SOCS-1 Protein Prevents Janus Kinase/STAT-dependent Inhibition of β Cell Insulin Gene Transcription and Secretion in Response to Interferon-γ

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In the pathogenesis of type I diabetes mellitus, activated leukocytes infiltrate pancreatic islets and induce β cell dysfunction and destruction. Interferon (IFN)-γ, tumor necrosis factor-α and interleukin (IL)-1β play important, although not completely defined, roles in these mechanisms. Here, using the highly differentiated βTet insulin-secreting cell line, we showed that IFN-γ dose- and time-dependently suppressed insulin synthesis and glucose-stimulated secretion. As described previously IFN-γ, in combination with IL-1β, also induces inducible NO synthase expression and apoptosis (Dupraz, P., Cottet, S., Hamburger, F., Dolci, W., Felley-Bosco, E., and Thorens, B. (2000) J. Biol. Chem. 275, 37672–37678). To assess the role of the Janus kinase/signal transducer and activator of transcription (STAT) pathway in IFN-γ intracellular signaling, we stably overexpressed SOCS-1 (suppressor of cytokine signaling-1) in the β cell line. We demonstrated that SOCS-1 suppressed cytokine-induced STAT-1 phosphorylation and increased cellular accumulation. This was accompanied by a suppression of the effect of IFN-γ on: (i) reduction in insulin promoter-luciferase reporter gene transcription, (ii) decrease in insulin mRNA and peptide content, and (iii) suppression of glucose-stimulated insulin secretion. Furthermore, SOCS-1 also suppressed the cellular effects that require the combined presence of IL-1β and IFN-γ: induction of nitric oxide production and apoptosis. Together our data demonstrate that IFN-γ is responsible for the cytokine-induced defect in insulin gene expression and secretion and that this effect can be completely blocked by constitutive inhibition of the Janus kinase/STAT pathway.

Development of type I diabetes is initiated by the infiltration of the islets of Langerhans by immune and inflammatory cells. The activation of leukocytes, following interactions with β cells autoantigens, results in the synthesis of cell-surface and secreted mediators such as the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-1β. These participate in the induction of the insulin-secreting β cell dysfunction and destruction (2). However, despite the fact that these cytokines have been found in the insulitis of non-obese diabetic mice and in the pancreas of type I diabetic patients, their exact role in the pathogenesis of type I diabetes remain unclear (for a review, see Ref. 3).

Insulitis in non-obese diabetic mice (4) as well as in patients with type I diabetes (5, 6) is mediated by T-helper type 1 (Th1) cells producing IFN-γ and IL-2. The role of IFN-γ in the development of type I diabetes has been evaluated in several studies, but its role in diabetes induction is not clearly defined. For instance, β cell injury was limited, and diabetes did not occur in the RIP-LCMV diabetic mouse model when the mice were deficient in IFN-γ (7). Similarly, Wang et al. (8) reported that non-obese diabetic mice lacking IFN-γ receptor showed a marked inhibition of insulitis and no signs of diabetes. In contrast, others have shown that deficiency in IFN-γ (9) or in IFN-γ receptor (10) impact only mildly on the onset of diabetes in non-obese diabetic mice.

Whether IFN-γ affects islet β cells directly or indirectly through the activation of other cells such as cytotoxic T-cells or through the up-regulation of other cytokotic factors is also not firmly established. In vitro, many direct effects of IFN-γ on β cells have been demonstrated, including the up-regulation of major histocompatibility complex class I (11, 12), intracellular cell adhesion molecule-1 (13), and iNOS (14). It has been proposed that NO production could cause β cells destruction. However, in human β cells, the role of NO is not established and may be in fact only partly, or not at all, involved in cell death (15–17). In mouse β cells or insulinomas, NO appears to be only one of the mechanisms by which these cells are destroyed (18, 19). Another effect of IFN-γ is to impair insulin secretion, as shown with rodent islets tested in vitro (12, 20). In human islets, however, no decrease in insulin mRNA and peptide levels has been observed, whereas a weak reduction in GSIS was measured (15).

