Mutant Cell Lines Unresponsive to Alpha/Beta and Gamma Interferon Are Defective in Tyrosine Phosphorylation of ISGF-3 α Components

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Received 9 September 1993/Returned for modification 6 October 1993/Accepted 23 December 1993

The 84-, 91-, and 113-kDa proteins of the ISGF-3 α complex are phosphorylated on tyrosine residues upon alpha interferon (IFN- α) treatment and subsequently translocate to the nucleus together with a 48-kDa subunit. In this study, we investigated the presence and the functional status of ISGF-3 α subunits and Tyk-2 and JAK1 tyrosine kinases in mutant HeLa cells defective in the IFN- α / β and - γ response. Stable cell fusion analysis revealed a single complementation group among one class (class B) of mutants. The class B mutants contain detectable level of mRNA and proteins of the 84-, 91-, and 113-kDa proteins, but neither the protein nor mRNA is inducible by IFN- α or - γ . The 91-kDa protein IFN- γ -activated factor fails to be activated into a DNA-binding state after IFN- α or - γ treatment. In addition, the 91-kDa protein is unable to localize in the nucleus after IFN- α and - γ treatment, and the 113-kDa protein fails to translocate after IFN- α treatment. Immunoprecipitation studies document a failure of phosphorylation of the 84- or 91-kDa proteins after IFN- α or - γ treatment. Similarly, no tyrosine-phosphorylated 113-kDa protein was detected after IFN- α treatment. The inability of class B mutants to phosphorylate the 84-, 91-, or 113-kDa protein on tyrosine residues correlated with the loss of biological response to IFN- α and - γ . The genetic defect appears to be the absence of the tyrosine kinase JAK1. Our data therefore confirm a recent report that JAK1 plays a critical early signaling role for both IFN- α / β and IFN- γ systems.

Alpha/beta and gamma interferon (IFN- α / β and - γ) are important cytokines with pleiotropic effects on cells ranging from antiviral and antiproliferative effects to augmentation of the immune response. Both IFN- α / β and IFN- γ stimulate transcription of distinct but overlapping sets of genes. The study of the transcriptional factors that regulate IFN-stimulated genes (ISG) has greatly facilitated the understanding of IFN signal transduction. The most extensively studied transcriptional factor is IFN-stimulated gene factor 3 (ISGF-3), which binds the IFN- α -stimulated response element (ISRE) of ISG-15 and directs IFN- α -mediated transcription (17, 18). The ISGF-3 complex has been shown to contain four protein subunits, the 84-, 91-, 113-, and the 48-kDa proteins (14). ISGF-3 α represents two populations of complexes, a p113-p91 complex and a p113-p84 complex. For IFN- α signal transduction, p84 and p91 appear to be identical and interchangeable (22). The ISGF-3 α complex translocates into the nucleus in response to IFN- α . All four proteins have been purified (9), and the full-length cDNAs have been cloned (10, 26, 30). The 84-, 91-, and 113-kDa proteins are latent cytoplasmic proteins that, upon activation by IFN- α , rapidly translocate into the nucleus and form a complex with the 48-kDa DNA-binding protein. This ISGF-3 complex then binds the ISRE and directs transcription of ISGs such as ISG-15 (14, 16). The 91-kDa

protein is also known as the IFN- γ -activated factor (GAF) that binds the IFN- γ activation sequence (GAS) in the promoter of the guanylate-binding protein (GBP) gene (5, 28). Despite the fact that p84 and p91 are alternatively spliced form of the same gene, only p91 is a major transcriptional regulator of GBP gene in response to IFN- γ stimulation (22).

Analysis of the nucleotide sequences of 84-, 91-, and 113-kDa protein genes revealed conserved SH2 and SH3 domains, which are motifs commonly found in proteins that interact with tyrosine kinases (8). The ISGF-3 α complex (84-, 91-, and 113-kDa proteins) is phosphorylated on tyrosine residues in response to IFN- α stimulation (8, 27). Similarly, IFN- γ activates the 91-kDa protein by tyrosine phosphorylation (28). It is known that phosphorylation of the ISGF-3 α subunits is crucial for complex formation (8, 27) and, in the case of the 91-kDa protein, binding of the GAS element (13, 28). However, it is not clear whether tyrosine phosphorylation is necessary for all of the biological activities of IFNs.

