Cells Previously Desensitized to Type 1 Interferons Display Different Mechanisms of Activation of Stat-dependent Gene Expression from Naïve Cells*

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Over the past decade, a wealth of knowledge has been obtained concerning the mechanisms by which interferons (IFNs) and other cytokines activate or down-regulate immediate early genes via the Jak/Stat pathway. In contrast, little information is available on interferon-activated gene expression in naïve cells compared with cells that have been desensitized and subsequently re-sensitized to the actions of these cytokines. In naïve cells, the ISG54 gene is activated via IFNβ-stimulated formation of ISGF3, a heterotrimeric DNA binding complex consisting of p48 (IRF9) and tyrosine-phosphorylated Stat1 and Stat2. In contrast, in previously desensitized cells IFNβ weakly stimulates the assembly of an ISG54-like complex that lacks Stat1, even though ISG54 mRNA induction is the same as in naïve cells. The lack of Stat1 tyrosine phosphorylation and DNA binding is due to increased activity of a protein-tyrosine phosphatase. In cells that do not express the tyrosine phosphatase Tc-PTP, the rate of Stat1 dephosphorylation is the same in naïve and previously desensitized cells. These results implicate Tc-PTP in a novel role in the regulation of type 1 interferon-stimulated gene expression.

The antiviral, antiproliferative, and immunomodulatory activities of type 1 interferons (IFNa/β)1 are controlled, in part, by a set of cellular genes that are rapidly induced upon binding of these cytokines to their specific cell surface receptors. IFNα/β-stimulated formation of ISGF3 mediates gene induction by binding to an interferon-stimulated response element (ISRE) located within the promoter of interferon-stimulated genes (ISGs) (1, 2). ISGF3 is composed of three proteins Stat1, Stat2, and p48 (IRF9). Stat1 and Stat2 are covalently modified by tyrosine phosphorylation after exposure of cells to IFNα/β and subsequently translocate to the nucleus where they interact with IRF9 (p48). The Jak1 and Tyk2 tyrosine kinases also are an integral component of these signaling cascades (3). They are activated by IFNα/β and are responsible for the tyrosine phosphorylation of Stat1 and Stat2. The mechanism governing interferon-induced transcriptional responses has now been extended to include a broad network of cytokine-regulated signaling systems that use tyrosine phosphorylation of Stat proteins to activate transcription of early response genes.

In addition to an increase in our knowledge of the activation of the Jak/Stat pathway, many studies have described mechanisms by which this signaling cascade is down-regulated. Protein-tyrosine phosphatases have been implicated in the inactivation of both the autophosphorylated Jaks as well as the nuclear-localized activated Stats (4–10). The SH2 domain-containing SOCS family of proteins (suppressors of cytokine signaling) are activated by a variety of cytokines including interferons and act as inhibitors of the Jak/Stat pathway. SOCS proteins bind to both tyrosine-phosphorylated receptors and Jaks, and mediate their ubiquitin-dependent degradation (11). In contrast to the SOCS proteins whose expression is up-regulated as a consequence of cytokine treatment, the PIAS (Protein Inhibitors of Activated STATs) proteins are constitutively expressed. This family of nuclear proteins binds to tyrosine-phosphorylated Stat dimers and prevents them from binding DNA (12, 13). They have an additional function as SUMO E3-ligases whose substrates include c-Jun and p53 (14, 15).

One aspect of cytokine regulation of the Jak/Stat pathway that has received little attention, is whether there are any changes in the regulation of immediate early genes in cells that have been previously exposed to a cytokine, have become refractory, and subsequently become re-sensitized to the cytokine. Understanding this process is significant in that in many pathophysiological settings cytokines can be repeatedly released in large amounts in response to both inflammatory and infectious insults. It is also relevant in terms of the therapeutic use of cytokines where the frequency of treatment of chronic conditions such as multiple sclerosis with IFNβ, should be optimized to intervals where cellular targets are not desensitized to actions of the drug. In order to understand the cellular effects of repeated cytokine exposure in greater detail, we have examined the IFNα/β-stimulated expression of ISG54. This gene is well known to be activated through an ISRE-dependent...
mechanism by type 1 interferons. It also has the advantage that the half-life of the mRNA is relatively short, so that re-induction of transcription can be easily monitored. The results from these studies clearly demonstrate that compared with naïve cells, cells previously desensitized to the actions of IFNβ show a dramatically altered response when re-exposed to this cytokine. Although previously desensitized cells display a robust induction of ISGs, this activation shows no detectable IFNβ-stimulated tyrosine phosphorylation of Stat1, and a reduced tyrosine phosphorylation of Stat2. The lack of Stat1 tyrosine phosphorylation is due to the tyrosine phosphatase, T-cell protein-tyrosine phosphatase (Tc-PTP).