The signal transduction pathway initiated by binding of IFN-γ to its receptor leads first to JAK1 and JAK2 activation and their association with the IFN-γ receptor (21–23). JAK kinases then phosphorylate IFN-γ receptor on specific tyrosines, which serve as docking sites for the transcription factor STAT-1. Upon activation by phosphorylation, STAT-1 molecules homodimerize and translocate into the nucleus to activate the transcription of target genes. Negative regulation

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1 The abbreviations used are: IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signaling; GSIS, glucose-stimulated insulin secretion; iNOS, inducible NO synthase; MTS, 3(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
of JAK/STAT signaling has just begun to be studied, and recently a family of proteins has been identified as the SOCS (suppressor of cytokine signaling) family. The first member of this family was denoted CIS, for cytokine-inducible SH2-containing protein, and has been shown to bind to phosphorylated tyrosines on multiple cytokine receptors (24, 25). Subsequently, three groups have independently identified the second family member denoted SOCS-1, JAB (JAK-binding protein), and SSI-1 (Stat-induced Stat inhibitor-1), respectively (26–28).

SOCS-1 has been shown to inhibit JAK kinase by binding to its activation loop, thus preventing the access of substrates and/or ATP to the enzyme catalytic pocket (29). Moreover, IFN-γ itself has been shown to be a potent inducer of SOCS-1 in a wide variety of cell lines (30, 31), suggesting that SOCS-1 acts as a negative feedback regulator of JAK/STAT signaling. Studies with SOCS-1 knockout mice have revealed its important role in negative regulation of IFN-γ action; animals deficient in SOCS-1 showed severe defects, the most prominent features being growth retardation, impaired T-cell and B-cell development, excessive IFN-γ responses, and early mortality (32–35).

In the present study, we evaluated the role of IFN-γ on inducing β cell dysfunction and apoptosis. We used as a model system the conditionally immortalized βTc-Tet cells. These cells display a normal glucose dose-dependent stimulation of insulin secretion, they can be growth-arrested in the presence of tetracycline, and, when transplanted in diabetic mice, they can maintain normoglycemia for several months (36). We previously genetically modified these cells to express the Bel-2 gene. These modified cells, referred to as CDM3D, display improved resistance to stress-induced apoptosis, show increased viability at high cell density, and grow more vigorously in cell culture than the parental cells (37). More recently, we further engineered the CDM3D cells for overexpression of proteins interfering with the IL-1β signaling pathway. These proteins were dominant-negative mutants of MyD88 (MyD88-Toll and MyD88-lpr), an adaptor protein linking the IL-1 receptor to downstream signaling molecules. We demonstrated an increased resistance of these cells to IL-1β/IFN-γ-induced iNOS expression and nitrite production and an increased resistance to cytokine-induced apoptosis but no resistance to impaired GSIS induced by cytokines (1).

Here we demonstrate that in CDM3D cells, IFN-γ alone is sufficient to induce a sustained, time- and dose-dependent decrease in insulin mRNA levels, peptide cellular content and glucose-stimulated insulin secretion. We show that the stable expression of SOCS-1 in CDM3D cells blocks IFN-γ-induced STAT-1 phosphorylation and increased cellular accumulation and prevents the negative effect of the cytokine on insulin gene expression and stimulated secretion. Furthermore, the continuous expression of SOCS-1 also blocks the cellular effects that
require the combined presence of IFN-γ and IL-1β: an increase in NO production and an induction of apoptosis. These data characterize the role of IFN-γ on β cell dysfunction and identify a molecular way of preventing these negative cytokine effects.

MATERIALS AND METHODS

Cell Culture—CDM3D cells are βTC-Tet cells (36) that have been modified to overexpress Bcl-2 (37). They were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 25 mM glucose and supplemented with 15% horse serum (Animex, BioConcept, Allschwil, Switzerland), 2.5% fetal bovine serum (Life Technologies, Inc.), 10 mM Hepes, 1 mM sodium pyruvate, 2 mM glutamine, at 37 °C with 5% CO2. For nitrite secretion measurements, medium was changed to RPMI 1640 (Life Technologies, Inc.), which has a lower level of nitrite content.

