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Differential tyrosine phosphorylation of the IFNAR chain of the type I interferon receptor and of an associated surface protein in response to IFN-α and IFN-β

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The human interferon α-receptor (IFNAR gene product) is a transmembranal protein of 557 amino acids with an intracytoplasmic domain of 100 amino acids containing four tyrosines. Antibodies to a C-terminal peptide (residues 521–536) were developed which efficiently immunoprecipitate the 105 kDa IFNAR protein from detergent extracts of human cells. We show that the IFNAR protein becomes tyrosine phosphorylated within 5 min after treatment of human myeloma U266 cells with IFN-α2, IFN-α8 or IFN-β. The IFNAR chain interacts with both IFN-α2 and IFN-β, as demonstrated by cross-linking. Among elements involved in signal transduction by type I IFNs, the tyrosine kinase Tyk2 but not Jak1, and the ISGF3 transcription factor subunit Stat2 (p113) but not Stat1 (p91), are found associated with the IFNAR protein. After IFN-β treatment for 5 min, a tyrosine-phosphorylated protein of ~95 kDa (β-PTyr) is found bound to IFNAR, but can be dissociated by denaturation. The β-PTyr protein is present on the cell surface, like IFNAR, as shown by extracellular biotin tagging. The ratio of β-PTyr to IFNAR tyrosine phosphorylation is much higher with IFN-β than with IFN-α2 or 8. Both are IFN dependent and abrogated by a monoclonal antibody which blocks IFNAR action. The β-PTyr component may represent an important difference in the action of IFN-β as compared with IFN-α in their shared receptor system.

Key words: IFNAR chain/β-PTyr/type I interferon receptor/tyrosine phosphorylation

Introduction

The biological effects of human type I interferons (IFN), characterized by induction of antiviral protection, inhibition of cell growth and expression of various IFN-inducible genes (e.g. MHC class I, 2′-5′ oligo A synthetase), are mediated by human-specific cell surface receptor proteins encoded by chromosome 21 (Revel *et al.*, 1976; Razziudin *et al.*, 1984). The >20 IFN-α subtypes and the more distantly related IFN-β share some receptor components since they bind to cells competitively (Aguet *et al.*, 1984; Merlin *et al.*, 1985; Flores *et al.*, 1991), but differences can be observed in the binding and activity of IFN-α subtypes (Pestka *et al.*, 1987; Hu *et al.*, 1993) and of IFN-β (Rosenblum *et al.*, 1990; Johns *et al.*, 1992). Such differences could be explained by interplays between the various subunits which form type I IFN receptor complexes, as revealed by ligand cross-linking (Razziudin *et al.*, 1984; Schwabe *et al.*, 1988; Colamonici and Pfeffer, 1991). The number of subunits, as well as their functions in binding and signal transduction by the IFN-α,β subtypes, is still unclear. By DNA transfection of mouse cells and selection for response to human IFN-α8 (αB2), the gene for one receptor component designated IFNAR was cloned and mapped in human chromosome 21q22.1 (Uze *et al.*, 1990; Lutfalla *et al.*, 1992). This IFNAR gene encodes a 557 amino acid long transmembranal glycoprotein, which confers to murine cells functional binding of human IFN-α8, but little response to IFN-α2 or IFN-β (Uze *et al.*, 1990). However, on human cells, the IFNAR protein appears to be involved in the action of many IFN-α,β subtypes since a monoclonal antibody (McAB 64G12) against the IFNAR extracellular domain inhibits IFN-α8 and IFN-α2 binding to Daudi cells and the response to these subtypes as well as to IFN-β (Benoit *et al.*, 1993). Furthermore, Chinese hamster ovary (CHO) cells expressing the human IFNAR cDNA respond to huIFN-β by (2′-5′) A synthetase induction and activation of transcription factors ISGF3 and IRF-1, whereas huIFN-α8 is inactive in contrast to the situation in murine cells (Abramovich *et al.*, 1994a). The range of IFNAR activity may, therefore, depend on interactions with other components contributed by the rodent cells and, on the human cell surface, IFNAR may similarly function as one component of larger receptor complexes.

Another IFN receptor protein was identified by affinity purification of urinary proteins on IFN-α2 and IFN-β, and its cloned cDNA found to encode a 331 amino acid long transmembrane protein, appearing in cells as a 102 kDa dimer of disulfide-linked 51 kDa chains (Novick *et al.*, 1994). This second receptor cDNA does confer human IFN-α2 cross-linking on transfected mouse cells, but no biological response, indicating that it requires other receptor components (Novick *et al.*, 1994). In addition to these cloned components, subunits of the type I IFN receptor were studied by using two distinct monoclonal antibodies: (i) McAB IFNaR3 which precipitates a 110 kDa protein (termed α-subunit) from human myeloma U266 cells, and 130, 210 kDa complexes after cross-linking to [^{125}I]IFNα2 (Colamonici *et al.*, 1990, 1992); (ii) McAB IFNaRβ1 recognizing a 100 kDa protein (termed β-subunit) in U266 cells, and able to block cell binding of IFN-α1,2,7,8,ω and IFN-β subtypes (Colamonici and Domanski, 1993). The proteins recognized by these McABs are encoded by the 21q22.1 segment which confers huIFN-α2 binding