To study IFN signal transduction, we generated two classes of HeLa mutants unresponsive to IFN- γ by using a novel metabolic selection scheme (19). One category of mutants (class A) exhibited loss of the induction of human HLA-DR α and invariant chain gene in response to IFN- γ induction. Surprisingly, another category of mutants, class B, exhibited the complete loss of all IFN- γ response as well as the IFN- α induction of HLA-B7, 9-27, ISG-15, and ISG-54 genes. Selected members of these class B mutants retained IFN- α induction of GBP, while all showed some degree of IRF-1 induction. The mutation in class B is interesting in that an apparent single gene defect could eliminate the response of IFN- α / β and IFN- γ .

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It is important to examine the functional status of ISGF-3 α components in B mutants and to determine whether the presence or absence of tyrosine phosphorylation of 84-, 91-, or 113-kDa proteins correlates with the IFN response. In this report, we describe complementation studies of all the members of the class B mutants. We also determined whether components of ISGF-3 α proteins are present in the B mutants, as well as the functional status of the ISGF-3 α proteins in response to IFNs and their ability to translocate upon activation. In addition, we examined two members, Tyk-2 and JAK1, of the Janus family of kinases, which has been implicated in signal transduction in selected cytokine receptors.

MATERIALS AND METHODS

Cells. The parental line 1D4, derived from the HeLa line, and class A (E2A1) and class B (E2D2, E2C6, E2A5, E2B1, E2A4, E1C3, and E2B6) mutants were described previously (19). All cells were cultured in Dulbecco minimal essential medium (DMEM) supplemented with 5% fetal bovine serum and L-glutamine. Biogen (Cambridge, Mass.) generously provided recombinant human IFN- γ (Immuneron). IFN- γ was used at a concentration of 500 U/ml, and recombinant human IFN- α 2 (Schering-Plough, Kenilworth, N.J.) was used at a concentration of 1,000 U/ml. Hygromycin (Calbiochem) was used at 200 μ g/ml, and puromycin (Sigma, St. Louis, Mo.) was used at 0.5 μ g/ml.

Flow cytometry. For fluorescence-activating cell sorting (FACS) analysis, cells were stained for HLA-DR surface expression with monoclonal antibody (MAb) L243, and secondary staining was performed with phycoerythrin-conjugated goat anti-mouse immunoglobulin G (Southern Biotechnology). The stained cells were analyzed with a FACStar and/or FAC-Scan (Becton Dickinson).

Stable cell fusion analysis. Each member of class A and B mutants was transfected individually with pBSpac (6) or EBO-pLPP (2). For each fusion, 5×10^6 hygromycin- or puromycin-resistant cells from each line were mixed and plated on a 10-cm-diameter tissue culture plate overnight in nonselection DMEM. The next day, cells were washed with prewarmed phosphate-buffered saline (PBS) or serum-free medium, and 1 to 2 ml of prewarmed polyethylene glycol (PEG 1000; diluted 50% [wt/vol] in serum-free DMEM and pH adjusted to 7.4 with 7.5% NaHCO₃; Koch-Light, Ltd., Hatfield, England) was gently and slowly layered on the tissue culture plate. The plate was incubated at 37°C for about 90 s, then 5 ml of prewarmed PBS or serum-free DMEM was layered on the plate, and excess PEG sucked off with a Pasteur pipette. This procedure was repeated twice without tilting the plate to avoid dislodging the cells from the plate. After two additional washes, the cells were allowed to recover overnight in fresh medium. Cells were then split 1:4 and plated with double-selection medium containing hygromycin and puromycin for 10 to 14 days. Stable colonies were pooled, and analysis was performed.

RNAse protection analysis and Northern (RNA) analysis. Cytoplasmic RNA was prepared by protocols described previously (25). The template used to generate antisense transcripts was derived by subcloning a 336-bp PCR fragment containing parts of exon 2 and intron 2 of the HLA-DR α gene in the antisense direction into *Xba*I and *Bam*HI sites of pSK-. Antisense transcripts were generated with T3 RNA polymerase after *Hind*III digestion. The γ -actin probe used, pSP64- γ actin, was described previously (7). Northern blots were probed with an internal fragment recognizing both 91- and 84-kDa transcripts and a fragment of 113-kDa cDNA; for

details, see reference 26. All blots were stripped and reprobed with γ -actin.

EMSA. The electrophoretic mobility shift assay (EMSA) was performed as previously described (19) with a Ly-6E GAS-like oligonucleotide (15) (5' CATGTTATGCATATTCCTGTAA GTG 3').

