific biological responses of prolactin (18–20). It can bind to the promoter sequence of the β-casein gene and regulate its expression in response to prolactin. Amino acid sequence analyses revealed that STAT5 shares amino acid sequences with other members in the STAT family and contains a SH2 and a SH3 domain (19). Two mouse STAT5 homologs have been identified (STAT5A and STAT5B) (21). The two proteins share 96% identity at the amino acid level. Recently, ligand binding to a number of the receptors of the cytokine receptor superfamily have been shown to stimulate the tyrosine phosphorylation of STAT5. These include receptors for interleukin (IL)-2, IL-3, IL-5, IL-6, erythropoietin (EPO), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), granulocyte-macrophage colony-stimulating factor, prolactin, and GH (21–27). Moreover, although both EPO and IL-2 can activate STAT5, EPO activates Jak2, whereas IL-2 activates Jak1 and Jak3 (22). Therefore, STAT5 can be activated through different signal transduction pathways by different receptors (22).

It has been suggested that the specific interactions between Jak5, STAT5, and transmembrane receptors contribute to the activation of distinct signaling pathways in response to different ligands (28). Jak kinases are constitutively associated with the Box-1 region of the cytokine receptor intracellular domain and become activated upon ligand-induced receptor dimerization (29–32). Several STAT members have been shown to be able to associate with the intracellular domains of receptors. STAT1α associates with tyrosine-phosphorylated interferon-γ receptor and epidermal growth factor receptor in a ligand-dependent manner (33, 34). STAT1α interacts with epidermal growth factor receptor through its SH2 domain (34). Interferon-α induces STAT2 association with the β2 subunit of the type I interferon receptor (35). STAT3 interacts with the β subunits for receptors for IL-6, CNTF, LIF, and OSM (gp130 and LIFR β), and a specific tyrosine-based motif in the receptor β subunit that confers the STAT3 association has been identified (36). Certain phosphotyrosine-containing peptides derived from the intracellular domain of the IL-4 receptor inhibit the activation of STAT6 (IL-4 STAT), suggesting a direct interaction of STAT6 with the IL-4R (37). Thus, it has been proposed that cytokine receptor cytoplasmic domains contain a docking site for selective binding of STATs, allowing them to be phosphorylated and activated by Jak5. Although STAT5 can be activated by a number of cytokines, no direct interaction of STAT5 with cytokine receptors has been reported. It has also been speculated that STAT5 may interact with transmembrane re-
ceptors before being phosphorylated in response to ligand binding (27). In the present study, we demonstrate that STAT5 can associate with GHR in a GH-dependent manner.

**EXPERIMENTAL PROCEDURES**

Materials—Mouse L cells that express porcine GHR (pGHR), designated L-pGHR cells, were established previously in our laboratory (42). Dulbecco’s modified Eagle’s medium (DMEM) and Nu-serum were purchased from Life Technologies, Inc. pGHR was a generous gift from Qi Shun-zhang (Beijing Agricultural University, Beijing, China). Protein A-agarose was purchased from Life Technologies, Inc. Protein molecular weight markers, Hybond-ECL nitrocellulose membranes, and ECL developing reagents were purchased from Amersham Corp. Horseradish peroxidase-conjugated phosphotyrosine antibody, PY-20, was purchased from ICN (Irvine, CA). Mouse STAT5 monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). STAT5 antibody was generously provided by Drs. G. Peter Frick (Lexington, KY) and Maurice Goodman of the University of Massachusetts Medical School (Worcester, MA). Monoclonal GHR antiserum was generously provided by Drs. G. Peter Frick and Dr. Donald I. Triess. (Lexington, KY).

The resulting DNA fragments from PCR were cloned using the EcoRI restriction enzyme digestion site (underlined sequences) were inserted into the downstream primers. Two STAT5 protein bands were sequenced and compared with the published sequences. In order to introduce a FLAG sequence to the N terminus of STAT5A and STAT5B, pMet-STAT5A-FLAG plasmid was used to replace the EcoRI fragment from the parental plasmids. The resulting plasmid pMet-STAT5A-FLAG is shown in Fig. 1.

