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Immunology

Characterization of three monoclonal antibodies that recognize the interferon α2 receptor

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ABSTRACT **The interferon system plays an important role in the control of viral infections and cell proliferation. These effects are mediated through the interaction of interferons with specific cell surface receptors. We report here the development of monoclonal antibodies against one of the subunits of the interferon α receptor. These antibodies detect a 110-kDa protein in surface-labeled cells and in Western blots, and 130- and 210-kDa bands after crosslinking to iodinated interferon α2. No other subunits are disulfide-linked to the 130-kDa subunit or are coprecipitated by these antibodies. Analysis by two-dimensional gel electrophoresis revealed that the pI of this subunit is 3.5–5.0. We suggest that the protein recognized by these monoclonal antibodies be named the α subunit of the interferon α receptor.**

Three types of interferons (IFNs) have been described: leukocyte or α (IFNα), fibroblast or β (IFNβ), and immune or γ (IFNγ). A wide variety of biological effects have been described for IFNs including antiviral activity; antiproliferative activity; stimulation of cytotoxic activities in lymphocytes, natural killer cells, and macrophages; and modulation of the major histocompatibility complex (1). Particularly important in the field of oncology is that some tumor cell lines are very sensitive to the antiproliferative effect of IFNs (2, 3). Furthermore, IFNα has proved to be effective in the treatment of hairy-cell leukemia and chronic myelogenous leukemia (4–9). A number of leukemia cell lines, primary leukemia cells from patients with acute lymphoblastic leukemia (10, 11), and gliomas (12) have deletions of chromosome 9 (band 9p22) that include the IFNα and -β genes. The loss of these genes could represent a critical event in the oncogenic process. Thus, IFNs could play a role not only in the treatment of certain types of leukemias but also in its development.

The effects of IFNs are mediated through interaction with cell surface receptors (13). IFNα and -β apparently share the same receptor structure, while IFNγ binds to a different surface molecule (14). Several normal and malignant human cells have been shown to bind ^{125}I-labeled IFNα to surface receptors. Even though the dissociation constants (K_d) are quite variable (ranging from 10^{-9} M to 10^{-11} M) (1, 13–22), there appear to be at least two classes of IFNα receptors: high and low affinity. Early attempts to characterize the protein structure of IFNα receptors by crosslinking methods showed a single band in SDS/PAGE at 140–150 kDa (including 20 kDa corresponding to ^{125}I-IFNα) (16, 23). While later reports confirmed these findings, IFNα receptor/^{125}I-IFNα complexes of 300, 230, and 80 kDa have also been reported (18, 23, 24). Crosslinking experiments performed with radiolabeled IFNβ showed two different complexes, 140 and 120 kDa (25).

Bands at 210, 130, 110, 75, and 55 kDa were seen in affinity crosslinking experiments performed with the myeloma cell lines U-266 and H929, which express high numbers of IFNα receptors. Displacement studies and affinity crosslinking experiments using different concentrations of ^{125}I-IFNα clearly demonstrated the presence of two classes of binding sites: low and high affinity (O.R.C., unpublished data). Thus, in the U-266 myeloma cells the 210- and/or the 130-kDa bands accounted for the high-affinity binding, and the 110-kDa band accounted for the low-affinity component (O.R.C., unpublished data). A gene that confers on mouse cells the ability to bind human IFNα has recently been cloned (27). This gene codes for a 64-kDa protein and has several potential sites for glycosylation. It is not known whether this gene corresponds to the 110- or 130-kDa band.

To characterize IFN receptors further, we have developed monoclonal antibodies (mAbs) against the IFNα receptor. In this report, we describe three mAbs that immunoprecipitate an ^{125}I-IFNα2/IFNα receptor complex of 130 kDa as well as a 210-kDa complex. All three antibodies fail to precipitate ^{125}I-IFNα2. A single 110-kDa band was recognized by these antibodies in Western blots and in immunoprecipitations of surface-iodinated U-266 and H929 cells. This product has a pI of 3.5–5.0 as determined by two-dimensional gel electrophoresis. We suggest that this protein be named the α subunit of the IFNα receptor.