Western blot (immunoblot) analysis. Crude whole cell extracts prepared as described previously (27) were fractionated on a 7% acrylamide gel and electroblotted onto nitrocellulose. The blot was blocked with 1 $\times$ TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.02% Tween 20) and 2.5% nonfat dry milk overnight and then incubated with a 1:2,000 dilution of antisera specific for 84- and 91-kDa proteins and a 1:1,000 dilution of an antiserum specific for the 113-kDa protein for 2 to 3 h. The JAK1 antiserum (32) was used at a 1:1,000 dilution. The secondary reagent was donkey anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Amersham) at a 1:2,000 dilution for 1 h. The Amersham enhanced chemiluminescence kit was used for detection.

Immunofluorescence. Cells were plated on chamber slides at low density (approximately 10^4 cells per cm²) and grown for 2 to 3 days. After induction with IFN for the times indicated, the cells were fixed in 4% paraformaldehyde as previously described (12) and then quenched in 50 mM NH₄Cl (pH 8.0) for 15 min. The samples were permeabilized with methanol stored at -20°C for 2 min and blocked with 10% normal goat serum in TBST. The primary rabbit polyclonal antisera were diluted in blocking buffer at 1:500 for 91T (anti-91-kDa protein) and 1:100 for anti-113 (anti-113-kDa protein) and allowed to bind for 2 h at 25°C. Goat anti-rabbit immunoglobulin G conjugated to a fluorescein isothiocyanate (Southern Biotechnology) secondary reagent was diluted at 1:150 in blocking buffer and incubated at 25°C for 50 min. After being washed in TBST, the cells were mounted in 2.5% DABCO [1,4-diazabicyclo(2.2.2) octane] in Mowiol and examined with the 63 $\times$ objective of a Zeiss Axiophot.

Immunoprecipitation and detection with an antiphosphotyrosine MAb. Crude whole cell extracts were immunoprecipitated with anti-91T or anti-113 as described by Schindler et al. (27). The immunoprecipitates were fractionated on a 7% acrylamide gel and immunoblotted with an antiphosphotyrosine antibody (PY20; ICN). The secondary antibody was donkey anti-mouse alkaline phosphatase used at a 1:1,000 dilution for 1 h. As a control, the filters were stripped and reprobed with antisera specific for 84-, 91-, and 113-kDa proteins. Immunoprecipitation with the anti-Tyk-2 antiserum was performed as described elsewhere (23).

RESULTS

Complementation analysis of class A and class B mutants.

To determine complementation groups, individual mutants were transfected with hygromycin or puromycin drug resistance markers. Various combinations of cells were then fused by using PEG. Stable synkaryons were selected by using hygromycin and puromycin, and surviving colonies were pooled and analyzed for HLA-DR expression. Stably fused cells generated between class A and class B mutants recovered normal IFN- γ -induced HLA-DR surface expression as well as IFN- γ -inducible steady-state HLA-DR α mRNA (Fig. 1a and b); however, no complementation was observed in the synkaryons generated in B $\times$ B crosses (Fig. 1c and d). The IFN- γ induction profile of major histocompatibility complex (MHC) class I antigens showed normal induction for A $\times$ B crosses (Fig. 1a and b) but no induction in B $\times$ B crosses (Fig. 1c and d). No positive complementation was seen when cells were

FIG. 1. Stable cell fusion analyses. Stable synkaryons generated by cell fusion (see Materials and Methods) were pooled and analyzed for IFN- γ induction of HLA-DR and MHC class I surface antigen, and steady-state mRNA transcripts were analyzed for HLA-DR α by RNase protection. In each panel, the two FACS profiles on the left are of cells stained with a MAb specific for HLA-DR (L243). C, untreated cells; IFN γ , profile of cells treated with 500 U of recombinant human IFN- γ for 48 h. For the HLA-DR staining, the baseline has been artificially moved to the right in order to detect minute changes in cellular profiles and does not indicate the basal level of staining on untreated cells. The two FACS profiles on the right are of cells stained with an MAb specific for human MHC class I antigens. The autoradiographs show RNase protection analyses performed to detect HLA-DR α transcripts and control γ -actin levels. Panels a to d show FACS staining and RNase protection analyses of E2A4 (class B) $\times$ E2A1 (class A) (a), E2B6 (B) $\times$ E2A1 (A) (b), E2B6 (B) $\times$ E2A4 (B) (c), and E2A4 (B) $\times$ E2B1 (B) (d).

