The Antiviral Response to Gamma Interferon

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Received 11 April 2002/Accepted 12 June 2002

A role for alpha/beta interferon (IFN- α/β) in the IFN- γ antiviral response has long been suggested. Accordingly, possible roles for autocrine or double-stranded-RNA (dsRNA)-induced IFN- α/β in the IFN- γ response were investigated. Use was made of wild-type and a variety of mutant human fibrosarcoma cell lines, including mutant U5A cells, which lack a functional IFN- α/β receptor and hence an IFN- α/β response. IFN- γ did not induce detectable levels of IFN- α/β in any of the cell lines, nor was the IFN- γ response per se dependent on autocrine IFN- α/β . On the other hand, a number of responses to dsRNA [poly(I) · poly(C)] and encephalomyocarditis virus were greatly enhanced by IFN- γ pretreatment (priming) of wild-type cells or of mutant cells lacking an IFN- α/β response; these include the primary induction of dsRNA-inducible mRNAs, including IFN- β mRNA, and, to a lesser extent, the dsRNA-mediated activation of the p38 mitogen-activated protein (MAP) kinase(s). IFN- γ priming of mRNA induction by dsRNA is dependent on JAK1 and shows biphasic kinetics, with an initial rapid (<30-min) response being followed by a more substantial effect on overnight incubation. The IFN- γ -primed dsRNA responses appear to be subject to modulation through the p38, phosphatidylinositol 3-kinase, and ERK1/ERK2 MAP kinase pathways. It can be concluded that despite efficient priming of IFN- β production, the IFN- α/β pathways play no significant role in the primary IFN- γ antiviral response in these cell-virus systems. The observed IFN- γ priming of dsRNA responses, on the other hand, will likely play a significant role in combating virus infection in vivo.

The interferons (IFNs) were identified first as antiviral agents. Both alpha/beta IFN (IFN- α/β) and IFN- γ have antiviral activity; in neither case is the basis for this fully understood. Inhibitions of viral uptake and uncoating, viral RNA and DNA replication, and viral protein synthesis and assembly have all been reported for different virus-cell systems. Accordingly, multiple mechanisms of action are accepted (reviewed in references 21, 22, and 26). At the molecular level, for both types of IFN, signaling through JAK/STAT pathways is essential, and the MX proteins, double-stranded RNA-dependent protein kinase (PKR), and the 2-5A system all have proven antiviral activity. Cells lacking all three of the latter, however, can still manifest an antiviral response (29). IFN- γ induces the synthesis of the STAT1 and p48 (ISGF3 γ /IRF-9) subunits of the transcription factor ISGF3, which is essential to the IFN- α/β response, and IFN- α/β induce STAT1, which is essential to the IFN- γ response (15, 22). Some synergism between the two pathways is therefore to be expected.

In human fibrosarcoma cells (HT1080), IFN- γ mediates a less profound inhibition of encephalomyocarditis (EMC) virus replication than IFN- α/β (10^2 - versus $>10^5$ -fold reduction in virus yield). Consistent with this, in a single-cycle growth experiment in this system, IFN- γ mediated a relatively modest

(about 25%) inhibition of viral RNA replication (2). Many years ago, in some of the original single-cycle growth experiments, the increased replication of RNA viruses in the presence of actinomycin D was attributed to the inhibition of early IFN induction or action (25). A priori, the IFN- γ response could be mediated, at least in part, through IFN- α/β . Indeed, results obtained with two mutants of JAK1 could readily be explained on this basis. The kinase-defective JAK1 (JAK1.KE) is inhibitory to the IFN- α/β response but selectively inhibits the antiviral response to IFN- γ (3). The second JAK1 mutant (JAK1. Δ B) shows a related phenotype: it is without an inhibitory effect on JAK/STAT signaling and the IFN- α/β and - γ responses in general, but it selectively inhibits both the antiviral response to IFN- γ and the induction of IFN- β in response to double-stranded RNA (dsRNA) (28). In these systems, therefore, the inhibition of the IFN- γ antiviral response could be, partially or wholly, through the inhibition of IFN- α/β action for JAK1.KE or through the inhibition of production for JAK1. Δ B. In more general terms, the antiviral effects of IFN- γ could, a priori, reflect, in addition to the direct induction of IFN-stimulated genes (ISGs), (i) the induction of IFN- α/β and, secondary to this, ISGs and/or (ii) the priming of viral (dsRNA) induction of ISGs directly or through IFN- α/β plus (iii) additional mechanisms yet to be identified. Consistent with this Takaoka et al. have recently identified a requirement for IFN- β in the IFN- γ response in mouse embryo fibroblasts (MEFs) (24).

Against this background, it was of interest to determine for a human system to what extent the primary IFN- γ response in general and the antiviral response to IFN- γ in particular are dependent on IFN- α/β . No evidence for any such requirement was, in fact, obtained. IFN- γ did not induce significant IFN-

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α/β , and wild-type primary general and antiviral responses to IFN- γ were obtained in cells lacking an IFN- α/β response. That said, it can reasonably be assumed that the observed IFN- γ priming of dsRNA responses likely plays a significant role in enhancing antiviral activity in vivo.