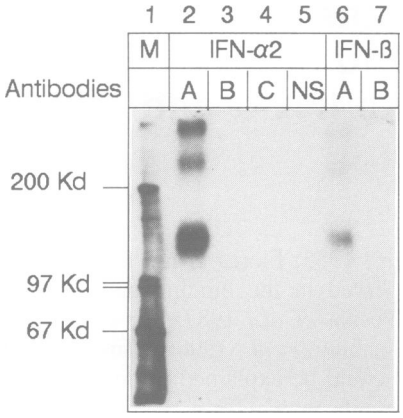

Fig. 1. Specific immunoprecipitation of [^{125}I]IFN-α and IFN-β cross-linked to the receptor IFNAR chain. Daudi cells were incubated at 10^7 cells/ml with 10^6 c.p.m./ml of radioiodinated IFN-α2 (lanes 2–5) or IFN-β (lanes 6 and 7) for 2 h at 4°C. After cross-linking with disuccinimide suberate (DSS), extracts of 3×10^7 cells made with Brij were precipitated with anti-IFNAR peptide A (lanes 2 and 6), pre-immune serum (lane 5) or control antibodies anti-IFNAR peptide B (lanes 3 and 7) or McAB 21.4 (lane 4), followed by SDS–PAGE and autoradiography. Lane 1 contains radioactive protein markers.

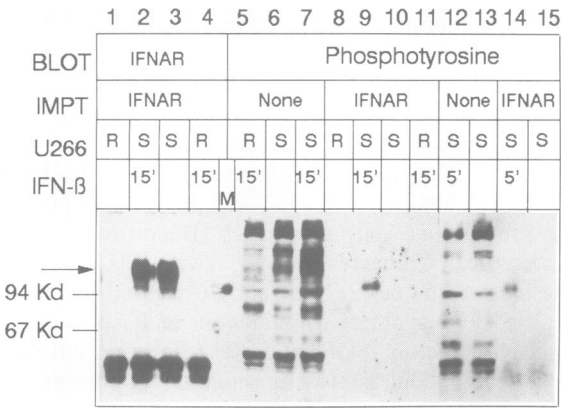

Fig. 2. Immunoprecipitation of the type I IFN receptor IFNAR chain and tyrosine phosphorylation. IFN-sensitive U266^S cells or IFN-resistant U266^R cells (2×10^7/ml) were treated with 2000 U/ml IFN-β for 15 or 5 min at 37°C, or left untreated as indicated. Extracts made with CHAPS were immunoprecipitated with anti-IFNAR peptide A antibodies (lanes 1–4, 8–11, 14, 15) or electrophoresed directly (lanes 5–7, 12–13), followed by immunoblotting with anti-IFNAR McAB 64G12 (lanes 1–4) or anti-phosphotyrosine McAB (lanes 5–15). Blots were developed by ECL.

and response to rodent cells (Langer *et al.*, 1990), but both McABs failed to precipitate the IFNAR gene product made with baculovirus (Colamonici and Domanski, 1993). This raised doubts about the role of the IFNAR protein in the receptor response to different IFN subtypes.

Another approach to evaluate the function of individual components of the IFN-α,β receptor complex is to study how each associates with identified elements of the signal transduction machinery (Pellegrini and Schindler, 1993). Tyrosine kinases Tyk2 and Jak1 are required for IFN-α action, and show IFN-induced tyrosine phosphorylation (Velazquez *et al.*, 1992; Muller *et al.*, 1993). The transcription factors ISGF3α-p91 (Stat1) and p113 (Stat2) become tyrosine phosphorylated in response to IFN, and assemble with ISGF3γ on the promoters of IFN-inducible genes (Fu

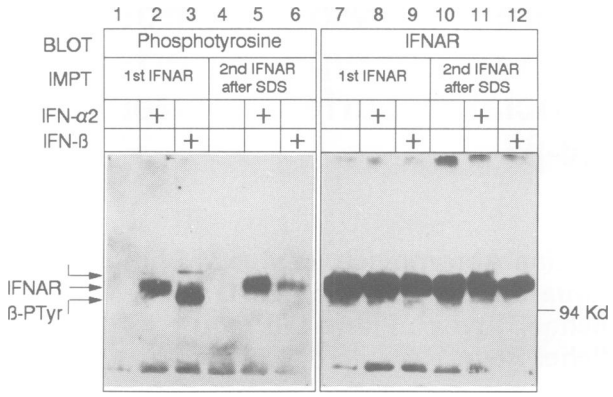

Fig. 3. IFN-dependent tyrosine phosphorylation of the IFNAR chain and of an IFN-β-specific associated protein. U266^S cells (8×10^7/5 ml) were treated with 2000 U/ml of either IFN-α2 or IFN-β for 8 min at 37°C, or left untreated. Cell extracts made with Brij were immunoprecipitated with anti-IFNAR peptide A antibodies. Protein A–beads with immunocomplexes from 1.5×10^7 cells were analyzed by electrophoretic immunoblotting with anti-phosphotyrosine McAB (lanes 1–3) and (after stripping) with anti-IFNAR McAB 64G12 (lanes 7–9). Beads from 2.5×10^7 cells were boiled with 0.3% SDS in 2% β-mercaptoethanol and the eluate was again precipitated with anti-IFNAR peptide A, and then analyzed by electrophoretic immunoblotting with anti-phosphotyrosine (lanes 4–6) and anti-IFNAR McAB (lanes 10–12). Development was by ECL. The bands corresponding to the IFNAR chain and to the associated protein tyrosine phosphorylated selectively after IFN-β (β-PTyr) are shown by arrows. The upper arrow indicates the position of associated Tyk kinase (see Figure 6).

et al., 1992; Darnell *et al.*, 1994). Association of the cloned 102/51 kDa receptor chain with Jak1 was observed (Novick *et al.*, 1994), whereas the McAB-defined α-subunit is associated with Tyk2 (Colamonici *et al.*, 1994) and undergoes IFN-induced tyrosine phosphorylation (Platanias and Colamonici, 1992). In the present study, we examined the function of the cloned IFNAR chain and show its association with Tyk2 but not Jak1, and with Stat2 but not Stat1. The IFNAR chain itself undergoes rapid tyrosine phosphorylation in response to IFN-α2, IFN-α8 and IFN-β. However, in line with recent results (Platanias *et al.*, 1994), IFN-β preferentially induces an additional tyrosine-phosphorylated protein which we show here to be a cell surface protein and in association with IFNAR.