mixed without PEG treatment (20). To improve the sensitivity of detection, RNase protection analysis was performed, and IFN- γ -inducible mRNA of HLA-DR α was again detected in the A $\times$ B crosses (Fig. 1a and b) but not in the B $\times$ B crosses (Fig. 1c and d). The negative complementation results for B $\times$ B crosses is not simply due to failure of generation of stable synkaryons, as demonstrated by the positive complementation by the A $\times$ B cross, which served as a positive control (Fig. 1a and b). In addition, transient heterokaryon analysis performed independently demonstrated no IFN- γ induction of HLA-DR α mRNA in B $\times$ B crosses, whereas mouse L929 cells were able to complement the defect in class B mutants (20). In addition, for the same A $\times$ B crosses shown in Fig. 1, IFN- α and γ induction of MHC class I genes remained normal as expected (Fig. 2a and b). However, the B $\times$ B crosses showed no induction of MHC class I genes by IFN- α or γ (Fig. 2c and d). These results show that all class B mutants belong to a single complementation group.

Table 1 illustrates all of the stable cell fusion analyses performed to date. Since class A mutants complement B mutants, each class is a distinct complementation group. When class B mutants were crossed to the 1D4 parental line, normal IFN- γ induction was observed, thereby ruling out a dominant suppressive mutation in class B mutants.

Class B mutants contain basal levels of ISGF-3 α compo-

nents but lack functional activity. Genes encoding the components of ISGF-3 α have been cloned, and both DNA probes and antisera are therefore available for Northern and immunoblot analyses, respectively. A series of Northern blot analyses performed with a probe common to both 84- and 91-kDa proteins revealed basal levels of steady-state mRNA in the parental line 1D4 and all class B as well as class A mutants (Fig. 3). In response to 14 h of IFN- γ treatment, both 84- and 91-kDa mRNA levels are strongly induced in the parental line or class A mutants (Fig. 3, lanes 1, 2, 17, and 18), but class B mutants have lost this response (lanes 3 to 16). IFN- α also fails to induce 84- and 91-kDa proteins in class B mutants, while the response of 1D4 and class A mutants is not affected (20). In a similar fashion, IFN- α strongly increased the steady-state mRNA level of the 113-kDa gene in 1D4 and class A mutant E1B6 (lanes 1, 2, 17, and 18), which is in the same complementation group as E2A1. However, class B mutants as a whole showed no induction (lanes 3 to 16). An identical result was also observed for IFN- γ (20).

A Western blot analysis (Fig. 4) was performed with whole cell extracts from parental line 1D4 and selected members of class B and class A mutants. The cells were induced with both IFN- α and IFN- γ prior to preparation of whole cell extracts, and the Western blot was probed with polyclonal antisera specific for 91-, 84-, and 113-kDa proteins. Figure 4 demon-

FIG. 2. IFN- α induction of MHC class I antigen in stable synkaryons. Cells were treated either with medium, IFN- α (1,000 U/ml for 24 h), or IFN- γ (1,000 U/ml for 48 h). The cells were stained with W6/32, which recognizes the conserved region of the human MHC class I antigen. The cells were analyzed on a FACScan. See the legend to Fig. 1 for a description of the stable cell fusions used.

strates that 1D4 along with members of class B and class A mutants expressed basal levels of 91-, 84-, and 113-kDa proteins, though levels varied from clone to clone (Fig. 4). IFN- γ treatment induced the levels of 91-, 84-, and 113-kDa proteins in 1D4 and class A mutant E2A1 (Fig. 4, lanes 3 and 15). In class B mutants, however, this IFN- γ response was absent (lanes 6, 9, and 12). Although no induction of 84-, 91-,

TABLE 1. Summary of complementation analyses of all class B mutants, class A mutant E2A1, and parental line 1D4^a

Class B mutant	Complementation with:								Parental. 1D4
	Class B							Class A, E2A1	
	E2D2	E2C6	E2A5	E2B1	E2A4	E1C3	E2B6		
E2D2	—	—	—	—	—	—	—	ND	+
E2C6	●	—	—	—	—	—	—	ND	+
E2A5	●	●	—	—	—	—	ND	ND	+
E2B1	●	●	●	—	—	—	—	ND	ND
E2A4	●	●	●	●	—	—	—	+	+
E1C3	●	●	●	●	●	—	—	+	+
E2B6	●	●	●	●	●	●	ND	+	+

^a Data were compiled from FACS staining and RNase protection analysis of pooled stable synkaryons similar to those presented in Fig. 1. +, positive complementation; —, negative complementation; ●, redundant combination; ND, not done.