MATERIALS AND METHODS

Cell culture. Human fibrosarcoma cell lines 2fTGH, U4A, U5A, complemented U5A (U5A/IFNAR2), 2C4, and γ 2A cells (13, 14, 17, 27) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U of penicillin per ml, and 50 μ g of streptomycin per ml. Resistant cells were maintained in medium containing 250 μ g of hygromycin (Calbiochem) per ml and/or 400 μ g of G418 (GibcoBRL/Life Technologies) per ml. The double-stranded polyribonucleotide poly(I)·poly(C) (polyIC) was obtained from Amersham and used throughout this study at 100 μ g/ml. Highly purified recombinant IFN- γ (4×10^7 IU/mg of protein) was a generous gift from G. Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria.

Antibodies. Antiphosphotyrosine 701-STAT1, anti-phospho-p38, and anti-p38 antibodies were from New England Biolabs, antiphosphoserine 727-STAT1 was from Upstate Biotech, and anti-STAT1 (C-111) was from Santa Cruz. Phycocerythrin (PE)-conjugated anti-HLA DR α was purchased from Becton Dickinson.

Cell lysis, Western blotting, and EMSA. Cells were lysed on ice with 50 mM Tris (pH 8.0)–0.5% NP-40–10% glycerol–150 mM NaCl–1 mM dithiothreitol–0.1 mM EDTA–0.2 mM sodium orthovanadate–0.5 mM phenylmethylsulfonyl fluoride–3 μ g of aprotinin per ml–1 μ g of leupeptin per ml, cell debris were removed by centrifugation, and whole-cell extracts were used for electrophoretic mobility shift assay (EMSA) or Western blotting, essentially as described previously (12). To detect phospho-p38 and p38, cells were lysed in 25 mM Tris (pH 7.6)–10% glycerol–2% sodium dodecyl sulfate according to the manufacturer's instructions.

RNase protection assays. Cytoplasmic RNA was isolated, using Tri Reagent (Elena Biosciences) as directed by the manufacturer, and protection assays were carried out as previously described (15). The CIITA probe was generated from a 350-bp *Hind*III fragment (nucleotides 2935 to 3285) from a human class II HLA transactivator (CIITA) cDNA (from R. Flavell) cloned into pGEM4.

Flow cytometry. Cells treated with medium only or 1,000 IU of IFN- γ per ml for 24 to 72 h were removed from the plates with buffered EDTA, washed in ice-cold phosphate-buffered saline, and incubated with anti-HLA DR α -PE for 45 min on ice in the dark. The cells were then washed three times with ice-cold phosphate-buffered saline, fixed in 1% *p*-formaldehyde, and analyzed on a fluorescence-activated cell sorter (FACS).

Antiviral assays. Cells seeded into 24-well plates at 2×10^5 cells/well were incubated overnight at 37°C, treated with serial dilutions of IFN- γ for 18 h, and challenged with EMC virus (0.3, 3, or 10 PFU/cell). Cells were fixed with formal saline and stained with Giemsa stain for live cells at 10 or 20 h postinfection.

IFN- β ELISA. Levels of secreted IFN- β in response to polyIC were measured by enzyme-linked immunosorbent assays (ELISA) (R&D Systems), essentially according to the manufacturer's instructions.

RESULTS

The IFN- γ response is not dependent on IFN- α/β . IFN- γ did not induce detectable IFN- α/β in wild-type or mutant U5A HT1080-based human fibrosarcoma cell lines. IFN- β was not detectable in the medium from such cells by standard ELISA assays (Fig. 1A [no polyIC] and data not shown). Moreover, when assayed on mutant IFN- γ response-negative γ 2A cells (to obviate any affect of carryover of IFN- γ), this medium did not induce an antiviral response, as would have been expected were the IFN- γ treatment to have induced significant (>1 U/ml) IFN- α or - β (Fig. 1B). Consistent with this, by very sensitive RNase protection assays, IFN- γ did not induce detectable IFN- β mRNA in these cells (see, for example, Fig. 4A, lanes 3, 7, and 11). In addition, in contrast to the situation in MEFs (24), no significant dependence of the IFN- γ response upon an IFN- α/β receptor was observed for the human fibrosarcoma

FIG. 1. IFN- γ does not induce IFN- α/β . (A) ELISA. IFN- γ does not induce detectable IFN- β (no polyIC, shaded bar; lower limit of detection 250 pg/ml) but primes IFN- β production in response to polyIC. Medium from IFNAR2 (IFN- α/β response)-negative U5A cells with (primed) or without (unprimed) pretreatment with IFN- γ (1,000 IU/ml for 18 h) and polyIC, as indicated, was assayed by standard ELISA for IFN- β (see Materials and Methods). (B) Antiviral assay. Conditioned medium from cells treated with IFN- γ was assayed for the presence of IFN- α/β by using JAK2 (IFN- γ response)-negative γ 2A cells. Wild-type cells (row 1) or γ 2A cells (row 2) were uniformly treated for 30 h with conditioned medium from wild-type cells preincubated with IFN- γ (1,000 IU/ml, 24 h). For positive and negative controls, γ 2A cells were treated with IFN- α (row 3) or IFN- γ (row 4) as indicated. Infection was with 0.3 PFU of EMC virus per cell, and cells were fixed and stained 24 h later. The results shown here and in all other figures are representative of those from at least three independent experiments. C, control; V, virus infected.