METHODS

Labeling of IFNα2. Human recombinant IFNα2 (2.2×10^8 units/mg) was kindly provided by Paul Trotta (Schering-Plough). IFNα2 was labeled with di[^{125}I]iodinated Bolton-Hunter reagent (New England Nuclear) according to the manufacturer's specifications, purified on a Bio-Gel P-6DG chromatography column (Bio-Rad), and stored in aliquots in 20% (vol/vol) glycerol/1% bovine serum albumin at −20°C. The recovery of ^{125}I-IFNα2 from the P-6DG columns was assessed for each iodination and ranged from 50% to 60%. The specific biological activity of ^{125}I-IFNα2 was determined in a proliferation assay using Daudi cells (Burkitt lymphoma cell line) in which the amount of ^{125}I-IFNα2 needed for 50% inhibition of [^{3}H]thymidine incorporation was calculated and compared with that of unlabeled IFNα2. IFNα2 labeled by this method retained 100% of the specific biological activity for at least 14 days after iodination. The maximal binding capacity of these preparations was 90% as calculated from the *y* intercept of regression lines in which the reciprocals of specific cpm and cell number were plotted (19). Specific

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Abbreviations: IFN, interferon; mAb, monoclonal antibody; NEPHGE, nonequilibrium pH-gradient gel electrophoresis.

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radioactivities ranged from 64 to 104 μCi/μg (1 μCi = 37 kBq).

Affinity Crosslinking of ^{125}I-IFNα2 to its Receptors. Affinity crosslinking was performed as described (26). Briefly, 3×10^8 NCI-H929 (H929 cells, kindly provided by Adi Gazdar, Navy Oncology Branch, National Cancer Institute; ref. 28) or U-266 human myeloma cells were washed twice in RPMI-1640/1% bovine serum albumin and incubated with 5 nM ^{125}I-IFNα2 for 1–2 hr at 4°C. After washing, the cells were resuspended in 8 ml of 138 mM NaCl/8.1 mM sodium phosphate/1 mM $MgCl_2$, pH 8.3, crosslinked with disuccinimidyl tartrate (300 μg/ml; Pierce) for 1 hr at 4°C, and lysed at a concentration of $0.5–1.0 \times 10^8$ cells per ml in 1% Nonidet P-40/300 mM NaCl/50 mM Tris, pH 7.4. This procedure yielded 2–3 μCi of crosslinked material that was used for the screening of antibodies directed against the various subunits of the IFNα receptor.

mAbs. These were produced as described (29). Briefly, two BALB/C mice were immunized four times at 14-day intervals with 10^7 U-266 cells intraperitoneally. Three days after the last booster injection, spleen cells from each mouse were separately fused with Sp2/0 myeloma cells. The clones were screened for binding to U-266 cells by flow cytometric analysis, and the positive clones were expanded to 1 ml and frozen. Pools of supernatants from seven clones were bound to protein G-Sepharose (Pharmacia) and then incubated with lysate ($1–3 \times 10^5$ cpm) obtained from crosslinking U-266 cells to ^{125}I-IFNα2 as described above. After the unbound ^{125}I-IFNα2/IFNα receptor complexes were removed by washing, the radioactivity associated with the protein G-Sepharose was measured, and those samples that had counts above background were analyzed by SDS/PAGE. The positive pools were individually screened to identify the positive clone. Three mAbs, termed IFNaR1, IFNaR2, and IFNaR3, were obtained. Crosslinking of purified mAbs to CNBr-activated Sepharose 4B (Pharmacia) was performed following the manufacturer's specifications.

Immunoprecipitation of Labeled Cell Surface Proteins with the mAbs. H929 cells were surface-labeled with ^{125}I by the lactoperoxidase method (30). After labeling, the cells were washed and lysed. Lysates were incubated with the indicated antibodies overnight, then incubated with protein G-Sepharose for 4 hr at 4°C and analyzed by SDS/8% PAGE under reducing and nonreducing conditions. Gels were exposed to x-ray film to detect labeled proteins.

