Previously, by a yeast 2-hybrid screen, we identified signal transducer and activator of transcription 5b (Stat5b) as a substrate of the insulin receptor (IR). We demonstrated that refeeding of fasted mice leads to rapid activation of Stat5 proteins in liver, skeletal muscle, and fat, suggesting that Stat5b is a physiological target of insulin. Here, we show that injection of glucose or insulin into fasted mice leads to robust activation of both Stat5a and Stat5b in skeletal muscle. In C2C12 myotubes, we find that insulin stimulates tyrosine phosphorylation of Stat5a and Stat5b by 3-5-fold. This degree of Stat5 activation in vitro is significantly lower than what we observe in vivo and inversely correlates with IRS-1/2 levels. We can recapitulate robust insulin activation of Stat5 in C2C12 cells by stable overexpression of the human IR (hIR). To identify insulin-activated genes that are Stat5 targets, we also overexpressed an IR mutant (LA-hIR) that signals normally for mitogen-activated protein kinase- and phosphatidylinositol 3-kinase-dependent pathways but is deficient in Stat5 signaling in response to insulin. We demonstrate that insulin induces the expression of SOCS-2 mRNA in the wild type hIR but not in the LA-hIR-overexpressing cells. The induction of SOCS-3 by insulin is reduced but not lost in the LA-hIR cells. Therefore, our results suggest that insulin induction of SOCS-2, and in part SOCS-3 mRNA expression, is mediated by Stat5 and can be independent of mitogen-activated protein kinase and phosphatidylinositol 3-kinase-signaling pathways.

Insulin plays a pivotal role in the regulation of glucose homeostasis and exerts numerous metabolic and proliferative responses in insulin-sensitive tissues (1). These effects are mediated by the binding of insulin and subsequent activation of the insulin receptor (IR) (2) tyrosine kinase (1). The activated receptor phosphorylates itself as well as several other intracellular substrates. Unlike several members of the receptor tyrosine kinase superfamily, which directly recruit and phosphorylate signaling/adapter proteins, the IR mainly signals by recruiting and phosphorylating members of the insulin receptor substrate family, IRS-1, -2, -3, and -4, Gab1 and -2, and p62dok-1, -2, and -3 (2, 3). These proteins then serve as multivalent docking sites for the recruitment of other Src homology domain 2-containing signaling proteins. Subsequently, at least two major signal transduction cascades are initiated including the mitogen-activated protein kinase- and phosphatidylinositol 3’-kinase-signaling pathways, which propagate the signal to various cytoplasmic and nuclear effectors (1, 4).

Unlike the IR, cytokine receptors do not possess intrinsic tyrosine kinase activity. Instead, they depend upon receptor-associated Janus kinase tyrosine kinases to initiate signaling after ligand binding (5–7). These Janus kinases become activated and phosphorylate themselves, the cytokine receptor to which they are bound, and Src homology domain 2-containing signaling/adapter proteins, including Stat transcription factors (6, 7). This family of transcription factors includes Stats1, -2, -3, -4, -5a, -5b, and -6, where Stat5a and Stat5b are two separate but homologous gene products (95% amino acid identity) (8). Stat transcription factor proteins are found latent in the cytoplasm. Phosphorylation of the activating tyrosine residue in the Stat proteins results in the nuclear translocation of Stat dimers and confers the ability of the Stat dimers to specifically bind to promoter sequences of target genes and to transactivate those genes (9). Like receptor tyrosine kinases, cytokine receptors also activate the mitogen-activated protein kinase- and phosphatidylinositol 3’-kinase-signaling pathways and in some instances through Janus kinase phosphorylation of IRS family members (2, 6). Activation of Stats is not limited to cytokine receptors, as several receptor tyrosine kinases, such as the epidermal growth factor receptor and the platelet-derived growth factor receptor, have been shown to activate one or more Stats family members through Janus kinase-independent and Janus kinase-dependent pathways (10–12).

