Communication

Growth Hormone Preferentially Induces the Rapid, Transient Expression of SOCS-3, a Novel Inhibitor of Cytokine Receptor Signaling*

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Four members (SOCS-1, SOCS-2, SOCS-3, and CIS) of a family of cytokine-inducible, negative regulators of cytokine receptor signaling have recently been identified. To address whether any of these genes are induced in response to growth hormone (GH), serum-starved 3T3-F442A fibroblasts were incubated with GH for various time points, and the expression of the SOCS gene family was analyzed by Northern blotting. GH stimulated the rapid, transient induction of SOCS-3 mRNA, peaking 30 min after the initiation of GH exposure and declining to basal levels by 2 h. Expression of the other SOCS genes (SOCS-1, SOCS-2, CIS) was also up-regulated by GH, although to a lesser extent than SOCS-3 and with differing kinetics. SOCS-3 expression was also strongly induced in 3T3-F442A cells treated with leukemia-inhibitory factor (LIF), with weaker induction of SOCS-1 and CIS being observed. The preferential induction of SOCS-3 mRNA was also observed in hepatic RNA isolated from the livers of mice that had received a single superphysiological dose of GH intraperitoneally. Co-transfection studies revealed that constitutive expression of SOCS-1 and SOCS-3, but not SOCS-2 or CIS, blocked GH-induced transactivation of the GH-responsive serine protease inhibitor 2.1 gene promoter.

The elucidation of the pivotal role played by the tyrosine kinase Jak2 in initiating signal transduction from the GH receptor has led to the identification of a number of intracellular pathways that mediate the cellular response to GH (1). However, the mechanism(s) by which signaling from GH receptor-activated Jak2 is attenuated is unclear. Ligand-induced tyrosine phosphorylation/activation of Jak2 by the erythropoietin (EPO) receptor, a member of the cytokine receptor superfamily that includes the GH receptor, is followed by the binding of the protein-tyrosine phosphatase SHP-1 to the cytoplasmic domain of the receptor (2). The recruitment of SHP-1 is accompanied by the dephosphorylation/inactivation of Jak2 and subsequent termination of EPO-induced cellular proliferation.

A similar role for SHP-1 in mediating the down-regulation of Jak2 following stimulation of cells with GH has been proposed (3), although whether SHP-1 can directly associate with the GH receptor remains to be established. Recently, a novel family of cytokine-inducible genes has been identified that appear to function as negative regulators of the JAK signaling pathway (4–7). Constitutive expression of one member, SOCS-1 (also referred to as SSI-1 and JAB) in the murine myeloid leukaemia M1 cell line blocked growth factor-induced differentiation and apoptosis and inhibited interleukin-6 (IL-6)-mediated tyrosine phosphorylation of the cell-surface receptor component, gp130, and the transcription factor, Stat3 (5, 6). SOCS-1 can interact with all four members of the JAK family of tyrosine kinases (6, 7), suppressing kinase activity and the subsequent tyrosine phosphorylation/activation of STAT factors (7). As a diverse array of cytokines appears to be able to induce expression of one or more members of this gene family (5), we sought to establish which, if any, of these genes might be a target for transcriptional activation by GH in vivo and in the classically GH-responsive cell line, 3T3-F442A fibroblasts (8).

**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant human GH was the generous gift of Dr. Ken Ho (Garvan Institute for Medical Research, Sydney, NSW); human insulin was obtained from Novo Nordisk Pharmaceuticals Pty Ltd. (North Rocks, NSW); leukemia-inhibitory factor (LIF) was obtained from AMRAD Pharmacia Biotech (Melbourne, Victoria). Radionucleotide ([α-32P]dCTP (3000 Ci/mmol) was from DuPont NEN (AMRAD Pharmacia Biotech). Hybrid-N+ membrane from Amersham Australia Pty Ltd (Melbourne), and GIGA prime DNA Labeling Kits from BresaLex Ltd. (Adelaide, SA). Cell culture media and reagents were from Life Technologies Pty Ltd (Melbourne).