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**Cell Culture and Transfection—**Mouse L cells were grown to confluence in T-75 flasks in DMEM containing 4.5 g/l glucose and 10% serum in an atmosphere of 5% CO2, 95% air. Before GH treatment, cells were incubated in DMEM minus serum for 2 h. pGHR was then added directly to the cells in serum-free medium. Cells were incubated at 37°C before cell lysis was performed. Establishment of stable cell lines which express STAT5A-FLAG and STAT5B-FLAG was performed using Lipo-fectin (Life Technologies, Inc.). In brief, mouse L cells were co-transfected with pMet-STAT5A or pMet-STAT5B plasmid and pRSV-neo plasmid (43). The transfected cells were cultured with DMEM supplemented with 10% Nu-serum and 400 µg/ml G418. Pools of G418-resistant cells were propagated for subsequent experiments.

**RESULTS**

**GH Induces Tyrosine Phosphorylation of Isoforms of STAT5—**Previously, we had demonstrated that GH can induce rapid and transient tyrosine phosphorylation of a group of proteins with the apparent molecular mass of approximately 95 kDa in mouse L cells stably expressing porcine receptors (L-pGHR) or mouse 3T3-F442A cells (38, 39). These proteins are termed pp93 and pp95/96. In this study, we examined whether these proteins could be recognized by antiseraum against STAT5. L-pGHR cells were treated with 500 ng/ml pGH for 5 min. Cell lysates from GH-treated and untreated L-pGHR cells were separated on a 4–12% gradient SDS-PAGE and subjected to Western blot analyses using antiseraum against STAT5 or phosphotyrosine (Fig. 2, panel A). Two STAT5 protein bands were observed. The STAT5 antibody can bind to both STAT5A and STAT5B proteins. Polyclonal GHR antiserum was generously provided by Drs. G. Peter Frick and Maurice Goodman of the University of Massachusetts Medical School (Worcester, MA). Monoclonal FLAG antibody was purchased from Transduction Laboratories (Lexington, KY). STAT5 antibody can bind to both STAT5A and STAT5B proteins. Poly-

**Fig. 1. Construction of pMet-STAT5A-FLAG plasmid expressed in mouse L cells.** PCR-cloned STAT5 DNA was ligated into an expression vector at the XhoI and EcoRI sites. The resulting plasmid, pMet-STAT5A-FLAG, contains metallothionein I promoter and bovine GH poly(A) signal. The FLAG sequence was inserted before the translation stop codon of the STAT5 A DNA by a second PCR which amplified the XhoI-EcoRI fragment from STAT5A DNA. Plasmid pMet-STAT5B-FLAG was constructed using the same strategy. MET-I, metallothionein I transcriptional regulatory sequence; Ori, replication origin; Amp, ampicillin resistance gene; I1G, M13 intergenic region; Poly A, bovine GH poly(A) addition signal.