Recently, by yeast two hybrid screen analysis using the kinase active cytoplasmic domain of the IR as a probe, we (13) and others (14) identified Stat5b as a direct substrate of the IR. We then provided the first evidence that insulin can stimulate the activation of Stats both in vitro and in vivo (13). We demonstrated that Stats (1, 3, 5a, and 5b) can be rapidly activated by insulin in two different IR-overexpressing cell lines and that perfusion of mouse livers with insulin selectively activates Stat5 proteins. Furthermore, we observed that refeeding of fasting mice, which causes post-prandial secretion of insulin, leads to the rapid activation of Stat5 proteins in liver, skeletal muscle, and fat, all physiologic target tissues of insulin action. Van Obberghen and co-workers (14) also find that insulin could stimulate tyrosine phosphorylation of Stat5b when co-ex-
pressed with the human IR in 293 cells. The potential importance of this pathway in insulin signaling is further supported by recent studies suggesting that Stat5b can play a role in the activation of glucokinase and suppressor of cytokine signaling-3 (SOCS-3) gene transcription by insulin (15, 16). In addition to being activated after refeeding, Stat5b has been shown to be activated by insulin in pmi28 mouse muscle cells and Kym-1 rhabdomyosarcoma cells (17, 18). The ability to detect insulin-stimulated Stat5b activation in both skeletal muscle in vivo and in vitro suggested that Stat5b might play an important role in insulin-regulated gene expression in muscle. Here, we show that injection of glucose or insulin into mice leads to robust activation of both 5a and 5b in skeletal muscle. To develop a model system to study the role of Stat5 in insulin signaling in skeletal muscle cells, we examined the activation of Stat5a and Stat5b by insulin in the myogenic C2C12 cell line. We demonstrate that insulin treatment results in a rapid 3–5-fold increase in tyrosine phosphorylation of both Stat5a and Stat5b in C2C12 myotubes but not in undifferentiated myoblasts.

Interestingly, Sawka-Verhelle et al. (14) mapped the site of interaction of Stat5 with the human IR to Y972 located in the juxtamembrane region of the IR by yeast two-hybrid analysis. This site contains a motif, pY972pYLSA (pY is phosphotyrosine), that is similar to other Stat5 recruitment sites in cytokine receptors (19–21). Overlapping this site is an NPEpY motif that recruits IRS-1 and Shc (22). In addition, by yeast 2-hybrid analysis, SOCS-3 competes with Stat5 for binding to this site as a part of the negative regulation of IR signaling via Stat5 (16). Because IR levels are equivalent in C2C12 myoblasts and myotubes, we speculate that the ability to detect Stat5 activation only in the myotubes is a consequence of reduced levels of IRS-1 and IRS-2 expression and increased levels of Stat5 expression compared with the myoblasts. In support of this hypothesis, the expression levels of IRS-1 and IRS-2 in isolated skeletal muscle are dramatically less than those observed in C2C12 cells. In contrast, IR expression is essentially equivalent in skeletal muscle and C2C12 cells. In lieu of significantly reducing the IRS-1 and IRS-2 content in C2C12 cells, we stably overexpressed the human IR (hIR) in C2C12 cells and obtained cell lines with an IRS:IR ratio that more closely approximates the ratio that exists in skeletal muscle. These cells respond to insulin with potent activation of Stat5 proteins and induction of putative Stat target genes such as C/EBP, SOCS1, SOCS2, and SOCS3 (23, 24).

To identify insulin-activated genes that are specific Stat5 targets, we took advantage of an IR mutant (LA-hIR) that signals normally for mitogen-activated protein kinase- and phosphatidylinositol 3-kinase-dependent pathways but is deficient in Stat5 signaling in response to insulin. We established cell lines expressing equivalent levels of either WT-hIR or LA-hIR, and we have demonstrated that insulin induces the expression of SOCS-2 mRNA in the WT-hIR but not in the LA-hIR-overexpressing cells. Interestingly, the induction of SOCS-3 by insulin is lost in LA-hIR myotubes but is only reduced in LA-hIR myoblasts. Therefore, our results suggest that in hIR cells Stat5 mediates the insulin induction of SOCS-2 and, in part, SOCS-3 mRNA expression.

**EXPERIMENTAL PROCEDURES**

**Animals**—6-week-old male C57/Black mice were fasted for 48 h before a bolus intraperitoneal injection of either glucose, insulin, or vehicle (phosphate-buffered saline). 10 min (glucose) or 25 min (insulin) after the injection, the mice were euthanized, and tissues were removed and frozen in liquid nitrogen. Tissue lysates were prepared as described previously (13).

**Reagents and Antibodies**—Insulin was a kind gift from Eli Lilly. Recombinant IGF-1 was obtained from GroPep (Adelaide, Australia). Bovine serum albumin and water-soluble dexamethasone were obtained from Sigma. Matrixtin-2 was obtained from Collaborative Biomedical Products (Bedford, MA). Chick embryo extract, HEPES, and TRizol were obtained from Life Technologies. Luciferase assay reagent was obtained from Promega (Madison, WI). The LHRR construct was a kind gift from Fabrice Gouilleux. The constructs containing the cDNAs for the SOCS/CIS genes were kind gifts from Douglas Hilton. The anti-phosphotyrosine monoclonal antibody 4G10 and anti-IRS-1 polyclonal antibody were obtained from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibodies recognizing the C terminus of mouse Stat5a (L20) or mouse Stat5b (C17) used for immunoprecipitations were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For immunoblot analysis, the monoclonal antibody S21520 recognizing both Stat5a and Stat5b was obtained from Transduction Laboratories (Los Angeles, CA). Generation and characterization of the monoclonal antibody recognizing the activated Stat5 (185E5) will be described elsewhere. Generation and characterization of polyclonal antisera recognizing the IR β-subunit was described previously (25). Horseradish peroxidase-conjugated secondary antibodies were obtained from CALTAG (Burlingame, CA). Monoclonal anti-phospho-p44/42 mitogen-activated protein kinase (Thr-202/Tyr-204) antibody, polyclonal antibody recognizing phospho-Akt (Ser473), and polyclonal antibody recognizing phospho-Stat3 (Tyr-705) were obtained from New England Biosabs (Beverly, MA).