MATERIALS AND METHODS

Cells and Culture—Human foreskin fibroblasts (HFFs), Tc-PTP−/− and wild-type mouse embryo fibroblasts obtained from matched litters were all maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen, Life Technologies, Inc.), and containing 2 mm t-glutamine, penicillin, and streptomycin (Invitrogen). Fresh human peripheral blood mononuclear cells (PBMC) were provided by Genetec Inc. RNA was isolated with RNeasy columns (Qiagen). ISG54 was overexpressed in human fibroblasts using T4 polynucleotide kinase (New England Biolabs, Inc.). Each 40-μl reaction mixture contained 5 ng of labeled oligonucleotides, poly (dI-dC) (containing 2 mM L-glutamine, penicillin, and streptomycin (Invitrogen). Both human IFNβ (Avonex) and murine IFNβ were obtained from Biogen Inc. Human and murine IFNγ were provided by Genetec Inc. RNA Assays—RNase protection assays were performed as previously described (17, 18). Briefly, total RNA was isolated with RNeasy columns (Qiagen). ISG54 was overexpressed in human fibroblasts using T4 polynucleotide kinase (New England Biolabs, Inc.). 10 μg of RNA and 25P-labeled probes were incubated in hybridization buffer (80% formamide, 40 mM PIPES (19), 400 mM NaCl, and 1 mM EDTA) overnight at 56 °C followed by digestion with T1 RNase (Ambion) for 1 h at 37 °C. After phenol:chloroform extraction and ethanol precipitation, protected RNA fragments were solubilized and subjected to electrophoresis on a 4.5% polyacrylamide-urea gel. RT-PCR was used to analyze ISG54 and GAPDH in wild-type and Tc-PTP-null MEFS. The primers for mouse ISG54 are: Forward, 5'-GATCCATGCCTCGGGAAAGGGAAACCGAAACTGAAGCC-3' and Reverse, 5'-AGATGATGACCCGTTTCTTC-3'. Primer conditions: 94 °C 5 min, 94 °C 1 min, 57 °C 1 min, 25 cycles Tc-PTP-null MEFS. The primers for mouse ISG54 are: Forward, 5'- AGAAGACGCGTTGAATAGTGA-3' and Reverse, 5'-TGGTGGAGGGCTTCTTTC-3'. Mouse GAPDH: Forward, 5'-TGGTGGAGGGCTTCTTTC-3' and Reverse 5'-AGATGATGACCCGTTTCTTC-3'; Primer conditions: 94 °C 5 min, 94 ° C 1 min, 57 ° C 1 min, 25 cycles 72 ° C 2 min (20).

Preparation of Cell Extracts—Cells were lysed by vigorously vortexing for 20 s in dounce buffer (20 mM Heps, 25 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 20% glycerol, 0.1% Nonidet P-40, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). After centrifugation (1500 × g) at 4 °C for 10 min, supernatants were collected as cytoplasmic extracts. Nuclear extracts were prepared by resuspension of the crude nuclei in nuclear extraction buffer (20 mM Heps, 300 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 20% glycerol, 0.1% Nonidet P-40, 10 mM β-glycerophosphate, 1 mM orthovandate, 25 mM NaF, 200 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) at 4 °C for 30 min, and the supernatants were collected as nuclear extracts after centrifugation at 4 °C for 10 min for 10 min. For whole cell extracts, cells were lysed in whole cell extraction buffer (20 mM Heps, 300 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 20% glycerol, 1% Nonidet P-40, 10 mM β-glycerophosphate, 1 mM orthovandate, 25 mM NaF, 200 μM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol). After centrifugation at 4 °C for 10 min, supernatants were collected. Protein concentration was measured by the Bio-Rad/Bradford protein assay.

Electrophoretic Mobility Shift Assays (EMSA)—Synthetic double-stranded oligonucleotides corresponding to the ISRE of the ISG54 promoter (5'-GATCCTATGGGAGGGAGTGATCAATTTC-3') and the IFN-γ response region (GRR) sequence of Pcy-R promoter (5'-AATTACGATGGTTCAAGGATGTAGATGGATATTCGACAAAAA-G-3') were used as probes. They were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs, Inc.). Each 40-μl reaction mixture contained 5 ng of labeled oligonucleotides, poly (dI-dC) (Amersham Biosciences), and equal amounts of protein in ISRE binding buffer (25 mM Heps, pH 7.0, 50 mM KCl, 1.25 mM MgCl₂, 0.125 mM EGTA, 0.625 mM dithiothreitol, 5% Ficoll, 0.025% Nonidet P-40) or GRR binding buffer (12.5 mM Tris-HCl, pH 7.4, 125 mM KCl, 6.25 mM MgCl₂, 1.25 mM dithiothreitol, 12.5% glycerol). The mixture was incubated at 25 °C for 30 min and then left on ice for 30 min. The DNA-protein complexes were subjected to electrophoresis on a 4.7% polyacrylamide gel in 0.25× Tris borate/EDTA at 285 V for 2.5 h and visualized by autoradiography (17).