Nonequilibrium pH-Gradient Gel Electrophoresis (NEPHGE). Immunoprecipitates obtained from surface-iodinated cells and from material crosslinked using ^{125}I-IFNα2 were run in NEPHGE in the first dimension (26) and reducing SDS/PAGE in the second dimension. Briefly, after immunoprecipitation and washing, protein G-Sepharose was resuspended in 9.5 M urea/2% Pharmalyte 3–10 (Pharmacia)/2% Nonidet P-40/5% 2-mercaptoethanol, incubated at 37°C for 3–4 hr, loaded on tube gels (4% polyacrylamide/9.2 M urea/2% Pharmalyte/2% Nonidet P-40), and overlaid with 9 M urea/1% Pharmalyte. The upper chamber buffer was 0.085% phosphoric acid and the lower chamber buffer was 10 mM NaOH. After electrophoresis for 6 hr at 500 V, the gels were soaked in 125 mM Tris, pH 6.8/2% SDS/10% glycerol/5% 2-mercaptoethanol and subjected to SDS/8% PAGE (second dimension). The second-dimension gel was then exposed to x-ray film.

Western Blotting. After SDS/PAGE, gels were equilibrated in 150 mM NaCl/40 mM Tris pH 8.0/10% methanol for 15 min and the proteins were transferred onto poly(vinylidene difluoride) membranes (Immobilon, Millipore) according to the manufacturer's instructions. Membranes were then blocked with 2% bovine serum albumin for 30 min in 10 mM Tris, pH 8.0/150 mM NaCl/0.05% Tween 20 (TBST), incubated for 1 hr with the indicated anti-IFNα receptor mAb, and washed three times in TBST. The secondary antibody (goat anti-mouse, alkaline phosphatase-conjugated) and the developing system were purchased from Promega and used according to the manufacturer's instructions.

