Regulation of Interferon-α Responsiveness by the Duration of Janus Kinase Activity*

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Daudi B lymphoblastoid cells are highly sensitive to the anti-growth and anti-viral effects of interferon (IFN). Unlike many cell lines, these cells show prolonged transcription of IFN-stimulated genes following treatment with IFN-α. This prolonged response correlated with the continued presence of the activated transcription factor, IFN-stimulated gene factor 3 (ISGF3). Pulse-chase labeling experiments indicated that the half-life of the phosphorylation of signal transducers and activators of transcription (Stat)1 and Stat2 was short (<2 h) although the turnover of the proteins themselves was slow (>24 h), indicative of a constitutive phosphatase activity. The administration of protein-tyrosine kinase inhibitors at any time point during IFN stimulation led to rapid inhibition of the response, indicating that tyrosine kinase activity was continuously required. Catalytic activity of Jak1 and Tyk2 kinases remained elevated for prolonged periods following stimulation. Continuous presence of IFN-α was necessary for maintaining prolonged activation of ISGF3 and of Janus kinases, an activity that was blocked by antibodies to IFN-α or by cycloheximide. Conditioned medium of IFN-α-stimulated cells was capable of stimulating STAT activation in naive cells. Taken together, these results suggest that the response to IFN-α is controlled by the duration of stimulated Janus kinase activity over the background of constitutive dephosphorylation and that this response can be sustained by autocrine secretion of IFN-α.

Interferons (IFNs) are cytokines with a wide variety of functions, including modulation of immune responses, inhibition of proliferation, and induction of an anti-viral state and of resistance to bacterial and parasitic infection. There are two types of IFN: type I IFN, comprised of many IFN-α genes and one IFN-β gene, and type II IFN, consisting of a single IFN-γ species. These two types of IFN produce different but somewhat overlapping responses in target cells following binding to two distinct receptors. The receptors for both types of IFN are composed of at least two transmembrane proteins involved in signal transduction. The binding of IFNs to their receptors causes receptor dimerization, triggering signal transduction by activation of intracellular tyrosine kinases of the Janus kinase (JAK) family.

The IFN-induced signal pathway has been elucidated recently (1, 2). During the response to IFN-α, Jak1 and Tyk2 tyrosine kinases are activated, leading to activation of their downstream substrates, signal transducers and activators of transcription (Stat1) and Stat2. Activated Stat1 and Stat2 assemble as a multimeric complex, the interferon-stimulated gene factor 3 (ISGF3) complex, with a member of interferon regulatory factor family termed ISGF3γ (3), and translocate into the nucleus where they bind to interferon-stimulated response elements in the promoters of IFN-α-stimulated genes. The response to IFN-γ is structured similarly; Jak1 and Jak2 are activated at IFN-γ receptors, leading to the phosphorylation of Stat1. Activated Stat1 forms homodimers as the γ-activated factor, translocates into the nucleus, and binds the γ-activated site elements in the promoters of IFN-γ-induced genes (4). The essential role of Stat1 in IFN signaling has been demonstrated by the IFN-resistant phenotypes of cells and animals missing the Stat1 gene product (5–7).

Upon IFN-α stimulation, Stat1 and Stat2 are activated by tyrosine phosphorylation within minutes, resulting in rapid production of ISGF3 (8). However, these responses are often transient. Following prolonged treatment of cells with IFN, the levels of phosphorylated STAT proteins decline to near pretreatment levels, leading to a decline in IFN-stimulated gene expression (9). The waning of induced gene expression is often accompanied by a refractory state during which cells remain unresponsive to addition of fresh inducer (10). This desensitization phenomenon demonstrates the tight regulation of IFN responses and is likely important to ensure the proper and controlled action of these potent cytokines. Mechanisms responsible for the control of IFN-induced responses probably operate on several levels. These include down-regulation and degradation of receptors (11), regulation of the activity of protein-tyrosine kinases and of protein-tyrosine phosphatases (PTPs) controlling the phosphorylation of STATs, the degradation of JAK and STAT proteins, for example, by proteasomes (12), and potentially the regulated nuclear transport of activated STATs (13). However, the precise mechanisms limiting the response to IFN remain undefined.