Western Blot Analysis—Proteins in nuclear extracts, cytoplasmic extracts, or whole cell extracts were separated in SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membranes. The membranes were probed with rabbit polyclonal antibody against either STAT1, phospho-STAT1 (PY 701) (Cell Signaling Technology), STAT2, or phospho-STAT2 (PY 889) (Upstate Biotechnology). Following hybridization, the membrane was washed and incubated for 30 min with peroxidase-conjugated anti-rabbit IgG and subsequently developed by chemiluminescence using the ECL Western blotting system (Amersham Biosciences).

Chromatin Immunoprecipitation PCR (ChIP)—ChIP were performed as described by Paulson et al. (21). The Stat2 antisera were purchased from Santa Cruz Biotechnology (C-20). Stat1 antibody was generated in rabbits as previously described (22).

RESULTS

To examine the effects of IFNα/β on naïve and previously desensitized cells, we used primary human fibroblasts because the transcriptional activation and inactivation of type 1 interferon-stimulated immediate early genes has been well characterized in these cells (23, 24). In these studies we have chosen the mRNA encoded by the ISG54 gene for analysis, because its expression is known to be regulated by IFNα/β-stimulated tyrosine phosphorylation of Stat1 and Stat2, and their subsequent binding to an ISRE in its promoter (25). Furthermore, the half-life of the ISG54 mRNA is short (see Fig. 1A). Cells exposed to IFNβ for 2 h show a robust induction of ISG54 expression which, in the continued presence of IFNβ, remains elevated for 16 h (23, 26). Incubation for an additional 6 h after removal of IFNβ from the media results in the complete disappearance of ISG54 mRNA (Fig. 1A, lane 4). Importantly, exposure of the same cells to IFNβ for 2 h yields an induction of ISG54 that is similar to that of naïve cells (Fig. 1A, compare lanes 2 and 5). Incubation of both naïve and previously desensitized cells with actinomycin D and IFNβ prevents the induction of ISG54 (Fig. 1B), indicating that the increase in ISG54 mRNA levels triggered by the second cytokine exposure results from a transcriptional re-induction rather than an IFNβ-mediated stabilization of previously transcribed mRNA. Nuclear extracts prepared from naïve fibroblasts incubated with IFNβ for 30 min contain the transcription factor complex ISGF3 (composed of Stat1 and Stat2) or GRR (which contains only Stat1) (22) (Fig. 1C, lane 1 versus 2). Surprisingly, compared with naïve cells, the extracts from which ISGF3 is capable of activating ISRE binding is very much diminished in cells incubated with IFNβ for 16 h followed by an additional 6 h without the cytokine, and subsequent re-stimulation with IFNβ for 30 min (Fig. 1C, compare lane 2 with 5). Moreover, we have not been able to detect any formation of a GRR binding complex in extracts prepared from previously desensitized cells (Fig. 1C, lower panel). To confirm the results of the EMSAs, we also directly examined tyrosine phosphorylation of Stat1 and Stat2 in the same nuclear extracts using phosphospecific antibodies (Fig. 1D). Naïve cells incubated with IFNβ for 30 min showed a significant amount of both tyrosine-phosphorylated Stat1 and Stat2 in the same nuclear extracts using phosphospecific antibodies (Fig. 1D). Naïve cells incubated with IFNβ for 16 h or those re-exposed to IFNβ after a 6 h recovery period showed no tyrosine phosphorylated Stat1 and Stat2 (Fig. 1D, lanes 3 and 5, upper panel). In contrast to Stat1, cells that had recovered for 6 h prior to re-exposure to IFNβ for 30 min showed clear induction of Stat2 tyrosine phosphorylation (Fig. 1D, lane 5). We have confirmed that the weak IFNβ-induced ISRE-binding complex seen in previously desensitized cells contains Stat2, but we have not been able to detect Stat1 in this complex (data not shown). As expected, there is an IFNβ-dependent nuclear translocation of these proteins, although there is some constitutive nuclear Stat1 in untreated cells (data not
FIG. 1. Expression of ISG54 mRNA and Stat activation in human fibroblasts exposed to IFNβ. A, primary human fibroblasts were incubated with IFNβ (1000 units/ml) for 2 h (lane 2), 16 h (lane 3), 16 h followed by 6 h in the absence of IFNβ (lane 4), or 16 h followed by 6 h without IFNβ prior to re-addition of IFNβ for an additional 2 h (lane 5). RNA was prepared, and ISG54 and actin mRNAs were analyzed by RNase protection. B, the same experiment as in A except fibroblasts were incubated with 1 µg/ml of actinomycin D (lanes 2 and 7) prior to the addition of IFNβ for 2 h. C, formation of ISGF3 and GRR binding complexes in nuclear extracts was analyzed by EMSA. Conditions of incubation were the same as in A except the second incubation with IFNβ was 30 min for lanes 2 and 5. D, IFNβ-stimulated tyrosine phosphorylation of Stat1 (upper panel) and Stat2 (lower panel) was analyzed by immunoblotting of nuclear extracts with phosphospecific Stat1 or Stat2 antisera. The blots were stripped and reprobed with antisera that recognize total Stat1 and Stat2, which are displayed below the phosphospecific blots. E, IFNβ-stimulated binding of Stat1 and Stat2 to the ISG54 promoter as analyzed by ChIP assays. Human fibroblasts were incubated with or without IFNβ as described in C. Lanes 1–5 show a PCR of 1% of the input chromatin used for each immunoprecipitate. Lanes 6–10 correspond to the immunoprecipitates with Stat2 (upper panel) and Stat1 (lower panel). This figure is representative of multiple experiments.
Desensitized Cells Display Activation of Stat-dependent Gene Expression

