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# Expression and signaling specificity of the IFNAR chain of the type I interferon receptor complex

STEFAN N. CONSTANTINESCU*, EDWARD CROZE†, ARUNA MURTI*, CHIANG WANG*, LEELA BASU*, DORIS HOLLANDER†, DEAN RUSSELL-HARDE†, MELISSA BETTS†, VICTOR GARCIA-MARTINEZ‡, JERALD E. MULLERSMAN*, AND LAWRENCE M. PFEFFER*§

*Department of Pathology (576 BMH), The University of Tennessee Health Science Center, 800 Madison Avenue, Memphis, TN 38163; †Department of Protein Biochemistry and Biophysics, Berlex Biosciences, Inc., 15049 San Pablo Avenue, Richmond, CA 94804; and ‡Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38101

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**ABSTRACT** **The IFNAR chain of the type I interferon (IFN) receptor ($IFN_IR$) undergoes rapid ligand-dependent tyrosine phosphorylation and acts as a species-specific transducer for type I IFN action. Using the vaccinia/T7 expression system to amplify IFNAR expression, we found that human HeLa-S3 cells transiently express high levels of cell surface IFNAR chains (≈250,000 chains per cell). Metabolic labeling and immunoblot analysis of transfected HeLa cells show that the IFNAR chain is initially detected as 65-kDa and 98-kDa precursors, and then as the 130-kDa mature protein. Due to variation in N-glycosylation, the apparent molecular mass of the mature IFNAR chain varies from 105 to 135 kDa in different cells. $IFN_IR$ structure was characterized in various human cell lines by analyzing $^{125}I$-labeled IFN cross-linked complexes recognized by various antibodies against $IFN_IR$ subunits and JAK protein-tyrosine kinases. Precipitation of cross-linked material from Daudi cells with anti-IFNAR antibodies showed that IFNAR was present in a 240-kDa complex. Precipitation of cross-linked material from U937 cells with anti-TYK2 sera revealed a 240-kDa complex, which apparently did not contain IFNAR and was not present in IFN-resistant HEC1B cells. The tyrosine phosphorylation and down-regulation of the IFNAR chain were induced by type I IFN in several human cell lines of diverse origins but not in HEC1B cells. However, of type I IFNs, IFN-β uniquely induced the tyrosine phosphorylation of a 105-kDa protein associated with the IFNAR chain in two lymphoblastoid cell lines (Daudi and U266), demonstrating the specificity of transmembrane signaling for IFN-β and IFN-α through the IFNAR chain.**

Interferons (IFNs) are proteins capable of interfering with the viral infection of cells, as well as inhibiting the proliferation of normal and transformed cells, regulating cell differentiation, and modulating the immune system. The four major antigenic types of IFNs (α, β, γ, and ω) are defined by the cellular source of their production. Type I IFNs (IFN-α, -β, and -ω) compete with each other for cellular binding to the type I IFN receptor ($IFN_IR$) and thus share at least some components of this multisubunit cell surface receptor, while the receptor for type II IFN (IFN-γ) is a distinct entity (1). Nearly all human cell lines and human tissues display the $IFN_IR$, varying in number from 500 to 20,000 high-affinity ($K_d \approx 50$ pM), and 2000 to 100,000 low-affinity ($K_d \approx 5$ nM) receptors per cell. Chemical cross-linking of iodinated IFN-α to human tumor cells has demonstrated that the $IFN_IR$ apparently is composed of ≈100-, 110-, and 135-kDa glycoprotein subunits (2–4). The cDNAs coding for two $IFN_IR$ chains have recently been cloned, and they have been named the IFNAR and IFNαβR subunits by the groups that isolated the cDNAs (5, 6).

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We and others previously demonstrated the central importance of the IFNAR chain in $IFN_IR$ structure and function by finding that the human (hu)IFNAR chain undergoes rapid ligand-dependent tyrosine phosphorylation, acts as a species-specific transducer for type I IFN action, and represents a 115- to 135-kDa subunit of the $IFN_IR$ (2, 7, 8). The huIFNαβR chain apparently represents the 100-kDa subunit of the $IFN_IR$, also undergoes ligand-dependent tyrosine phosphorylation, and may be involved in ligand binding (6, 9). Studies with monoclonal antibodies (mAbs) directed against hu$IFN_IR$ components suggest the existence of accessory proteins that may modulate the specificity of binding and signal transduction by the $IFN_IR$ (10, 11). Somatic cell genetics show that both binding and transducing chains of $IFN_IR$ map to human chromosome 21 (12–14). In addition, mAbs generated to $IFN_IR$ components react with chromosome 21-encoded proteins (15, 16). IFNs elicit their effects by transducing a signal to the nucleus that results in selective stimulation of a family of early genes, termed the IFN-stimulated genes (ISGs) (17–20). ISG transcriptional activation is mediated by the protein-tyrosine kinase (PTK)-dependent phosphorylation of latent cytoplasmic transcriptional activators, termed the STAT proteins for signal transducers and activators of transcription (21, 22). Central to the IFNα-activated PTK pathway are two Janus PTKs, JAK1 and TYK2 (23, 24), which apparently mediate the tyrosine phosphorylation of STATs, as well as $IFN_IR$ subunits.