**Cell Culture and Transfections**—Mouse skeletal muscle C2C12 myoblasts and myotubes were induced to grow in growth medium: Dulbecco's modified Eagle's medium supplemented with 0.2% bovine serum albumin and 25 mM HEPES, and 0.2% gentamicin. Myoblasts were cultured in growth medium: Dulbecco’s modified Eagle’s medium supplemented with 0.2% bovine serum albumin and 25 mM HEPES and, in part, SOCS-3 mRNA expression.

For immunoblot analysis, the monoclonal antibody S21520 recognizing both Stat5a and Stat5b was obtained from Transduction Laboratories (Los Angeles, CA). Generation and characterization of the monoclonal antibody recognizing the activated Stat5 (185E5) will be described elsewhere. Generation and characterization of polyclonal antisera recognizing the IR β-subunit was described previously (25). Horseradish peroxidase-conjugated secondary antibodies were obtained from CALTAG (Burlingame, CA). Monoclonal anti-phospho-p44/42 mitogen-activated protein kinase (Thr-202/Tyr-204) antibody, polyclonal antibody recognizing phospho-Akt (Ser473), and polyclonal antibody recognizing phospho-Stat3 (Tyr-705) were obtained from New England Biosabs (Beverly, MA).

**Cell Culture and Transfections**—Mouse skeletal muscle C2C12 myoblasts and myotubes were induced to grow in growth medium: Dulbecco’s modified Eagle’s medium containing 15% heat-inactivated fetal bovine serum, 0.5% chick embryo extract, 25 mM HEPES, and 0.2% gentamicin. For myoblast cultures, C2C12 cells were grown to 80% of confluence on tissue culture dishes, washed with phosphate-buffered saline (PBS), and placed in serum starvation media: Dulbecco’s modified Eagle’s medium supplemented with 0.2% bovine serum albumin and 25 mM HEPES for 16 h before treatment with insulin or diluent (PBS). For the generation of myotubes, C2C12 cells were grown to confluence on tissue culture dishes coated with Matrigel, washed with PBS, and placed in differentiation medium (DM): Dulbecco’s modified Eagle’s medium containing 2% heat-inactivated horse serum and 25 mM HEPES for 3 days. Well differentiated cultures (>60% of the plate multinucleated myotubes) were then washed with PBS and placed in serum starvation media for 16 h before treatment with insulin or diluent (PBS). For generation of WT-IR and LA-hIR cell lines, C2C12 cell and LA-hIR cell lines were plated at 1.5 × 105 cells/35-mm tissue culture dish. After 16 h, the cells were transfected with 2.0 µg of pEF/hIR/Neo or pEF/LA-hIR/Neo. Twenty-four h after transfection, the cells were trypsinized and plated at low density. Cells were selected for 14 days in neomycin (G418) before screening isolated colonies by analysis of lysates for the expression of hIR. For transient transfections, WT-IR cells were plated at 3 × 105 cells/80-mm tissue culture dish. After 16 h, the cells were washed and transfected with 3.0 µg of LHRR luciferase reporter plasmid, 1.5 µg of CMV-β-gal, and either 1.5 µg of pKRS empty vector or 0.75 µg of pKRSStat5aFLAG and pKRSStat5bFLAG with 18 µl of Fugene (Roche Molecular Biochemicals) according to the manufacturer directions. Twenty-four h after transfection, the transfected cells were split onto Matrigel-coated 6-well plates. Six h later, the cells were replaced in serum starvation media for 16 h before treatment with dexamethasone and/or insulin or diluent (PBS). After stimulation with insulin for 6 h, cells were harvested in 1× reporter lysis buffer from Promega. After normalization for β-galactosidase activity, lysates were assayed for luciferase activity using the luciferase assay kit from Promega.