The treatment conditions prior to preparation of RNA (A and C) and whole cell extracts (B) are identical to those described in Fig. 2. Incubation of cells with IFNβ for 30 min induced an increase in the binding of both Stat1 and Stat2 to the ISG54 promoter (lanes 6 and 7). After 16 h Stat1 was not associated with the promoter while there was still a small amount of Stat2 binding (lane 8). This is of interest since under these conditions there is likely a very low level of ISG54 transcription (data not shown). After removal of IFNβ for 6 h both Stat1 and Stat2 binding to the promoter were undetectable (lane 9). Incubation of previously desensitized cells with IFNβ induced an association of Stat2, but not Stat1 with the promoter. The amount of Stat2 bound to the promoter of previously desensitized cells varied from experiment to experiment, and ranged from 40 to 80% of naïve cells (data not shown). The amount of Stat2 that bound to the ISG54 promoter in previously desensitized cells correlated well with the degree to which Stat2 was tyrosine phosphorylated in previously desensitized cells. These results establish that primarily Stat2 and not Stat1 binds to the endogenous ISG54 promoter in previously desensitized cells.

The experiments described above were performed using primary human fibroblasts. To determine whether the same pattern of transcriptional responses is seen in other primary cells, a similar experiment was performed using primary human peripheral blood monocytes. Monocytes isolated from healthy volunteers were purified by elutriation and treated as outlined above. IFNβ-stimulated induction of ISG54 mRNA showed a similar pattern in primary monocytes as was seen in primary fibroblasts. Cells incubated for 16 h with IFNβ, followed by a 6-h recovery period, showed a full transcriptional re-induction of the gene when re-exposed to IFNβ (Fig. 2A, compare lanes 2 and 5). Whole cell extracts prepared from monocytes under similar conditions were probed for tyrosine-phosphorylated Stat1 and Stat2 (Fig. 2B). As expected, both Stats showed increased tyrosine phosphorylation after 30 min of incubation with IFNβ (Fig. 2B, lane 2, upper panels), but only Stat2 tyrosine phosphorylation could be detected after 16 h in the presence of IFNβ followed by a 6 h recovery period, and re-treatment with IFNβ (Fig. 2B, lane 5, upper panels). Interestingly, Stat1 and Stat2 displayed residual tyrosine phosphorylation after 16 h of incubation with IFNβ (lane 3), which differs from the pattern of tyrosine phosphorylation of these Stats seen in fibroblasts (see Fig. 1D). Probing the blots for total Stat1 and Stat2 (lower panels) revealed that the amount of Stat1 is increased in cells incubated with IFNβ for 16 h as interferons induce Stat1 gene expression (27).