**Cell Culture—**3T3-F442A fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, and 50 mg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. For hormone induction experiments, cells were grown to confluence and serum-starved overnight in DMEM containing 1% (w/v) bovine serum albumin. Human GH (500 ng/ml), LIF (100 units/ml), or insulin (56 ng/ml) were added, and cell culture was maintained for different time intervals.

**Animals and GH Administration—**Female C57BL/6 mice (7 weeks old) were injected intraperitoneally with GH (10 μg/10 g of body weight) and were subsequently sacrificed at different time points. Livers were removed, snap frozen in liquid nitrogen, and stored at −75 °C.

**RNA Extraction and Northern Blotting—**Cells were rinsed twice with ice-cold phosphate-buffered saline; total RNA was extracted from cell cultures and liver samples using guanidinium thiocyanate-phenol (9). Total RNA was fractionated by electrophoresis through agarose-formaldehyde gels and transferred and subsequently fixed onto Hybond-N+ membranes as described (10). Membranes were hybridized at 63 °C overnight with random primed, radiolabeled probes derived from AMRAD Pharmacia Biotech, and were subsequently washed twice with ice cold 1% (w/v) bovine serum albumin, 0.1% (v/v) Tween-20, and 0.1% (w/v) formaldehyde, followed by two washes with 1× SSC for 15 min each at 55 °C. Membranes were exposed to X-ray film.
from full-length cDNA inserts encoding SOCS-1, SOCS-2, SOCS-3, or CIS (5). Membranes were then washed at high stringency (0.1% SDS at 60 °C) and exposed to Kodak BioMax MS film with an intensifying screen at −70 °C. Where blots were reprobed, membranes were first stripped in boiling 0.1% SDS prior to prehybridization.

**Cell Transfection and Reporter Gene Assays**—Chinese hamster ovary (CHO) cells were maintained in Ham’s F-12 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin. The cells were transfected as described previously (11). Briefly, serum-starved cells were transfected using the calcium phosphate procedure with 1.5 μg of rat GH receptor-encoding plasmid, 3 μg of β-galactosidase internal control reporter plasmid, 1.5 μg of the GH-responsive, serine protease inhibitor (Spi) 2.1 promoter-chloramphenicol acetyltransferase (CAT) construct, and 1 μg of Flag epitope-tagged SOCS-1, SOCS-2, SOCS-3, or CIS expression vectors. After overnight culture in the absence or presence of GH (25 nM), cell extracts were prepared, and CAT and β-galactosidase activities were measured. Equivalent expression of the various SOCS and CIS proteins was confirmed by Western blotting portions of extracts from transfected cells with the Flag-specific monoclonal antibody, M2.

**RESULTS**

**GH Preferentially Induces SOCS-3 mRNA Expression in 3T3-F442A Fibroblasts**—In the absence of GH, Northern blotting of total RNA isolated from serum-starved 3T3-F442A cells detected low levels of SOCS-3 and SOCS-1 mRNA (Fig. 1). Transcripts corresponding to SOCS-2 and CIS were only observed upon prolonged exposure. Upon the addition of GH, induction of SOCS-3 expression was apparent at the first time point examined (15 min), peaking at 30 min before declining rapidly over subsequent time points (Fig. 1). This pattern of induction has been confirmed independently using both total and poly(A)§ mRNA (data not shown). The induction of SOCS-1, by comparison, was much less pronounced although with similar kinetics (Fig. 1). Both CIS and SOCS-2 mRNA were up-regulated in response to GH treatment (Fig. 1); with respect to CIS, induction peaked 60 min after hormonal exposure, and although the abundance of transcript had declined at time points assayed thereafter, expression remained significantly above basal levels. Similarly, while the induction of SOCS-2 mRNA exhibited delayed kinetics compared with other SOCS genes, elevated levels of the SOCS-2 message persisted to the final time point assayed (4 h).