In the present study, we report that the IFNAR chain is initially made as an ≈65-kDa precursor and is modified by the addition of carbohydrate to produce the 130-kDa cell surface protein. In a variety of cells expressing the IFNAR chain, the apparent molecular mass varies dramatically from 105 to 135 kDa due to differential glycosylation. While precipitation of $^{125}I$-labeled IFN ($^{125}I$-IFN) cross-linked material from Daudi cells with anti-IFNAR mAb showed that IFNAR was present in a 240-kDa complex, precipitation of cross-linked material from U937 cells with anti-TYK2 sera revealed the existence of a 240-kDa $^{125}I$-IFN-containing complex, which was not precipitated by anti-IFNAR mAb. Type I IFN induced the IFNAR chain phosphorylation in various IFN-sensitive cell lines, but only IFN-β induced the tyrosine phosphorylation of a 105-kDa protein associated with the IFNAR chain in Daudi and U266 lymphoblastoid cells. In U937 cells, IFN-α/β (but not IFN-γ) induced the tyrosine phosphorylation of the IFNAR chain, as well as its down-regulation. These data demonstrate the high specificity of transmembrane signaling through the IFNAR chain.

## MATERIALS AND METHODS

**Cells.** Human Daudi, U266, H929 and U937 cells were maintained at 2.5–10 × $10^5$ cells per ml in RPMI-1640 medium

Abbreviations: IFN, interferon; $IFN_IR$, type I IFN receptor; hu-, human; mAb, monoclonal antibody; PTK, protein-tyrosine kinase; BCS, bovine calf serum; IU, international reference unit(s); BRAP, β receptor-associated protein.

§To whom reprint requests should be addressed.

containing 10% defined bovine calf serum (BCS; HyClone). Human HeLa-S3 cells were grown in stirred suspension culture in spinner medium supplemented with 10% BCS. Human astroglioma (provided by E. Benveniste, University of Alabama, Birmingham) and HEC1B (American Type Culture Collection) cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% BCS. Mouse NIH 3T3 cells were infected with retroviruses containing the pLXSN vector (25) carrying the IFNAR chain cDNA (provided by G. Uzé, Institut de Genetique Moleculaire, Montpellier, France). As a control, cells were infected with retrovirus not carrying a cDNA insert. Stable transfectants were selected for geneticin resistance and were maintained in DMEM and 10% BCS containing geneticin.

**IFN and mAbs.** Recombinant human IFN-$\alpha$ (IFNCon1) and IFN-$\beta$1b (Betaseron) were provided by Amgen Biologicals and Berlex Biosciences, respectively. IFN activities are expressed in international reference units (IU)/ml as assayed by protection against the cytopathic effect of vesicular stomatitis virus (VSV) on human fibroblasts, using the National Institutes of Health human IFN-$\alpha$ standard for reference. mAbs directed against the extracellular domain of the IFNAR chain have been described previously (2).

**The Vaccinia/T7 Expression System.** HeLa-S3 cells were infected at a high multiplicity (10 plaque-forming units per cell) with recombinant vaccinia/T7 hybrid virus, which codes for the bacteriophage T7 RNA polymerase, and after 1 hr were transfected in the presence of Lipofectamine (BRL) with the IFNAR cDNA (provided by G. Uzé) inserted into the pcDNA3 vector, which contains T7 promoter and terminator regions (26).

**IFN and Anti-IFNAR mAb Binding to Cells.** IFNCon1 was iodinated by using Iodo-Gen (Pierce) to specific activities of 75–100 Ci/g (1 Ci = 37 GBq) as described (27). Iodinated IFN preparations were routinely >95% biologically active in antiviral and antiproliferative assays. The anti-IFNAR 4B1 mAb was iodinated to 75 Ci/g by using Iodo-Gen. Saturation curves were obtained and Scatchard analyses were performed as described (27).