FIG. 3. Detection of basal levels of steady-state mRNA transcripts of the 84-, 91-, and 113-kDa proteins. The top panel is the Northern blot of various mutants and parental line 1D4 uninduced and induced with 500 U of IFN- γ for 14 h. The filter was probed with a fragment of 91-kDa sequence also common to the 84-kDa protein. In the next panel, the same filter was stripped and probed with γ -actin as a control. Another filter with total RNAs from cells untreated and induced with 200 U of human IFN- α was probed with a probe specific for the 113-kDa protein. Again, the same filter was stripped and probed with γ -actin for control of RNA loading.

and 113-kDa proteins was observed with short-term (30-min) IFN- α treatment, 12-h IFN- α treatment induced both steady-state mRNA and protein levels but not in the class B mutants (20). These results show that the class B mutants express basal levels of 84-, 91-, and 113-kDa proteins but fail to up-regulate the ISGF-3 α components after IFN- α and - γ induction.

Although up-regulation of ISGF-3 components was defective in class B mutants, we considered that the DNA-binding

FIG. 4. Immunoblot of the 84-, 91-, and 113-kDa proteins. Three class B mutants (E2A4, E2C6, and E2D2), one class A mutant (E2A1), and the 1D4 parental line were untreated and induced with IFN- α (200 U) for 30 min or IFN- γ (500 U) for 14 h. Primary antiserum specific to the 113-kDa protein was mixed with antisera specific for 84- and 91-kDa proteins in appropriate dilutions (see Materials and Methods).

FIG. 5. EMSA to determine the presence of activated p91. The probe used is a 25-mer containing a GAS-like sequence in the 5' regulatory region of the murine LY-6E gene (see Materials and Methods). Whole cell extracts were made with cells induced with IFN- α for 20 min, induced with IFN- γ for the same time, or untreated. Lane 1 contains probe only; lanes 2 to 10 contain probe mixed with whole cell extracts. Lane 11, in addition to probe and whole cell extracts, contains a 50-fold excess of specific DNA competitor (the unlabeled LY-6E GAS-like site), while lane 12 contains a nonspecific DNA competitor (the octamer sequence of HLA-DR α). Lanes 13 to 15 contain antisera specific for p91, p113, and IRF-1, respectively.

properties of ISGF-3 components might remain intact. To test this possibility, an EMSA was performed with a GAS-like site from the murine Ly-6E 5' regulatory region. This GAS-like element was shown to bind IFN-activated 91-kDa protein (15). The EMSA performed with the whole cell extracts from 1D4, E2A4, and E2B1 demonstrates nonspecific bands in untreated and IFN-treated cells (Fig. 5). A specific and faster-migrating complex (below the low-mobility band near top of the gel) can be observed after IFN- α and - γ treatment of 1D4 cells. This specific band was inhibited with a specific oligonucleotide (Fig. 5, lane 11) but not with a nonspecific oligonucleotide (lane 12) and was shifted with an antiserum specific for 91-kDa protein (lane 13) but not with nonspecific antisera (lanes 14 and 15). No induction of the specific band was observed in two of the class B mutants after similar treatment with IFN- α and IFN- γ (lanes 5 to 10). Despite the presence of p91 (Fig. 4, lanes 4 to 12), p91 fails to be activated in these mutants. Identical results were obtained for all class B mutants (20). These data demonstrate a defect in the ability of class B mutants to activate p91.

Components of ISGF-3 α fail to translocate in response to IFN- α and - γ . Immunofluorescence staining was performed to examine the intracellular distribution of 91- and 113-kDa proteins in class B mutants. Parental line 1D4 (Fig. 6a to c) and class B mutant E2B1 (Fig. 6d to f) were stained with an antiserum specific for the 91-kDa protein. The 91-kDa protein was stained prominently in the cytoplasm and nuclei (Fig. 6a

and d) of untreated 1D4 and E2B1 cells. In 1D4 cells, one can see the rapid nuclear localization of the 91-kDa protein after treatment with IFN- α or IFN- γ (Fig. 6b and c); however, the class B mutant E2B1 failed to show any nuclear localization (Fig. 6e and f). Similarly, the 113-kDa protein appears predominantly in the cytoplasm of untreated 1D4 and E2B1 cells (Fig. 7a and d). The 113-kDa protein demonstrates clear nuclear localization in the 1D4 cell line after IFN- α treatment (Fig. 7b) but not after IFN- γ treatment (Fig. 7c). The E2B1 mutant exhibits a similar translocation defect for the 113-kDa protein after IFN- α stimulation (Fig. 7e). The defects in the IFN- α - and IFN- γ -induced nuclear localization of 91- and 113-kDa proteins were also observed in other class B mutants (20).