The observation that IFNβ does not stimulate tyrosine phosphorylation of Stat1 in previously desensitized cells, would lead to the prediction that genes that are activated by IFNβ through Stat1 GRR binding would not be induced in such cells. To test

1, except that IFNγ (10 ng/ml)was used for re-stimulation in C. A, ISG54, FcγR1, and actin mRNAs were analyzed by RPA as in Fig. 1. Immunoblots were probed with anti-phosphoStat1 antisera (upper panel) or anti-phosphoStat2 antisera (lower panel). Both blots were reprobed for total Stat1 or Stat2 as described in Fig. 1. This figure is representative of three experiments.
Desensitized Cells Display Activation of Stat-dependent Gene Expression

Fig. 3. IFNβ-stimulated accumulation of ISGF3 in cytoplasmic and nuclear fractions of naïve and previously desensitized human fibroblasts. A, nuclear and cytoplasmic extracts were prepared from cells incubated with IFNβ for 5, 10, 15, or 30 min (lanes 1–5 and 11–15). Alternatively, cells were incubated with IFNγ for 16 h and an additional 6 h in the absence of IFNβ, and re-exposed to the cytokine for 5, 10, 15, or 30 min (lanes 6–10 and 16–20). B, immunoblots of nuclear extracts from cells incubated with or without IFNβ for 30 min were probed with anti-phospho Stat1 (upper panels) or anti-phospho Stat2 (lower panels) antibodies. This figure is representative of two experiments.

this hypothesis we examined IFNβ-induced expression of FcγR1 mRNA in primary human monocytes (Fig. 2C). The promoter region of the FcγR1 gene, which is activated primarily by IFNγ and, to a lesser extent, by IFNα/β, harbors a GRR which binds tyrosine-phosphorylated Stat1 (18, 28). Monocytes were incubated for 2 h with either IFNβ or IFNγ, or 16 h with IFNβ followed by a 6-h recovery period, and re-stimulation with IFNβ or IFNγ for an additional 2 h. FcγR1 mRNA was then analyzed by RNase protection using actin as an internal control. In naïve cells, both IFNβ and IFNγ stimulated the expression of FcγR1 (Fig. 2C, lane 1 versus 2 and 4). The induction of FcγR1 RNA by IFNβ is less than that by IFNγ because this gene is activated by Stat1, and IFNγ stimulates tyrosine phosphorylation of Stat1 far more effectively that IFNβ in primary monocytes. Cells that were incubated with IFNβ for 16 h and subsequently allowed to recover for 6 h showed no FcγR1 mRNA (data not shown). Re-exposure of such cells to IFNβ failed to induce the expression of FcγR1 (lane 3). In contrast, IFNγ did induce the RNA, albeit to a lesser degree than in naïve cells (Fig. 2C, compare lanes 4 and 5). These results strongly support the notion that the IFNβ-stimulated transcriptional re-induction in previously desensitized cell is restricted to genes under the control of the ISRE enhancer.

There are several potential mechanisms that could account for the selective decrease in the tyrosine phosphorylation of Stat1 compared with Stat2 in previously desensitized fibroblasts. One possibility is a selective inhibition of the upstream signaling events leading to Stat1 tyrosine phosphorylation. Conversely, a change in the relative activities of the respective tyrosine phosphatase(s) that dephosphorylate these proteins could also explain the observed results. To examine these possibilities, cytoplasmic and nuclear extracts were prepared from naïve and previously desensitized fibroblasts incubated with IFNβ for different times. EMSAs were then performed to assay formation of ISRE-binding complexes (Fig. 3A). As previously described (29, 30), ISGF3 can be observed in the cytoplasm of IFNα/β-treated cells within 10 min (Fig. 3A, left panel, lane 3). Approximately the same amount of ISGF3 can also be seen in the cytoplasm of previously desensitized cells re-exposed to IFNβ for 10 min (Fig. 3A, left panel, lane 4). However, whereas a robust ISRE-binding activity exists in the nuclei of IFNβ-stimulated naïve cells (Fig. 3A, right panel, lanes 3–5), very little or no ISGF3 is detectable in the nucleus of previously desensitized cells (Fig. 3A, right panel, lanes 7–10). Furthermore, Western blotting for nuclear tyrosine-phosphorylated Stat1 and Stat2 in naïve or previously desensitized cells incubated with IFNβ revealed that only tyrosine-phosphorylated Stat2 was present in the nuclei of previously desensitized cells (Fig. 3B, compare lanes 1 and 2 with 3 and 4, and 5 and 6 with 7 and 8). These results suggest that formation of ISGF3 is not diminished in previously desensitized cells compared with naïve cells. Rather, it appears that the ability of these cells to accumulate the activated complex in the nucleus is impaired, suggesting the elevated activity of a Stat1-specific tyrosine phosphatase in previously desensitized cells.