Because 3T3-F442A fibroblasts express functional receptors for LIF and insulin (12), the ability of these ligands to influence SOCS gene expression was examined. LIF treatment strongly induced SOCS-3 mRNA expression, with the response appearing maximal after a 15-min stimulation with growth factor (Fig. 2). Interestingly, the subsequent down-regulation of SOCS-3 transcripts was significantly delayed by comparison with GH-treated cultures, with elevated levels of transcripts persisting at the final time point (4 h) assayed. Both SOCS-1 and CIS mRNAs were induced in response to LIF, albeit to a much lesser extent than SOCS-3, and returned to basal levels by 4 h (Fig. 2). No induction of SOCS-2 was observed (data not shown), while no member of the SOCS family gene was induced in cell cultures treated with insulin (data not shown).

**SOCS-3 Is Induced by GH in Vivo**—Strikingly, analysis of hepatic RNA isolated from the livers of mice injected with a single dose of GH confirmed the preferential activation of SOCS-3 mRNA expression by GH (Fig. 3). A marked elevation of SOCS-3 expression was observed around 60 min following GH injection, declining thereafter. Of the other SOCS family genes, clear induction of CIS expression was found 30 min after hormone injection, peaking at 60 min, and remaining elevated even after 24 h (Fig. 3). In contrast, SOCS-1 and SOCS-2 were only weakly induced by GH.

**SOCS-1 and -3 Block Transactivation of a GH-responsive Promoter Element**—To explore the functional consequences of GH-induced expression of the different SOCS family genes, we determined the ability of GH to transactivate the GH-responsive Spi 2.1 promoter, linked to a CAT reporter gene, in CHO cells co-transfected with the various SOCS-encoding plasmids. In cells that were not transfected with SOCS expression constructs, GH induced a 3.4-fold increase in CAT activity from the Spi 2.1 promoter (Fig. 4). While constitutive expression of CIS had no effect on the hormonal responsiveness of the Spi 2.1 promoter, expression of either SOCS-1 or SOCS-3 ablated the ability of GH to transactivate reporter gene activity from the same promoter. Paradoxically, co-expression of SOCS-2 re-
resulted in the superinduction of CAT activity in response to GH, giving a 7–9-fold increase above basal levels of activity.

**DISCUSSION**

The identification of SOCS-3 as a major transcriptional target of GH action raises a number of questions, in particular how might SOCS-3 act to regulate signaling by the GH receptor. SOCS-1 can bind directly to Jak2 and Tyk2 (6, 7), and it is capable of inhibiting the activity of all four members of the JAK kinase family (6, 7). As Jak2 is the principal effector of GH action at a cellular level, it would be anticipated that enforced expression of SOCS-1 in transfected CHO cells would block GH-mediated transactivation of the Spi 2.1 promoter (11) and normalized against bor presence (1) as an internal control), alone or together with expression vectors encoding individual SOCS family members. Following transfection, extracts were prepared from cells cultured in the absence (−, light bars) or presence (+, dark bars) of GH, as described (11). CAT activity was determined and normalized against β-galactosidase activity to control for transfection efficiency. The basal level of CAT activity, in the absence of GH and the different SOCS constructs, was given the value of 1. The results presented correspond to the mean values of three independent experiments, with the error bars representing the S.D. of the mean.

FIG. 4. Overexpression of different SOCS genes can influence GH-induced transactivation of the Spi 2.1 promoter. CHO cells were transfected with the Spi 2.1-CAT reporter construct, in conjunction with plasmids expressing the rat GH receptor and β-galactosidase (as an internal control), alone or together with expression vectors encoding individual SOCS family members. Following transfection, extracts were prepared from cells cultured in the absence (−, light bars) or presence (+, dark bars) of GH, as described (11). CAT activity was determined and normalized against β-galactosidase activity to control for transfection efficiency. The basal level of CAT activity, in the absence of GH and the different SOCS constructs, was given the value of 1. The results presented correspond to the mean values of three independent experiments, with the error bars representing the S.D. of the mean.

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