**Immunoprecipitation and Western Blotting**—Tissue or cells were lysed in radioreimmunoprecipitation buffer (200 mM NaCl, 50 mM NaF, 50 mM Tris, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM benzamidine, 5 µg/ml pepstatin A, 5 mM microcystin) for 20 min at 4 °C. Lysates were cleared of insoluble material by centrifugation. Cleared lysates were immunoprecipitated overnight at 4 °C with either anti-Stat5A or anti-Stat5B antibodies, incubated with protein-A-Sepharose, washed four times in radioreimmunoprecipitation buffer, boiled in SDS-PAGE sample buffer containing 100 mM dithiothreitol, and subjected to SDS-PAGE (7.5% gel).
acrylamide). Alternatively for Western blotting of whole cell lysates, cleared radioimmune precipitation buffer lysates containing equal protein were boiled in SDS-PAGE sample buffer and subjected to SDS-PAGE (7.5% acrylamide). After electrophoretic transfer of proteins to nitrocellulose, the membranes were blocked overnight at 4 °C in either bovine serum albumin blocking buffer (3% bovine serum albumin in PBS, 0.1% Tween 20) for anti-phosphoysrin Western blots or milk blocking buffer (4% nonfat dried milk in PBS, 0.1% Tween 20) for all other Western blots. Blocked membranes were incubated with primary antibodies in the appropriate blocking buffer for 2 h at room temperature. The membranes were washed extensively, incubated with anti-mouse IgG-horseradish peroxidase or anti-rabbit IgG-horseradish peroxidase in PBS, 0.1% Tween 20, 4% nonfat dried milk for 1 h, washed extensively, developed with Supersignal ECL reagent from Pierce, and exposed to film (Kodak X-Omat AR).

Electrophoretic Mobility Shift Analysis—Whole cell extracts were prepared as described previously (26). Electrophoretic mobility shift analysis was performed on the whole cell extracts as described previously (26) with 32P-radiolabeled double-stranded oligonucleotide probes containing high affinity binding sites for Stat5 (β-casein gene promoter (8)).

RNA Extraction and Northern Analysis—After treatment with insulin, C2C12 or hIR64 cells were rinsed twice with ice-cold PBS, and total RNA was extracted using TRIZol (Life Technologies). Total RNA was fractionated by electrophoresis on agarose-formaldehyde gel, transferred to Hybond XL membrane (Amer sham Pharmacia Biotech) using the Northern Max kit (Ambion), and fixed by UV cross-linking. Membranes were hybridized at 68 °C overnight (except for SOCS-1 at 75 °C) with 32P-radiolabeled antisense riboprobe derived from full-length cDNA inserts encoding CIS, SOCS-1, SOCS-2, or SOCS-3 (27) prepared with the Strip-EZ RNA kit (Ambion). Membranes were washed at high stringency (0.1× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS at 68 °C) and exposed to Kodak X-Omat AR-5 film with an intensifying screen at −70 °C. For reprobing, membranes were stripped according to manufacturer directions for the Strip-EZ RNA kit (Ambion).

RESULTS

Insulin Activates Stat5a and Stat5b in Skeletal Muscle in Vivo—We have shown that refeeding of fasting mice leads to increases in the tyrosine phosphorylation of Stat5b in liver, adipose tissue, and skeletal muscle (13). Although these data suggest that physiological levels of insulin released after feeding are capable of stimulating Stat5 activation, we considered the possibility that peptides secreted by the gut after feeding might be responsible for stimulating the tyrosine phosphorylation of Stat5 proteins. We therefore bypassed the feeding response and increased circulating insulin levels by direct injection of glucose into fasted mice. We detect strong increases in the tyrosine phosphorylation of both Stat5a and Stat5b in skeletal muscle within 15 min of glucose injection (Fig. 1). As expected, direct injection of insulin into fasted mice also leads to rapid increases in the tyrosine phosphorylation of both Stat5a and Stat5b in skeletal muscle (Fig. 1). Similar results were obtained in fat and liver.