HT1080-derived cells used here. Activation of STAT1 in response to IFN- γ is dependent on phosphorylation, and it can be monitored by activation of DNA binding (EMSA) or phosphorylation of tyrosine residue 701 and serine residue 727. By each of these three assays, STAT1 activation in response to IFN- γ was comparable in (i) wild-type 2fTGH cells, (ii) the mutant U5A cell line which lacks the IFNAR2 subunit of the IFN- α/β receptor and hence an IFN- α/β response, and (iii) complemented U5A cells stably expressing transfected IFNAR2 (U5A/IFNAR2 cells) (Fig. 2). The induction of IFN- γ -inducible genes in response to IFN- γ was similarly comparable in all three of these cell lines (for example, Fig. 4A, CIITA, GBP1 and IRF-1, lanes 3, 7, and 11, and data not shown), as was the induction of class II HLAs (the cell surface expression of which was monitored by FACS [Fig. 3]) and the antiviral response (see Fig. 10). Thus, despite the potential for synergism between the responses to the two types of IFNs, there is no absolute requirement for such synergism for a primary IFN- γ response.

IFN- γ primes the induction of IFN- β and ISG mRNAs in response to virus and dsRNA in wild-type and IFN- α/β re-

FIG. 2. STAT1 activation in response to IFN-γ is not dependent on autocrine or induced signaling through the IFN-α/β receptor. The kinetics (at 100 IU/ml) and dose response (at 15 min) of STAT1 activation for IFN-γ in wild-type (2fTGH), IFNAR2 (IFN-α/β-response)-negative U5A, and complemented U5A (U5A/IFNAR2) cells were monitored in whole-cell extracts by EMSA with an hSIE probe (A) and by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and Western blotting with antibody to STAT1 PTyr701 (B) or PSer727 (C). Protein levels were monitored by reprobing with specific antibody to STAT1 (D) (see Materials and Methods). The enhanced signals at 18, 24, and 48 h in panel D reflect the de novo synthesis of STAT1 in response to IFN-γ.

sponse-negative cells. dsRNA induces IFN-β and, through it, ISGs. It also induces ISGs (1) and a number of additional genes directly through a distinct mechanism(s) (5). PolyIC has been widely used as a model dsRNA in the study of such responses. The IFI56K gene is an example of a gene highly inducible in response to IFN-β and directly in response to polyIC and/or dsRNA (the dsRNA-direct response). It is only minimally induced in response to IFN-γ (see, for example, reference 21 and Fig. 4A, lanes 3, 7, and 11, and Fig. 5, lanes 3 to 8). Accordingly, it was used here to monitor the IFN-γ priming of the dsRNA-direct response in wild-type cells versus IFNAR2-negative U5A cells, which lack any response to IFN-α/β.

As determined by RNase protection assay, IFN-γ pretreatment primed the induction of IFN-β mRNA in response to polyIC comparably in wild-type 2fTGH, mutant U5A, and complemented U5A (U5A/IFNAR2) cells (Fig. 4A). Consis-

tent with this, the production of IFN-β per se in response to dsRNA was also primed by pretreatment with IFN-γ (Fig. 1A). In addition, IFN-γ primed the dsRNA-direct response of the IFI56K gene as manifest by the enhanced response of this gene to polyIC in U5A cells (Fig. 4A and 5). IFN-γ pretreatment, therefore, primes both the pathway leading to the induction of IFN-β mRNA and the dsRNA-direct pathway leading to the induction of mRNAs for ISGs such as the IFI56K gene.

On the basis of current evidence, viral dsRNA appears to be responsible, in large part at least, for the viral induction of IFN-β and ISGs, but additional or alternative players cannot be excluded. Indeed, the IFI56K gene can be induced in response to Sendai virus in a mutant cell line which lacks a dsRNA response (8). Accordingly, it was of interest to ask if the induction of IFN-β and IFI56K mRNAs by virus also shows priming by IFN-γ. It is, in fact, clear that IFN-γ pretreatment

FIG. 3. Induction of class II HLA in response to IFN- γ is unaffected in IFNAR2 (IFN- α/β response)-negative U5A cells. Wild-type (2fTGH), U5A, and complemented U5A (U5A/IFNAR2) cells, with or without treatment with IFN- γ (1,000 IU/ml) for 24, 48, or 72 h as indicated on the left, were harvested, stained with an anti-HLA DR α PE-conjugated antibody, and analyzed for class II HLA expression (open peaks) by FACS. Shaded peaks correspond to cells stained with an isotype-matched irrelevant PE-conjugated antibody.

also primes the induction of both IFN- β and IFI56K mRNAs in response to EMC virus in both wild-type 2fTGH cells (Fig. 4B) and U5A cells (see, e.g., Fig. 8B). IFN- γ , therefore, primes both the IFN- β and IFI56K (dsRNA-direct) responses similarly for virus and dsRNA (polyIC).

Although the priming by IFN- γ of the response of the IFI56K gene to dsRNA (IFI56K in Fig. 4A, lane 8 versus lane 6, and Fig. 5, lanes 9 to 14 versus lanes 3 to 8) cannot be dependent on a positive feedback loop through the production of IFN- β in the IFN- α/β response-negative U5A cells, it may be very modestly augmented by a feedback loop through IFN- β triggered by polyIC in both the complemented U5A/IFNAR2 and wild-type (2fTGH) cells (Fig. 4A, IFI56K, lanes 4 and 12 versus lane 8). Overall it can be concluded that although there is no dependence of the IFN- γ response per se on IFN- α/β , the priming by IFN- γ of the response to virus infection (dsRNA) will likely be of importance in vivo.