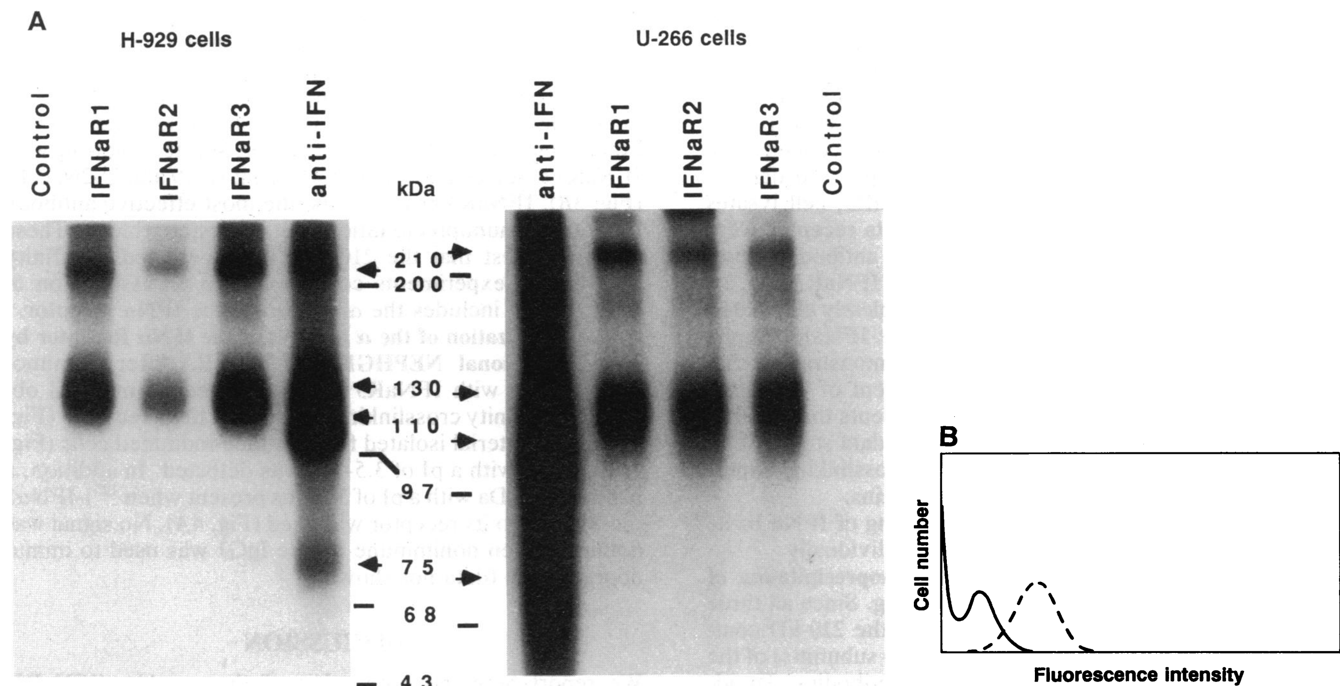

FIG. 1. (*A*) Immunoprecipitation of the 130- and 210-kDa complexes in affinity crosslinked material by mAbs directed against the IFNα receptor. The polyclonal serum (rabbit) against IFNα (anti-IFN) was purchased from ICN. Nonimmune mouse IgG1 (Coulter) was used as a control. (*B*) Flow cytometric analysis of U-266 cells with the anti-IFNα receptor mAb IFNaR3. U-266 cells (5×10^5) were incubated with 100 μl of IFNaR3 culture supernatant (dotted line) or 5 μg of nonimmune mouse IgG1 (solid line) for 30 min on ice, washed, incubated with a 1:40 dilution of fluorescein-conjugated goat anti-mouse IgG (Boehringer Mannheim), washed, and analyzed in an Epics cell sorter (Coulter).

RESULTS

Precipitation of ^{125}I-IFNα2/Receptor Complexes by mAbs Against the IFNα Receptor. The two fusions yielded 300 clones that showed binding to U-266 cells by flow cytometric analysis. Pools of supernatants from seven clones were bound to protein G-Sepharose, incubated with lysates containing ^{125}I-IFNα2/receptor complexes, washed, and assayed for radioactivity in a γ counter. Those pools that showed counts above background (300 cpm) were analyzed by SDS/PAGE to determine which subunits of the IFNα receptor were recognized by these antibodies. Three positive clones, IFNaR1, IFNaR2, and IFNaR3 [all three expressed the IgG1(κ) isotype] were obtained and underwent two or three rounds of subcloning by limiting dilution until 100% positivity was reached. IFNaR1 and -2 were isolated from the first fusion, whereas IFNaR3 came from the second fusion. All three mAbs precipitated 130- and 210-kDa complexes (the apparent molecular masses include the 20 kDa corresponding to ^{125}I-IFNα2) from U-266 and H929 lysates obtained after crosslinking ^{125}I-IFNα2 to its receptors (Fig. 1*A*). When a rabbit polyclonal anti-IFNα antibody was used for immunoprecipitation, additional bands at 110, 75, and sometimes 55 kDa were seen (Fig. 1*A*). All three antibodies were unable to precipitate free ^{125}I-IFNα2, confirming that they recognize an epitope on the IFNα receptor (data not shown). As expected, U-266 cells were positive with all three mAbs when analyzed by flow cytometry; IFNaR3 produced the strongest signal (Fig. 1*B*).