Analysis of Insulin and SOCS-1 Expression by Northern Blot—Total RNA was isolated and analyzed by Northern blot as described previously using specific probes prepared by random-primer labeling (38). Densitometry scanning of the blots was performed using the Bio-Rad phosphorimaging device, IMAGE FX.

Preparation of Lentiviral Vectors and Infection of CDM3D Cells—The human SOCS-1 cDNA, kindly provided by Dr. R. W. Furlanetto (Rochester, NY), contains a FLAG epitope. It was subcloned into a modified SIN-18-phosphoglycerate kinase-woodchuck hepatitis virus vector (SIN-18-PGK-WHV) (39, 40) that contains a neomycin resistance gene downstream of an internal ribosome entry site from encephalomyocarditis virus, kindly provided by Dr. N.Déglon (University Hospital, Lausanne, Switzerland). High titer stocks of lentiviral vectors packaged by the multiply attenuated lentivirus CMV-MR9.1 and pseudotyped with vesicular stomatitis vector (plasmid pMD-G) were prepared by transient transfection of 293T cells as described (37, 41, 42). SOCS-1 virus titer was determined by p24 enzyme-linked immunosorbent assay (PerkinElmer Life Sciences) according to the manufacturer’s instructions. CDM3D cells were transduced with a multiplicity of infection of 10–20. Selection of the pool of infected cells was initiated 48 h after infection by adding 800 μg/ml of G418 for 1 week, followed by 400 μg/ml of the drug for an additional week.

Insulin Secretion—Cells were plated in 24-well dishes at a density of 105 cells/well 48 h before incubation with cytokines. Following cytokine exposure for 48 h, cells were then incubated for 1 h in Hepes-buffered Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.5% bovine serum albumin with 2.8 mM glucose and 200 μM isobutylmethylxanthine (Sigma). The medium was changed, and cells were incubated again for 1 h in Hepes-buffered Krebs-Ringer bicarbonate buffer/0.5% bovine serum albumin containing 2.8 or 16.7 mM glucose and isobutylmethylxanthine. Secreted insulin was quantitated by radioimmunoassay (Linco Research, Labodia, Yens, Switzerland) as described (37, 43). Intracellular insulin was measured in acid-ethanol cell lysates. Briefly, cells were lysed in 250 μl of 75% ethanol, 1.5% concentrated hydrochloric acid. Aliquots of cell lysates were also analyzed for DNA content (44) to normalize the secretion data. The lysates in acid ethanol were neutralized with ½% volume of 1 M Na2CO3, and DNA content was determined by fluorescence using a Fluoroskan-II microplate fluorometer (Lab systems, Helsinki, Finland) with an excitation filter set at 355 nm and an emission filter set at 460 nm.

Cytokine-induced Nitrite Accumulation—Nitrite accumulation in the conditioned culture medium was detected spectrophotometrically (at 540 nm) by the Griess reaction in the presence of 1 mM sulfanilamide and 0.1 M HCl (45). The concentrations (pmol of NO2/mg protein) were measured by the Griess reaction in the presence of 1 mM sulfanilamide and 0.1 M HCl (45).

Fig. 3. IFN-γ-dependent tyrosine phosphorylation and accumulation of STAT-1 is suppressed in CDM3D expressing SOCS-1. Western blot analysis of tyrosine-phosphorylated STAT-1 (PY-STAT-1) was performed with cytoplasmic and nuclear fractions from the indicated cell lines stimulated with IFN-γ (200 units/ml), IL-1β (20 units/ml) plus IFN-γ (200 units/ml; I/F), and IL-1β plus TNF-α (200 units/ml; I/T) for 3 h (A) or 72 h (B). The membrane was stripped and reprobed with a rabbit polyclonal anti-STAT-1 antibody. Tyrosine-phosphorylated STAT-1 was detected in cytoplasmic and nuclear extracts from CDM3D cells treated with IL-1β or in combination with IL-1β, although no protein accumulation was observed in SOCS-1-expressing cells. The data presented in A are representative of three independent experiments, and the data shown in B are the results of one experiment.
calculated from the absorption before (A1) and after (A2) the addition of 70 mM naphthylethylenediamine and compared with a standard curve derived from NaNO₂ (0–20 μM). The values shown are the means ± S.D. of three independent experiments performed in triplicate.