Kinetics of priming. The kinetics of priming for both the IFN- β and the IFI56K (dsRNA-direct) mRNA responses were monitored in IFNAR2/IFN- α/β response-negative U5A cells to exclude any possibility of secondary signaling through IFN- α/β . Interestingly, when assayed by RNase protection for IFN- β and IFI56K mRNAs (Fig. 5) or by ELISA for IFN- β per se (Fig. 1A and data not shown), overnight treatment with IFN- γ was required to generate an optimal response. Some priming can, however, be detected with pretreatment for as

little as 30 min. Thus, IFN- γ may affect the dsRNA responses both directly (for example, through priming of the activation of p38 mitogen-activated protein [MAP] kinases, see below) and through the induced synthesis of signaling intermediates (for example STAT1 and IRF-1, implicated in the dsRNA-direct and IFN- β pathways, respectively, of the dsRNA response) (5, 21).

IFN- γ priming of the dsRNA responses is dependent on JAK1. Data obtained from work with mutant cell lines and knockout mice defective in elements of JAK/STAT signaling have established an essential role for such signaling in the IFN- γ response. Although it is essential, JAK/STAT signaling is not necessarily sufficient to mediate all aspects of the IFN- γ response. Indeed, there is clear evidence for a role for additional JAK-dependent signaling in, for example, STAT1-negative cells and mice (6, 18, 19), and the possibility of ancillary non-JAK1-dependent signaling cannot be excluded. Accordingly, it was of interest to determine whether IFN- γ priming is JAK dependent or would reveal a novel, non-JAK pathway. In U4A cells, which lack JAK1 and, therefore, any JAK-mediated responses to IFN- γ , the induction of both the IFN- β and IFI56K mRNAs in response to polyIC were, as expected (14), comparable to those in wild-type cells. The priming of these responses by IFN- γ was, however, dependent on the presence of JAK1 (Fig. 6, lanes 6 to 8 versus 14 to 16).

IFN- γ pretreatment enhances the activation of a p38 MAP kinase(s) in response to dsRNA. p38 MAP kinases have been implicated in both the IFN- γ and dsRNA responses (7, 9, 11). Phosphorylation of p38 can indeed be seen in response to both stimuli in the wild-type and mutant U5A human fibrosarcoma cells when they are grown in strictly endotoxin-free medium with low serum concentrations to reduce background levels of constitutive activation (Fig. 7A, lanes 4 to 6, for polyIC and 7B, lanes 3 to 6, for IFN- γ). Pretreatment of cells with IFN- γ for 18 h enhanced the response to 1 to 6 h of treatment with polyIC (100 μ g/ml) (Fig. 7A). Progressively enhanced signals were also observed in response to polyIC (100 μ g/ml, 3 h) on IFN- γ pretreatment from 1 to 6 h (Fig. 7B). The enhanced signals appear to be greater than the sum of the IFN- γ and the polyIC responses, consistent with some degree of IFN- γ priming of the dsRNA response. No such effect was obtained with EMC virus (data not presented). Whether or not this represents a true difference in the responses to dsRNA and virus or simply a quantitative difference remains to be established. Phosphatidylinositol 3-kinase (PI3K) and the p38 MAP kinases have both been implicated in the classical antiviral and ISG responses to IFN- γ . Roles for these kinases (Fig. 8 and 9A) and ERK1 and ERK2 (Fig. 9B) in priming by IFN- γ were therefore investigated.

Role for the p38 MAP kinase in the IFN- γ -primed induction of IFN- β and IFI56K mRNAs in response to dsRNA or virus in IFN- α/β response-negative U5A cells. In order to investigate a possible role for the p38 MAP kinase(s) in the primed dsRNA and dsRNA-direct responses, advantage was taken of the availability of well-characterized inhibitors of these kinases (in particular, SB202190). To monitor the dsRNA-direct response of the IFI56K gene, the experiments were carried out in U5A cells, which are unresponsive to IFN- α/β . The addition of a known effective concentration (1 μ M) of SB202190 inhibitor to IFN- γ -primed cells 30 min prior to polyIC substantially and

FIG. 4. IFN- γ primes the induction of IFN- β and ISG mRNAs in response to dsRNA (polyIC, 100 μ g/ml for 2 h) (A) and EMC virus (10 PFU/cell for 8 h) (B). (A) Expression of IFN- β and IFI56K mRNAs in wild-type (2fTGH), IFNAR2 (IFN- α/β response)-negative U5A, and complemented U5A (U5A/IFNAR2) cells was monitored by RNase protection assay (6% gel; 13 μ g of RNA/lane) together with CIITA, IRF1, and GBP mRNAs as predominantly IFN- γ -inducible controls. For IFN- β mRNA, the levels induced in response to polyIC (P) in unprimed (lanes 2, 6, and 10) versus primed (IFN- γ at 1,000 IU/ml for 18 h; lanes 4, 8, and 12) cells were similar in all three cell lines. The slight reduction in the level of the IFI56K mRNA in response to polyIC in the primed IFN- α/β response-negative U5A cells (lane 8 versus lanes 4 and 12) likely reflects the absence of secondary signaling through polyIC-induced IFN- β to this most highly IFN- α/β -inducible mRNA. (B) Induction of IFN- β and IFI56K mRNAs by EMC virus (V) also shows priming by IFN- γ (as for panel A) comparable to that seen for the response to polyIC (lanes 5 and 6 versus lanes 2 and 3). Actin mRNA was monitored here and for subsequent figures as a loading control. C, control; P, polyIC; γ , IFN- γ ; γ P, IFN- γ followed by polyIC; V, virus infected.