**TA cloning kit (Invitrogen, San Diego, CA). The 2.3-kilobase pair**

**STAT5A and STAT5B DNA fragments were isolated by digestion with restriction enzymes XbaI and EcoRI and subcloned into an expression vector (Fig. 1). The resulting plasmids, pMet-STAT5A and pMet-STAT5B, contain the mouse metallothionein I promoter sequence which directs transcription. The cloned STAT5A and STAT5B DNA fragments were sequenced and compared with the published sequences. In order to introduce a FLAG sequence to the C terminus of STAT5A and STAT5B, PCR fragments were used to amplify the XhoI and EcoRI fragments from pMet-STAT5A plasmids. The DNA sequence encoding the FLAG peptide (DYKDDDDK) was introduced into the downstream PCR primers directly upstream of the translational stop codon. The downstream primers also contain an EcoRI site to facilitate the subcloning of the PCR fragments. The resulting PCR fragments with the FLAG sequence insertion were digested with restriction enzymes XhoI and EcoRI and used to replace the XhoI-EcoRI fragment from the parental plasmids. The resulting plasmid pMet-STAT5A-FLAG is shown in Fig. 1.
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with apparent molecular masses of 89 and 95 kDa could be detected in control L-pGHR cells (Fig. 2, panel A, lane 1). Upon GH treatment, a third STAT5 band with an apparent molecular mass of approximately 95 kDa (Fig. 2, lane 1) appeared accompanied with a decrease in intensity of the 89-kDa STAT5 band (Fig. 2, panel A, lane 2). When probed with phosphotyrosine antiserum, the pp93 and pp95/96 could be observed only in GH-treated L-pGHR cells. The migration of pp93 and pp95/96 correlated with the 93- and 95-kDa STAT5 bands observed (Fig. 2, panel A, lane 2). Prolonged treatment with GH resulted in complete disappearance of the 89- and 93-kDa STAT5 bands (data not shown). The time course of the appearance of the 93-kDa STAT5 band correlated with that of pp93 (data not shown), suggesting that the pp93 observed in GH-treated L-pGHR cells is the 93-kDa STAT5 protein. Therefore, two isoforms of STAT5 protein are present in mouse L cells, and both of them can undergo GH-induced tyrosine phosphorylation. GH can induce an increase in apparent molecular mass of the 89-kDa STAT5 protein which may be due to phosphorylation of tyrosine residues.

In order to confirm that the tyrosine-phosphorylated proteins observed in L-pGHR cells are antigenically related with STAT5, immunoprecipitation using STAT5 antiserum was performed. L-pGHR cells were treated with 500 ng/ml pGH for 10 min, and the cell lysates were immunoprecipitated with STAT5 antiserum. The resulting immune complexes from both GH-treated and untreated cells were subjected to Western blot analyses using STAT5 antiserum. The resulting immune complexes were subjected to Western blot analyses using STAT5 (lanes 1 and 2) and phosphotyrosine (lanes 3 and 4) antiserum. Arrows on the right side of the figure indicate the migration of pp93 and pp95/96 (Fig. 2). Upon GH treatment, GHR phosphorylation increased dramatically in the L-pGHR cells (Fig. 3, panel A, lane 3). Upon GH treatment, GH-induced tyrosine phosphorylation of pp93 and pp95/96 could be observed only in GH-treated L-pGHR cells. Neither tyrosine-phosphorylated GHR nor STAT5 signal was observed in parental L cells (Fig. 3, lanes 1 and 2).

GH-induced tyrosine phosphorylation of pp93 and pp95/96 was also observed in mouse 3T3-F442A cells which possess endogenous mouse GHR (mGHR) and can undergo GH-dependent differentiation to adipocytes. 3T3-F442A cells were treated with 500 ng/ml pGH for 10 min. Cell lysates were prepared with non-denaturing conditions and subjected to immunoprecipitation using GH antiserum as described above. Cell lysates and immunoprecipitates were subjected to Western blot analyses (Fig. 4). In the cell lysates (Fig. 4, lanes 1 and 2), GH-induced tyrosine phosphorylation of cellular proteins (Fig. 4, panel A) and an up-shifted 89-kDa STAT5 band (Fig. 4, panel B) were observed, similar to that observed in L-pGHR cells. GH-induced tyrosine phosphorylation of mGHR was observed in GH-treated 3T3-F442A cells (Fig. 4, panel A, lanes 3 and 4). Longer exposure of the same blot revealed that tyrosine-phosphorylated STAT5 was co-precipitated with mGHR (Fig. 4, panel A, lanes 5 and 6). The identity of STAT5 was confirmed by blotting with STAT5 an-
Fig. 4. STAT5 can be co-precipitated with mouse GHR in 3T3-F442A cells. GH-treated and untreated 3T3-F442A cells were lysed under non-denaturing conditions and IPs with GHR antisera were performed. The cell lysates (lanes 1 and 2) and GHR immunoprecipitates (lanes 3 and 4) were resolved on a 4–12% SDS-PAGE and subjected to Western blotting using phosphotyrosine (panel A) and STAT5 (panel B) antisera. Lanes 5 and 6 represent a longer exposure of lanes 3 and 4 of the phosphotyrosine blot. GH treatments are indicated on the top of the panels. Antisera used for Western blotting are indicated on the left of the panels. Arrows on the right represent the migration of tyrosine-phosphorylated GHR and co-precipitated STAT5 present in GH-treated 3T3-F442A cells.