The observation that IFNβ-stimulated formation of ISGF3 could be detected in about the same amounts in the cytoplasm, but not in the nuclei of re-sensitized cells suggests an enhanced nuclear tyrosine phosphatase activity that dephosphorylates Stat1, and to a lesser extent, Stat2. To further examine this possibility, we assayed the rate of Stat1 dephosphorylation in naïve and previously desensitized cells under pulse-chase type conditions. IFNβ does not induce tyrosine phosphorylation of Stat1 in previously desensitized cells, thus we used IFNγ to induce Stat1 tyrosine phosphorylation. Naïve or previously

2 A. C. Larner, G. Feldman, and D. Finbloom, unpublished observations.
desensitized human fibroblasts (incubated with IFNγ for 16 h followed by a 6 h recovery period), were incubated with IFNγ for 30 min. Staurosporin (1 μM) was then added to cells to prevent further activation of Stat1 and whole cell extracts were prepared 5, 10, or 20 min later. Tyrosine phosphorylation of Stat1 was assayed for the EMSAs instead of whole cell extracts. This figure is representative of multiple experiments.

Whole cell extracts were prepared at various times after the addition of staurosporin and Stat1 DNA binding was assayed by EMSA using a probe corresponding to the GRR element as a probe. B, the same experiment was performed as above except nuclear extracts were used for the EMSAs instead of whole cell extracts. This figure is representative of multiple experiments.

The enhanced PTPase activity toward Stat1 in cells exposed to IFNγ for extended periods suggests that these cells would also show altered sensitivity to IFNγ. The results presented in Fig. 2C support this model since IFNγ induction of FcγR1 RNA is diminished in monocytes incubated with IFNβ for 16 h compared with naive cells. To examine this in greater detail, human fibroblasts were incubated with or without IFNβ for 16 h, allowed to recover for 6 h and then stimulated with different doses of IFNγ for 30 min. Nuclear extracts were prepared and analyzed for Stat1 activation by EMSA as described in the legend to Fig. 1. This figure is representative of two experiments.

Two tyrosine phosphatases have been reported to be responsible for dephosphorylating Stat1 (10, 32). One is SHP2, which was identified using an antibody array (32), and the other, Tc-PTP, was purified from nuclear extracts of HeLa cells using tyrosine-phosphorylated Stat1 as a substrate (10). At the moment it is not evident whether these PTPases function to simultaneously dephosphorylate Stat1 or whether they each function in different physiological conditions. To examine this issue, we compared the rates of Stat1 dephosphorylation in naive and previously desensitized Tc-PTP-deficient immortalized MEFs (10, 32). MEFs were subject to pulse-chase type experiments as previously outlined. Extracts were prepared at various times after the addition of staurosporin and immunoblots probed with anti phospho-Stat1 antiserum. In wild-type MEFs there was a rapid disappearance of tyrosine-phosphorylated Stat1 after the addition of staurosporin (Fig. 6A, lanes 2–5). In cells that were incubated overnight with IFNβ, allowed to recover for 6 h and then exposed to IFNγ, the rate of Stat1 tyrosine dephosphorylation was enhanced (Fig. 6A, lanes 7–10). Similar results were seen in Tc-PTP−/− cells that were reconstituted with the appropriate PTPase (10, 32) and data not shown. A similar pattern of Stat1 dephosphorylation was seen in naive and previously desensitized SHP2-null cells (data not shown). In contrast to wild-type MEFs, little Stat1 dephosphorylation was observed in Tc-PTP−/− cells that were reconstituted with the appropriate PTPase (10, 32) and data not shown. A similar pattern of Stat1 dephosphorylation was seen in naive and previously desensitized SHP2-null cells (data not shown). In contrast to wild-type MEFs, little Stat1 dephosphorylation was observed in Tc-PTP−/− cells that were reconstituted with the appropriate PTPase (10, 32) and data not shown. A similar pattern of Stat1 dephosphorylation was seen in naive and previously desensitized SHP2-null cells (data not shown).
still was detectable in previously desensitized cells, we wanted to examine whether the expression of Tc-PTP altered tyrosine phosphorylation of Stat2. Tc-PTP-null cells or their reconstituted counterparts were incubated with or without IFN/α for 16 h prior to preparing cell extracts to analyze tyrosine phosphorylation of Stat1 and Stat2 (Fig. 7). While we were unable to detect tyrosine phosphorylation of Stat1 in previously desensitized reconstituted cells re-exposed to IFN/α (Fig. 7A, lane 2 versus 5), a re-induction of Stat1 tyrosine phosphorylation equal to the initial response was observed in the absence of TcPTP (Fig. 7A, lane 7 versus 10). IFN/α-stimulated tyrosine phosphorylation of Stat2 was equally strong in naïve compared with previously desensitized cells (Fig. 7B, lane 2 versus 5), regardless of TcPTP expression (Fig. 7B, lanes 2, 5, 7, and 10). These results not only confirm that activation of Stat1 occurs in previously desensitized cells that do not express Tc-PTP, they also emphasize that expression of Tc-PTP selectively controls the tyrosine phosphorylation of Stat1 without altering Stat2 activation.