partially inhibited the induction of the IFN- β and IFI56K mRNAs, respectively (Fig. 8A). A similar effect of inhibition of p38 activation on the IFN- γ -primed responses to EMC virus infection was observed, although an effect of the SB202190 inhibitor on EMC virus infection per se cannot be excluded (Fig. 8B). The specific inhibition of the p38 MAP kinases by 1 μ M SB202190 has a limited half-life. Nevertheless, a quantitatively similar inhibition of the dsRNA response was observed on addition of 1 μ M SB202190 prior to 18 h of priming with IFN- γ (data not presented). Such an effect would be consistent at least with a role for the p38 MAP kinases in priming as well as in the primed responses. Taken together, the data indicate a role for a pathway(s) involving p38 in both of the IFN- γ -primed dsRNA-triggered pathways (to IFN- β and IFI56K) and that an additional role for p38 in the priming of these responses by IFN- γ cannot be excluded.

FIG. 5. Kinetics of IFN- γ priming of dsRNA responses. The kinetics of IFN- γ priming for both the IFN- β and IFI56K (dsRNA-direct) mRNA responses were monitored in IFNAR2 (IFN- α/β response)-negative U5A cells to exclude any possibility of secondary signaling through IFN- α/β . Treatment of U5A cells with IFN- γ (1,000 IU/ml), with or without subsequent treatment with polyIC (100 μ g/ml, 2 h), was for the times indicated; mRNA expression was monitored as described for Fig. 4.

FIG. 6. IFN- γ priming of dsRNA responses is dependent on JAK1. IFN- γ priming of both the IFN- β and the IFI56K (dsRNA-direct) mRNA responses to polyIC was monitored in wild-type (2fTGH) and JAK1-negative U4A cells by RNase protection (as described for Fig. 4) in comparison with CIITA, IRF-1, and GBP mRNAs as predominantly IFN- γ -inducible controls. IFN- γ (1,000 IU/ml) treatment with or without subsequent polyIC (100 μ g/ml, 2 h) treatment was for the times indicated.

Modulation through additional signaling pathways. The PI3K and classical ERK1/ERK2 MAP kinase pathways can also be activated in response to the IFNs, most probably to a variable extent in different cell types (4, 10, 16). The possible modulation of the IFN- γ -primed dsRNA responses on inhibition of these pathways was therefore investigated. Use was again made of well-characterized inhibitors of these pathways to obtain an indication of any possible modulatory effects. Similarly, the experiments utilized U5A cells in order to be able to monitor the dsRNA-direct response of the IFI56K mRNA. Interestingly, the PI3K inhibitor LY294002 and the ERK1/ERK2 inhibitor PD98059, when introduced at known effective concentrations (20 and 50 μ M, respectively), had opposite effects. The PI3K inhibitor partially inhibited and the ERK1/ERK2 inhibitor minimally but significantly stimulated both IFN- γ -primed dsRNA responses (IFN- β and IFI56K mRNAs) (Fig. 9). Both inhibitors were without effect on the IFN- γ -mediated induction of the CIITA or IRF-1 mRNAs, i.e., on the IFN- γ response per se (Fig. 9). Results identical to those obtained with PD98059 were obtained using U0126, another MAP kinase-specific inhibitor (data not shown). Taken together, the data indicate that IFN- γ priming of mRNA induction by dsRNA is biphasic, JAK1 dependent, and, most likely, subject to complex modulation through cross talk with a variety of additional signaling pathways which may well vary with cell type and status.

The primary antiviral response to IFN- γ is not dependent on an IFN- α/β response. Accepting that IFN- γ treatment efficiently primes the induction of IFN- β and, both directly and indirectly, ISGs and that it can also enhance the IFN- α/β response through the induction of, for example, STAT1 and p48, it remained of interest to determine the extent of the dependence, if any, of the primary IFN- γ antiviral response upon IFN- α/β . Accordingly, the IFN- γ antiviral responses to EMC virus in wild-type cells, U5A cells lacking an IFN- α/β response, and complemented U5A/IFNAR2 cells were compared. No difference in the antiviral response between the cell types was observed in classical cytopathic effect assays involving either one (10 h) or two (20 h) rounds of virus replication (Fig. 10 and data not presented). The cytopathic effect of EMC virus in these cells is inhibited by zVAD, a pan-caspase inhibitor, and therefore is primarily apoptotic (20; A. P. Costa-Pereira and I. M. Kerr, unpublished data). Thus, in this context, pretreatment with IFN- γ is antiapoptotic. We have noted that such an antiapoptotic effect does not necessarily reflect an inhibition of virus replication (B. Strobl, S. J. Newman, and I. M. Kerr, unpublished data). Accordingly, virus replication per se was monitored in a parallel series of experiments in which the yields of progeny virus at 10 and 20 h were assayed on L929 mouse cells. Once again, no difference in IFN sensitivity was observed for the different cell types (data not shown). Accordingly, despite efficient priming of IFN- β production,

FIG. 7. IFN- γ pretreatment enhances the activation of p38 MAP kinases in response to dsRNA in wild-type (2fTGH) and IFNAR2 (IFN- α/β response)-negative U5A cells. (A) Pretreatment of cells with IFN- γ (1,000 IU/ml) for 18 h enhances the response to polyIC (1 to 6 h, 100 μ g/ml). (B) Pretreatment of cells with IFN- γ (1,000 IU/ml) for 1 to 6 h progressively increases the response to polyIC (100 μ g/ml, 3 h). The phosphorylation of p38 (upper panels) was monitored by Western blotting of whole-cell extracts with a phospho-specific p38 antibody (see Materials and Methods). Membranes were stripped and subsequently reprobbed with an anti-p38 antibody as a loading control (lower panels).