Results

Immunoprecipitation of IFNAR and its IFN-induced tyrosine phosphorylation

Antipeptide-A antibodies were produced against residues 521–536 near the carboxy-terminal end of the IFNAR protein intracytoplasmic domain. These antibodies could precipitate IFNAR from Brij or CHAPS extracts of human cells. Figure 1 shows that [^{125}I]IFN-α2 as well as [^{125}I]IFN-β, bound and cross-linked to human Daudi cells, were immunoprecipitated as a complex of ~130 kDa by these anti-IFNAR peptide A antibodies. The reaction was specific and demonstrated that the IFNAR receptor chain does interact with both IFN-α2 and IFN-β subtypes. The same anti-IFNAR peptide A antibodies also precipitated specifically larger 210–220 kDa complexes of cross-linked IFN and even larger aggregates, which represent

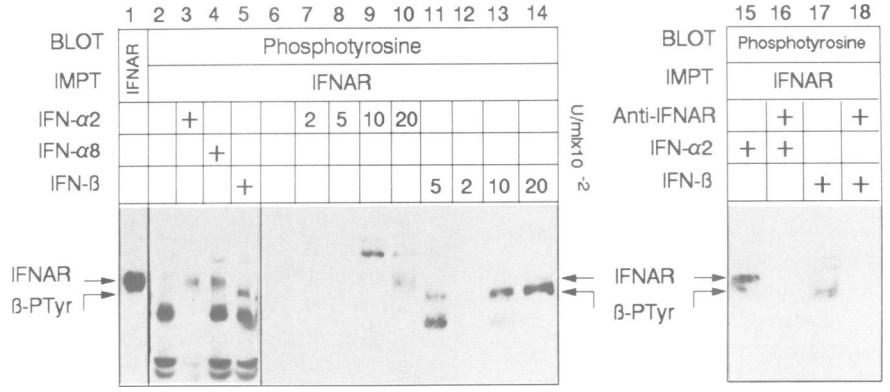

Fig. 4. Comparison of IFN-α2, IFN-α8 and IFN-β, and dose dependence for tyrosine phosphorylation of IFNAR and associated β-Ptyr protein. U266^S cells (2×10^7 in 1.5 ml) were treated with 2000 U/ml of the indicated IFN subtype for 8 min at 37°C. Cell extracts made with Brij were immunoprecipitated with anti-IFNAR peptide A antibodies and subjected to electrophoretic immunoblotting with anti-phosphotyrosine McAB (lanes 2–5) or for an aliquot (equivalent of 10^6 cells) with anti-IFNAR McAB (lane 1). Development was by ECL. In lanes 6–14, the cells were treated with concentrations of IFN-α2 or IFN-β indicated in U/ml$\times10^{-2}$. Lanes 16 and 18 show the effect of adding neutralizing anti-IFNAR McAB 64G12 (50 μg/ml) for 20 min prior to, respectively, IFN-α2 (compare with lane 15) and IFN-β (compare with lane 17).The bands of the IFNAR chain and of the associated protein tyrosine phosphorylated selectively after IFN-β (β-PTyr) are shown by arrows.

associations of IFN and IFNAR with other components of the receptor system (Colamonici *et al.*, 1992). In the absence of bound IFN, the IFNAR chain immunoprecipitated from human myeloma U266^S cells was identified as a 105 kDa protein (Figure 2, lanes 2 and 3). Its apparent size was slightly larger (110 kDa) in Daudi cells (not shown). The IFNAR protein was not seen in detergent extracts of the variant U266^R cells, which are resistant to IFN action and lack the mRNA for the transmembrane IFNAR protein (Abramovich *et al.*, 1994b).

Signaling by the IFN type I receptor involves tyrosine phosphorylation of tyrosine kinases Jak1 and Tyk2, and of the Stat1 and Stat2 components of the ISGF3α transcription complex (Darnell *et al.*, 1994). Analysis of the total tyrosine-phosphorylated proteins (PTyr) showed several IFN-β induced bands migrating more slowly than IFNAR (Figure 2, lane 7 compared with lanes 3 and 6). These bands did not appear in the U266^R variant cells (lane 5) and were seen at 15 min but not at 5 min after IFN addition (lane 12 compared with lane 7). After immunoprecipitation by anti-IFNAR, a PTyr band induced by IFN was already detected at 5 min (Figure 2, lane 14). This was not seen in the U266^R variants even at 15 min (lane 11 versus 9). In order to investigate whether this tyrosine-phosphorylated protein is the IFNAR chain or an associated factor, we eluted the proteins bound to the anti-IFNAR antibodies by boiling in sodium dodecyl sulfate (SDS) and repeated the immunoprecipitation with anti-IFNAR. Figure 3 shows that phosphotyrosine was found in the re-precipitated IFNAR band and was completely dependent on IFN treatment. Therefore, both IFN-α2 and IFN-β induced rapid tyrosine phosphorylation of the IFNAR component.