**Cross-Linking of $^{125}$I-IFN to Cell Surface Receptors, Immunoprecipitations, and Immunoblot Analyses.** Cells were incubated at $5 \times 10^6$ per ml in binding medium (RPMI-1640 medium containing 5% BCS and 20 mM Hepes, pH 7.4) with 200 pM $^{125}$I-IFNCon1 for 2 hr at 15°C. Cells were washed in 4°C phosphate-buffered saline (PBS) and incubated with 25 mM disuccinimidyl suberate (Pierce) for 30 min at 15°C. Samples were lysed in buffer containing 1% Nonidet P-40 and used for immunoprecipitation. Affinity cross-linked samples were immunoprecipitated overnight either with anti-IFNAR mAb or with polyclonal mouse serum directed against JAK1, JAK2, or TYK2 (Upstate Biotechnology, Lake Placid, NY), and immune complexes were collected on staphylococcal protein A-Sepharose beads (2). Proteins were eluted in SDS/sample buffer and electrophoresed on 7.5% or 5–15% gradient polyacrylamide gels containing SDS (3). Double immunoprecipitations of cross-linked material were performed on eluates in sample buffer; precipitates were diluted and concentrated as described (28) a total of three times to remove SDS and mercaptoethanol and were reprecipitated with anti-IFNAR mAb.

For tyrosine phosphorylation studies, $2 \times 10^8$ cells were treated with IFN-$\alpha$ or -$\beta$ (5000 IU/ml) at 37°C for the indicated periods of time and then washed with ice-cold PBS and lysed for 20 min in lysis buffer [50 mM Tris·HCl, pH 7.4/150 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/15% (vol/vol) glycerol] containing 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, soybean trypsin inhibitor at 5 $\mu$g/ml, leupeptin at 5 $\mu$g/ml, and benzamidine at 1.75 $\mu$g/ml (22). Samples were centrifuged ($12,000 \times g$, 15 min) at 4°C and supernatants were immunoprecipitated with anti-IFNAR mAb or control mouse serum overnight at 4°C. Immune complexes were collected by using protein A beads and were eluted in sample buffer. Samples were run on SDS/7.5% PAGE, transferred to Immobilon (Millipore), and probed with anti-phosphotyrosine mAb (Oncogene Sciences Ab-2, dilution 1:500), followed by anti-mouse IgG coupled with horseradish peroxidase (Amersham). Blots were developed by using enhanced chemiluminiscence (ECL; Amersham). The blots were stripped and reblotted with antibodies against the immunoprecipitated protein to validate that there were equal amounts of protein in each lane.

## RESULTS

**Synthesis of the IFNAR Chain in HeLa Cells Transfected with IFNAR cDNA and Infected with Recombinant Vaccinia/T7 Hybrid Virus.** The vaccinia/T7 expression system in human HeLa-S3 cells was used to characterize the synthesis of the huIFNAR chain because of the relatively low basal and IFN-induced levels of IFNAR synthesis. For example, anti-IFNAR immunoblot analysis of Daudi cells demonstrated that after IFN-induced down-regulation the cell surface expression of IFNAR chains increased linearly for ≥24 hr, with full recovery requiring ≈36 hr, while IFNAR mRNA levels in Northern blots remained constant (data not shown). Thus, HeLa cells were infected with recombinant vaccinia/T7 hybrid virus and then were transfected with the IFNAR chain inserted into the pcDNA3 vector. Transfected HeLa cells expressed very high cell surface levels of the IFNAR chain (≈250,000 chains per cell, based on a transfection efficiency of ≈10%), as determined by binding assays with $^{125}$I-labeled anti-IFNAR 4B1 mAb. Fig. 1 shows that the IFNAR chain was synthesized in HeLa cells in the vaccinia/T7 system as ≈65-kDa and 98-kDa proteins at 4 hr after transfection. The latter was the predominant protein species as determined by [$^{35}$S]methionine metabolic labeling and immunoprecipitation of HeLa cells. By 6 hr after transfection, the IFNAR appeared as the mature 130-kDa protein (revealed by pulse–chase or long-term labeling experiments), which has mobility identical to that of the endogenous IFNAR chain from HeLa cells. Inhibition of N-linked glycosylation by tunicamycin pretreatment (Fig. 1) blocked the appearance of the 98- and 130-kDa immunoreactive bands, indicating that the 98-kDa protein rep-