FIG. 8. Role of p38 MAP kinase(s) in the IFN- γ -primed induction of IFN- β and IFI56K mRNAs in response to dsRNA (A) or EMC virus (B) in IFNAR2 (IFN- α/β response)-negative U5A cells. U5A cells, with or without priming with IFN- γ (1,000 IU/ml for 18 h), were treated with a p38-specific inhibitor (1 μ M SB202190) for 30 min and then with polyIC (100 μ g/ml) (A) or with EMC virus (10 PFU/cell) (B) for the times indicated. mRNA expression was monitored by RNase protection assay (as described for Fig. 4). Inhibition of p38 significantly and detectably inhibited the IFN- γ -primed polyIC (panel A, lanes 11 to 16) and EMC virus (panel B, lanes 13 to 20)-mediated induction of IFN- β and IFI56K mRNAs. The inhibitions of the IFN- γ -primed responses to polyIC at 1, 2, and 3 h (panel A, lanes 11, 12, and 13 versus 14, 15, and 16) were 1.7-, 1.3-, and 2.3-fold, respectively, for the IFN- β mRNA and 1.1-, 1.3-, and 1.5-fold, respectively, for the IFI56K mRNA, and those to EMC virus infection for the four time points (panel B, lanes 13 to 16 versus lanes 17 to 20) were 1.4-, 1.7-, 1.5-, and 1.6-fold, respectively, for the IFN- β mRNA and 1.2, 1.6, 0.9-, and 1.2-fold, respectively, for the IFI56K mRNA. The RNase protections were scanned by a phosphorimager, and all values were corrected relative to the corresponding internal actin control. For each type of experiment here and in Fig. 9, similar values were obtained in two or more independent experiments. The overall average inhibitions observed for the IFN- γ -primed responses to polyIC for the IFN- β and IFI56K mRNAs were 2-fold and 1.5-fold, respectively, for the SB202190 inhibitor and 1.85 and 1.5-fold, respectively, for the LY292004 inhibitor (see Fig. 9A). The average stimulation for the PD59058 inhibitor was approximately twofold for both mRNAs (see Fig. 9B). C, control; γ , IFN- γ ; SB, SB202190; γ /SB, IFN- γ followed by SB202190.

this plays no significant role in the primary antiviral response to IFN- γ .

DISCUSSION

Takaoka et al. have presented persuasive evidence that in MEFs the IFN- γ response is substantially augmented through autocrine IFN- α/β and that cross-recruitment and phosphorylation of the IFNAR1 subunit of the IFN- α/β receptor occurs in response to IFN- γ in these cells (24). We have been unable to obtain evidence for or against recruitment or phosphorylation of IFNAR1 in response to IFN- γ in the HT1080-based human cell systems used here (H. Is'harc and I. M. Kerr, unpublished data). In a potentially analogous but inverse situation, cross-phosphorylation of the IFNGR1 subunit of the IFN- γ receptor in response to activation of erythropoietin/gp130 receptor chimeras in HT1080-based cell lines is ob-

served. Importantly, however, such receptor cross-phosphorylation plays no part in the IFN- γ -like response observed (23), a result which emphasizes that even if IFNAR1 were recruited and phosphorylated in response to IFN- γ , proof of necessity of this for the IFN- γ antiviral response would require cells lacking IFNAR1. Such IFNAR1-negative human cells are not available. It is therefore accepted that a requirement for IFNAR1 per se, as opposed to requirements for an intact IFN- α/β receptor and IFN- α/β response, cannot be rigorously excluded. This leaves open the question as to whether there is any absolute requirement for an IFN- α/β response to sustain any unique aspect of the IFN- γ response per se. It is clear that an additional signal(s), over and above those required for the majority of the IFN- γ response, is required for the antiviral response(s) and, as outlined in the introduction, cogent arguments can be made in favor of IFN- α/β as the required signal. This does not, however, appear to be the case at least for the

FIG. 9. A role for PI3K and the ERK MAP kinases in IFN- γ -primed dsRNA responses. IFNAR2 (IFN- α/β response)-negative U5A cells were treated with IFN- γ (1,000 IU/ml, 18 h) and either 20 μ M LY292004 (A) or 50 μ M PD59058 (B) for 30 min prior to polyIC (100 μ g/ml, 2 h) treatment. IFN- β and IFI56K mRNA expression was monitored by RNase protection in comparison with CIITA, IRF-1, and actin mRNAs as described for Fig. 4. The inhibitions of the IFN- γ -primed responses of the IFN- β and IFI56K mRNAs in the presence of the LY294002 inhibitor (panel A, lane 4 versus 8, corrected for actin controls) were 1.7- and 1.5-fold, respectively. The stimulations of the IFN- γ -primed dsRNA responses of the IFN- β and IFI56K mRNAs in the presence of the PD98059 inhibitor (panel B, lanes 4 versus 8, corrected for actin controls) were 2.05- and 1.8-fold, respectively. C, control; P, polyIC; γ , IFN- γ ; γ P, IFN- γ followed by polyIC.

human fibrosarcoma-EMC virus system. Results with the U5A cells lacking an IFN- α/β response clearly exclude any such absolute requirement for an IFN- α/β response in the IFN- γ response in these cells.