Tyrosine phosphorylation of Stat1 remains elevated in Tc-PTP-null cells even after incubation with IFN/α for 16 h followed by a 6 h wash (see Figs. 6 and 7). We wanted to examine whether the continued presence of tyrosine phosphorylated Stat1 in Tc-PTP-null cells correlated with continued expression of ISG54. RNAs were prepared from naïve or previously desensitized cells reconstituted with Tc-PTP and Tc-PTP-null cells. ISG54 expression was assayed by RT-PCR (Fig. 7C). In a manner similar to human fibroblasts and monocytes, treatment of these cells with IFN/α for 2 h stimulated ISG54 RNA accumulation (Fig. 7C, lane 2 and 7) compared with GAPDH, which was used as an internal control. ISG54 RNA remained present in cells continuously exposed to IFN/α for 16 h, and disappeared after incubation of cells in the absence of IFN/α for 6 h. Interestingly, disappearance of ISG54 RNA occurred in both wild-type and Tc-PTP-null cells (Fig. 7C, lane 4 and 9), even though Tc-PTP-null cells continued to contain significant amounts of tyrosine-phosphorylated Stat1. The re-induction of
Desensitized Cells Display Activation of Stat-dependent Gene Expression

ISG54 was also the same in both the reconstituted and TcPTP-null cells (Fig. 7C, lanes 5 and 10). These results suggest that although tyrosine-phosphorylated Stat1 is needed to initially induce the transcription of ISG54 in naive cells, once transcription has been initiated it is not needed for the continued expression of the gene, and it plays no significant role in initiating the transcription of the gene in previously desensitized cells.

DISCUSSION

Under physiological and pathological conditions, levels of cytokines and growth factors can fluctuate. The mechanisms by which cells respond to these stimuli and down-regulate an initial response is well documented. Much less is known about how cells that have seen a particular stimulus respond to the same stimulus upon re-exposure. Previous studies examining IFNβ-stimulated genes indicate that cells become desensitized after incubation with IFNβ for about 8 h (24, 33). The experiments in this report demonstrate changes in the mechanisms by which IFNβ induces ISRE-dependent genes in cells that have been previously exposed to IFNβ. To our surprise, the nature of the response in previously desensitized cells compared with naive cells is significantly different in two aspects. 1) The amount of the ISRE-binding complex formed in previously desensitized cells is far less than that seen in naive cells under conditions where the induction of a well-described IFNβ-activated ISRE-dependent gene (ISG54) is the same. 2) The relative ability of IFNβ to stimulate the tyrosine-phosphorylation of Stat1 is far less than the IFNβ-stimulated tyrosine-phosphorylation of Stat2 in previously desensitized cells (see Figs. 1 and 2), and this result correlates well with the binding of these transcription factors to the endogenous ISG54 promoter as analyzed by ChIP assays (Fig. 1E). In fact, in most experiments we have not detected any IFNβ-mediated activation of Stat1 in re-stimulated cells. This observation is also confirmed when we examined induction of the Stat1-dependent gene FcγR1 in human monocytes (see Fig. 2C). While IFNβ activation of ISG54 is the same in naive and previously desensitized monocytes, we were not able to detect any IFNβ-induced expression of FcγR1 RNA in previously desensitized cells. These experiments suggest that there are different rates of recovery of previously desensitized cells in their ability to activate sets of early response genes induced by Stat1 compared with those induced by Stat1 and Stat2. It also should be noted that we have not seen any changes in the expression of these proteins in naive compared with previously desensitized cells (data not shown).

Published studies have documented a dose-dependent relationship between the levels of induction of interferon-stimulated genes and activation of Stat1 in naive cells (18). We have confirmed these observation in human fibroblasts (data not shown). The ‘‘ISGF3’’ transcription complex that we observed in previously desensitized cells does contain Stat2 as determined by antibody supershift analysis, but we have not been able to detect Stat1 in the complex (data not shown). Several reports have indicated that Stat2 is the primary component of the ISGF3 transcription complex responsible for inducing the expression of ISRE-driven genes. Deletions of the C-terminal region of Stat2 abrogate the transcriptional activity of ISGF3 (34). Recently it has been demonstrated that an IRF9-STAT2 hybrid can both induce ISRE gene expression and allow for an antiviral response in the absence of type 1 interferons (35). Our results indicate that Stat2 can also activate ISRE-dependent genes in the absence of tyrosine phosphorylation of Stat1 in a variety of cells under conditions that are physiological and likely occur within the setting of innate immune responses. The fact that there is no difference in the pattern of IFNβ-stimulated ISG54 RNA expression in wild type compared with TcPTP-null cells highlights the probable importance of Stat2 and lack of importance of Stat1 in cells repeatedly exposed to these cytokines (Fig. 7C).

