Transcriptional Regulation of Insulin-like Growth Factor-I by Interferon-γ Requires STAT-5b*

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Insulin-like growth factor (IGF)-I, a growth factor important for cell proliferation, cellular differentiation, and multiple metabolic functions, is regulated in vivo by growth factors and cytokines, but the mechanism(s) of regulation at the cellular level is not well understood. We now demonstrate, employing primary human dermal fibroblasts (CF), that the multipotent cytokine, interferon-γ (IFN-γ), can up-regulate IGF-I mRNA expression and that this regulation occurs via activation of the signal transducer and activator of transcription-5b (STAT-5b) pathway. IFN-γ (100 units/ml) treatment of CF cells resulted in a preferential, time-dependent activation of STAT-5b, although both STAT-5a and STAT-5b isoforms are present. The activated STAT-5b translocated to the nucleus within 30 min of treatment and induced an increase in IGF-I mRNA of 6 ± 1.0-fold, 3 h post-treatment, with a further increase to 8 ± 1.7-fold at 5 h. In contrast, IFN-γ treatment of primary human dermal fibroblasts with a nonfunctional STAT-5b (PF cells) resulted in activation of only STAT-5a and an increase of the IGF-I mRNA level of 1.7 ± 0.6-fold, 5 h post-treatment. The IFN-γ-induced regulation of the interferon regulatory factor-1 gene, whose expression is dependent on activated STAT-1, was the same between CF and PF cells. In summary, our results provide evidence of the following in human primary dermal fibroblasts: (a) IFN-γ preferentially activates STAT-5b, but, in the absence of a functional STAT-5b, STAT-5a is activated; (b) IFN-γ time-dependently up-regulates IGF-I mRNA expression; (c) the regulation of IGF-I requires an active STAT-5b, and activated STAT-5a cannot substitute for an inactive STAT-5b; and (d) STAT-5b has an essential role in the transcriptional up-regulation of IGF-I.

Interferon-γ (IFN-γ) is a cytokine with pleiotropic effects on immune (e.g. macrophages) and nonimmune (e.g. fibroblasts and epithelial) cells. The biological effects, which include regulation of cell proliferation, immune surveillance, and tumor suppression (1, 2), are mediated through the regulation of gene expression, via the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling pathways. The JAK-STAT-1 pathway has been shown to induce expression of gene products that are responsible for the majority of the pleiotropic effects of IFN-γ (3). Signal transduction is initiated when IFN-γ binds to a heterodimeric complex of IFN-γ receptors consisting of two IFN-γR1 and two IFN-γR2 subunits per cell. Association of Jak1 and Jak2 subsequently phosphorylates Tyr410 (in humans) on the IFN-γR1 subunits, creating docking sites for STAT-1 (4, 5). The recruited STAT-1, which binds through its v-src homology-2 domain, is tyrosine-phosphorylated by Jak-2, dimerizes, and translocates to the nucleus, where the activated dimer functions as a transcription factor (6). In addition to STAT-1, STAT-3 and STAT-5a/b can also be activated by IFN-γ (7, 8). The biological consequences of IFN-γ-induced activation of STAT-3 and -5, however, are not well understood.

We recently demonstrated that normal, human, primary dermal fibroblasts responded to growth hormone (GH) as well as IFN-γ (9). GH treatment induced the up-regulation of insulin-like growth factor-I (IGF-I), a secreted peptide important for somatic growth (10), and of IGF-binding protein-3 (IGFBP-3) mRNA through activation of the JAK-STAT signaling pathways (9). In particular, STAT-5b was implicated as a critical factor for the transcriptional regulation process, based on analysis of dermal fibroblasts from a patient with severe GH insensitivity resulting from an autosomal recessive mutation in the STAT-5b gene. As a consequence, the GH signaling pathway was disrupted, and IGF-I and IGFBP-3 expression was dysregulated, thereby demonstrating the importance of STAT-5b for GH-induced regulation of IGF-I and IGFBP-3 (9). These results raised the question of whether other cytokines capable of activating STAT-5b can also regulate IGF-I mRNA.