To determine whether IFNaR1, IFNaR2, and IFNaR3 recognize the same epitope on the receptor present in the 130-kDa complex, the antibodies were affinity-purified on a protein G-Sepharose column and coupled to CNBr-activated Sepharose. Crosslinked material containing ^{125}I-IFNα2/IFNα receptor complexes was incubated with IFNaR1-Sepharose and IFNaR3-Sepharose in the presence or absence of soluble IFNaR3 and IFNaR1, respectively, and analyzed by SDS/PAGE. Both IFNaR1-Sepharose and IFNaR3-Sepharose precipitated the 130- and 210-kDa complexes, but no signal was obtained in the presence of an excess of soluble IFNaR3 and IFNaR1, respectively (Fig. 2). A similar result was obtained when IFNaR3-Sepharose was incubated with ^{125}I-IFNα2 receptor complexes in the presence of soluble IFNaR2 (data not shown). These results suggest that IFNaR1, IFNaR2, and IFNaR3 recognize the same epitope or very closely spaced epitopes. To demonstrate further the specificity of mAb IFNaR3, cell lysates obtained after crosslinking ^{125}I-IFNα2 to its receptor were first immunoprecipitated with anti-IFNα antibodies (preclearing) and then immunoprecipitated with IFNaR3 (Fig. 2). Preclearing with anti-IFNα antibodies completely eliminated the signal for the 130-kDa band (compare IFNaR3/Sepha with anti-IFN + IFNaR3 in Fig. 2), demonstrating that IFNaR3 recognizes specifically a component of the IFNα receptor. In Fig. 2 the anti-IFN lane represents the material precipitated in the preclearing step. These data suggest that the 130- and 110-kDa bands detected in crosslinking experiments corresponded to two different proteins.

All three mAbs failed to block the binding of IFNα to its receptors (data not shown) when tested individually.

Detection of a 110-kDa Band by Immunoprecipitation of Surface-Iodinated Cells and Western Blotting. Since all three antibodies precipitated both the 130- and the 210-kD complexes, it was necessary to determine which subunit(s) of the IFNα receptor was recognized by these antibodies. To address this question, H929 cells were surface-labeled with ^{125}I by the lactoperoxidase method. After iodination, the cells were lysed and immunoprecipitates obtained with IFNaR1, IFNaR2, and IFNaR3 were analyzed by SDS/PAGE under reducing conditions. The three mAbs precipitated a 110-kDa band (Fig. 3*A*) that corresponded to the 130-kDa band in crosslinking experiments with labeled IFNα2. We propose to name the subunit precipitated by the IFNaR1, IFNaR2, and IFNaR3 mAbs the α subunit of the IFNα receptor, and we will refer to this subunit as such hereafter to avoid confusion with the 110-kDa band detected in affinity crosslinking experiments. There were no differences when samples were analyzed under reducing or nonreducing conditions (Fig. 3*A*), indicating that the α subunit is not disulfide-linked to any other component of the IFNα receptor. Western blotting with IFNaR3 detected a 110-kDa band from H929 and U-266 cells (Fig. 3*B*). IFNaR3 proved to be the most effective antibody in both immunoprecipitations and Western blots. These results suggest that the 210-kDa band detected in affinity crosslinking experiments corresponds to an association of proteins that includes the α subunit of the IFNα receptor.

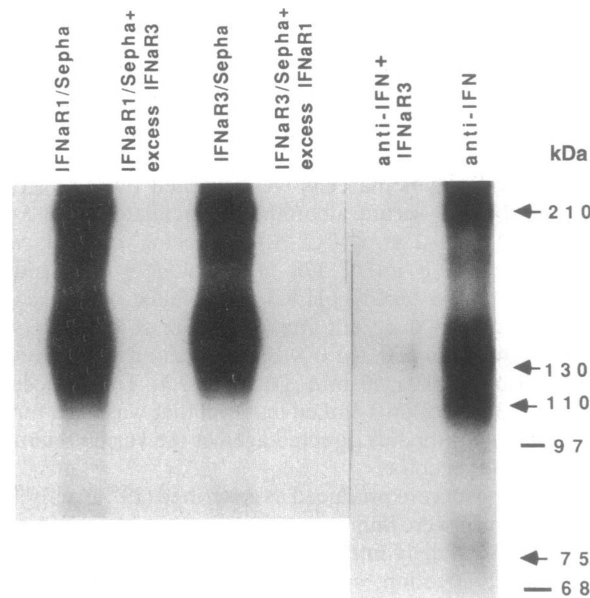

FIG. 2. IFNaR1 and IFNaR3 recognize the same or very closely spaced epitopes of the IFNα receptor. IFNaR1 and IFNaR3 coupled to CNBr-activated Sepharose (IFNaR1/Sepha and IFNaR3/Sepha, respectively) were incubated with IFNα receptors crosslinked to ^{125}I-IFNα2 in the presence (IFNaR1/Sepha + IFNaR3 and IFNaR3/Sepha + IFNaR1) or absence of soluble IFNaR3 or IFNaR1, respectively. The soluble antibodies prevented association of the labeled complexes with the Sepharose-conjugated antibodies, indicating that both mAbs recognize the same epitope or very closely spaced epitopes. Preclearing of the crosslinked material with anti-IFNα antibodies also prevented IFNaR3 immunoprecipitation of 130- and 210-kDa bands (anti-IFN + IFNaR3).