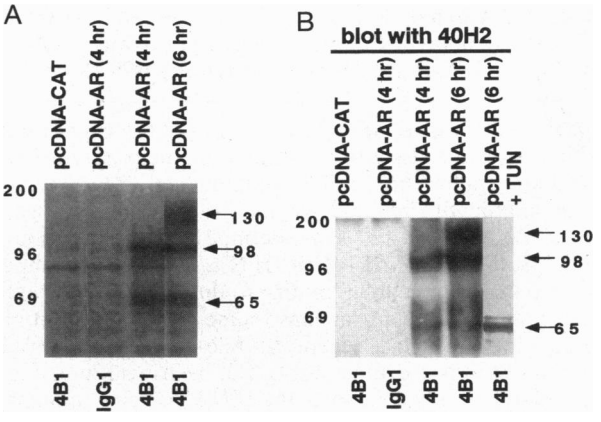


FIG. 1. IFNAR chain synthesis in vaccinia-infected HeLa cells. HeLa cells were infected with the recombinant vaccinia/T7 hybrid virus and then were transfected with the IFNAR chain or chloramphenicol acetyltransferase (CAT) inserted into the pcDNA3 vector in the absence or presence of tunicamycin (TUN; 10 $\mu$g/ml). At 4 hr after transfection, the cells were labeled for 15 min, or 2 hr with [$^{35}$S]methionine, lysed in Nonidet P-40 buffer, immunoprecipitated with 4B1 anti-IFNAR mAb or control IgG1 as indicated at the bottom, and analyzed by SDS/7.5% PAGE followed by phosphorimage analysis (*A*), or followed by immunoblotting with 40H2 anti-IFNAR (*B*). Molecular size markers (69, 96, and 200 kDa) and the 65-, 98-, and 130-kDa anti-IFNAR-reactive bands are indicated.

resents a partially glycosylated precursor of the mature 130-kDa glycoprotein. Precipitation with anti-IFNAR mAb of cells transfected with chloramphenicol acetyltransferase or precipitation with control mouse IgG1 of cells transfected with IFNAR resulted in no specific immunoreactive bands (Fig. 1).

**Heterogeneity in the Molecular Mass of the IFNAR Chain in Human and Mouse Cells Expressing the IFNAR Chain, and Role of N-Linked Glycosylation.** We have previously reported that the IFNAR chain has a molecular mass of ≈135 kDa in Daudi and HeLa cells (2). However, a recent study in U266 cells suggested that the 110-kDa subunit of the $IFN_IR$ (denoted the $\alpha$ subunit) represents the IFNAR chain (8). We therefore next examined the molecular mass of IFNAR in various cell lines and in stable transfectants expressing the IFNAR chain. Fig. 2 shows that in U266 cells IFNAR has a mass of 110 kDa, while IFNAR in H929 cells migrates as a 125-kDa protein. In mouse NIH 3T3 transfectants IFNAR migrates as a 105-kDa protein. These results suggest that there is variable cell-type dependent posttranslational modification of IFNAR, most probably reflecting differential glycosylation. To directly assess if the IFNAR chain is heavily glycosylated, we used N-glycanase to remove N-linked carbohydrates. As shown in Fig. 2*B*, extensive N-glycanase treatment of Daudi or U266 cells reduced a fraction of IFNAR to ≈75 kDa (as measured by the leading edge of the band), although the mass of the mature IFNAR chain differed markedly in these cells (135 and 110 kDa, respectively). Thus, the marked discrepancy between the predicted protein molecular mass of the IFNAR and the mature chain observed in cells largely reflects N-linked glycosylation. The IFNAR chain has 12 potential sites for N-linked glycosylation in its extracellular domain. We cannot exclude the possible contribution of O-linked glycosylation in the processing of IFNAR.

**Specificity of Tyrosine Phosphorylation of the IFNAR Chain.** We previously reported that treatment with IFN-$\alpha$ or IFN-$\beta$ induced tyrosine phosphorylation of IFNAR in Daudi lymphoblastoid cells within 1 min, with kinetics paralleling that of the IFN-activated PTKs JAK1 and TYK2 (2). Ligand-induced tyrosine phosphorylation of IFNAR was blocked by the tyrosine kinase inhibitors genistein and staurosporine. We next examined the type I IFN-induced tyrosine phosphorylation of IFNAR in cell lines of diverse origins (astrocytes, HEC1B embryonic carcinoma cells, U266 myelocytic leukemia cells, and U937 histiocytic leukemia cells). As shown in Fig. 3, rapid ligand-dependent tyrosine phosphorylation of the IFNAR was observed in astrocytes, U266 cells, and U937 cells.