Limited data exist on the effects of cytokines on IGF-I expression. In murine macrophages, IFN-γ was shown to transcriptionally down-regulate IGF-I (11) through a STAT-1-dependent mechanism (12), and IFN-α/β down-regulated IGF-I mRNA expression in rat glioma cells (13). More recently, tumor necrosis factor-α (TNF-α) was demonstrated to similarly down-regulate IGF-I mRNA in rat myoblasts (C2C12) (14). Interestingly, the down-regulation was suggested to be mediated via a Jun N-terminal kinase pathway, but independent of STAT-5 phosphorylation (14). We now demonstrate that, in contrast to the murine cell models, IFN-γ, similar to GH, up-regulates IGF-I mRNA in human dermal fibroblasts and that this up-regulation is STAT-5b-dependent.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies directed against tyrosine-phosphorylated STAT-1 and tyrosine-phosphorylated STAT-5 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against STAT-5a (L-
20), STAT-5b (G-2), STAT-1, and interferon regulatory factor-1 (IRF-1) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-STAT-5a/b polyclonal antibody (OPA1-03010) was from Affinity BioReagents (Golden, CO); and goat serum (for blocking in immunofluorescence), anti-FLAG M2 antibody, and anti-FLAG-M2-agarose gel (for immunoprecipitation) from Sigma. Secondary antibodies (anti-mouse IgG and anti-rabbit IgG) were obtained from Amersham Biosciences. For immunofluorescence, Hoechst 33342 (Molecular Probes, Inc., Eugene, OR), goat anti-mouse IgG, or anti-rabbit IgG conjugated to fluorescein (Molecular Probes) was used.

Cell Culture—Primary fibroblast cultures, established from skin biopsies taken from a GH-insensitive patient (PF cells) and a normal 30-year-old female (CF cells), have been previously described (9). PF cells from a patient homozygous for a missense mutation, that resulted in a Pro to Ala substitution at amino acid 630 (A630P) in the STAT-5b gene (9). Additional normal human dermal fibroblasts from a 31-year-old female were purchased from BioWhittaker (Walkersville, MD) (NHDF 8560). The fibroblasts were cultured as previously described (9). COS-7 cells were from ATCC and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). For all experiments, cells were serum-starved 24 hr prior to treatment with either IFN-γ (Roche Applied Science) or recombinant human GH (a generous gift from Genentech, Inc., South San Francisco, CA).

Immunofluorescence Analysis—Poly-L-lysine-coated, eight-chamber slides (Nalge Nunc International, Rochester, NY) were seeded with 3000 cells/chamber. After 24 hr, the cells were washed with phosphate-buffered saline and serum-starved overnight. Duplicate chambers were treated with IFN-γ (10 ng/ml) or GH (1 μg/ml; 500 ng/ml gave similar results) for the times indicated, and the reactions were terminated by washing with phosphate-buffered saline. Cells were fixed with 100% ice-cold methanol (10 min, −20 °C) and processed according to the manufacturer's recommendations (Cell Signaling Technology, Beverly, MA). Primary antibodies employed were anti-phospho-STAT-1 (anti-p-STAT-1) (1:500 dilution) and anti-pSTAT-5 (1:500 dilution). Secondary antibodies were used at a 1:500 dilution. Hoechst (1:1000 dilution) was used for nonspecific staining of the nucleus. Immunofluorescence was observed as previously described (15).

Plasmids—The FLAG tag was introduced into the STAT-5b cDNA sequences by PCR-amplifying ~400 bp of the N-terminal STAT-5b cDNA using 5'-ggtaggactaacaagcacgactgagggtgtggtagacaagctcagc- gcagctccaa-3' (forward primer; FLAG sequence is underlined) and 5'-acagctgcagctctaaagaa-3' (reverse primer). The resultant PCR product was cloned into pCR2.1-TOPO (TOPO TA Cloning; Invitrogen), and the correct insertion of the FLAG tag was confirmed by sequencing. The FLAG tag was introduced into the STAT-5b cDNA sequences by PCR-amplifying ~400 bp of the N-terminal STAT-5b cDNA using 5'-ggtaggactaacaagcacgactgagggtgtggtagacaagctcagc- gcagctccaa-3' (forward primer; FLAG sequence is underlined) and 5'-acagctgcagctctaaagaa-3' (reverse primer). The resultant PCR product was cloned into pCR2.1-TOPO (TOPO TA Cloning; Invitrogen), and the correct insertion of the FLAG tag was confirmed by sequencing.