Fig. 5. Expression of STAT5A-FLAG and STAT5B-FLAG in L-pGHR cells. Cell lysates from L-pGHR cells and pools of STAT5A-FLAG- and STAT5B-FLAG-transfected cells were subjected to Western blot analyses using FLAG (panel A), STAT5 (panel B), and phosphotyrosine (panel C) antisera. Cell types and GH treatments are indicated on the top of the panels. Antisera used for Western blotting are indicated on the left of the panels. Arrows on the right indicate the migration of STAT5A-FLAG and STAT5B-FLAG proteins.

Fig. 6. STAT5A-FLAG but not STAT5B-FLAG can be co-precipitated with GHR. Cell lysates were prepared from L-pGHR cells and pools of STAT5A-FLAG- and STAT5B-FLAG-transfected cells under the non-denaturing conditions. IPs with GHR antisera were performed, and the resulting immune complexes analyzed by Western blotting using phosphotyrosine (panel A and C) and FLAG (panel B) antisera. Cell types and GH treatments are indicated on the top of the panels. Antisera used for IP and Western blotting are indicated on the left of the panels. Arrows on the right represent the migration of tyrosine-phosphorylated GHR and STAT5A-FLAG present in the GHR immune complexes.

GHR immunoprecipitate from GH-treated STAT5A-FLAG-transfected cells than that in the GH-treated L-pGHR cells (Fig. 6, panel A, lane 4). When the same blot was probed with FLAG antisera, the FLAG signal was observed only in the GHR immunoprecipitates from STAT5A-FLAG-transfected cells (Fig. 6, panel B, lane 4), suggesting STAT5A, but not STAT5B, can undergo GH-dependent association with the pGHR. To confirm that the STAT5B-FLAG proteins expressed did not undergo GH-induced tyrosine phosphorylation, immunoprecipitation was performed using FLAG antisera. The FLAG immunoprecipitates were subjected to Western blot analyses using phosphotyrosine antisera. Only STAT5A-FLAG was found to be phosphorylated in response to GH treatment (Fig. 6, panel C, lane 4). Western blot using FLAG anti-
serum revealed that both STAT5A-FLAG and STAT5B-FLAG were successfully immunoprecipitated (data not shown). Therefore, STAT5B not only failed to associate with pGHR upon GH treatment, but also failed to undergo tyrosine phosphorylation, suggesting a strong correlation between GHR association and tyrosine phosphorylation of STAT5A.

DISCUSSION

Previously in our laboratory, three intracellular proteins, pp93 and pp95/96, have been revealed to undergo GH-induced tyrosine phosphorylation in GH-treated mouse L cells stably expressing pGHR (L-pGHR cells) (38–40). In the present study, we report that these proteins are tyrosine-phosphorylated STAT5 proteins based on the following. (i) STAT5 protein is present as two isoforms with molecular masses of 89 and 95 kDa, respectively, in untreated L-pGHR cells. Upon GH treatment, the 89-kDa STAT5 protein band becomes weaker, and a third STAT5 protein with a molecular mass of 93 kDa becomes evident (Fig. 2, panel A). GH treatment of L-pGHR cells for longer than 30 min results in a diminishing of both 89- and 93-kDa STAT5 bands and an appearance of a stronger 95/96-kDa STAT5 band. The 93-kDa STAT5 band co-migrates with the pp93 observed in GH-treated cells and the 95-kDa STAT5 protein doublet also co-migrates with pp95/96 (Fig. 2, panel A). (ii) The time course of pp93 phosphorylation and the appearance of 93-kDa STAT5 are correlated, i.e. both peak within 5 min of GH treatment and disappear in 30 min (39). (iii) STAT5 proteins precipitated from GH-treated L-pGHR cells are tyrosine-phosphorylated (Fig. 2, panel B). (iv) In cell lysates prepared from GH-treated L-pGHR cells, removal of STAT5 protein by immunoprecipitation also resulted in a decrease of pp95/96 (data not shown). Therefore, we conclude the pp93 and pp95/96 observed in GH-treated L-pGHR cells are isoforms of STAT5.