Human Daudi lymphoblastoid cells are highly sensitive to the biological effects of IFN. This high sensitivity is at least partially due to a prolonged rather than transient transcriptional response to IFN in which IFN-stimulated gene expression remains induced for greater than 24 h post-treatment (14). However, the mechanisms for maintaining this response are still unknown. We have examined the mechanisms underlying...
the prolonged response to IFN-α in Daudi cells. We found that the level of phosphorylated STAT protein in Daudi cells is prolonged following continued treatment with IFN-α, and that this prolonged phosphorylation was strictly dependent on continued activity of JAK protein-tyrosine kinases. Moreover, neither tyrosine dephosphorylation nor protein turnover appeared to be impaired in Daudi cells, suggesting that the maintenance of phosphorylated STAT protein is regulated primarily at the level of active phosphorylation. Interestingly, we found that the continued activity of JAK kinases and of STAT phosphorylation were dependent on the induced secretion of autocrine IFN.

MATERIALS AND METHODS

Cell Culture and Antibodies—Daudi (ATCC), a human lymphoblastoid cell line, and FS2, a human fibroblast cell line, were maintained in RPMI 1640 and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal calf serum. Polyclonal rabbit antisera against Jak1, a gift from Dr. Martin Seidel, Ligand Pharmaceuticals Inc., and Tyk2, a gift from Dr. Sandra Pellegrini, Pasteur Institute, France (15), were used for immunoprecipitation. Monoclonal antibodies against Jak1, phosphotyrosine (Py-20) purchased from Transduction Labs (Lexington, Kentucky), 4G10 purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and Tyk2 (from Dr. Sandra Pellegrini) were used in Western blot analysis. Rabbit antisera against Stat1 and Stat2 were gifts from Dr. Chris Schindler, Columbia University, New York, NY (16). Rabbit polyclonal antisera against IFN-α (Interferon Sciences Inc., New Brunswick, NJ) was used for neutralizing IFN-α. Recombinant IFN-α-2a was a gift from Hoffmann-La Roche and was used at 500 units/ml.

Electrophoretic Mobility Shift Assay (EMSA)—Gel shift assays were performed as described previously (8). In brief, a double-stranded 32P-labeled DNA probe containing interferon-stimulated response element sequence from the human ISG15 gene (17) was incubated with cell extracts, fractionated on a non-denaturing polyacrylamide gel, and autoradiographed.

Immunoprecipitation and Western Blot Analysis—Total cell extracts were prepared by lysing cells in 1% Triton X-100, 300 mM NaCl, 50 mM HEPES, pH 7.6, 1 mM Na3VO4, 1 mM dithioreitol, 1 mM phenylmethanesulfonyl fluoride, and 5 μg/ml aprotinin and leupeptin. Cytoplasmic extracts were prepared by lysing cells in 0.25% Nonidet P-40, 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 3 mM MgCl2, 10% glycerol, 1 mM Na3VO4, 1 mM dithioreitol, 1 mM phenylmethanesulfonyl fluoride, and 5 μg/ml aprotinin and leupeptin. Nuclear extracts were prepared by incubating nuclei in 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, and protease inhibitors. Immunoprecipitation and immunoblotting were performed as described previously (18).

In Vivo 32P Pulse-Chase Labeling—Cells were preincubated in phosphate-free Dulbecco’s modified Eagle’s medium containing 5% dialyzed fetal bovine serum for 1 h, followed by addition of 1 μCi/ml [35S]methionine and incubation for 4 h (18). Labelled cells were treated with IFN-α, 500 units/ml, for 30 min, washed three times with cold 1× phosphate-buffered saline, and incubated for various times in medium without label.

[35S]Methionine Pulse-Chase Labeling—Cells were preincubated in methionine-free Dulbecco’s modified Eagle’s medium containing 5% dialyzed fetal bovine serum for 1 h, followed by addition of 1 μCi/ml [35S]methionine and incubation for 2 h (13). Labelled cells were washed, incubated in label-free medium, and then treated with IFN-α (500 units/ml) for different times.