Transfection Experiments—COS-7 cells were grown to ~50% confluence in 100-mm plates. Cells were transiently transfected with vector, pcDNA3.1, or vector carrying F-STAT-5b or F-STAT-5b(A630P), using TransIT-TL (Mirus, Madison, WI). After a 24-h transfection, cells were washed and serum-starved (Dulbecco's modified Eagle's medium with 0.1% bovine serum albumin) for a further 24 hr prior to treatment with IFN-γ (100 units/ml). Total RNA and cell lysates were collected 1 hr post-treatment. In some cases, total cell lysates (~320 μg) from the transfected COS-7 cells were immunoprecipitated with anti-FLAG-M2 agarose gel by standard methods prior to Western immunoblot analysis.

RESULTS

IFN-γ Specifically Activates STAT-5b in Normal Dermal Fibroblasts—We recently demonstrated that IFN-γ robustly activates the JAK/STAT signaling pathway in normal human dermal fibroblasts (CF cells), leading to phosphorylation of not only STAT-1 but also STAT-3 (9). The effect of IFN-γ on the STAT-5 signaling pathway in these cells, however, was not known. CF cells were therefore treated with IFN-γ (100 units/ml), and the activation of STAT-5 was examined. The activation of STAT-1 was confirmed, with pSTAT-1 readily detected within 15 min of IFN-γ treatment (Fig. 1A). STAT-5, like STAT-1 (and STAT-3; data not shown), was rapidly tyrosine-phosphorylated, and phosphorylation was sustained for at least 120 min (Fig. 1B). Interestingly, compared with growth hormone-treated CF cells (9), the activation of STAT-5 by IFN-γ was considerably more robust (Fig. 1C). Increasing concentrations of GH did not enhance phosphorylation with a panel of anti-STAT-5 antibodies (Fig. 1D). As shown, STAT-5a and STAT-5b were successfully separated, and the
Fig. 1. IFN-γ activates tyrosine phosphorylation of STAT-5. Primary dermal fibroblasts were treated with 100 units/ml IFN-γ or 500 ng/ml GH, and cell lysates were collected at the times indicated. Western immunoblot analysis was performed to determine the phosphorylation status of STAT-1 and STAT-5. A, IFN-γ-treated cells were immunoblotted for tyrosine phospho-STAT-1 (upper panel); both the 91- and 84-kDa pSTAT-1 forms were detected. The blot was then stripped and reprobed with antibody against total STAT-1 (bottom panel). B, immunoblot of IFN-γ-treated cells; phospho-STAT-5 (upper panel) was stripped and reprobed using antibody specific for STAT-5b. The tyrosine phospho-STAT-5 antibody cannot distinguish between pSTAT-5a and pSTAT-5b. C, cells were treated with increasing concentrations of GH or with IFN-γ for 30 min, and cell lysates were immunoblotted for pSTAT-5. The nonspecific immunoreactive band (arrow) indicates that protein loading was equivalent. D, immunoblot analysis for the STAT-5 isoform that is activated by IFN-γ in cell lysates from CF cells. 250 µg of cell lysates (untreated or IFN-γ-treated, 60 min) were loaded into single preparative wells, size-fractionated by 7% SDS-PAGE, and electroblotted to nitrocellulose membranes, and membranes were cut into strips. Each strip was immunoblotted with the indicated antibody and reassembled prior to enhanced chemiluminescence treatment. Correct alignment of the reassembled blot was achieved by marking the blot with reference lines drawn prior to cutting of the membranes for immunoblotting. STAT-5a and STAT-5b bands are indicated by arrows.