Insulin Activates Stat5a and Stat5b in C2C12 Myotubes but Not Myoblasts—Tyrosine phosphorylation of Stat5b in response to insulin is detectable in several in vitro cell models including Kym-1 rhabdomyosarcoma (18) and pmi28 mouse muscle cells (17) as well as in 3T3-L1 adipocytes (16) and HepG2 cells (15) when these cells are pretreated with protein tyrosine phosphatase inhibitors. Stat5b, but not Stat5a, is activated in Kym-1 rhabdomyosarcoma cells (18), which is in contrast to the in vivo activation of both Stat5a and Stat5b in the skeletal muscle that we observe. To determine whether insulin activates both Stat5 proteins in skeletal muscle cells in vitro, we performed experiments with the myogenic C2C12 cell line. These cells are maintained in culture as myoblasts in the presence of high concentrations of serum. When C2C12 cells are grown to confluence and high serum-containing medium was removed, the myoblasts withdraw from the cell cycle and begin a myogenic program of differentiation. After 3 days in low serum, the myoblasts have already fused to form multinucleated myotubes that express muscle-specific genes at high levels. To determine whether insulin stimulates Stat5a or Stat5b either as proliferating myoblasts or at any point during the differentiation of myoblasts to myotubes, we treated C2C12 myoblasts or C2C12 cultures at 1, 2, 3, or 4 days in low serum media with or without insulin. We analyzed the control and insulin-stimulated lysates by immunoprecipitation with antibodies recognizing either Stat5a or Stat5b followed by blotting with a phosphorylation state-specific monoclonal antibody that recognizes both Stat5a and Stat5b phosphorylated on the activating tyrosine. We observed reproducible insulin-induced 3–5-fold increases in the tyrosine phosphorylation of both Stat5a and Stat5b in C2C12 myotubes with little or no effect detected in the undifferentiated myoblasts (Fig. 2). Indeed, the ability of insulin to activate Stat5 appears to be correlated with the level of differentiation in the C2C12 myotubes (Fig. 2A).

To determine whether the activation of Stat5 in skeletal muscle occurs at doses of insulin that do not result in activation of the IGF-1 receptor, we treated C2C12 myotubes with varying concentrations of insulin for 10 min. We detected insulin activation of Stat5a and Stat5b in C2C12 myotubes at doses of insulin below levels that activate the IGF-1 receptor (Fig. 2B). To confirm this result, we treated C2C12 myotubes with 200 ng/ml recombinant insulin growth factor-1 for 10 min. Analysis of recombinant IGF-1-stimulated lysates with anti-phospho-Stat5 antibodies showed no detectable increases in Stat5 phosphorylation in response to IGF-1 (data not shown). To determine the kinetics of Stat5 activation in response to insulin, we treated C2C12 myotubes with insulin for several time points and assayed cell lysates for Stat5 tyrosine phosphorylation. As shown in Fig. 2B, we detected Stat5 activation in myotubes at the earliest time point tested (2 min). This response peaked at 10 min, and the decline was evident by 20 min. These results
suggest that the Stat5 activation by insulin in myotubes is rapid and transient.

**Insulin Activation of Stat5 in C2C12 Cells Stably Overexpressing the hIR**—We were surprised that the Stat5 activation in response to insulin in C2C12 cells was relatively weak compared with the response we observed in skeletal muscle in vivo (Fig. 1). Since Stat5 and IRS-1 are recruited to the same phosphorylated residue on the IR (Tyr(P)-972) (14, 22), we speculated that IRS-1 and Stat5 might be competing for recruitment to limited amounts of IR in the C2C12 cells. We therefore performed biochemical analysis of C2C12 myoblasts and myotubes with regard to the expression levels and insulin-stimulated tyrosine phosphorylation levels of several IR substrates (Fig. 3). Although IR β-subunit expression and insulin-stimulated tyrosine phosphorylation are equivalent in C2C12 myoblasts and myotubes, IRS-1 protein expression is extremely high in the C2C12 myoblasts, which could block efficient recruitment of Stat5 proteins to the IR in the myoblasts. Consistent with this observation, in C2C12 myotubes, where insulin-stimulated Stat5 activation is detected, IRS-1 expression is reduced 3–5-fold, and Stat5 expression is increased 4–5-fold. Nevertheless, the level of Stat5 activation by insulin in C2C12...
myotubes is significantly less than that observed in skeletal muscle. We therefore performed a direct comparison of the levels of IR, IRS-1, IRS-2, and Stat5 proteins in lysates from isolated skeletal muscle, C2C12 myoblasts, and C2C12 myotubes (Fig. 3A). Isolated skeletal muscle, C2C12 myoblasts, and C2C12 myotubes express equivalent levels of IR protein. In contrast, the expression of IRS-1 and IRS-2 is dramatically lower in skeletal muscle compared with the immortalized C2C12 cells. These data are consistent with our hypothesis that the high expression levels of IRS proteins prevent efficient recruitment and activation of Stat5 proteins by limiting amounts of IR.

Based on these observations, we hypothesized that increasing the IR content in these cells by stable overexpression of the IR would result in cell lines in which the activation of Stat5 approaches the levels we observe in vivo. To test our hypothesis, we generated several clones of C2C12 cells that stably overexpress the hIR that are capable of differentiating into myotubes. As shown in Fig. 3B, in a representative clone (HIR64), insulin activates Stat5 proteins 25–40-fold in both hIR myoblasts and myotubes, approaching the response to insulin we observed in skeletal muscle in vivo. Further biochemical analysis in regard to other IR substrates revealed that, compared with C2C12 myoblasts, the hIR myoblasts display increased IRS-1 tyrosine phosphorylation in response to insulin despite reduced IRS-1 expression. In addition, insulin-stimulated mitogen-activated protein kinase activation is also increased in the hIR myoblasts, as evidenced by increases in the amounts of activated extracellular-regulated kinase-1 and -2.