In Vitro Kinase Activity Assay—Jak1 or Tyk2 immunoprecipitated from total cell extracts were incubated with 15 μl of in vitro kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 0.1 mM MnCl2, 0.1 mM Na3VO4) containing 20 μM ATP for 30 min at room temperature. The reaction was stopped by adding protein sample buffer and heated at 95 °C for 5 min. The reaction mixture was fractionated on 8.5% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane, and phosphorylation was detected using anti-phosphotyrosine antibodies (19). As a control, reaction mixtures lacking ATP were processed in parallel.

RESULTS

Prolonged IFN-α Response in Daudi Cells—Since Daudi cells maintain transcription of IFN-stimulated genes during prolonged treatment with IFN, we studied the kinetics of activation of ISGF3 and of Stat1 and Stat2 phosphorylation. Cells were treated with recombinant IFN-α-2a for 30 min, 2 h, 4 h, and 24 h, and the kinetics of ISGF3 activation were determined using EMSA. For comparison, ISGF3 was also analyzed in FS2 cells, a human fibroblast cell line that displays a typically transient response. ISGF3 was detected after stimulation for 30 min in FS2, and the complex dramatically decreased after 4 h (Fig. 1A, upper panel), indicative of a transient response to IFN-α. Following 24 h of continuous treatment with IFN, no active ISGF3 was detected in either cytoplasmic or nuclear extracts (data not shown). A similar phenomenon was seen in other human cell lines, such as HeLa S3 and 2TGH (data not shown). In contrast, Daudi cells showed prolonged activation of ISGF3. Activated ISGF3 was detected after 30 min of IFN-α treatment and was maintained for greater than 24 h (Fig. 1A, lower panel, and data not shown). The level of active ISGF3 in extracts of Daudi cells treated with IFN for 24 h was ~ 70% of the level observed following 30 min of treatment. As previously reported (20), a basal activation of ISGF3 was also observed in Daudi cells in the absence of exogenous IFN-α stimulation (Fig. 1A, lower panel, lane 1).

To confirm that Stat1 and Stat2 phosphorylation was prolonged in response to IFN-α, the kinetics of tyrosine phosphorylation of these two proteins were followed by immunoprecipitating with anti-Stat1 (Fig. 1B, left panel) or Stat2 (Fig. 1B, right panel) antibody followed by blotting with anti-Tyr(P) antibody. Stat1 and Stat2 were tyrosine-phosphorylated after stimulation for 10 min, and the phosphorylation was main-
The administration of IFN-α results in prolonged tyrosine phosphorylation of Stat1 and Stat2, which is observed throughout the treatment period. This result suggested that protein-tyrosine phosphatases constantly counteract the loss of active STAT protein due to dephosphorylation. This prompted us to examine the requirement for continuous kinase activity using antibodies against phosphotyrosine (left panel). The levels of Jak1 or Tyk2 proteins were assessed by Western blotting (right panel).

The levels of Jak1 or Tyk2 proteins were assessed by Western blotting (right panel). The signals, however, disappeared in both compartments after 4 h (Fig. 2A), even though protein levels of Stat1 and Stat2 were still comparable throughout the experiment (data not shown). This result indicated that the phosphorylation of activated Stat1 and Stat2 turned over very rapidly, although the proteins themselves were relatively stable.

The short half-life (<2 h) of phosphorylated STATs suggested that protein-tyrosine phosphatases constantly counteracted ligand-dependent tyrosine phosphorylation. Alternatively, protein degradation could be responsible for the rapid turnover of activated STAT proteins. To distinguish these possibilities, we performed 35S-methionine pulse-chase labeling to determine the half-life of Stat1 and Stat2 proteins following IFN treatment. Daudi cells were labeled with 35S-methionine for 1.5 h, followed by treatment with genistein (300 μM) and incubated for 30 min followed by treatment with genistein (300 μM) and incubated for 30 min to 24 h as indicated (h). Cytoplasmic extracts were assayed for ISGF3.

The rapid turnover of phosphorylated Stat1 and Stat2 was in marked contrast to the near constant levels of active ISGF3 observed throughout the treatment period. This result suggested that new protein was being constantly phosphorylated in vivo, although the proteins themselves were relatively stable.