Selective effect of IFN-β on a tyrosine-phosphorylated cell-surface protein associated with IFNAR

A difference was noted between IFN-α and IFN-β effects in the first anti-IFNAR immunoprecipitate which show that IFN-β induces a major PTyr band migrating faster

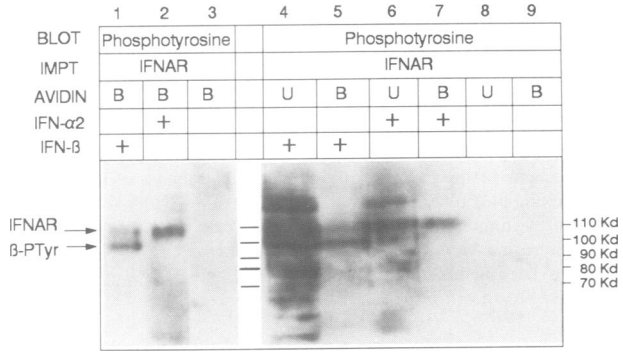

Fig. 5. The IFNAR-associated β-PTyr protein, tyrosine phosphorylated after IFN-β, is a cell surface protein. U266^S cells (13×10^7 cells) were treated with 2000 U/ml of IFN-α2 or IFN-β for 5 min at 37°C, or left untreated. The intact cells were subjected to biotinylation with NHS-SS-Biotin (see Materials and methods) for 10 min at 4°C and washed. Cell extracts were made with Brij and immunoprecipitates with anti-IFNAR peptide A antibodies collected on protein A−beads. The beads were boiled with 1% SDS in 50 mM Tris−HCl (pH 8.2) and the eluate (0.15 ml) supplemented with streptavidin−agarose beads. The streptavidin-bound proteins (B) from 3×10^7 cells were electrophoresed in SDS and immunoblotted with anti-phosphotyrosine McAB (lanes 1–3, 5, 7 and 9). The streptavidin-unbound supernatant (U) from equivalent amounts of cells was analyzed in parallel (lanes 4, 6 and 8). Development was by ECL. The biotinylated bands corresponding to the IFNAR chain tyrosine phosphorylated by IFN-α (lane 2) and IFN-β (lane 1), and to the associated protein tyrosine phosphorylated selectively after IFN-β (β-PTyr) (lane 1), are shown by arrows.

than IFNAR with an apparent size of 95 kDa (Figure 3, lane 3). This protein, designated β-PTyr, was removed after SDS treatment and re-precipitation with anti-IFNAR (lane 6), demonstrating that it is an associated protein differing from IFNAR. The β-PTyr band was not readily visible after treatment with IFN-α2 (lane 2) and the ratio of β-PTyr to IFNAR tyrosine phosphorylation was strikingly different with IFN-α2 and with IFN-β. The same difference was observed when either IFN-α8 or IFN-α2 were compared with IFN-β: both IFN-α induced mainly the PTyr band corresponding to IFNAR (Figure 4, lanes 3 and 4 compared with lane 1), whereas IFN-β

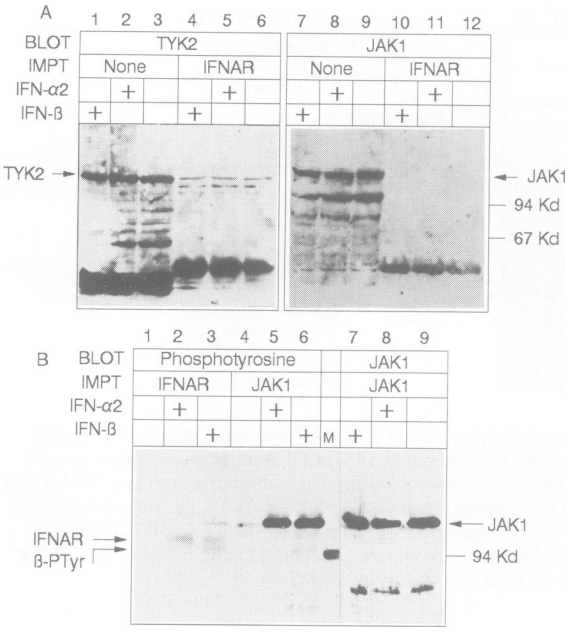

Fig. 6. Association of Tyk2, but not Jak1, with the receptor IFNAR chain. (**A**) U266^S cells (1.5×10^7/3 ml) were treated with 2000 U/ml of the indicated IFN subtype for 8 min at 37°C or left untreated. Cell extracts made with Brij were precipitated with anti-IFNAR peptide A antibodies (lanes 4–6, 10–12) or an aliquot was analyzed directly (lanes 1–3, 7–9) by SDS electrophoretic imunoblotting with antibodies to Tyk2 (lanes 1–6) or to Jak1 (lanes 7–12) on the same stripped blot. Development was by ECL. (**B**) In a similar experiment, cell extracts were immunoprecipitated with anti-Jak1 antibodies (lanes 4–9) and analyzed by immunoblotting with anti-Jak1 (lanes 7–9) or with anti-phosphotyrosine McAB (lanes 4–6), followed by ECL. The tyrosine-phosphorylated bands immunoprecipitated by anti-IFNAR peptide A antibodies are shown in parallel (lanes 1–3).

induced mainly the faster β-PTyr band (lane 5). The β-PTyr band could be visualized in cells treated with 500 U/ml IFN-β, and faintly already at 200 U/ml (Figure 4, lanes 11–14 compared with lane 6). Under these conditions, the IFNAR tyrosine phosphorylation was seen at 2000 U/ml IFN-α (lane 10). The phosphorylation of both IFNAR and β-PTyr was blocked by neutralizing anti-IFNAR McAB (Figure 4, lanes 15–18), confirming that IFNAR is essential for the response of the cells to both IFN-α and IFN-β subtypes. The upper PTyr bands (which correspond to associated kinases, see below) were also absent when the IFNAR function was blocked.