The respective specific isoform of STAT-5 that was phosphorylated could be determined. Under uninduced conditions, neither STAT-5a nor STAT-5b was activated (top panel). Upon IFN-γ treatment (bottom panel), it was apparent that only STAT-5b was phosphorylated (Fig. 1D).

IFN-γ-induced Nuclear Localization of pSTAT-5—Activated STATs are known to translocate from the cytoplasm to the nucleus. Immunofluorescent analysis of treated and untreated CF fibroblasts indicated that within 30 min of IFN-γ treatment, pSTAT-5 accumulated in the nucleus, as did pSTAT-1 (Fig. 2A). Unlike pSTAT-1, which remained nuclear for at least 90 min, depletion of nuclear pSTAT-5 was observed at 90 min. It is noted that for growth hormone-treated cells, the immunofluorescence analysis was not sufficiently sensitive to detect nuclear pSTAT-5 (Fig. 2B). The results correlated with the robust activation of STAT-5b by IFN-γ, compared with that by growth hormone. Altogether, these results suggest that in dermal fibroblasts, of the two STAT-5 isoforms, IFN-γ preferentially activates STAT-5b, which subsequently translocates to the nucleus.

IFN-γ Up-regulates IGF-I mRNA Expression—Recently, we demonstrated that the transcriptional regulation of IGF-I by growth hormone in CF cells was STAT-5b-dependent (9). We therefore hypothesized that since IFN-γ preferentially activates STAT-5b over STAT-5a, IGF-I may be a target gene for IFN-γ-activated pSTAT-5b. Preliminary analysis by semiquantitative RT-PCR amplification supported this hypothesis, with IGF-I mRNA observed to be up-regulated 3 and 5 h post-IFN-γ treatment (Fig. 3A). IRF-1, a STAT-1-dependent transcription factor known to be up-regulated by IFN-γ (17), was up-regulated within 1 h. In contrast, expression of IGFBP-3 mRNA was not altered by IFN-γ treatment (Fig. 3A).

RTQ-PCR confirmed that IGF-I mRNA was up-regulated upon IFN-γ treatment. As shown in Fig. 3B, IGF-I mRNA progressively increased in a time-dependent manner, with 6 ± 1.0-fold (S.E.) and 8 ± 1.7-fold induction 3 and 5 h post-treatment, respectively. Similar results were obtained from a second (commercially purchased) normal dermal fibroblast cell line (data not shown), demonstrating that it was not a phenomenon unique to our CF cells. In cells treated with IFN-γ for 24 or 48 h, IGF-I mRNA levels were similar to those of untreated cells (data not shown), suggesting that the up-regulation is transient. The up-regulation of IGF-I mRNA, furthermore, was not dependent on de novo protein synthesis, since the induction was not inhibited by cycloheximide treatment (data not shown). Altogether, the results demonstrate that IGF-I is a
target gene of IFN-\(\gamma\) signal transduction and that the increase in IGF-I mRNA is likely to be the result of IFN-\(\gamma\) activation of STAT-5b (see below).

**FIG. 2. Immunofluorescence of pSTAT-1 and pSTAT-5 in IFN-\(\gamma\)-treated cells.** Primary dermal fibroblasts were treated with IFN-\(\gamma\) for the times indicated, fixed, and labeled as indicated under “Experimental Procedures.” A, nuclear localization of pSTAT-1 and pSTAT-5 over time. In untreated cells over the time course, neither nuclear pSTAT-1 nor pSTAT-5 was detected (data not shown). B, comparison of GH treatment with IFN-\(\gamma\) treatment on the nuclear localization of pSTAT-1 and pSTAT-5, 30 min post-treatment.