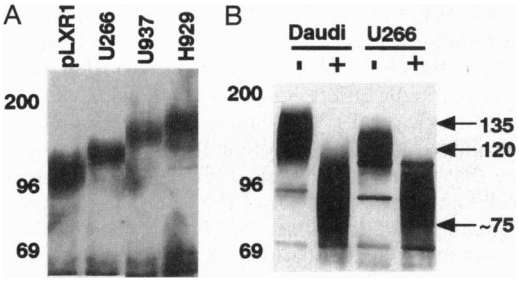


FIG. 2. Heterogeneity in the molecular mass of the IFNAR chain in human and mouse cells expressing the IFNAR chain and in N-linked glycosylation of the IFNAR chain. (*A*) Mouse NIH 3T3 cells transfected with pLXSN vector containing IFNAR (pLXR1) or human U266, H929, and U937 cells were lysed in Nonidet P-40 buffer, immunoprecipitated with 4B1 anti-IFNAR mAb, and analyzed by SDS/7.5% PAGE followed by immunoblotting with 40H2 anti-IFNAR mAb. (*B*) Daudi cells or U266 cells were lysed, immunoprecipitated with 4B1 mAb, treated with N-glycanase (Boehringer Mannheim) for 18 hr at 37°C according to the supplier's recommendations, and analyzed by SDS/7.5% PAGE followed by immunoblotting with 40H2 mAb. Molecular size markers are indicated in kDa.

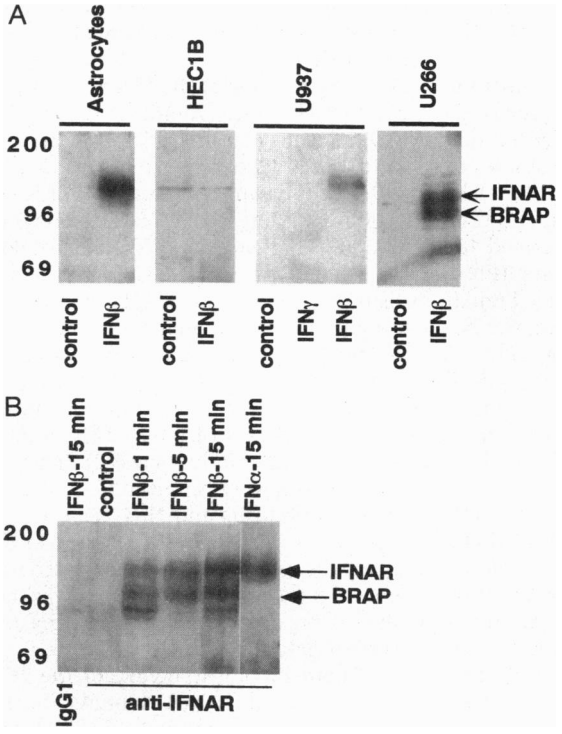


FIG. 3. Type I IFN-induced tyrosine phosphorylation of the IFNAR chain and $\beta$ receptor-associated protein (BRAP) in human cells. Astrocytes, HEC1B, U266, and U937 cells (*A*) were incubated in the presence or absence of IFN-$\beta$ (5000 IU/ml for 10 min), or Daudi cells (*B*) were incubated with IFN-Con1 or IFN-$\beta$ for the indicated times, lysed, immunoprecipitated with 4B1 anti-IFNAR chain mAb (or mouse IgG1 as a control), and analyzed by SDS/7.5% PAGE followed by immunoblotting with anti-phosphotyrosine mAb and detection by enhanced chemiluminescence.

However, no discernible tyrosine phosphorylation of IFNAR was found upon type I IFN addition to the HEC1B cell line, which is completely resistant to the stimulation of gene expression by type I IFNs (29). Stripping of the blots and reprobing with anti-IFNAR mAb demonstrated that the tyrosine phosphorylated protein was indeed IFNAR, and that HEC1B cells express IFNAR (data not shown). The antiviral response to IFN-$\alpha$ and IFN-stimulated gene expression in the human monocytic U937 cell line is restored by IFN-$\gamma$ pretreatment, although normal levels of activated Stat1$\alpha$ and Stat2 are rapidly induced in U937 cells by IFN-$\alpha$ (30). Therefore, ligand-induced tyrosine phosphorylation of IFNAR has been observed in several cell lines where the early signaling pathway through the $IFN_IR$ is intact (Daudi lymphoblastoid cells, HeLa epithelioid tumor cells, astrocytes, U266 cells, and U937 cells) but not in HEC1B cells, which apparently have an early signaling defect.