In order to investigate whether β-PTyr is a surface protein with an extracellular domain or an intracytoplasmic protein, U266^S cells were biotinylated from the outside (Ip *et al.*, 1992) and the anti-IFNAR immunoprecipitated PTyr proteins were examined for biotinylation. As expected, the IFN-α2-induced phosphorylated IFNAR band was recovered in the biotinylated fraction (streptavidin-bound; Figure 5, lanes 4 and 7). The IFN-β-induced β-PTyr band was clearly seen in the biotinylated fraction along with IFNAR (lanes 1 and 5). On the other hand, the IFN-induced PTyr bands migrating slower than IFNAR lacked biotin and were in the unbound fraction (lanes 4 and 6). Therefore, we can conclude that the β-PTyr 95 kDa protein is present on the cell surface in association with the IFNAR chain of the type I IFN receptor.

Jak/Tyk tyrosine kinases and Stat proteins associated with IFNAR

In order to identify which of the signal transducing components may be associated with the IFNAR chain, the immunoprecipitates were examined by blotting with antibodies to Jak and Stat proteins. A band migrating at 130 kDa as Tyk2 and recognized by anti-Tyk2 antibodies was found in the anti-IFNAR immunoprecipitate (Figure 6A, lanes 4–6). Tyk2 was associated with IFNAR even without IFN treatment (lane 6). Tyk2 is phosphorylated after IFN (Velazquez *et al.*, 1992) and probably corresponds to the upper IFN-induced PTyr bands of 130 kDa associated with IFNAR (Figures 3–5). On the other hand, Jak1 was not detected in association with IFNAR either before or after IFN treatment (Figure 6A, lanes 10–12). We verified that Jak1 was present and tyrosine phosphorylated in response to both IFN-α and IFN-β in these U266^S cells by examining immunoprecipitates obtained with anti-Jak1 antibodies (Figure 6B, lanes 4–9). Interestingly, a 95 kDa band resembling β-PTyr could be precipitated by anti-Jak1 (Figure 6B, lane 6 compared with lane 3).

Blots with anti-Stat1 (p91) antibodies showed that no Stat1 is associated with IFNAR (Figure 7A, lanes 3 and 4). A shift-up of Stat1 in the non-precipitated extracts was induced by IFN-β in U266^S; this was not observed in U266^R cells (not shown). The Stat1 protein could also be recovered from the supernatants of anti-IFNAR immunoprecipitations (lanes 5 and 6), and phosphorylation induced by both IFN-α and IFN-β was clearly observed (lanes 7–9). In contrast to Stat1, the association of Stat2 (p113) with IFNAR could be observed, a protein precipitated and recognized on blots by anti-Stat2 antibodies being present in the anti-IFNAR-bound proteins (Figure 7B, lanes 10–12). The association of Stat2 and IFNAR appears to be IFN independent, whereas tyrosine phosphorylation of Stat2 was dependent on either IFN-α or IFN-β (lanes 13–15).

The large cross-linked complexes of IFNAR with other receptor components are preferentially tyrosine phosphorylated

After cross-linking of labeled IFN-α2 or IFN-β on the surface of cells and immunoprecipitation by anti-IFNAR, as in Figure 1, several large SDS-resistant complexes of >200 kDa are observed in addition to the 130 kDa complex, corresponding to the expected size of an IFNAR–IFN complex. These large IFNAR complexes, probably denoting interactions with the extracellular domains of other surface proteins, appear to be highly tyrosine phosphorylated. When extracts of U266^S cells cross-linked with unlabeled IFN-α2 or IFN-β were immunoprecipitated with anti-IFNAR and blotted with anti-phosphotyrosine, a stronger signal was observed in the large complexes than in the 130 kDa complex (Figure 8, lanes 4 and 6). In contrast, the 130 kDa complex contained most of the bound IFNs when cross-linking with [^{125}I]IFN was carried out in parallel (lanes 7 and 8). Blotting with anti-IFNAR confirmed the presence of the IFNAR protein in the >200 kDa cross-linked complexes (not shown). The large PTyr complexes were seen only after cross-linking, and after IFN treatment. The 110 kDa tyrosine-phosphorylated IFNAR band tended to decrease