IFN-\(\gamma\) Activates STAT-5a in Dermal Fibroblasts Carrying Mutant STAT-5b(A630P)—To elucidate whether IFN-\(\gamma\)-induced phosphorylation of STAT-5b is responsible for regulation
of IGF-I mRNA, we compared the response of CF cells with that of a human primary dermal fibroblast cell line carrying a naturally occurring, nonfunctional STAT-5b (9). We recently characterized these novel fibroblasts, designated PF cells, and demonstrated that they carry a homozygous missense mutation in the STAT-5b gene, which results in expression of an aberrant STAT-5b protein (schematically presented, Fig. 4A) (9). The mutated protein, designated STAT-5b(A630P), contains an Ala630→Pro substitution within the v-src homology-2 domain. Unlike wild-type STAT-5b, the STAT-5b(A630P) is poorly detected by immunoblotting techniques employing STAT-5b-specific antibody (Fig. 4B, top panel) and could not be activated by the growth hormone receptor signaling pathway (9). The PF cells, however, had concentrations of STAT-5a comparable with that in CF cells (Fig. 4B, bottom panel). In addition, IFN-γ induced a phosphorylation pattern (STAT-1, STAT-3, and extracellular signal-regulated kinase 1/2) in PF cells identical to that observed in CF fibroblasts (9). The PF cells were, therefore, ideal for determining whether the up-regulation of IGF-I mRNA by IFN-γ, like growth hormone, is STAT-5b-dependent.

Treatment of PF cells with IFN-γ unexpectedly resulted in a detectable phospho-STAT-5 time-dependent pattern similar to that observed in CF fibroblasts (Fig. 4C, top panel). This intriguing result suggested that either STAT-5a was now phosphorylated (unlike in CF cells) or that the mutant STAT-5b(A630P) could be activated by select cytokines such as IFN-γ but not GH (Fig. 4C, bottom panel) (9). Detailed analysis of the phospho-STAT-5 product in PF cells (Fig. 4D) indicated the former hypothesis was correct (i.e. STAT-5a was preferentially phosphorylated in PF cells).

Recombinant FLAG-STAT-5b(A630P) Is Not Activated by IFN-γ—To further support the observation that mutant STAT-5b(A630P) cannot be activated by IFN-γ (9), the STAT-5b cDNAs, cloned from CF and PF cells (9), were N-terminally FLAG-tagged for expression in the COS-7 cell system. COS-7 cells have undetectable STAT-5b, and our initial characterization of COS-7 cells indicated that IFN-γ, but not growth hormone, induced the JAK/STAT signaling cascade (9) (data not shown), confirming the presence of sufficient functional endogenous IFN-γ receptors but not growth hormone receptors.

COS-7 cells were transfected with vector (pcDNA3.1) or vector carrying either wild-type FLAG-STAT-5b or FLAG-STAT-5b(A630P) and treated with IFN-γ. The efficiency of transfection and mRNA expression was equivalent, as determined by limiting RT-PCR analysis (Fig. 5A). As shown in Fig. 5B (lanes 1 and 2), IFN-γ induced phosphorylation of endogenous STAT-5a in nontransfected cells. An equivalent pSTAT-5 pattern was detected in COS-7 cells transfected with pcDNA3.1 (Fig. 5B, lanes 3 and 4). Overexpression of wild-type FLAG-STAT-5b resulted in low, basal, constitutive phosphorylation of the recombinant STAT-5b (Fig. 5B, lane 5). Treatment with IFN-γ (1 h) significantly increased detectable phospho-FLAG-STAT-5b (Fig. 5B, lane 6). In contrast, cells transfected with FLAG-STAT-5b(A630P) cDNA generated a phospho-STAT-5 pattern (Fig. 5B, lanes 7 and 8) similar to untransfected and vector-transfected cells. Interestingly, the recombinant human FLAG-STAT-5b proteins (both wild-type and mutant) were readily distinguishable from endogenous STAT-5a, since the recombinant proteins ran at a higher molecular weight on SDS-PAGE than does the endogenous protein. Immunoblotting with anti-STAT-5a indicated that total protein loading was the same and that endogenous STAT-5a was unaffected by the transfection process.
Sequential stripping and reprobing of the pSTAT-5 blot with anti-FLAG antibody and anti-STAT-5b-specific antibody (Fig. 5B) indicated that the mutant STAT-5b protein was consistently less well immunodetected than wild-type (a -4-fold difference in detection). This difference in detection may account for the inability to detect phospho-FLAG-STAT-5b(A630P). Therefore, increased loading of cell lysates from COS-7 cells overexpressing FLAG-STAT-5b(A630P) (immunologically equivalent to cell lysates of FLAG-STAT-5b protein) were analyzed, and as shown in Fig. 5C, phosphorylated mutant STAT-5b(A630P) was still not detectable. Finally, the overexpressed recombinant proteins were concentrated by immunoprecipitation with anti-FLAG antibody, but, again, only phosphorylated wild-type STAT-5b was detectable (Fig. 5D).