Class B mutants are defective at the level of phosphorylation of 91- and 113-kDa proteins. To determine whether the critical event for activation of the ISGF-3 α complex and the subsequent IFN- α and - γ biological response is the tyrosine phosphorylation of 84-, 91-, and 113-kDa proteins, we investigated the ability of class B mutants to phosphorylate the components of the ISGF-3 α complex in response to IFNs. Whole cell extracts from the class B mutants and the parental line 1D4 were prepared with and without IFN- α and - γ treatment. The whole cell extracts were then immunoprecipitated with antisera specific for the 91-kDa protein (Fig. 8A) and the 113-kDa protein (Fig. 8B). The Western blot was prepared with the immunoprecipitated protein and probed with an antiphosphotyrosine antibody to detect tyrosine-phosphorylated 91- and 113-kDa proteins. The anti-phosphotyrosine antibody detected tyrosine-phosphorylated 91-kDa proteins (Fig. 8A, lanes 8 and 9) in 1D4 after 20 min of IFN- α and - γ treatment. However, this antibody failed to detect phosphorylated 91-kDa proteins from immunoprecipitates of class B mutants E2C6, E2B1, and E1C3 after treatment with IFN- α or - γ (lanes 1 to 7), although the same filter when stripped and reprobed with polyclonal antisera to 84- and 91-kDa proteins showed the presence of non-tyrosine-phosphorylated 91-kDa protein (Fig. 8A, bottom filter). When the whole cell extracts were immunoprecipitated with an antiserum to the 113-kDa protein, the antiphosphotyrosine antibody detected a tyrosine-phosphorylated complex of 84-, 91-, and 113-kDa proteins in the IFN- α -treated 1D4 cell line (Fig. 8B, lane 9). The ISGF-3 α complex is not responsive to IFN- γ , and this finding is confirmed by the absence of phosphorylated 113-, 91-, and 84-kDa protein complex in the immunoprecipitates (lane 8) of 1D4. Again, class B mutants failed to phosphorylate the 113-kDa protein in response to IFN- α , and no tyrosine-phosphorylated 84- and 91-kDa proteins were observed (lanes 1 to 7), although basal levels of nonphosphorylated 113-kDa proteins were detected in the immunoprecipitates (Fig. 8B, bottom filter). These data clearly demonstrate the defect in the tyrosine phosphorylation of the ISGF-3 α complex in class B mutants. Furthermore, IFN- α was not able to promote complex formation between p84 and p113 or p91 and p113 (lanes 1, 3, and 5).

Class B mutants demonstrate no IFN- α -induced tyrosine phosphorylation of Tyk-2 and absence of JAK1 tyrosine kinase. In a recent report, Müller et al. convincingly demonstrated the defect in the IFN-unresponsive mutant U4A as that of the JAK1 tyrosine kinase (23). The U4A mutant is also defective in tyrosine phosphorylation of the Tyk-2 and JAK2 tyrosine kinases. Since U4A is very similar to our class B mutants, we set out to test the phosphorylation state of Tyk-2. In Fig. 9, whole cell extracts were prepared from cell lines untreated and treated with IFN- α for 5 min. The whole cell extracts were immunoprecipitated with a Tyk-2 antiserum then

FIG. 6. Immunofluorescence analyses to detect nuclear translocation of the p91 in response to IFNs. The cells were stained with an antiserum specific for p91. (a to c) 1D4 cells; (d to f) class B mutant E2B1. Cells were untreated (a and d), treated with IFN- α for 20 min (b and e), and treated with IFN- γ for 20 min (c and f).

probed with an antiphosphotyrosine MAb. Parental cell line 1D4 showed no tyrosine phosphorylation of Tyk-2 in the absence of IFN- α treatment (Fig. 9, lane 1). Upon IFN- α induction, tyrosine-phosphorylated Tyk-2 appeared (lane 2). Interestingly, tyrosine-phosphorylated Tyk-2 appeared in untreated as well as treated class B mutants E1C3, E2A4, E1C3 $\times$ E2A4, E2B1, and E2C6 (lanes 3 to 12). The level of tyrosine-phosphorylated Tyk-2 appeared to be higher in E1C3

and E2A4 and lower in E2B1 and E2C6. The variation in tyrosine-phosphorylated Tyk-2 was not due to variation in immunoprecipitation, as a stripped filter probed with the antiserum to Tyk-2 demonstrated nearly equal levels in all lanes (Fig. 9, bottom).