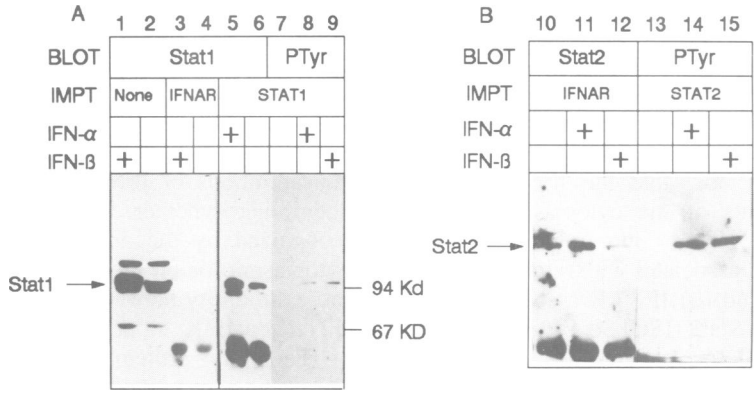

Fig. 7. Association of Stat2, but not Stat1, with the receptor IFNAR chain. (**A**) U266^S cells (1×10^7) were treated with 2000 U/ml of the indicated IFN for 8 min at 37°C or left untreated. From cell extracts made with Brij−buffer mix, a 1/10 aliquot was analyzed directly (lanes 1 and 2), whereas 9/10 of the extract was immunoprecipitated with anti-IFNAR peptide A antibodies (lanes 3 and 4) and subjected to SDS electrophoretic immunoblotting with antibodies to Stat1 (p91) (lanes 1–4). The supernatant unbound to the anti-IFNAR was precipitated with anti-Stat1 and immunoblotted with either anti-Stat1 (lanes 5 and 6) or anti-phosphotyrosine (lanes 7–9). (**B**) U266^S cells (2.5×10^7) were treated as in (A) and the extracted proteins immunoprecipitated with anti-IFNAR peptide A antibodies were eluted from the beads as in Figure 3 and the eluate re-immunoprecipitated with antibodies to Stat2 (p113) and immunoblotted with anti-Stat2 (lanes 10–12). Extracts from similar cell amounts were first precipitated with anti-Stat2, eluted and re-precipitated with anti-Stat2, and then immunoblotted with anti-phosphotyrosine (lanes 13–15). All developed by ECL.

after cross-linking, suggesting that a sizeable portion of the tyrosine-phosphorylated IFNAR enters into complexes with other surface receptor components. On the other hand, the associated β-PTyr band preferentially induced by IFN-β was not significantly reduced after extracellular cross-linking (Figure 8, lanes 5 and 6), suggesting that it is not as readily cross-linked to the IFNAR chain as other components.

Discussion

Functions of the IFNAR chain

The availability of an antipeptide antibody to the cloned IFNAR chain, which can immunoprecipitate this 105 kDa protein from detergent extracts of human cells, has allowed it to be established that the IFNAR chain is associated with both IFN-α2 and IFN-β after cross-linking. It also established that the IFNAR protein becomes rapidly tyrosine phosphorylated in response to IFN-α2 , IFN-α8 and IFN-β. Re-precipitation after boiling in SDS and reducing conditions demonstrates that this occurs on the IFNAR chain itself and not on an associated protein of the same size. This established that, in human cells, the IFNAR chain is directly involved in the action of different type I IFN subtypes. That IFNAR was required for binding of IFN-α8 as well as IFN-α2, and for the antiviral action of IFN-α2,8,w and IFN-β, could be deduced from neutralization with a monoclonal antibody to the extracellular domain of IFNAR (Benoit *et al.*, 1993). This McAB also inhibited ISGF3 and IRF-1 activation by IFN-α2 and IFN-β in Hep-2 cells (B.Cohen, M.Tovey and M.Revel, unpublished). In addition, the U266^R cell variant, lacking the IFNAR gene transcript encoding the transmembranal protein, has lost the response to IFN-α2 or IFN-β (Abramovich *et al.*, 1994b, and unpublished data). On the other hand, IFNAR probably acts together with other components of the human type I IFN receptor since, when expressed by itself in mouse cells, the IFNAR action is limited to huIFN-α8 (Uze *et al.*, 1990). This does not mean that IFNAR is specific for IFN-α8, as proposed

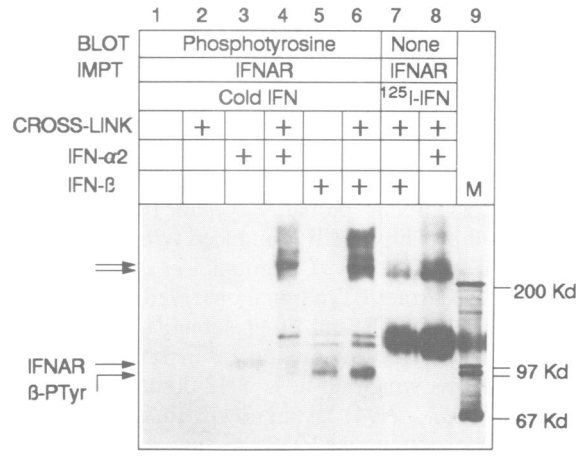

Fig. 8. Tyrosine phosphorylation of cross-linked complexes immunoprecipitated by anti-IFNAR. U266^S cells (6×10^7) were treated with 2000 U/ml of unlabeled IFN-α2 or IFN-β for 5 min at 37°C, or left untreated. From each, three-quarters of the cells were treated with the DSS cross-linker (lanes 2, 4 and 6) and a quarter were not cross-linked (lanes 1, 3 and 5). Equivalent amounts of Brij extracts were immunoprecipitated with anti-IFNAR peptide A antibodies, followed by electrophoretic immunoblotting with anti-phosphotyrosine and ECL development. On the same gel, Brij extracts of U266^S cells reacted with [^{125}I]IFN-β or IFN-α2 as in Figure 1, and cross-linked before precipitation with anti-IFNAR peptide A, were analyzed by autoradiography (lanes 7 and 8) with radioactive marker proteins (lane 9).

(Colamonici *et al.*, 1994) and, indeed, in hamster cells IFNAR confered responses to huIFN-β and not to IFN-α8 as in murine cells (Abramovich *et al.*, 1994a). Rather, it is likely that to respond to various human type I IFNs, rodent cells require several components of the human IFN receptor which can be provided by chromosome 21 (Slate *et al.*, 1978), a 21q22.1 3 mb segment (Langer *et al.*, 1990) and even a YAC clone from this region including the IFNAR gene (Soh *et al.*, 1994). An additional 21q22.2-3 segment is needed for full response (Hertzog *et al.*, 1994).