Preparation of Cytoplasmic and Nuclear Extracts—At determined times, cytokine-stimulated or untreated cells were lysed on ice in 200 μl of buffer A (20 mM Tris, pH 7.8, 10 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM dithiothreitol) with 4 mM Na₃VO₄ and 10 mM NaF, complemented with 50 μg/ml of phenylmethylsulfonyl fluoride and 2 μg/ml of aprotinin. Cell lysates were incubated for 15 s in 50 μl of buffer A with 2.5% Nonidet P-40 and centrifuged at 4 °C for 15 s in a microcentrifuge (2000 rpm). The recovered supernatant constitutes the cytoplasmic extract. The pelleted nuclei were resuspended for 15 min in 100 μl of buffer A with 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 300 mM NaCl and centrifuged at 4 °C for 15 min in a microcentrifuge (13,000 rpm), the recovered supernatant corresponding to the nuclear extract. The amount of total proteins in the cytoplasmic and nuclear fractions was determined by colorimetric dosage with the Micro BCA protein assay reagent (Pierce).

Western Blot Analysis—Cytoplasmic and nuclear extracts were immunoassayed as described previously (46) to detect both total and tyrosine-phosphorylated STAT-1 proteins, using a rabbit polyclonal anti-STAT-1 antibody (diluted 1:200) and a mouse monoclonal anti-phospho-STAT-1 antibody (diluted 1:100), respectively (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The SOCS-1 protein level was determined in cell lysates prepared in 5% SDS, 5 mM EDTA, and 80 mM Tris, pH 6.8, with 50 μg/ml of phenylmethylsulfonyl fluoride and 2 μg/ml of aprotinin. SOCS-1 protein was detected in immunoblot using the anti-FLAG-M2 mouse monoclonal antibody (Sigma).

Transient Transfection and Luciferase Assays—Cells were seeded in 24-well dishes at a density of 10⁵ cells/well 48 h before transfection with the indicated plasmids using the Lipofectamine 2000 reagent (Roche Molecular Biochemicals). A total of 1 μg of DNA was transfected, which consisted of 0.8 μg of insulin-luciferase reporter plasmid containing fragment –326 to +30 of the human insulin gene promoter linked to luciferase (47) (kindly provided by Dr. A. Abderrahmani, University Hospital, Lausanne, Switzerland) and 0.2 μg of a β-galactosidase reporter plasmid (driven by the cytomegalovirus promoter), which was used to correct for transfection efficiency. 72 h after transfection, cells were stimulated with cytokines for 18 h, and relative activity of luciferase and β-galactosidase was determined as described (1).

Cytotoxicity Assay—Two days before induction of apoptosis, cells were seeded in a polylysine-treated 96-well microtiter plate (10⁶ cells/well). The medium was changed, and the cells were left untreated or treated for 36 h with a combination of TNF-α, IL-1β, and IFN-γ (10⁴ units/ml each). Cytokine-induced cell death was assessed using a cytotoxicity assay based on tetrazolium dyes (CellTitre 96® Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI). MTS tetrazolium compound is bioreduced by metabolically active cells into a colored formazan product that is proportional to the number of viable cells. The medium of the determined cell lines. After 2 ho fincubation at 37 °C, the absorbance at 490 nm was recorded using an enzyme-linked immunosorbent assay plate reader, and the percentage of viable cells was determined.