Next, we examined whether any JAK1 could be detected in the class B mutants. JAK1 was detectable in both 1D4, HeLa, U937, and 2fTGH cell lines by Western blotting (Fig. 10, lanes

FIG. 7. Immunofluorescence analyses to detect intracellular p113. The cells were stained with an antiserum specific for p113. (a to c) 1D4 cells; (d to f) class B mutant E2B1. Cells were untreated (a and d), treated with IFN- α for 20 min (b and e), or treated with IFN- γ for 20 min (c and f).

1 and 7 to 9). The 130-kDa JAK1 protein was either absent or drastically reduced in class B mutants E1C3, E2A4, E1C3 $\times$ E2A4, E2B1, and E2C6 (lanes 2 to 6). A more rapidly migrating band not present in the parental line was observed in E2A4, E1C3 $\times$ E2A4, E2B1, and E2C6 mutants (lanes 3 to 6). It is possible that these bands are truncated JAK1 fragments, but further work will be required to establish this. PCR

detection of JAK1 message revealed correct-size bands in mutants E2B1 and E2C6 (3), suggesting that the JAK1 gene is transcribed in these cells. The data presented above demonstrate that class B mutants lack or have severely reduced levels of full-size JAK1 protein. Future transfection experiments will be carried out to determine whether JAK1 can complement class B mutants.

FIG. 8. Immunoprecipitation and immunoblotting to detect tyrosine-phosphorylated species of the 84-, 91-, and 113-kDa proteins. Whole cell extracts were prepared from cells untreated or induced with IFN- α or IFN- γ for 20 min. Immunoprecipitates were prepared with an antiserum specific for the 91-kDa (A) or 113-kDa (B) protein. The Western blots prepared with immunoprecipitates were first probed with an antiphosphotyrosine (anti p-tyr) antibody (top) and then stripped and reprobbed with a mix of antisera specific for 84-, 91-, and 113-kDa proteins as a control (bottom).

DISCUSSION

Tyrosine phosphorylation has emerged as a major component of signal transduction for both IFN- α (4, 8, 11, 15, 27, 31) and IFN- γ (13, 15, 24, 28). The data presented here report a clear correlation between the disruption of tyrosine phosphorylation of ISGF-3 α components and the loss of biological activities of IFN- α and - γ (Fig. 4 to 9). In all of the class B mutants tested, the ISGF-3 α complex (84, 91, and 113 kDa) showed defective tyrosine phosphorylation and nuclear translocation after IFN treatment (Fig. 8). It is not known whether tyrosine phosphorylation is the signal required for nuclear translocation, but in the absence of phosphorylation, ISGF-3 α is unable to be assembled into an active complex (8, 27). In the absence of phosphorylation, the 91-kDa protein (GAF), though present in the mutants, is not activated into a GAS-binding form in response to either IFN- α or IFN- γ treatment (Fig. 4 and 5). It appears that in order for IFN- γ to activate p91, tyrosine phosphorylation must occur. However, this is not the case with IFN- α , as certain class B mutants were able to induce GBP without tyrosine-phosphorylated GAF (E1C3 [Fig. 8A, lane 5]; E2B1 [Fig. 8A, lane 3]). This observation suggests that IFN- α regulation of the GBP gene may include a signaling pathway independent of ISGF-3 α tyrosine phosphorylation. Using a DNA element from the GBP gene containing both ISRE and GAS, Igarashi et al. (13) have shown binding of multiple IFN- γ -induced complexes with biochemical characteristics distinct from those of ISGF-3, and additional complexes may exist for IFN- α . Therefore, additional regulatory factors may be involved in the regulation of GBP.

The IFN- γ induction of HLA-DR α , like that of ISGF-2

(IRF-1), is a late process requiring protein synthesis (1). The 5' regulatory region of HLA-DR α contains no ISRE or GAS site. However, GAF may be a critical component in this pathway, being activated to induce the cycloheximide-sensitive intermediate required for HLA-DR α regulation (29). From the data presented here, tyrosine phosphorylation appears to be an early and a necessary signaling mechanism for both MHC class I and II antigens. A study demonstrating that protein tyrosine kinase inhibitors can block IFN- γ induction of HLA-DR α in a concentration-dependent manner (24) supports the hypothesis that protein tyrosine phosphorylation is required for IFN- γ regulation of HLA-DR α .