Altogether, these results demonstrate that mutant STAT-5b(A630P) is not activated by IFN-γ treatment.

**IGF-I mRNA Is Not Up-regulated by IFN-γ in PF Cells**—Since IFN-γ, like GH, cannot activate mutant STAT-5b(A630P), we determined whether the IFN-γ-induced transcriptional up-regulation of IGF-I expression was affected in PF cells. Compared with CF cells, where induction of IGF-I mRNA was 8 ± 1.7-fold (see also Fig. 3B), up-regulation of IGF-I mRNA was not detected in PF cells treated with IFN-γ (Fig. 6A). Over the 5-h time course, induction of IGF-I mRNA levels remained less than 2-fold in PF cells. Similar results were obtained with CF and PF cells treated with other cytokines such as IFN-α/β (data not shown). In contrast, IFN-γ induced similar phospho-STAT-1 profiles (Fig. 6B) and equivalent up-regulation of IRF-I, at the mRNA (Fig. 6C) and protein levels (Fig. 6D) in CF and PF cells. Altogether, the results are
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**Figure 5.** Human recombinant mutant STAT-5b(A630P) is not activated by IFN-\(\gamma\) when overexpressed in COS-7 cells. Expression vector, pcDNA3.1, carrying N-terminally FLAG-tagged wild-type STAT-5b cDNA (WT) or mutant STAT-5b(A630P) cDNA (A630) was transfected into COS-7 cells, and total RNA, as well as cell lysates, were collected after IFN-\(\gamma\) treatment (100 units/ml, 60 min). A, ethidium-stained agarose gel of PCR products. RT reactions of total RNA collected were employed for limiting PCR amplification. RT reactions were diluted 1:9, 1:49, and 1:99 prior to PCR amplification of FLAG-STAT-5b and 18S. B, Western immunoblot analysis of cell lysates (30 \(\mu\)g/lane) for pSTAT-5. The immunoblot was stripped and reprobed with, consecutively, anti-FLAG, anti-STAT-5b, and anti-STAT-5a. C, immunoblot analysis for phosphorylation of mutant FLAG-STAT-5b(A630P). Immunologically equivalent concentrations of FLAG-STAT-5b (30 \(\mu\)g) and FLAG-STAT-5b(A630P) (~120 \(\mu\)g) were immunoblotted for pSTAT-5. D, total cell lysates (320 \(\mu\)g) were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted for phospho-STAT-5, stripped, and reprobed with anti-FLAG antibody. Note that the \(\alpha\)-pSTAT-5 was not completely stripped in lane 4.