In contrast, there is little augmentation of insulin-stimulated phosphatidylinositol 3'-kinase activation, as shown by nearly equivalent levels of activated protein kinase B in hIR64 cells compared with C2C12 cells.

**Insulin Induces Stat5 DNA Binding Activity and Transactivates a Stat5 Reporter Gene in hIR Cells**—To verify that the insulin-stimulated tyrosine phosphorylation of Stat5 results in functional DNA binding, we performed electrophoretic mobility shift analysis with insulin-treated whole cell extracts incubated with a Stat5 DNA binding site (β-casein promoter) (Fig. 4A). Stat5-specific DNA complexes were detected in both hIR myoblasts and myotubes, confirming the results obtained in Fig. 3. To investigate whether insulin-activated Stat5 proteins are capable of transactivation in hIR-overexpressing cells, we performed transient transfections with a luciferase reporter gene construct driven by six copies of the Stat5 binding site located upstream of a minimal thymidine kinase promoter. Insulin stimulates Stat5 reporter gene expression in hIR myoblasts by up to 3-fold with endogenous levels of Stat5 and by 35-fold when Stat5 proteins are co-transfected (Fig. 4B). In contrast, C-terminal truncation mutants of Stat5 (Stat5a/5bΔCT), which lack the transactivation domain, do not support insulin stimulation of the Stat5 reporter.

The glucocorticoid receptor (GR) has been shown to physically interact with both Stat5a and Stat5b and augment the relatively weak transcriptional activation potential of Stat5 proteins (28–31). We therefore tested whether the potent synthetic glucocorticoid, dexamethasone (DEX) has any effect in this construct. When DEX and insulin were added together, however, a synergistic induction of luciferase was observed. Consistent with the idea that the Stat5-GR interaction recruits potent transactivation domains to Stat5, the addition of DEX allows the Stat5a/5bΔCT proteins to activate the Stat5 reporter gene in an insulin-stimulated fashion (29). The synergistic effect of DEX is also apparent in the absence of co-transfected Stat5 expression vectors. Therefore, a significant fraction of the endogenous Stat5 proteins are likely to be interacting with the GR, and this may be important for genes that are synergistically regulated by insulin and glucocorticoids, such as glucokinas (32). Although the Stat5-GR interaction may lead to significant increases in the activation of Stat5 target genes, this interaction has also been shown to lead to a decreased ability of the GR to transactivate a simple GRE-containing reporter gene (28). Therefore, it is possible that insulin-stimulated Stat5 could play a role in the inhibitory effect of insulin on several glucocorticoid-inducible genes in vivo.

**Insulin Induces the mRNA Expression of Several Members of the SOCS/CIS Family of Signaling Proteins in hIR Myoblasts and Myotubes**—Recently two genes were described whose regulation by insulin is mediated in part by Stat5, glucokinas, and SOCS-3 (15, 16). SOCS-3 is a member of a novel family of cytokine-inducible genes, the CIS/SOCS genes, which were initially isolated as suppressors of cytokine signaling (24, 27). Emanuelli et al. (16) demonstrate that the insulin induction of SOCS-3 in COS-7 cells is enhanced by Stat5B expression. To determine whether insulin induces the expression of SOCS-3 or other members of the SOCS family in hIR myoblasts...
or myoblasts or myotubes stimulated for 0, 0.5, 1, 2, and 4 h with 100 nM insulin and performed Northern analysis with antisense riboprobes for CIS, SOCS-1, SOCS-2, and SOCS-3. As shown in Fig. 5, insulin strongly induces all four SOCS/CIS family members in hIR myoblasts and induces SOCS-1, SOCS-2, and SOCS-3 but not CIS in hIR myotubes. As reported previously, the kinetics of activation among the family members differ as SOCS-1 and SOCS-3 are rapidly induced, whereas CIS and SOCS-2 show slower induction kinetics (33).