However, while both IFNCon1 and IFN-$\beta$ induced tyrosine phosphorylation of the IFNAR chain, only IFN-$\beta$ induced the tyrosine phosphorylation of a 105-kDa protein in Daudi and U266 cells (Fig. 3). The IFN-$\beta$-induced tyrosine phosphorylation of the 105-kDa protein BRAP in Daudi cells is detectable within 1 min and is maximal at 5 min. Of all type I IFNs tested (IFN-$\alpha$1, -$\alpha$2, -$\alpha$6, -$\alpha$7, -$\alpha$8, -Con1, -$\omega$, and -$\beta$) at concentrations up to 500,000 IU/ml, only IFN-$\beta$ induced BRAP phosphorylation (data not shown). These findings suggest that IFN-$\beta$ interacts with the $IFN_IR$ differently from other type I IFNs. Interestingly, while the molecular mass of IFNAR is lower in U266 cells (110 kDa) compared to Daudi cells (135 kDa), the mass of BRAP is the same in both cell lines. In addition, while the IFNAR chain was tyrosine phosphorylated in U937 cells within minutes of treatment with IFN-$\alpha$ or -$\beta$, IFN-$\gamma$ did not induce tyrosine phosphorylation of

IFNAR (Fig. 3). Interestingly, both type I and II IFNs activate the JAK1 tyrosine kinase in U937 cells, while only type I IFNs activate TYK2 (data not shown).

**Ligand-Induced Down-Regulation of the IFNAR Chain.** We have previously shown that IFNAR is rapidly down-regulated by IFN treatment of Daudi cells (2). In Table 1, we expanded these studies to show that IFN treatment (IFNCon1 at 20,000 IU/ml for 18 hr) of U937 cells down-regulated 90% of IFNAR chains and IFN binding sites as detected by binding assays with $^{125}$I-labeled 4B1 mAb and $^{125}$I-IFNCon1. IFN-$\gamma$ did not down-regulate IFN or 4B1 binding sites in U937 cells (data not shown). Similar levels of IFN-induced IFNAR chain and IFN binding site down-regulation were obtained with astrocytes as well as Daudi, HeLa, and U266 cells, while IFN treatment of HEC1B cells did not down-regulate 4B1 or IFN binding sites. This cell line is completely resistant to the antiviral, antiproliferative, and gene-inducing effects of type I IFN, although HEC1B cells express IFN and 4B1 binding sites (2). In a recent study IFNAR and the $\alpha$ subunit were reported to be the same protein (8). However, we show, using anti-IFNAR mAbs, that the IFNAR chain is efficiently (90%) down-regulated in U937 cells (Table 1) and rapidly tyrosine phosphorylated upon IFN-$\alpha$ or -$\beta$ treatment (Fig. 3), while the $\alpha$ subunit in U937 cells was reported not to be down-regulated by IFN or to become tyrosine phosphorylated (31, 32).

**Role of the IFNAR Chain in the Structure of the IFN$_I$R Complex.** To define the role of IFN$_I$R subunits in IFN-activated signaling pathways, we analyzed $^{125}$I-IFN cross-linked complexes recognized by various antibodies against IFN$_I$R subunits and JAK PTKs. We previously showed that in Daudi cells $^{125}$I-IFN cross-linking and precipitation with the anti-IFNAR 4B1 mAb detected a 240-kDa complex and a diffuse 130- to 150-kDa complex (2). To directly demonstrate the presence of the IFNAR chain in these complexes, we analyzed them by double precipitation using the 4B1 mAb. Fig. 4 shows that in Daudi cells both complexes contain IFNAR because they are reprecipitated with 4B1 mAb even after denaturation of immunoprecipitates by heating in the presence SDS and 2-mercaptoethanol. In contrast, 4B1 mAb precipitated a diffuse 130- to 150-kDa complex but not a 240-kDa complex from U937 and HEC1B cells, in agreement with previous studies (4). However, anti-TYK2 sera selectively precipitated a 240-kDa complex from U937 cells but not from HEC1B cells (Fig. 4). The 240-kDa complex was not precipitated in U937 cells by anti-JAK1 sera or by control rabbit IgG. Thus, the 240-kDa complex formed in U937 cells is physically coupled to the TYK2 PTK that triggers part of the IFN-$\alpha$ signal. This TYK2-coupled complex apparently does not contain IFNAR, since it was not precipitated by any of five different anti-IFNAR mAbs. In Daudi cells, there are several complexes of ≈240 kDa which were precipitated by antibodies against TYK2 (slower migrating), or JAK1 and IFNAR (faster migrating). Our finding that anti-TYK2 does not immunoprecipitate a 240-kDa complex from IFN-resistant HEC1B cells provides further evidence of an early signaling defect in these cells.