The dsRNA response is complex, involving the induced expression of many hundreds of genes (5) and more than one mechanism (1, 8). Some of the genes are IFN inducible; some are not (5). The most intensively studied and best understood mechanism is that of the induction of IFN- β , for which the promoter has been minutely analyzed and signaling is at least partially understood. Induction appears to involve, at least in part, the activation of PKR by dsRNA; this leads, by mechanisms yet to be fully defined, to the activation of NF- κ B, which, together with IRF-1 (for dsRNA) and IRF-3 (for virus), appears to be the major transcription factor required (21). In contrast, the dsRNA-direct response of the IFI56K gene shows a major requirement for STAT1 (1). Clearly, although much is known, the signaling pathways involved remain to be fully defined. Similarly, the priming of the dsRNA responses by IFN- α/β is well established, but the mechanisms remain to be fully defined. Recent analyses by Harcourt and Offermann have served to emphasize the complexities involved. They have concluded from experiments involving expression profiling and the use of inhibitors of signaling pathways that IFN- α priming enhances activation of the p38 and ERK pathways in response

FIG. 10. The primary antiviral response to IFN- γ is not dependent on an IFN- α/β response. Wild-type (2fTGH), IFNAR2 (IFN- α/β -response)-negative U5A, and complemented U5A (U5A/IFNAR2) cells were incubated for 18 h at 37°C with the indicated concentrations of IFN- γ , infected where shown with EMC virus (0.3 PFU/cell in duplicate [upper two rows] or 3 PFU/cell in duplicate [lower two rows]) and stained for live cells at 20 h postinfection. C, control; V, virus infected.

to polyIC, thereby enhancing the expression of some but not all induced genes (9).

Here we have been concerned with priming through IFN- γ , not IFN- α , of both viral and dsRNA responses. Readout was through the assay of the induced expression of IFN- β and IFI56K mRNAs as indices of at least two distinct dsRNA response pathways. To the extent studied, with the possible exception of priming of the p38 MAP kinase, the results with virus were similar to those with polyIC (Fig. 4 and 8). It would appear that IFN- γ primes, in response to virus, both dsRNA response pathways leading to the induction of IFN- β mRNA and the dsRNA-direct pathway leading to ISGs. Priming of the dsRNA response is JAK1 dependent (Fig. 6). A likely partial dependence of the primed dsRNA responses on the p38 MAP kinase is indicated, and a similar dependence for the priming element (prior to the addition of dsRNA) of this response cannot be excluded (Fig. 7 and 8). The results of the survey of the effects of the pharmacological inhibitors of the p38, PI3K, and ERK pathways suggest that the IFN- γ -primed dsRNA pathways are subject to modulation by such inhibitors and likely, therefore, by these pathways themselves. Such cross talk may well vary substantially with cell type and status. Thus, despite the complexities of the systems involved, some cogent conclusions can be drawn from the results reported here. It is clear that IFN- γ substantially primes aspects of the viral and dsRNA responses, that this priming is JAK dependent, and that the primed responses are subject to complex modulation through additional pathways. In addition, in contrast to the data for mouse cells (24), despite the efficiency with which IFN- γ primes the induction of IFN- β in response to virus and dsRNA, in the human cell systems investigated here, the primary IFN- γ antiviral response showed no dependence on an IFN- α/β response. The importance, if any, of the dsRNA-direct response in this regard remains to be established. It is, however, reasonable to assume that the priming by IFN- γ of dsRNA responses triggered by virus likely plays a major role in enhancing the IFN- γ antiviral response in vivo.

ACKNOWLEDGMENTS

We are heavily indebted to G. Adolf for the IFN- γ used in this study and to R. Flavell for the CIITA probe.