RESULTS

IFN-γ Reduces Insulin mRNA Levels and Glucose-stimulated Insulin Secretion—We previously reported that βTc-Tet cells expressing Bel-2, referred to as CDM3D cells, had impaired GSIS when exposed to IL-1β and IFN-γ. We proposed that this was mostly due to an effect of IFN-γ on total insulin content (1), because IL-1β alone had no effect, whereas IFN-γ induced the full inhibition. Here we first evaluated the dose dependence of the IFN-γ inhibitory effect. Fig. IA shows the insulin secreted by CDM3D cells pre-exposed to different concentrations of cytokines for 18 h and then kept in the presence of 2.8 or 16.7 mM glucose for 1 h. IFN-γ markedly inhibited insulin secretion at both basal and high glucose concentrations. A very significant effect was detected with doses as low as 5 units/ml. The defect in insulin secretion was correlated with a parallel decrease in cellular insulin content (Fig. 1B). Secretion of insulin, expressed as a percentage of insulin content, was not reduced by IFN-γ treatment (not shown), suggesting that the defect in secretory response may be due to a decrease in insulin content.
A separate role of IFN-γ directly on inhibition of glucose-stimulated insulin secretion can, however, not be excluded.

Next, to determine whether the IFN-γ effect on cellular insulin content correlated with a decrease in insulin mRNA level, CDM3D cells were treated with IFN-γ or with IFN-γ plus IL-1β for 48 h, and insulin mRNA was assessed by Northern blot analysis. Fig. 4A shows that IFN-γ alone or in combination with IL-1β reduced the insulin mRNA content by ~70%. To determine the rate at which IFN-γ reduced the cellular insulin mRNA content, cells were exposed for different periods of time to IFN-γ, and the insulin to actin mRNA levels were determined. Fig. 4B shows that the insulin mRNA levels decreased progressively to reach a minimal value after 48 h. Treatment for up to 5 days did not induce a more extensive reduction of this mRNA level. Because the half-life of the insulin mRNA in these cells is ~24 h (not shown), this indicates that the inhibitory effect of IFN-γ is probably taking place immediately after the addition of IFN-γ to the cells.

**Lentivirus-mediated Transfer of the Human SOCS-1 Gene in CDM3D Cells**—To evaluate whether the IFN-γ pathway in CDM3D cells could be regulated by the suppressor of cytokine signaling-1, we overexpressed the SOCS-1 protein with a recombinant lentivirus. Pools of transduced cells were selected in the presence of G418 and SOCS-1 transcripts, and proteins were detected by Northern and Western blot analysis (Fig. 2). We then verified that these cells were still able to secrete insulin in response to glucose. A 4–6-fold stimulation of insulin secretion was observed in response to high glucose concentrations (data not shown and see Fig. 6).

**SOCS-1 Blocks IFN-γ-induced Tyrosine Phosphorylation and Nuclear Accumulation of STAT-1**—To evaluate the role of SOCS-1 on IFN-γ-induced activation of the JAK/STAT signaling pathway, we assessed the level of STAT-1 tyrosine phosphorylation in CDM3D and SOCS-1 CDM3D cells exposed to IFN-γ in the presence or the absence of IL-1β. Fig. 3 shows that phospho-STAT-1 could be easily detected in the cytosol and nuclear fraction of CDM3D cells as early as 3 h (Fig. 3A) and up to 72 h after initiation of treatment (Fig. 3B). At 72 h of treatment, a large induction of total STAT-1 was observed in both cytosolic and nuclear fractions of control cells. In contrast, in SOCS-1 CDM3D cells, no phospho-STAT-1 could be detected at either time of analysis, and only a very small increase in total STAT-1 was visible. The band migrating slightly below STAT-1 is probably STAT-1β (an alternative splicing product of STAT-1 lacking the C-terminal 38 amino acid residues).

**SOCS-1 Prevents IFN-γ-induced Reduction of Insulin mRNA**—To determine whether SOCS-1 overexpression could suppress the decrease in insulin mRNA induced by IFN-γ, SOCS-1 CDM3D cells were treated with IFN-γ, alone or in combination with IL-1β. Fig. 4A shows that expression of SOCS-1 protected cells exposed for 48 h to IFN-γ alone (150 units/ml) or to IFN-γ in the presence of IL-1β at 10 units/ml. SOCS-1 similarly provided a complete protection against IFN-γ-mediated suppression of insulin mRNA expression for up to 5 days (Fig. 4B).