In this study, we demonstrate that GH induces the association of STAT5 with GHR in a GH-dependent manner. In particular, we have shown that STAT5 can be co-immunoprecipitated only from GH-treated L-pGHR cells (Fig. 3) and 3T3-F442A cells (Fig. 4), but not from untreated cells. J AK2 is reported to be activated by GH-GHR interaction and is likely to be the kinase that phosphorylates STAT5 in response to GH treatment (19, 20). Previously in our laboratory, a pGHR analog in which all of the eight intracellular tyrosine residues substituted with phenylalanine were expressed in mouse L cells. The resulting cell line (MYF 8) can undergo GH-induced phosphorylation and activation of J AK2 to a similar level as observed in L-pGHR cells. However, GH cannot induce tyrosine phosphorylation of STAT5 in these cells. Moreover, pGHR analogs that contain a single tyrosine residue at positions 487, 534, 566, or 627 can restore GH-induced tyrosine phosphorylation of STAT5 in these cells. However, pGHR analogs also co-migrate with pp95/96 (Fig. 2, panel A).

92-kDa protein, and each possesses similar DNA binding specificities (19). Two mouse cDNAs encoding two STAT5 homologs, STAT5A and STAT5B, have been cloned from a library prepared from MC/9 cells (a mouse mast cell line) (21). According to the published sequence of STAT5A and STAT5B, we cloned the two STAT5 cDNAs from BALB/c mouse liver. The cDNAs were sequenced and showed the same sequence as published STAT5A and STAT5B (21). Since the exogenous STAT5 proteins contain an inserted FLAG sequence, they could be detected by FLAG antiserum (Fig. 5). As expected, the STAT5A-FLAG shows a higher molecular mass than STAT5B-FLAG. The 95-kDa STAT5 protein is likely to be STAT5A, since it shows similar molecular masses as STAT5A-FLAG, and both can undergo GH-induced tyrosine phosphorylation and interaction with GHR (Figs. 5 and 6). This result also shows that the addition of the FLAG did not affect phosphorylation of the protein by the kinase. In contrast to the 95-kDa STAT5 protein, the 89-kDa protein underwent GH-induced phosphorylation and a noticeable shift of molecular mass. These GH-induced events were not observed in STAT5B-FLAG-expressing cells, suggesting the 89-kDa STAT5-related protein is not STAT5B. The 89-kDa STAT5 protein in mouse L cells may be another isoform of STAT5. We cannot explain why the STAT5B-FLAG cannot associate with GHR and undergo GH-induced tyrosine phosphorylation, since the sequence of the SH2 domain of the two isoforms of STAT5 are identical (21).

Based on the observations made in this study and the past studies of several laboratories, the following model for GH signal transduction can be envisioned (Fig. 7). One GH molecule interacts with two GHRs on the cell surface through binding site 1 and site 2. This interaction induces the dimerization of the GHRs. The dimerization of GHR brings into close juxtaposition two inactive J AK2 molecules that are associated with

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the Box-1 motif of GHR. The two JAK2 transactivate each other, and this event leads to the phosphorylation of one or a combination of GHR tyrosine residues at the position of 487, 534, 566, and 627. The phosphotyrosine residue(s) in the GHR intracellular domain provides the site(s) for STAT5 docking though its SH2 domain. Alternatively, an adaptor molecular may bind to the phosphotyrosine residue(s) of GHR, thereby providing a site for STAT5 to bind. The recruitment of STAT5 to the GHR brings STAT5 into close proximity with activated JAK2, thereby resulting in tyrosine phosphorylation and activation of STAT5. Tyrosine-phosphorylated STAT5 then dissociates from the GHR and associates with either another STAT5 or other activated transcription factors. The complex then translocates into the nucleus, interacts with the corresponding DNA sequence, and regulates gene expression.

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