**Protein-tyrosine Kinase Activity Is Constantly Required for Maintaining a Prolonged Response**—The administration of phosphatase inhibitors such as sodium vanadate or pervanadate to cells causes the accumulation of tyrosine phosphorylated STATs even in the absence of IFN stimulation (21–23), suggesting tyrosine phosphatases are required to maintain STAT proteins in a latent state. To test the involvement of phosphatases in the prolonged response, the turnover rate of phosphorylated STATs was measured using in vivo pulse-chase labeling. Daudi cells were labeled with 32Porthophosphate for 1.5 h, followed by treatment with IFN-α for 30 min. Cells were then washed and incubated in the absence of label for 0, 30 min, 2 h, 4 h, 8 h, and 12 h. Stat1 and Stat2 proteins were immunoprecipitated, and phosphorylated protein was detected by autoradiography. 35S-Labeled, activated Stat1 (Fig. 2A, left panel) and Stat2 (right panel) were observed in both cytoplasmic (Fig. 2A, upper panel) and nuclear (lower panel) extracts at the beginning of the chase process (Fig. 2A, lane 2 in each panel). The signals, however, disappeared in both compartments after 4 h (Fig. 2A), even though protein levels of Stat1 and Stat2 were still comparable throughout the experiment (data not shown). This result indicated that the phosphorylation of activated Stat1 and Stat2 turned over very rapidly, although the proteins themselves were relatively stable.

The half-life of Stat1 and Stat2 was determined by autoradiography. The levels of Stat1 (Fig. 2B, left panel) and Stat2 (Fig. 2B, right panel) were found to be comparable during the course of the experiment, indicating that both Stat1 and Stat2 protein stability is considerably greater than the relatively short half-life of the tyrosine phosphate (Fig. 2A).

The rapid turnover of phosphorylated Stat1 and Stat2 was in marked contrast to the near constant levels of active ISGF3 observed throughout the treatment period. This result suggested that new protein was being constantly phosphorylated to counteract the loss of active STAT protein due to dephosphorylation. This prompted us to examine the requirement for continued kinase activity. Cells were treated for 30 min with IFN-α to activate ISGF3. Then, the tyrosine kinase inhibitor genistein was added to block further JAK kinase activity. ISGF3 formation was detected after the initial 30 min of treatment; however, complex formation was significantly blocked following 2 h of treatment with genistein and was undetectable following incubation for 24 h (Fig. 2C). A similar phenomenon was observed using another kinase inhibitor, staurosporine, while the protein kinase C inhibitor, H7, had no inhibitory effect (data not shown). These results show that continuous protein tyrosine kinase activity was required for maintaining a prolonged response to IFN.

**Kinase Activity of Both Jak1 and Tyk2 Is Prolonged**—The observation that continuous protein kinase activity was required for sustaining the IFN-α response in Daudi cells prompted us to study directly the kinase activity of Jak1 and
The preceding results indicated that the absence of IFN-α for 30 min, followed by treatment with IFN-α for the indicated times (h). Cytoplasmic extracts were analyzed for ISGF3. A, effect of CHX. Cells were pretreated with CHX (50 μg/ml) for 15 min, and then incubated in the absence of IFN for 30 min of treatment. Augmented levels of kinase activity were maintained for 24 h (Fig. 3, left panel). Similarly, both Jak1 and Tyk2 exhibited elevated kinase activity toward exogenous substrates 24 h after treatment of cells with IFN (data not shown), suggesting that the kinase activity of Jak1 and of Tyk2 were prolonged in Daudi cells in response to IFN-α.

Continuous Presence of IFN-α Is Required for Maintaining a Prolonged Response—The preceding results indicated that the prolonged response to IFN-α was maintained by continued JAK kinase activity. We next considered whether this continued kinase activity required continuous ligand stimulation. Cells were pulsed with IFN-α for 30 min, then incubated in the absence of IFN-α for 30 min, 2 h, 4 h, 8 h, and 24 h. Cytoplasmic extracts were analyzed by EMSA. ISGF3 was found to be maintained for 24 h in the absence of exogenous IFN. However, there was a transient decrease followed by a reaccumulation of ISGF3 (Fig. 4A). This profile suggested that the transient loss of ISGF3 might be due to the turnover of activated STAT proteins as observed during pulse-chase labeling (Fig. 2A). However, active ISGF3 was restored at later times, presumably through the action of induced factors.