With a similar selection method, McKendry et al. have also selected for IFN- α mutants that are defective for both IFN- α and - γ signal transduction (U3 and U4) (21). Recently, it has been shown that mutants in the U3 complementation group lack both p84 and p91 (22). Complementation of the IFN- α pathway was observed with transfection of either p84 or p91 full-length cDNA, whereas only transfection of p91 was able to restore IFN- γ responsiveness (22). This finding suggests strongly that p91 is involved in both IFN- α/β (ISGF-3) and IFN- γ (GAF) signaling pathways. p84 and p91 are, however, unlikely to be defective in our class B mutants, as demonstrated by the basal level of p84 and p91 in the mutants and the observation that transfection of the p91 full-length cDNA in an expression vector into selected B mutants failed to restore IFN- α or - γ responsiveness (20). The genetic defect in class B mutants appears to be in the JAK1 tyrosine kinase. Müller et al. first showed that U4A mutants lack JAK1 and that they can be complemented by both murine and human JAK1 (23). The

FIG. 9. Immunoprecipitation and immunoblotting to detect tyrosine-phosphorylated Tyk-2. Cells were untreated or treated with IFN- α for 5 min, and whole cell extracts were prepared. The whole cell extracts were immunoprecipitated with an anti-serum to Tyk-2, and the immunoprecipitates were immunoblotted with a MAb specific for antiphosphotyrosine (anti-P-Tyr) (top). The blot was then stripped and reprobed with a Tyk-2 antibody as a control (bottom).

independent findings of the importance of JAK1 for the IFN response underscores the crucial role of this enzyme in IFN signaling. Additionally, in their studies of the U4A mutants, tyrosine phosphorylation of Tyk-2 and JAK2 appears to require an intact JAK1 (23). Similarly, IFN- α -induced tyrosine

FIG. 10. Immunoblot to detect JAK1. Whole cell extracts were fractionated on a 7% polyacrylamide gel and blotted onto nitrocellulose. The filter was probed with an antiserum specific for JAK1 (32).

phosphorylation of JAK1 required Tyk-2, and IFN- γ -induced tyrosine phosphorylation of JAK1 required JAK2. This reciprocal dependence between JAK1, JAK2, and Tyk-2 is intriguing and yet puzzling. Since neither JAK1 nor Tyk-2 mutants are downstream from each other, a coactivation mechanism probably exists whereby each kinase cross-phosphorylates the other. It is not clear whether this cross-phosphorylation occurs in the presence of receptor dimerization in response to ligand binding. Our studies show that some of our class B mutants have a basal level of tyrosine-phosphorylated Tyk-2; we do not yet understand why this differs from the situation for U4A, which also has a defect in JAK1 but shows no phosphorylation of Tyk-2. It is possible that these class B mutants have a partial defect in JAK1. Additional studies are needed to determine the presence of truncated JAK1 protein.

The genetics of IFN mutants have proven to be a critical link to assigning structure and function to the signaling components identified by biochemistry. Although IFN- α/β and IFN- γ receptors have been cloned, multimeric receptor subunits are yet to be identified. In addition, how the respective IFNs transmit signals to JAK1, JAK2, and Tyk-2 tyrosine kinases remains unknown. As JAK1 and Tyk-2 are highly similar at the molecular level, the question can be raised as to the domain that is responsible for receptor-kinase interaction and the ligand-specifying domain. Class B mutants will serve as valuable hosts for dissection of the structure domains of both JAK1 and Tyk-2, using JAK1/Tyk-2 chimera molecules. Class B mutants and U4 will be invaluable in dissecting the immediately early events of IFN signaling and unraveling the interdependence of JAK1, Tyk-2, and JAK2. It is hoped that the identification of exact mutations of the JAK1 gene in various class B mutants will provide additional knowledge about the structure and functions of the tyrosine kinase in early IFN signal transduction. In addition, knowledge of the precise mutation in JAK1 will help to explain the functional differences in individual mutants with respect to the IFN- α regulation of the GBP gene.

ACKNOWLEDGMENTS

We thank Mercedes Rincon-Rincon, Cheong-Hee Chang, and Alfred Bothwell for critical reading of the manuscript. In addition, we thank Adrian Lunn for expert advice on immunofluorescence and Fran Manzo for expert secretarial help. We are also grateful to Tom Taylor and Jennifer Denning for expert technical help. The antiserum to Tyk-2 was generously provided by John Krolewski.

This work was supported by the Howard Hughes Medical Institute and in part by the James S. McDonnell Foundation (C.S.) and Warner Lambert grant 649556 (C.S.). J.E.L. is a Howard Hughes Medical Institute predoctoral fellow. R.A.F. is an Investigator of the Howard Hughes Medical Institute.

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