To determine whether the protective effect was at the transcriptional level, we assessed whether cytokine-induced reduction in insulin transcription was blocked in the presence of SOCS-1. To address this question CDM3D and SOCS-1 CDM3D cells were transiently transfected with an insulin-luciferase reporter gene and stimulated with cytokines for 18 h before luciferase assays were performed. As shown in Fig. 5, insulin promoter-driven luciferase activity was decreased by 42 ± 5.9% in CDM3D cells exposed to IFN-γ at 150 units/ml and by 44 ± 6.5% in cells treated both with IL-1β (10 units/ml) and IFN-γ (150 units/ml). An almost complete protection against cytokine-mediated decrease in insulin reporter activity was achieved in SOCS-1 CDM3D cells. Analysis of insulin mRNA stability in CDM3D cells, left untreated or treated with IFN-γ in the presence of actinomycin D, did not show any destabilizing effect of the cytokine, confirming that the decrease in insulin mRNA is at the transcriptional level (data not shown).

**SOCS-1 Protects CDM3D Cells against Cytokine-induced Suppression of Glucose-stimulated Insulin Secretion**—To determine whether expression of SOCS-1 also protected cytokine-treated cells from impaired GSIS, we performed secretion experiments in cells exposed for 48 h to IFN-γ, alone or in...
combination with IL-1β or IL-1β and TNF-α. The cells were then incubated in the presence of 2.8 or 16.7 mM glucose. A, at low and high glucose concentrations in CDM3D cells, GSIS is markedly reduced by IFN-γ alone or by the combinations of cytokines, whereas cytokine-induced reduction of secretion was prevented in cells expressing SOCS-1. *, p < 0.005; ¶, p < 0.05 versus untreated cells. B, intracellular insulin content was strongly reduced (60–70%) in CDM3D cells exposed to cytokines, whereas expression of SOCS-1 prevented this inhibitory effect. *, p < 0.0001 versus untreated cells; ¶, p < 0.0001 versus IFN-γ-treated cells; §, p < 0.005 versus untreated cells. C, secretion of insulin at 16.7 mM glucose, expressed relative to insulin content, was not significantly modified following treatment with cytokines. The data are the means ± S.E. of two independent experiments, each done in triplicate.

**SOCS-1 Prevents Cytokine-induced iNOS mRNA Expression, NO Secretion, and Apoptosis**—We previously showed that induction of iNOS mRNA and nitrite production by CDM3D cells required the combined action of IL-1β and IFN-γ and that iNOS expression was completely suppressed by blocking IL-1 intracellular signaling pathway (1). Here, we show that the presence of SOCS-1 similarly blocked the cytokine-mediated induction of iNOS mRNA accumulation and NO production. Indeed, SOCS-1 CDM3D cells, in contrast to parental CDM3D cells, exposed for 24 h to IFN-γ alone or in combination with IL-1β did not express iNOS mRNA as assessed by Northern blot analysis (Fig. 7A) and did not increase nitrite production (Fig. 7B).
Cytokine-induced apoptosis of βTc-Tet cells was partially prevented by the expression of Bcl-2. Additional blocking of the IL-1β intracellular signaling pathway conferred an increased resistance to apoptosis (1). Here, we evaluated whether blocking the IFN-γ signaling pathway would also confer an increased resistance to apoptosis induced by a combination of the three cytokines. Fig. 8 shows that SOCS-1 CDM3D cells exposed for 36 h to 10^3 units/ml of TNF-α, IL-1β, and IFN-γ were almost completely protected from apoptosis. This was in contrast to CDM3D cells in which a 40% reduction of viability was observed.

DISCUSSION

In this study, we report that IFN-γ strongly inhibits insulin gene expression, induces a reduction in cellular insulin content, and decreases glucose-stimulated insulin secretion. The effect of IFN-γ involves activation of the JAK/STAT pathway because overexpression of the suppressor or cytokine SOCS-1 prevented IFN-γ-dependent: (i) tyrosine phosphorylation and increased cellular accumulation of STAT-1, (ii) decrease in insulin gene transcription and intracellular insulin content, and (iii) inhibition of GSIS. Furthermore, up-regulation of iNOS, increase in NO production, and apoptosis, which require the combined presence of IL-1β and IFN-γ, were also suppressed by stable expression of SOCS-1.