To test if the synthesis of new proteins might account for these induced factors, cells were treated with IFN in the presence of CHX. Daudi cells were first pretreated with CHX (50 μg/ml) for 15 min followed by treatment with IFN-α for 15 min, 30 min, 2 h, 4 h, and 8 h. ISGF3 formation in both cytoplasm (Fig. 4B, upper panel) and nucleus (lower panel) was gradually reduced and eventually disappeared after 8 h of CHX treatment. Tyrosine phosphorylation of Stat1 and its associated Stat2 was also diminished after CHX treatment for 8 h (Fig. 4C, upper panel), correlating with the loss of ISGF3 in EMSA. However, no significant change in the levels of Stat1 protein was detected during treatment with CHX (Fig. 4C, lower panel), indicating that degradation of Stat1 is not a significant mechanism for removal of activated STATs and that its synthesis does not account for CHX sensitivity. Taken together, these results suggested that continuous presence of IFN-α is required to maintain the prolonged activation of STAT proteins, maintaining JAK kinase activity through the action of induced factors.

Secretion of IFN-α Is Induced in Daudi Cells following IFN-α Treatment—It has been shown that Daudi cells secrete low levels of IFN-α (20). To test if secretion of IFNs was induced in response to IFN treatment and thus contributed to the reactivation of ISGF3 during the IFN-α withdrawal experiment, anti-IFN-α antisera were employed. Daudi cells were first pulsed with IFN-α for 30 min, and then incubated in the absence of exogenous IFN-α but in the presence of anti-IFN-α antisera (100 units/ml) for the indicated times (h). Cytoplasmic extracts were analyzed for ISGF3. A, effect of IFN-α antisera. Cells were treated with IFN-α for 1 h, washed, re-freshed with medium, and incubated in the absence of IFN-α for 24 h. The conditioned medium was collected and incubated with fresh Daudi cells for the indicated times (h). Conditioned medium from cells never exposed to IFN-α was used for treating cells for 24 h (lane 1, C). Cytoplasmic extracts were analyzed for ISGF3.

To further confirm that secretion of endogenous IFN-α is required for maintaining a prolonged response in the absence of exogenous IFN-α.

Duration of JAK Kinase Activity Controls IFN-α Responsiveness

Fig. 4. A, effect of IFN-α withdrawal. Cells were left untreated or pulsed with IFN-α for 30 min, washed three times, and incubated in the absence of IFN-α for the indicated times (h). Cytoplasmic extracts were analyzed for ISGF3. B, effect of CHX. Cells were pretreated with CHX (50 μg/ml) for 15 min, followed by treatment with IFN-α for the indicated times (h). Cytoplasmic (upper panel) or nuclear (lower panel) extracts were analyzed for ISGF3. C, total extracts from cells treated as described in B were immunoprecipitated (IP) with anti-Stat1 antibody and analyzed with anti-Tyr(P) (P-Tyr) antibody (upper panel) or with anti-Stat1 antibody (lower panel).
Duration of JAK Kinase Activity Controls IFN-α Responsiveness

Regulation of tyrosine phosphorylation (activation) and dephosphorylation (deactivation) of STAT proteins controls IFN-mediated signaling and biological activities. Transient activation of STAT proteins results in rapid responses, whereas prolonged activation leads to prolonged levels of gene induction and enhanced biological activities. The regulation of tyrosine phosphorylation and dephosphorylation is orchestrated by the coordinated action of PTPs and protein-tyrosine kinases (24, 25). Modulation of the function of either PTPs or protein tyrosine kinases results in imbalanced activity, leading to altered signal transduction and response. For example, an EPO receptor-associated phosphatase SH-PTP1 (HCP, PTP1C, SHP-1) regulates EPO signaling (26). Cells expressing mutant EPO-R lacking SH-PTP1, show increased tyrosine phosphorylation of Jak1 and Stat1 upon IFN-α stimulation (27). The region of STAT proteins involved in the interaction with phosphatases is further suggested by the constitutive activation and enhanced tyrosine phosphorylation of an amino-terminally deleted version of Stat1 when overexpressed in cells. While this result suggests that the amino terminus is crucial for modulating STAT activity through tyrosine dephosphorylation (28), whether tyrosine dephosphorylation is constitutive or ligand-dependent has not been determined.