**C2C12 Cells Overexpressing the LA-hIR Mutant Are Deficient in Stat5 Signaling in Response to Insulin**—To examine the role of Stat5 in insulin-regulated transcription in C2C12 muscle cells, we overexpressed a mutant hIR (LA-hIR) that is deficient in Stat5 signaling. We generated this mutant as part of a detailed analysis of the amino acid residues critical for Stat5 recruitment to the YLSA motif in the juxtamembrane region of the IR. A schematic of the mutations is shown in Fig. 6A. We screened several WT-hIR and LA-hIR myoblast clones and identified a matched pair that expresses equivalent amounts of the WT-hIR (hIR18) or the mutant LA-hIR (LA2E). Compared with the WT-hIR18 myoblasts, the LA-hIR clone LA2E myoblasts display a greater than 50-fold reduction in Stat5 activation in response to insulin (Fig. 6B). In contrast, insulin-stimulated tyrosine phosphorylation of IRS-1 and activation of extracellular-regulated kinase-1 and -2 and protein kinase B are essentially equivalent. These results suggest that we can use these cell lines as a tool to examine the role of Stat5 in insulin-regulated transcription in C2C12 cells either in proliferating myoblasts or in differentiatated myotubes.

**Insulin Induction of SOCS-2 and SOCS-3 Is Mediated by Stat5**—To determine whether Stat5 is involved in insulin stimulation of SOCS-2 or SOCS-3 mRNA expression, we isolated total RNA from serum-starved WT-hIR(hIR18) myoblasts and myotubes or LA-hIR(LA2E) myoblasts and myotubes stimulated for 0, 0.5, 1, 2, and 4 h with 100 nM insulin. For comparative purposes, we also isolated total RNA from serum-starved C2C12 or WT-hIR(hIR64) myoblasts and myotubes stimulated for the same time points with 100 nM insulin. We performed Northern analysis on each set of RNAs with either an antisense riboprobe for SOCS-2 or SOCS-3, and the results are shown in Fig. 7. We detected similar patterns of SOCS-2 induction in response to insulin in both WT-hIR clones, although the basal levels in the myotubes of hIR18 were slightly higher. In contrast, the SOCS-2 induction in response to insulin in the LA-hIR (LA2E) clone was reduced to levels observed in C2C12 cells. These results provide compelling evidence that SOCS-2 mRNA induction by insulin in hIR cells involves Stat5 activation.

Like SOCS-2, we observed similar patterns of insulin-stimulated SOCS-3 induction in the two WT-hIR clones, although again, basal levels in the myoblasts of hIR18 were slightly higher. Unlike SOCS-2, the insulin-stimulated SOCS-3 induction in response to insulin in the LA-hIR (LA2E) myoblasts was reduced to levels observed in C2C12 cells only in the myotubes. The insulin-stimulated SOCS-3 induction in the LA-hIR myoblasts was blunted (~50%) but not reduced completely, suggesting that transcription factors other than Stat5 are required.
for insulin-stimulated SOCS-3 transcription in myoblasts. Nevertheless, these results show that Stat5 is involved in SOCS-3 mRNA induction by insulin especially in the LA-hIR myotubes.

DISCUSSION

Insulin exerts its pleiotrophic effects on metabolism, proliferation, and differentiation by several mechanisms (1, 3, 4). These include changing the activity and the intracellular localization of its effector proteins as well as regulating gene expression at the transcriptional level (1, 3, 4). Recent progress in identifying the factors involved in insulin regulation of gene expression indicate that the Forkhead (FKHD)- and sterol-regulated enhancer-binding protein (SREBP) families of transcription factors play critical roles in the regulation of key metabolic enzymes such as fatty acid synthase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and glucokinase (34–39). We and others have identified a novel signaling pathway involving activation of Stat transcription factors by insulin (13, 14). Injection of insulin into mice activates Stat5a and Stat5b in skeletal muscle, suggesting that Stat5 proteins are physiological substrates of insulin-stimulated signaling pathways. Nevertheless, the specific functional roles of Stats in insulin action remain to be determined. To address this question we have engineered an in vitro model system for skeletal muscle such that we can now identify potential insulin-regulated genes that require Stat5 function. We show evidence that induction of SOCS-2 mRNA expression by insulin in muscle cells overexpressing the IR is mediated by Stat5. Thus, we have identified an insulin target gene in IR-overexpressing muscle cell lines that is clearly regulated by a Stat protein. We also confirm that the induction of SOCS-3 mRNA expression by insulin in these cells is mediated in part by Stat5.