Our experiments show that the IFNAR chain interacts

with a number of other proteins. First, the IFNAR protein can be cross-linked on the surface of human cells into large complexes of >200 kDa, which are highly tyrosine phosphorylated in response to IFN-α2 or IFN-β and contain these IFNs. These complexes probably represent interactions with the extracellular domain of other receptor components. Second, we show that the IFNAR chain is associated with elements of the cytoplasmic signal transduction machinery. The immunoprecipitations demonstrate that (i) IFNAR associates with tyrosine kinase Tyk2, but not with Jak1 and (ii) IFNAR associates with transcription factor subunit Stat2 (ISGF3α-p113), whereas no binding with Stat1 (ISGF3α-p91) could be seen. Thus, the IFNAR chain would be constitutively binding only one of the two tyrosine kinases required for IFN-α action (Muller *et al.*, 1993) and only one of the two ISGF3α subunits that are activated in response to IFN (Fu *et al.*, 1992; Darnell *et al.*, 1994). Possibly, the interaction with another receptor chain may bring together the missing Jak1 and Stat1 elements. In this respect, it is interesting that the recently cloned 102/51 kDa IFN-α,β receptor was shown to be constitutively associated with Jak1 (Novick *et al.*, 1994). Colamonici *et al.* (1994) also reported that Tyk2 is associated with the 110 kDa α-subunit and that this protein, as well as the 100–110 kDa β-subunit, becomes tyrosine phosphorylated in response to IFN-α2 (Platanias and Colamonici, 1992; Colamonici *et al.*, 1994). The relationships between these different proteins and their possible interactions should obviously be further clarified. The large type I IFN receptor complexes visualized after cross-linking probably contain two or more of these receptor proteins and IFN-induced tyrosine phosphorylation of several chains (Colamonici *et al.*, 1994) may account for the strong PTyr signal observed. The function of the PTyr in IFNAR and other subunits of the type I IFN receptor in their interaction or in binding Jak/Tyk and Stat proteins which all have SH2 domains (reviewed by Darnell *et al.*, 1994) is unknown. In the case of an IFN-γ receptor subunit, one PTyr was shown to be involved in the binding of Stat1 (Greenlund *et al.*, 1994).

A cell-surface tyrosine phosphoprotein selectively involved in the IFN-β response

A 95–100 kDa tyrosine-phosphorylated protein, β-PTyr, was found to be specifically associated with IFNAR in cells responding to IFN-β. The β-PTyr band appeared as rapidly as the tyrosine phosphorylation of IFNAR at 5 min after IFN, earlier than associated signal transduction elements which peaked at 15 min. The ratio of β-PTyr to IFNAR tyrosine phosphorylation was always much higher after IFN-β than after IFN-α2,8 treatment. The concentration of IFN-β needed to see β-PTyr was 200–500 U/ml, whereas IFNAR phosphorylation required 2000 U/ml of IFN-α2 under the same conditions. Antibodies blocking IFNAR action abrogated both β-PTyr and IFNAR tyrosine phosphorylation. The β-PTyr protein is not another form of IFNAR since it was dissociated from IFNAR by SDS, but it is another cell-surface protein since it could be tagged by extracellular biotinylation in the same way as IFNAR itself. Other IFNAR-associated PTyr bands (such as the 130 kDa band identified as Tyk2) were not tagged by biotin under the same conditions. β-PTyr could be another component of the receptor which is either selectively tyrosine phosphorylated after IFN-β or becomes more tightly associated to IFNAR by IFN-β than by IFN-α. Colamonici *et al.* (1994) also recently observed a p100 tyrosine-phosphorylated band immunoprecipitated with the α-subunit, but not the β-subunit, after IFN-β but not after IFN-α2 or ω. These studies used very high concentrations of IFN-β (20 000 U/ml) and it was not determined whether the protein is directly or indirectly recognized by the antibody, or whether the protein is cytoplasmic or on the cell surface. However, this is one more similarity between the α-subunit defined by McAB IFNaR3 and the cloned IFNAR chain.

The β-PTyr protein may represent an important difference which can be observed in the functions of IFN-β and IFN-α at the receptor level. Another difference is that cells lacking Tyk2 have lost the response to IFN-α, but still respond to IFN-β (Velazquez, 1992; Watling *et al.*, 1992). A preferential response to IFN-β has been observed in a number of human tumor cells, including gliomas (Rosenblum *et al.*, 1990) and melanomas (Johns *et al.*, 1992). The IFN-β glycoprotein is only 30% homologous to the closely related IFN-α subtypes and, despite sharing some receptor subunits with IFN-α, the action of the IFN-β glycoprotein may involve additional receptor components which allow cells to respond discriminately to this IFN species.

Material and methods

Cell cultures, interferons and antibodies

Human myeloma $U266^S$ cells were cultured in RPMI 1640 (Bio-Lab, Israel) with 10% heat-inactivated fetal calf serum (FCS) at 37°C in 5% CO_2. The $U266^R$ cells are an IFN-α,β-resistant derivative of U266 lacking the transmembranal IFNAR mRNA (Abramovich *et al.*, 1994b). Human Daudi lymphoblastoid cells were used in some experiments. Human recombinant (*Escherichia coli*) IFN-α8 (α-B) and IFN-α2 (α-A), 2×10^8 IU/mg, were kind gifts from Dr M.Grutter (Ciba-Geigy) and from Dr C.Weismann, respectively. Human recombinant IFN-β, 5×10^8 IU/mg, produced as described from hamster CHO cells (Chernajovsky *et al.*, 1984), was obtained from InterPharm (Ares-Serono group, Israel). The hexadecapeptide DEDHKKYSSQTSQDSG, corresponding to amino acids 521–536 of the cloned IFNAR protein (Uze *et al.*, 1990), was used coupled to keyhole limpet hemocyanin for immunizing rabbits as described previously (Chebath *et al.*, 1987). This antiserum, designated anti-IFNAR peptide A antibodies (Ab 631), was used throughout for immunoprecipitation of the membrane IFNAR chain of the type I IFN receptor. Antiserum obtained against peptide B from an N-terminal region of IFNAR was used as control. Neutralizing anti-IFNAR monoclonal antibody McAB 64G12 derived from mice immunized with COS cell-produced IFNAR was described previously (Benoit *et al.*, 1993). McAB 21.4 was obtained from mice immunized with *E.coli*-produced IFNAR (Novick *et al.*, 1992). Commercial anti-peptide antibodies to tyrosine kinases Jak1 and Tyk2, and anti-phosphotyrosine McAB 49-10 were purchased from UBI (Lake Placid, NY). Antibodies to Stat1 (p91L) were produced by immunizing rabbits with peptide 478–730, and for Stat2 (p113) with peptide 669–808 (residues according to Fu *et al.*, 1992) using the pGEX-3X method (Pharmacia), and were kind gifts of Drs S.Harroch and J.Chebath (this department).