Data presented in this report demonstrate that the unusually prolonged IFN-α response of Daudi cells requires continued protein-tyrosine kinase activity. Jak1 and Tyk2 remain active for prolonged periods following IFN treatment, and addition of protein-tyrosine kinase inhibitors at any point during the response leads to a rapid cessation of STAT activity. The need for continued kinase activity appears to be due to the opposing action of one or more PTPs. The half-life of STAT phosphorylation was found to be short (<2 h), reflecting the constitutive activity of PTPs. This rapid turnover of phosphorylation is sufficient to explain the loss of activated STAT proteins observed in the presence of tyrosine kinase inhibitors. It would also account for the limited duration of STAT activation observed in more typical cell lines that do not maintain prolonged kinase activity (e.g. FS2). Therefore, it is likely that the regulation of JAK activity, rather than changes in the rate of dephosphorylation, is the primary mode for controlling the duration of IFN-α responses.

An additional mechanism for the deactivation of STAT proteins besides dephosphorylation could be protein degradation. Given the relatively stable nature of Stat1 and Stat2 protein, such degradation would need to be targeted specifically for phosphorylated protein to explain the loss of activated STATs in the absence of significant loss of total protein. Indeed, specific degradation of phosphorylated Stat1 in IFN-γ treated HeLa cells through a ubiquitin-proteasome-mediated pathway has been recently reported (12). However, protein degradation does not appear to be responsible for the observed turnover of phosphorylated Stat1 or Stat2 in IFN-α-treated Daudi cells. First, treatment of cells with proteasome inhibitors did not prevent the turnover of phosphorylated STATs (data not shown). Second, we estimate that approximately 50% of total cellular Stat2 and 15–20% of cellular Stat1 are phosphorylated in Daudi cells treated with IFN-α for 30 min (Stat1 is more abundant than Stat2). This estimate was based on the fraction prolonged response to IFN. These results suggested that autocrine production and secretion of IFNs were required for maintaining STAT phosphorylation.

**Discussion**

**FIG. 6. Anti-IFN-α antisera block prolonged activation of Jak1, Stat1, and Stat2 in Daudi cells.** A. Cells were pulsed with IFN-α for 1 h, washed, refreshed with medium containing 500 units/ml anti-IFN-α antisera, and incubated for the indicated times (h). The positive control (lane 2) was IFN-α treatment for 10 min without addition of anti-IFN-α antibody. Total cell extracts were prepared, immunoprecipitated (IP) with anti-Stat1 (left panel), or anti-Stat2 (right panel), followed by blotting with anti-Tyr(P) (P-Tyr) antibody (upper panel). The membranes were stripped and reblotted with anti-Stat1 and Stat2 antibodies (lower panel). B. Jak1 immunoprecipitates were assayed for in vitro kinase activity. The membrane was first blotted with anti-Tyr(P) antibody (left panel), then stripped and rebotted with anti-Jak1 antibody (right panel).

Daudi cells for 30 min, 2 h, 4 h, and 24 h, which were subsequently analyzed by EMSA. Conditioned medium was able to activate ISGF3 formation at 30 min, and this activation was maintained for 24 h (Fig. 5B). Conditioned medium from cells that had never been exposed to IFN-α showed no such activity (Fig. 5B, lane 1). ISGF3 complex formation was used as a bioassay to quantify the amount of IFN-α secreted in IFN-α-stimulated Daudi cells. Activity equivalent to about 0.5 unit/ml recombinant human IFN-α-2a was produced from 1 × 10⁷/ml cells previously pulsed with IFN-α (data not shown).