Table 1. Number of anti-IFNAR 4B1 mAb and IFNCon1 binding sites on various human cell lines

| Cell line | IFNAR | | IFN binding | |
|---|---|---|---|---|
| | No. of sites | % down-regulation | No. of sites | % down-regulation |
| Daudi | 10,200 | 95 | 3,800 | 95 |
| U266 | 7,300 | 89 | 3,400 | 91 |
| HEC1B | 2,300 | 5 | 1,300 | 5 |
| U937 | 3,200 | 84 | 2,700 | 81 |
| Astrocytes | 25,000 | 90 | 30,500 | 90 |

Adherent cells (HEC1B) plated in 24-well plates ($10^5$ cells per well) or suspension cells (Daudi, HeLa-S3, and U937; $2 \times 10^6$ cells per ml) were incubated with $^{125}$I-labeled ligand for 4 hr at 4°C, or 100 min at 15°C, respectively. Specific binding was determined and the data were transformed into Scatchard plots. The SEM in the number of sites was ≤15%. For down-regulation studies, cells were treated overnight with IFNCon1 at 20,000 IU/ml.

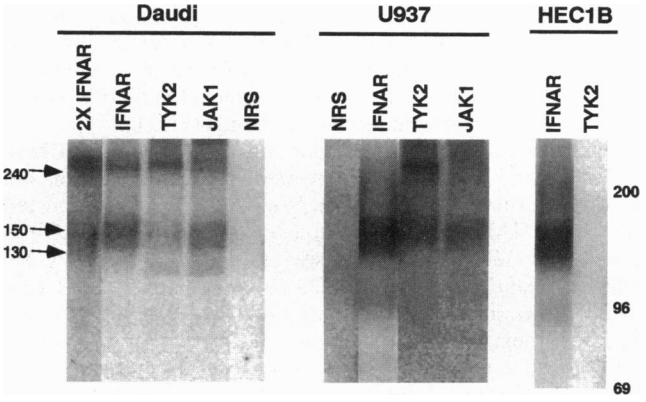


FIG. 4. TYK2 PTK is selectively associated with the 240-kDa affinity-cross-linked IFN-IFNAR complex. $^{125}$I-IFNCon1 was affinity cross-linked to Daudi, U937, or HEC1B cells, and cell lysates were prepared, immunoprecipitated with anti-IFNAR mAb, polyclonal mouse serum directed against JAK1 or TYK2, or control serum (mouse IgG1 or rabbit polyclonal), and analyzed by SDS/7.5% PAGE. Molecular size markers are indicated.

## DISCUSSION

In this report we have characterized the expression and signaling of huIFNAR with mAbs generated to the baculovirus-expressed ectodomain of this protein (2). Precipitation and blotting with anti-IFNAR mAb of lysates from a variety of human cell lines showed that IFNAR has an apparent molecular mass that varies from 115 to 135 kDa, depending on the cell line examined. By [$^{35}$S]methionine metabolic labeling and immunoprecipitation of HeLa cells in the vaccinia/T7 system, we show that the IFNAR chain was synthesized as an ≈65-kDa protein (tunicamycin-insensitive), which is consistent with the theoretical translation product of ≈64 kDa, as well as an intermediately glycosylated, tunicamycin-sensitive 98-kDa form of IFNAR. At later times, the chain was further processed to yield the mature, tunicamycin-sensitive 130-kDa glycoprotein, which has mobility identical to that of the endogenous IFNAR chain from HeLa cells. The 65-, 98-, and 130-kDa forms were all detected with anti-IFNAR mAb by blotting or precipitation of metabolically labeled cell lysates, suggesting that the anti-IFNAR mAb was directed to an epitope present in the unprocessed IFNAR protein. We then determined the role of N-linked glycosylation in human cells that synthesize IFNARs of markedly different mobilities—i.e., Daudi (135 kDa) and U266 (110 kDa) cells. N-glycanase treatment of lysates from either cell line reduced the mass of the IFNAR chain to ≈75 kDa, indicating that differential N-linked glycosylation is responsible for the cell line-specific mobility of IFNAR chain. Furthermore, since N-glycanase did not reduce the IFNAR chain to the size of the unmodified protein (≈65 kDa), O-linked glycosylation (or other posttranslational modifications) may also contribute to the processing of the mature IFNAR chain.