**DISCUSSION**

The mechanism of regulating IGF-I expression by cytokines in humans has been difficult to study, in part because of the lack of an appropriate cell model. Only two human cell models have been reported. In human umbilical vein endothelial cells, interleukin-1 (IL-1) was shown to induce a transient transcriptional 4.5-fold up-regulation of IGF-I (18), but the mechanism(s) was not demonstrated. In another study, primary dermal fibroblasts were used to investigate the effects of IFN-\(\gamma\) on IGF-I mRNA expression (19). No regulation was detected, but the study was complicated by the presence of excess fetal bovine serum (10%) in the experimental conditions. The majority of such studies, however, have employed rodent cell models, where cytokines appear to have inhibitory effects on IGF-I mRNA expression (11, 13, 14, 20–22). For example, IFN-\(\gamma\) significantly inhibited IGF-I mRNA transcription in a time- and dose-dependent manner in mouse macrophages (11, 12), and in rat glioma cells, IFN-\(\alpha/\beta\) also down-regulated IGF-I mRNA expression (13). In contrast, no alterations in IGF-I mRNA were observed in IFN-\(\gamma\)-treated rat aortic vascular smooth muscle cells (21) or C2C12 cells (14, 22). Similarly, the cytokine TNF-\(\alpha\) also inhibited IGF-I expression, as was demonstrated in vascular smooth muscle cells (85% reduction at 24 h) (21) as well as C2C12 cells (14, 22). Interestingly, the inhibitory effects of IFN-\(\alpha/\beta\), IFN-\(\gamma\), and TNF-\(\alpha\) were sensitive to cycloheximide, suggesting that de novo protein synthesis is required. Other cytokines tested (IL-6 and IL-1\(\beta\)) in these same rodent cells did not alter IGF-I mRNA expression (21, 22). The mechanism(s) of these inhibitions are not known, although recently, the Jun N-terminal kinase pathway was implicated in the TNF-\(\alpha\)-induced down-regulation of IGF-I expression in C2C12 cells (14), and the IFN-\(\gamma\)-induced down-regulation of IGF-I expression in primary mouse macrophages appeared to require STAT-1 (12). In the same bone marrow-derived mouse macrophages, Th2 cytokines such as IL-4 and IL-13 up-regulated IGF-I expression in a STAT-6-dependent manner (12).

An exception to the transcriptional down-regulation of IGF-I mRNA by cytokines is the effects of GH. GH, a class I cytokine, induced the up-regulation of IGF-I mRNA in both rodents and humans. For example, IFN-\(\alpha/\beta\) and IFN-\(\gamma\) (9) cytokines. With IFN-\(\gamma\)-treatment, the increase in IGF-I mRNA was time-dependent but transient, with a 6 ± 1.0-fold induction observed by 3 h post-treatment and a further increase to 8 ± 1.7-fold 5 h after treatment. This is contrary to that observed in rodent macrophages (11), most likely reflecting cell type and/or species differences. The -fold induction was significantly higher than that observed when the dermal fibroblasts were treated with GH (1.8 ± 0.6-fold, 3 h post-treatment (9)). Further, de novo protein synthesis was not required (data not shown), suggesting that positive and negative regulations of IGF-I expression are via different mechanisms. IFN\(\alpha/\beta\) also up-regulated IGF-I mRNA expression to
levels similar to that observed for IFN-γ (data not shown). Thus, IGF-I mRNA in human dermal fibroblasts not only is readily detectable, but the signaling pathways leading to its regulation are apparently intact.

The differences in induction of IGF-I mRNA expression between IFN-γ and GH correlated with the degree the JAK-STAT signaling pathway was activated. We previously demonstrated that the phosphorylation of extracellular signal-regulated kinase 1/2 by GH and IFN-γ was similar, whereas the activation of STAT-1 and STAT-3 by IFN-γ was considerably more robust than that observed with GH (9). The same trend for the activation of STAT-5 was observed in this report. It is not clear at present whether this is due to a higher concentration of functional IFN-γ receptors compared with GH receptors. Not only was pSTAT-5 more readily detected when cells were treated with IFN-γ, but immunocytochemical analysis of treated fibroblasts indicated that pSTAT-5 and pSTAT-1 localized to the nucleus after IFN-γ, but not GH, treatment. The lack of detectable nuclear pSTAT-5 (and pSTAT-1) under GH-induced conditions indicates that our immunofluorescent technique was not sufficiently sensitive to detect low concentrations of nuclear phosphorylated STAT proteins. Indeed, nuclear extracts of GH-treated fibroblasts suggest that pSTAT-5 is present in the nucleus (data not shown).