IFN-γ induces a sustained and dose-dependent inhibition of insulin mRNA and peptide levels in CDM3D cells. A significant decrease in secreted insulin is already detected with concentrations as low as 5 units/ml. The reduction by ~50% of the cellular insulin content reached with maximally active doses of IFN-γ is closely correlated with the reduction in insulin transcript and with the transcriptional activity of an insulin promoter-luciferase reporter construct. This is finally reflected on the secretory potential of the treated cells. Because secretion expressed as a percentage of the total cellular insulin content

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FIG. 7. SOCS-1 protein blocks iNOS mRNA accumulation and NO secretion induced by cytokines. A, Northern blot analysis indicated that the presence of SOCS-1 suppressed iNOS mRNA accumulation upon 48 h of exposure to 10 units/ml of IL-1β plus 150 units/ml of IFN-γ (I/F). The blot was stripped and reprobed with a mouse β-actin probe to control for gel loading. B, nitrite accumulation was measured in cells stimulated for 19 h with the same concentration of cytokines as in A and with 100 units/ml of TNF-α. These results demonstrated that SOCS-1 can inhibit iNOS mRNA and NO production in cytokine-treated CDM3D cells. The data are the means ± S.E. of three independent experiments, each performed in triplicate.

FIG. 8. Recombinant SOCS-1 protects CDM3D cells against cytokine-induced apoptosis. The cells were exposed for 36 h to 10^3 units/ml of TNF-α, IL-1β, and IFN-γ before measuring the viability. The results showed that blocking IFN-γ intracellular signaling with recombinant SOCS-1 protects CDM3D cells against cytokine-induced apoptosis. The data are the means ± S.E. of three independent experiments, each performed in triplicate. *, p < 0.005 versus untreated cells.
was not modified by IFN-γ, this indicates that the negative effect of this cytokine on insulin secretion was mediated, at least in part, by a decrease in hormone content. A possible additional role of IFN-γ on inhibition secretion cannot, however, be excluded.

These results also indicate that the IFN-γ effect is independent of the presence of other cytokines such as IL-1β. These data are consistent with previous in vitro evidence suggesting that IFN-γ might directly affect β cell function and viability. However, species-specific regulations of β cell function by IFN-γ have been described. For instance, a decrease in glucose-stimulated insulin secretion was observed in primary mouse islets treated with high concentrations of IFN-γ (2000 units/ml) (48, 49), whereas incubation of rat islets with IFN-γ led to reduced insulin accumulation without any changes in insulin secretory response to 16.7 mM glucose (50). In human islets, prolonged exposure to IFN-γ alone did not induce a decrease in insulin transcript and peptide levels, whereas only a weak reduction in GSIS was observed (15). In addition to these different susceptibilities between islets from different species, the effect of cytokines may also depend on whether islets of Langerhans or purified β cells or β cell lines are studied. Indeed, the presence of non-β cells within intact islets, such as endothelial cells or passenger leukocytes, which are sensitive to different cytokines, may also indirectly modulate β cell function. In this context, our data with a pure β cell line displaying highly differentiated function certainly provide important information on the cellular effect of IFN-γ.

Stimulation of CDM3D cells with IFN-γ results in the tyrosine phosphorylation of the transcription factor STAT-1 and the appearance of the phosphorylated form in the nuclear fraction. Moreover, expression of STAT-1 is markedly induced in CDM3D cells as evidenced after a 72-h exposure to IFN-γ. Such an increase in STAT-1 content after stimulation with this cytokine has also been observed in NIH-3T3 and M1 cells (31), suggesting a positive feedback regulation of STAT-1 synthesis. The molecular mechanisms by which IFN-γ mediates inhibition of transcription of the insulin gene are not known. It has been suggested in a recent report that IFN-γ-induced inhibition of c-Myc expression relies on the consensus γ-activated sequence element to which STAT-1 homodimers bind and that STAT-1 might interact with a co-repressor bound elsewhere in the c-Myc promoter (51). In the rat insulin 1 gene promoter, Galsgaard et al. (52) reported the presence of a γ-activated sequence element, providing a possible initial explanation for IFN-γ-mediated inhibitory effect.