We provide several lines of evidence that signaling through the huIFNAR chain displays high IFN specificity. Only type I IFN induces detectable tyrosine phosphorylation of the IFNAR chain in U937 cells, which are sensitive to type I and II IFNs, although these IFNs all activate the JAK1 PTK. Similar specificity is observed for the tyrosine phosphorylation of erythropoietin and interleukin 3 receptors by their respective ligands, although both ligands activate JAK2 and phosphorylate common protein targets (33). In addition, we show that only IFN-$\beta$ induces the tyrosine phosphorylation of the

105-kDa $IFN_IR$-associated BRAP protein in lymphoblastoid cells, although both IFN-$\alpha$ and IFN-$\beta$ induced IFNAR tyrosine phosphorylation in these cells. These rapid ligand-dependent phosphorylation events are inhibited by the PTK inhibitors staurosporine and genistein. While the apparent mobility of the IFNAR chain is slightly lower in U266 cells as compared to Daudi cells (110 and 135 kDa, respectively), the mobility of BRAP is similar in these cells, showing that BRAP and IFNAR are distinct proteins. In addition, BRAP is not detected by anti-IFNAR or anti-IFN$\alpha\beta$R mAb. The phosphorylation of BRAP is highly dependent on cell type, since IFN-$\beta$ induces tyrosine phosphorylation of IFNAR but not of BRAP in U937 cells, human fibroblasts, or HeLa cells, or even in human astrocytes, which express high levels of the IFNAR chain and are highly responsive to IFN-$\beta$.

Furthermore, we found that of all type I IFNs tested only IFN-$\beta$ induced BRAP phosphorylation. We cannot assess the role of IFNAR tyrosine phosphorylation in inducing BRAP phosphorylation, since we detect BRAP through its association with the IFNAR chain. A protein of similar molecular mass becomes tyrosine phosphorylated and associated with the $IFN_IR$ upon IFN-$\beta$ treatment of U266 cells (9, 34). The phosphorylation of BRAP appears to be highly cell-type specific. For example, IFN-$\beta$ did not induce BRAP phosphorylation in human astrocytes, which express relatively high numbers of IFNAR chains (20,000 per cell), while IFN-$\beta$ induces rapid IFNAR tyrosine phosphorylation. BRAP does not correspond to any of the known subunits of the $IFN_IR$ complex, since it is not detected by antibody directed against IFNAR or the recently cloned IFN$\alpha\beta$R chain (data not shown). Presumably, BRAP is a transmembrane protein which becomes tyrosine phosphorylated and associates with the IFNAR chain only after IFN-$\beta$ binding to the $IFN_IR$ complex. In any case, we propose that the ligand-induced IFNAR tyrosine phosphorylation serves as docking sites for signaling proteins. Interestingly, while IFNAR, JAK1, and TYK2 are all tyrosine phosphorylated with a maximum at 5 min, Stat2 and Stat1$\alpha$ are tyrosine phosphorylated with a maximum at 60 min. This might be due to sequential tyrosine phosphorylation of the STAT proteins by PTKs other than JAK1 or TYK2, or their sequestration into a phosphatase-inaccessible compartment (i.e., accumulation in the nucleus). Nevertheless, these data show that IFN-induced tyrosine phosphorylation is temporally regulated. The specificity of signaling through the IFNAR chain is further demonstrated by the findings that (*i*) type I IFNs, but not IFN-$\gamma$, induce the down-regulation of IFNAR chains and IFN-$\alpha/\beta$ binding sites, as well as IFNAR tyrosine phosphorylation; and (*ii*) type I IFN did not down-regulate IFNAR chains or IFN binding sites in IFN-resistant HEC1B cells.

In addition, using the vaccinia/T7 expression system in HeLa cells, we have also found that the 98- and 130-kDa forms of IFNAR are basally tyrosine phosphorylated (data not shown), suggesting that high expression induces IFNAR oligomerization and ligand-independent phosphorylation. In other high-expression systems (i.e., pcDNA3-transfected COS cells, baculovirus-infected insect cells) basal phosphorylation was found only when the substrate (receptor subunit) and the kinase were both overexpressed. However, since the IFNAR is a transducer subunit, homodimerization of this chain and possibly its association with other receptor subunits might trigger phosphorylation. The interaction of the IFNAR chain with itself and/or with other receptor chains has also been shown by its presence in high molecular mass complexes as defined by affinity cross-linking as well as by its coprecipitation with a 110-kDa cell surface protein (2). The presence of the huIFNAR chain in the 150- and 240-kDa cross-linked complexes has been demonstrated in double-immunoprecipitation experiments.

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