**Autocrine Production of IFN-α Prolongs the Activation of JAK-STAT Pathway—**Since anti-IFN-α antibody blocked prolonged activation of ISGF3 in Daudi cells, the effect of this antibody on the activation of Stat1 and Stat2 was examined. Daudi cells were pulsed with IFN-α and treated with anti-IFN-α antibody for 4 and 24 h. As a control, cells were treated with IFN-α for 10 min in the absence of neutralizing antibody. Activated Stat1 and Stat2 were monitored by using antibodies for immunoprecipitation followed by blotting with anti-Tyr(P) antibody. The results showed that tyrosine phosphorylation of Stat1 and Stat2 appeared after 10 min of stimulation and was readily blocked by treatment with antibody for 4 h (Fig. 6A, upper panel) even though protein levels were comparable during the 24 h of treatment (Fig. 6A, lower panel). The kinase activity of Jak1 was also transient under these conditions. Jak1 was no longer autophosphorylated after 4 h of antibody treatment (Fig. 6B, left panel). Further, the ability to phosphorylate exogenous substrate was also abrogated (data not shown), indicating that neutralization of secreted IFN-α inhibited the...
of STAT protein that is translocated to the nucleus and the relative intensities of anti-phosphotyrosine signals in cytoplasmic and nuclear extracts (data not shown). Given this large fraction of the cellular pool of STAT protein that is activated, significant loss of protein would be expected to be observed following 4 h of treatment if protein degradation played a major role in turnover. In contrast, little or no turnover of total Stat1 or Stat2 was observed by 32P-labeled pulse-chase labeling (Fig. 2B) while significant turnover of phosphorylation was observed (Fig. 2A). Therefore, although we cannot completely exclude a role for protein degradation in the turnover of phosphorylated STAT proteins, we conclude that a constitutive PTP contributes the major activity. Similar conclusions implicating the dephosphorylation of activated Stat1 in the absence of significant protein degradation have been recently reported for IFN-γ-treated cells (13).

Mechanisms underlying the prolonged kinase activity of Jak1 and Tyk2 in Daudi cells are still unknown. The continuous requirement of ligand stimulation in maintaining a prolonged response suggests that receptor-mediated signaling is involved. Although evidence has suggested that IFN-α-induced receptor down-regulation and degradation occurred rapidly in Daudi cells (11), the retention of ligand-receptor-kinase complexes within endosomes might contribute to prolonged kinase activity similar to that seen in the epidermal growth factor-induced response of liver parenchyma (29). Alternatively, recycling of a small portion of high affinity receptors in Daudi cells could also maintain the kinase activity. Interestingly, the administration of neutralizing antibody inhibited this prolonged response (Fig. 4B), and conditioned medium collected from IFN-α-stimulated Daudi cells contained IFN-α (Fig. 4C), suggesting the continuous production and secretion of IFN-α. This autocrine loop of IFN-α not only contributes to a prolonged response, it also increases the sensitivity to IFN-α owing to the basal activation of the signaling machinery. It has been shown that specific IFN-α mRNAs are constitutively present at low levels in organs of normal humans (30), indicating that the constant production of IFN-α could also provide an important host defense mechanism against invading pathogens. Thus, the involvement of autocrine secretion and production of IFN-α in maintaining a prolonged response may play a significant role in vivo.

Although Daudi cells have been shown to secrete low amounts of IFN-α constitutively (20), we show here that IFN-α secretion is induced to higher levels in Daudi cells in response to IFN-α. The finding that CHX treatment blocked the prolonged response (Fig. 4) suggested the requirement for new protein synthesis. The IFN detected in conditioned medium was indeed produced by the cells and could not be ascribed to residual exogenous IFN from the treatment pulse. Neutralization with anti-IFN monoclonal antibodies demonstrated that the secreted IFN was a mixture of IFN-α subtypes rather than the recombinant IFN-α-2a used for exogenous treatment (data not shown).

IFN-γ also has been shown to induce prolonged activation of Stat1 and continued transcriptional activation of an IFN-γ-inducible gene, such as GBP (31, 32). However, there are several different aspects that distinguish the IFN-α-induced prolonged response. First, the IFN-γ-induced response is stable to removal of ligand, while the response induced by IFN-α is dependent on continuous presence of IFN-α. Second, the response to IFN-γ is sensitive to protein kinase C inhibitors while the IFN-α-induced response is not affected by these kinase inhibitors. Third, the response to IFN-γ is only partially affected by CHX treatment, but the continued response of Daudi cells to IFN-α is totally blocked by CHX. The differences between IFN-γ and IFN-α-induced prolonged response suggest that a unique mechanism may be involved in the regulation of the IFN-α response in Daudi cells.

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