The level of insulin stimulation of Stat5a and Stat5b tyrosine phosphorylation in skeletal muscle is quite robust. Consistent with this observation, insulin activation of Stat5b has been reported in two muscle cell models, Kym-1 rhabdomyosarcoma (18) and pmi28 mouse muscle cells (17), although at significantly lower levels than we have observed in vivo. Similarly, the insulin activation of Stat5 proteins in C2C12 cells is relatively weak and observed only in myotubes. We explored potential reasons for the differential ability of insulin to stimulate Stat5 activation in skeletal muscle versus cell culture models. Although the expression levels of the IR β-subunit are equivalent in isolated skeletal muscle, C2C12 myoblasts, and myotubes, we discovered important differences. First, the levels of IRS-1 and IRS-2 protein expression are highest in the C2C12 myoblasts, at least 5-fold lower in the C2C12 myotubes, and dramatically lower in the skeletal muscle. Second, the expression of Stat5a and especially Stat5b are significantly higher in the myotubes compared with the myoblasts. Based on the observation that the recruitment sites on the IR for IRS-1 and Stat5 are partially overlapping (14, 22), we speculated that these two proteins are competing for recruitment to the IR. Therefore, high levels of IRS-1 expression in the immortalized C2C12 myoblasts (as well as many other immortalized cell lines compared with their primary counterparts (40–42)) could effectively block Stat5 recruitment to the IR. This model predicts that reducing the IRS:IR ratio either by IRS-1 antisense or IR overexpression would allow for insulin-stimulated Stat5 activation. Consistent with this model, stable overexpression of the human insulin receptor in C2C12 cells confers the ability of these cells to respond to insulin with levels of Stat5 activation that approach the levels observed in vivo. We have characterized several other known insulin-signaling pathways in these stable hIR overexpressing cell lines. Although the ability to augment insulin-stimulated Stat5 activation is the most dramatic result of increased levels of IR, the activation of other signaling pathways by insulin are also enhanced (for example, extracellular-regulated kinase-1). In contrast, protein kinase B activation in response to insulin is relatively unaffected. This result suggests that any transcriptional response above that of C2C12 cells is not likely to be due to phosphatidylinositol 3'-kinase and more likely due to Stat5 and extracellular-regulated kinase 1 or other pathways we have not yet identified.

In the hIR cell lines, insulin stimulates the expression of four putative Stat target genes from the SOCS/CIS family of proteins: SOCS-1, SOCS-2, SOCS-3, and CIS (24, 27). Insulin activation of a SOCS gene is not novel, as Emanuelli et al. (16) have recently demonstrated that insulin induces SOCS-3 mRNA expression in 3T3-L1 adipocytes. In these cells, however, they did not observe insulin induction of either SOCS-2 or CIS mRNA expression, suggesting the possibility of tissuespecific insulin regulation of SOCS/CIS genes. Emanuelli et al. (16) suggest that the insulin induction of SOCS-3 is mediated in part by Stat5 because there are Stat5 binding sites present in the SOCS-3 promoter, and insulin-stimulated expression of SOCS-3 in COS cells is enhanced by co-transfection of Stat5b. To determine whether insulin activation of SOCS-2 and SOCS-3 in hIR cells is mediated by Stat5, we compared the induction of these genes in a cell line that expresses WT-IR with a cell line expressing the mutant LA-hIR that does not
activate Stat5 but is otherwise equivalent to the WT-hIR. Insulin stimulation of SOCS-2 mRNA expression is not observed in either the LA-hIR myoblasts or myotubes. These results demonstrate that insulin induction of SOCS-2 mRNA expression in the hIR cells is mediated by Stat5. Insulin stimulation of SOCS-3 is reduced in LA-hIR myoblasts and not observed at all in LA-hIR myotubes. Thus, the insulin-stimulated expression of SOCS-3 in hIR cells is also mediated, at least in part, through Stat5 proteins. Our results support and extend those of Emanuelli et al. (16), who showed that co-transfection of Stat5b enhances the ability of insulin to stimulate SOCS-3 gene expression in COS cells transiently transfected with the IR. Furthermore, SOCS-3 was also found to directly interact with Tyr(P)-972 in the IR and prevent activation of Stat5b in co-transfection assays (16). SOCS-2 has also been reported to interact with the IR (43). These data are provocative, as hyperinsulinemia clearly leads to insulin resistance in vivo (4). The overall importance of SOCS-2 and SOCS-3 in normal regulation of insulin action remains unclear. The gigantism phenotype of the SOCS-2 knockout mice suggests that SOCS-2 functions as an inhibitor of the growth hormone/IGF-1 axis (44), whereas the early lethality in the SOCS-3 knockout due to excessive fetal liver hematopoiesis (45) precludes an evaluation of its role as a physiological inhibitor of insulin action.

Several studies suggest that CIS is a Stat5 target gene. A tandem pair of high affinity Stat5 binding sites is present in the proximal promoter of the CIS gene and is required for activation (46, 47). We therefore suspect that this will also be the case for insulin activation of CIS mRNA expression. In contrast, the expression of SOCS-1 is most potently stimulated by cytokines that predominantly activate Stat1 and Stat3 (24). The insulin induction of SOCS-1 is less likely to be mediated by Stat5. We are now in the process of determining the role of Stat5 in the induction of these and other insulin target genes.

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Insulin Induction of SOCS-2 and SOCS-3 mRNA Expression in C2C12 Skeletal Muscle Cells Is Mediated by Stat5
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