If the IFN-γ signaling in β cells depends on the JAK/STAT pathway, stable overexpression of SOCS-1 in CDM3D cells should block it. We indeed demonstrated a complete suppression of the tyrosine phosphorylation of STAT-1 induced in response to IFN-γ and an inhibition of STAT-1 accumulation, which was especially visible at the 72-h time point. Furthermore, SOCS-1 blocked the effect of IFN-γ on reducing the insulin mRNA and insulin peptide intracellular levels. Importantly, this protected the cells against the decrease in GSIS induced by this cytokine. These data therefore strongly suggest that the IFN-γ signaling pathway requires JAK kinase phosphorylation of STAT-1, a result in agreement with a recent report showing that IFN-γ treatment of mouse and rat islets was associated with prolonged tyrosine phosphorylation of STAT-1 (53).

That SOCS-1 could confer resistance to IFN-γ was previously reported when studying NIH-3T3 and M1 cells overexpressing SOCS-1, but not SOCS-2 or SOCS-3. In these cells, IFN-γ was no longer able to induce the tyrosine phosphorylation and DNA binding activity of STAT-1 (31). Sakamoto et al. (31) also found that IFN-γ-resistant clones derived from LoVo and Daudi cells expressed high level of constitutive SOCS-1 and showed reduced JAK and STAT-1 phosphorylation upon IFN-γ treatment. SOCS-1 and SOCS-3 proteins display overlapping activities, and both are induced by and inhibit the actions of cytokines such as IL-2, IL-6, and IFN-α (31, 54–56). However, SOCS-1 appears to be considerably more active than SOCS-3 in the regulation of the IFN-γ intracellular signaling pathway (31, 55). Consistent with these in vitro studies, the phenotype of SOCS-1-deficient mice revealed a key role for SOCS-1 in modulating IFN-γ action that could not be compensated by SOCS-3 in vivo (34). SOCS-1 seems thus to act as a critical regulator of cellular sensitivity to IFN-γ, and we demonstrated in this study that overexpressed SOCS-1 can efficiently and in a prolonged manner block IFN-γ-mediated intracellular signaling and prevent impaired insulin regulation in mouse β cells in response to IFN-γ action.

We previously showed in CDM3D cells that the production of nitrite, associated with the increase in iNOS mRNA and protein levels, required the combined presence of IL-1β and IFN-γ (1). We further demonstrated that expression of dominant-negative mutants of MyD88, an adapter protein participating in IL-1β signaling, is sufficient to reduce cytokine-induced nuclear factor κB transcriptional activity as well as iNOS expression and NO production. In the present study, stable expression of SOCS-1 leads to the complete suppression of iNOS mRNA induction and nitrite accumulation in response to cytokines.

Thus, whereas IL-1β and IFN-γ are needed simultaneously for nitrite production, blocking either the IL-1β or IFN-γ intracellular signaling pathway is sufficient to completely prevent the iNOS response. Therefore, both pathways must converge to the iNOS gene to activate its transcription, but the exact mechanisms involved are not yet known. A similar situation was observed for induction of apoptosis in CDM3D cells. Expression of proteins interfering with the IL-1β signaling pathway protected the cells against cytokine-induced apoptosis; an almost complete protection was also conferred by SOCS-1 expression. Taken together our data indicate that cytokines can activate distinct pathways to induce β cells dysfunctions and apoptosis. Impaired secretory response to glucose is solely mediated by IFN-γ, whereas cell apoptosis requires at least the combined presence of IL-1β and IFN-γ. The present and previously published data indicate furthermore that the deleterious effect of cytokines may be blocked by overexpression of proteins interfering with specific signaling pathways. Ultimately, these observations may pave the way to the genetic engineering of tolerance in β cells to be transplanted in type I diabetic patients.
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SOCS-1 Protein Prevents Janus Kinase/STAT-dependent Inhibition of β Cell Insulin Gene Transcription and Secretion in Response to Interferon-γ
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