There are several possibilities that might account for the full activation of ISG54 under conditions where there is reduced activation of Stat2 and the lack of activation of Stat1. The chromatin in the regions of interferon-stimulated genes might remain relaxed even after the transcriptional response has dissipated. Under these conditions, even a reduced activation of Stat2 (Stat2 provides the primary transcriptional activation domain of ISGF3) and binding to the ISG54 promoter could be sufficient to stimulate a full response. The other possibility is that another IFNβ-regulated transcription factor(s) complements or substitutes for ISGF3 to drive the response in previously desensitized cells. Although we have no evidence for this scenario, it is known that the interferon regulatory factors (IRFs) bind ISREs, and IRF1 and IRF3 can activate or contribute to the activation of ISGs including ISG54 (36, 37). We are presently exploring whether either or both of these mechanisms account for full activation of ISG54 in previously desensitized cells.

The other clear difference in IFNβ-stimulated signaling in re-sensitized cells compared with naive cells is that tyrosine phosphorylation of Stat1 is absent or very diminished. Decreased IFNβ-stimulated tyrosine phosphorylation of Stat1 could be due to either decreased production of tyrosine-phosphorylated protein or enhanced dephosphorylation of Stat1. Our results indicate that in previously desensitized cells there is an elevated PTPase activity that dephosphorylates Stat1 (Figs. 4 and 5). It has been known for several years that a nuclear PTPase plays an important role in down-regulating IFN-stimulated gene expression (4). Recently, two PTPases have been described to be involved in dephosphorylating Stat1 (10, 32). Using cells that do not express Tc-PTP, we demonstrated that decreased levels of phosphorylated Stat1 in previously desensitized cells are likely due to the expression of this PTPase. Tc-PTP is ubiquitously expressed and has two isoforms. TC48, (the 48-kDa isoform), is localized to the endoplasmic reticulum, and TC45 is primarily targeted to the nucleus, although there is a small fraction in the cytoplasm (38). Although we see enhanced dephosphorylation of Stat1 in nuclear fractions of previously desensitized cells, cytoplasmic fractions also show enhanced activity (data not shown). This is not surprising since both nuclear and cytoplasmic Tc-PTP have been reported to dephosphorylate Stat1 (10). We are investigating whether the activity or subcellular localization of Tc-PTP is affected by extended exposure to IFNβ. We have detected no changes in the total amount of Tc-PTP in cells incubated with IFNβ for extended periods (data not shown). It has been reported that methylation of arginine 31 of Stat1 enhances its interaction with Tc-PTP and decreases its interaction with Pias1, a negative regulator of Stat1-dependent transcription (39). It is therefore possible that previously desensitized cells will contain altered levels of arginine methylated Stat1 and/or changes in its affinity for Pias1. Although we have not completely eliminated the role of SOCS protein expression in blunting IFNβ-stimulated tyrosine phosphorylation of Stat1 in previously desensitized cells, the fact that tyrosine phosphorylation of Stat2 is not nearly as inhibited as tyrosine phosphorylation of Stat1 argues for SOC proteins playing a less prominent if any role in the phenomenon that we are studying (see Fig. 7). The PTPase responsible for dephosphorylating Stat2 has not been defined although it does not appear to be Tc-PTP (Fig. 7).

It is notable that cells exposed to IFNβ for extended times...
show a blunted response to IFNγ with regard to activation of Stat1, especially at suboptimal doses of IFNγ (Fig. 5). IFNγ-induced expression of FcR-RII is also decreased in primary monocytes previously treated with IFNβ (see Fig. 2C). Although the physiological significance of extended exposure of cells to type I interferons is not fully appreciated, there have been a variety of reports which indicate that type I interferons can inhibit the effects of IFNγ with respect to induction of MHC Class II and inducible nitric-oxide synthase expression on macrophages and endothelial cells (40–43). Furthermore, the ability of IFNγ to inhibit histoplasmosis infection of macrophages is blunted by IFNα/β (44). IFNβ activation of Tr-PTP, resulting in attenuated responses to IFNγ, is a plausible mechanism to explain these observations. We speculate that the ability of IFNβ to regulate the actions of IFNγ will also occur with other cytokines that activate common sets of Stats. The ability of prolonged exposure to one cytokine to attenuate responses to another cytokine by a post-receptor down-regulation of Stat activation is a novel mechanism of cytokine “cross-talk.” Such a mechanism may contribute to altered patterns of gene regulation in mammals that are repeatedly exposed to bursts of cytokine release under conditions of infection and inflammation.

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