REFERENCES

- Bandyopadhyay, S. K., G. T. Leonard, Jr., T. Bandyopadhyay, G. R. Stark, and G. C. Sen. 1995. Transcriptional induction by double-stranded RNA is mediated by interferon-stimulated response elements without activation of interferon-stimulated gene factor 3. *J. Biol. Chem.* **270**:19624–19629.
- Briscoe, J. 1996. Ph.D. thesis. University of London, London, United Kingdom.
- Briscoe, J., N. C. Rogers, B. A. Witthuhn, D. Watling, A. G. Harpur, A. F. Wilks, G. R. Stark, J. N. Ihle, and I. M. Kerr. 1996. Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *EMBO J.* **15**:799–809.
- David, M., E. Petricoin III, C. Benjamin, R. Pine, M. J. Weber, and A. C. Lerner. 1995. Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. *Science* **269**:1721–1723.
- Geiss, G., G. Jin, J. Guo, R. Bumgarner, M. G. Katze, and G. C. Sen. 2001. A comprehensive view of regulation of gene expression by double-stranded RNA-mediated cell signaling. *J. Biol. Chem.* **276**:30178–30182.
- Gil, M. P., E. Bohn, A. K. O'Guin, C. V. Ramana, B. Levine, G. R. Stark, H. W. Virgin, and R. D. Schreiber. 2001. Biologic consequences of Stat1-independent IFN signaling. *Proc. Natl. Acad. Sci. USA* **98**:6680–6685.
- Goh, K. C., S. J. Haque, and B. R. Williams. 1999. p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J.* **18**:5601–5608.
- Guo, J., D. J. Hui, W. C. Merrick, and G. C. Sen. 2000. A new pathway of translational regulation mediated by eukaryotic initiation factor 3. *EMBO J.* **19**:6891–6899.
- Harcourt, J. L., and M. K. Offermann. 2001. Multiple signaling cascades are differentially involved in gene induction by double stranded RNA in interferon-alpha-primed cells. *Eur. J. Biochem.* **268**:1373–1381.
- Hu, J., S. K. Roy, P. S. Shapiro, S. R. Rodig, S. P. Reddy, L. C. Platanias, R. D. Schreiber, and D. V. Kalvakolanu. 2001. ERK1 and ERK2 activate CCAAAT/enhancer-binding protein-beta-dependent gene transcription in response to interferon-gamma. *J. Biol. Chem.* **276**:287–297.
- Iordanov, M. S., J. M. Paranjape, A. Zhou, J. Wong, B. R. Williams, E. F. Meurs, R. H. Silverman, and B. E. Magun. 2000. Activation of p38 mitogen-activated protein kinase and c-Jun NH(2)-terminal kinase by double-stranded RNA and encephalomyocarditis virus: involvement of RNase L, protein kinase R, and alternative pathways. *Mol. Cell. Biol.* **20**:617–627.
- Lillemeier, B. F., M. Koster, and I. M. Kerr. 2001. STAT1 from the cell membrane to the DNA. *EMBO J.* **20**:2508–2517.
- Lutfalla, G., S. J. Holland, E. Cinato, D. Monneron, J. Reboul, N. C. Rogers, J. M. Smith, G. R. Stark, K. Gardiner, K. E. Mogensen, et al. 1995. Mutant USA cells are complemented by an interferon-alpha beta receptor subunit generated by alternative processing of a new member of a cytokine receptor gene cluster. *EMBO J.* **14**:5100–5108.
- Muller, M., J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. G. Harpur, G. Barbieri, B. A. Witthuhn, C. Schindler, et al. 1993. The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. *Nature* **366**:129–135.
- Muller, M., C. Laxton, J. Briscoe, C. Schindler, T. Improta, J. E. Darnell, Jr., G. R. Stark, and I. M. Kerr. 1993. Complement of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathways. *EMBO J.* **12**:4221–4228.
- Nguyen, H., C. V. Ramana, J. Bayes, and G. R. Stark. 2001. Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of STAT1 on serine 727 and activation of gene expression. *J. Biol. Chem.* **276**:33361–33368.
- Pellegrini, S., J. John, M. Shearer, I. M. Kerr, and G. R. Stark. 1989. Use of a selectable marker regulated by alpha interferon to obtain mutations in the signaling pathway. *Mol. Cell. Biol.* **9**:4605–4612.
- Ramana, C. V., M. P. Gil, Y. Han, R. M. Ransohoff, R. D. Schreiber, and G. R. Stark. 2001. Stat1-independent regulation of gene expression in response to IFN-gamma. *Proc. Natl. Acad. Sci. USA* **98**:6674–6679.
- Ramana, C. V., M. P. Gil, R. D. Schreiber, and G. R. Stark. 2002. Stat1-dependent and -independent pathways in IFN- γ -dependent signaling. *Trends Immunol.* **23**:96–101.
- Schwarz, E. M., C. Badorff, T. S. Hiura, R. Wessely, A. Badorff, I. M. Verma, and K. U. Knowlton. 1998. NF-kappaB-mediated inhibition of apoptosis is required for encephalomyocarditis virus virulence: a mechanism of resistance in p50 knockout mice. *J. Virol.* **72**:5654–5660.
- Sen, G. C. 2001. Viruses and interferons. *Annu. Rev. Microbiol.* **55**:255–281.
- Stark, G. R., I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber. 1998. How cells respond to interferons. *Annu. Rev. Biochem.* **67**:227–264.
- Strobl, B., V. Arulampalam, H. Is'harc, S. J. Newman, J. F. Schlaak, D. Watling, A. P. Costa-Pereira, F. Schaper, I. Behrmann, K. C. Sheehan, R. D. Schreiber, F. Horn, P. C. Heinrich, and I. M. Kerr. 2001. A completely foreign receptor can mediate an interferon-gamma-like response. *EMBO J.* **20**:5431–5442.
- Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. *Science* **288**:2357–2360.
- Taylor, J. 1964. Inhibition of interferon action by actinomycin. *Biochem. Biophys. Res. Commun.* **14**:447–451.
- Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh. 2000. Viral subversion of the immune system. *Annu. Rev. Immunol.* **18**:861–926.
- Watling, D., D. Guschin, M. Muller, O. Silvennoinen, B. A. Witthuhn, F. W. Quelle, N. C. Rogers, C. Schindler, G. R. Stark, J. N. Ihle, et al. 1993. Complement by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature* **366**:166–170.
- Williams, T. M. 2001. Ph.D. thesis. University of London, London, United Kingdom.
- Zhou, A., J. M. Paranjape, S. D. Der, B. R. Williams, and R. H. Silverman. 1999. Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. *Virology* **258**:435–440.