IFN-γ, like GH, specifically activated STAT-5b. This is contrary to suggestions that, of the two isoforms, STAT-5a is preferentially activated by IFN-γ (7). In human dermal fibroblasts, both STAT-5a and STAT-5b are present, although the relative amount of each is not known. In our human dermal fibroblast cell line that lacked wild-type STAT-5b (PF cells), it was STAT-5a that was equivalently activated by IFN-γ. Since activation of STAT-5a/b involves initial docking of the relevant STAT-5 to Tyr440 in the STAT recruitment site (SRS) on IFN-γR1 (8), our results suggest an absolute preference for recruitment of STAT-5b over STAT-5a by the IFN-γ receptors. This preference may be due to intrinsic properties in the structure(s) of the receptor and/or the STAT-5 that dictate docking specificities. Another possibility is that the ratio of STAT-5b to STAT-5a is high in the fibroblasts, and STAT-5b has, therefore, a correspondingly higher probability of being recruited. Investigations are currently under way to evaluate the various possibilities. Significantly, in the PF cells, even as IFN-γ activates
STAT-5a when wild-type STAT-5b is absent, the GH signaling system, in the same PF cells, does not activate STAT-5a. Hence, in our cell system, STAT-5a cannot substitute for STAT-5b. Furthermore the results suggest that the recruitment of specificity in the STAT-5a/b to receptors. Overall, tyrosine phosphorylation of STAT-5 in response to IFN-γ is clearly dependent on the cell type, since it has been shown that pSTAT-5 is not detected in HeLa cells, despite the expression of both STAT-5 isoforms (7). In C2C12 cells, GH apparently activates both STAT-5a and STAT-5b (24).

The up-regulation of IGF-I mRNA induced by GH and IFN-γ in CF cells correlated with the level of activated STAT-5b induced by these cytokines. This, together with a mutant STAT-5b(A630P) that is not activated by IFN-γ (both in dermal fibroblasts and when overexpressed in COS-7 cells (present work) (9)) and the lack of IFN-γ-induced regulation of IGF-I mRNA in PF cells, confirmed that phosphorylated STAT-5b is critical for the up-regulation of IGF-I mRNA. In addition, the lack of detectable pSTAT-5a in CF cells and the preferential activation of STAT-5a by IFN-γ in PF cells suggest that pSTAT-5a is unlikely to participate in the regulation of IGF-I mRNA, at least in human dermal fibroblasts. Our results affirm the importance of STAT-5b in up-regulating IGF-I expression, based on cumulative data from rodents (27–30) and humans (9). Other STATs, such as STAT-6, could also be involved (12), although IFN-γ is not known to activate STAT-6. In cases where IFN-γ does not appear to regulate IGF-I expression (C2C12 (22) and vascular smooth muscle cells (21)) or down-regulates IGF-I (11), the phosphorylation status of STAT-5b was not determined. For the TNF-α-induced down-regulation of IGF-I expression observed in C2C12 cells, it was shown that no pSTAT-5 was detected, and, when co-treated with GH, TNF-α was still able to down-regulate IGF-I, even in the presence of pSTAT-5 (14). It was unclear, however, which isoform of STAT-5 was phosphorylated.

It is apparent that the functions of STAT-5a and STAT-5b are not interchangeable. This is most clearly demonstrated in our use of the PF cells. The PF cells were derived from a patient (work) (9)) and the lack of IFN-γ-induced down-regulation of IGF-I by IFN-γ and/or Raf-1 pathways, as was shown for c-myc (33). Further studies employing the PF cells will aid in elucidating the biological importance of STAT-1-independent STAT-5b-dependent, IFN-γ-regulated genes.

In summary, we have demonstrated the following in human primary dermal fibroblasts: (a) IFN-γ, like GH, specifically activates STAT-5b, but, unlike GH, STAT-5a can be recruited and activated by IFN-γ receptor complexes in the absence of a functional STAT-5b; (b) IFN-γ, like GH, time-dependently up-regulates IGF-I mRNA expression; (c) the regulation of IGF-I mRNA requires an active STAT-5b, and pSTAT-5a cannot substitute for an inactive STAT-5b; and (d) STAT-5b has an essential role in the transcriptional up-regulation of IGF-I.

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