IFN-treated cell extracts, immunoprecipitations and immunoblotting

Routinely, $U266^S$ cells at $1.5–2\times10^7$ cells/ml were treated with 2000 IU/ml of IFN-α2, IFN-α8 or IFN-β for 5–8 min at 37°C. The cells were washed at 4°C with phosphate-buffered saline (PBS) containing 1 mM sodium orthovanadate and extracted with 0.2 ml/10^7 cells of Brij–buffer mix [1% Brij, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 1 mM sodium vanadate, 10 mM sodium molybdate]. After 30 min at 4°C, the supernatant was recovered by centrifugation in an Eppendorf minifuge for 15 min. For immunoprecipitation, extracts from 2×10^7 cell

aliquots were adjusted to 0.75 ml with Brij−buffer mix and anti-IFNAR peptide A antiserum was added at 1:150 dilution (when indicated anti-Stat or anti-Jak or Tyk were used at 1:200). After shaking for 18 h, 0.05 ml of a 50% suspension of protein A−beads (IPA-400 fast-flow, Repligen) was added for 1 h, all at 4°C. The beads were washed three times with Brij−buffer mix and the SDS-eluted proteins after boiling in reducing conditions (10% β-mercaptoethanol) were subjected to 7.5% PAGE in SDS. Transfer onto nitrocellulose (Schleicher and Schuell BA85) was as described previously (Chebath *et al.*, 1987), followed by blocking with PBS, 0.05% Tween-20, 5% low-fat milk for 1 h, and incubation with anti-phosphotyrosine McAB (or anti-IFNAR McAB) in blocking solution for 2 h, all at room temperature. After six washings with PBS, 0.05% Tween, the blots were developed with an enhanced chemiluminescence kit (ECL, Amersham) using horseradish peroxidase coupled to protein A or sheep anti-mouse Ig.

For consecutive immunoprecipitations, the protein A−beads after the first immunoprecipitation were washed with Brij−buffer mix, then with 50 mM Tris−HCl (pH 7.5), 150 mM NaCl, and resuspended in 0.3 ml of 0.3% SDS, 2% β-mercaptoethanol in Tris/NaCl (pH 8.2) for 20 min at 4°C before boiling for 5 min. The supernatant was diluted to 3 ml with Brij−buffer mix and the second antibody added for 2 h at 4°C, followed by fresh protein A−beads as above. In indicated experiments, the cell extracts were obtained with 10 mM CHAPS detergent as detailed before (Abramovich *et al.*, 1994a).

IFN cross-linking and biotinylation of surface proteins

Radioiodination of IFN-α2 was carried out by a modification of the chloramine T procedure as described (Novick *et al.*, 1992) using 420 μCi of Na [^{125}I] for 10 μg IFN with 1 mg/ml chloramine T for 20 s at 4°C, yielding ~4×10^7 c.p.m./mg. The IFN-β was first dialyzed against PBS and similarly labeled with 110 μCi of Na [^{125}I] for 2 μg. For cross-linking, Daudi cells (1.8×10^8) were washed and resuspended in 18 ml of RPMI 1640, 1% FCS and 10^6 c.p.m./ml of [^{125}I]IFN-α2 or IFN-β. After 2 h at 4°C, the cells were centrifuged and resupended in 18 ml of PBS with 1 mM disuccinimide suberate for 20 min at 4°C. The cells were washed in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl and extracts made with Brij−buffer mix as above for immunoprecipitation with anti-IFNAR peptide A antibodies. When tyrosine phosphorylation was measured, 1 mM sodium vanadate was added to the cross-linking reaction.

For biotinylation, 13×10^7 U266^S cells treated with IFN-α2 or IFN-β for 5 min were washed and resuspended in 4 ml PBS, 1 mM sodium vanadate, then supplemented with 1 mg/ml sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (Immunopure NHS-SS-Biotin, Pierce Chemical Company) for 10 min at 4°C with gentle shaking. After washing with 100 mM Tris−HCl (pH 7.5), 150 mM NaCl, cell extracts were made with Brij−buffer mix and immunoprecipitated with anti-IFNAR peptide A antibodies as above. The protein A−beads were washed with Brij−buffer mix, then with 50 mM Tris−HCl (pH 7.5), 150 mM NaCl and resuspended in 0.15 ml of Tris−HCl (pH 8.2) containing 1% SDS. After boiling for 5 min, 0.04 ml of a 50% suspension of streptavidin−agarose (Immunopure immobilized streptavidin, Pierce Chemical Company) was added and, after 1 h at 4°C, the beads were centrifuged, washed with Brij−buffer mix and the SDS-eluted proteins after boiling in reducing conditions were subjected to gel electrophoresis and immunoblots as above. The streptavidin supernatant (non-biotinylated) proteins were similarly analyzed.

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