Characterization of the α Subunit of the IFNα Receptor by Two-Dimensional NEPHGE–SDS/PAGE. After immunoprecipitation with IFNaR3 was performed on material obtained by affinity crosslinking ^{125}I-IFNα2 to its receptor (Fig. 4*A*) or on material isolated from surface-iodinated cells (Fig. 4*B*), a band with a pI of 3.5–5.0 was detected. In addition, a band at 210 kDa with a pI of 5.0 was present when ^{125}I-IFNα2 crosslinked to its receptor was used (Fig. 4*A*). No signal was obtained when nonimmune mouse IgG1 was used to immunoprecipitate (data not shown).

DISCUSSION

We report here the production of three mAbs (IFNaR1, IFNaR2, and IFNaR3) that recognize what we have termed the α subunit of the IFNα receptor. These mAbs were obtained through a double screening procedure: (*i*) determination of those clones producing antibodies that recognize antigens expressed on the surface of U-266 cells and (*ii*)

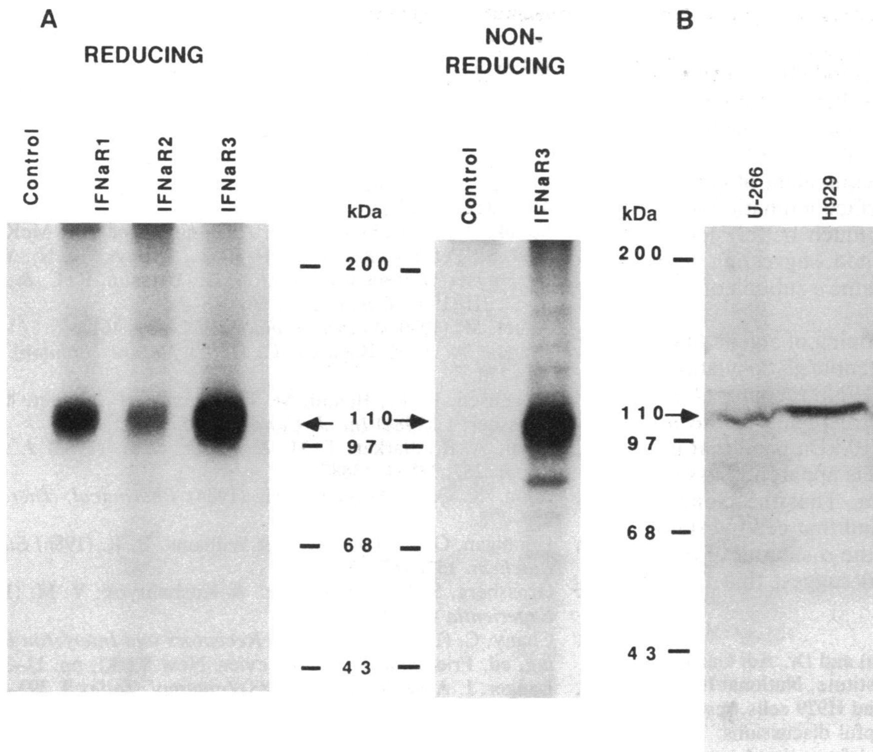

FIG. 3. Detection of a 110-kDa band by the mAbs IFNaR1, IFNaR2, and IFNaR3 in immunoprecipitations of surface-iodinated cells and in Western blots. (*A*) H929 cells were surface-iodinated and immunoprecipitates were performed. Nonimmune mouse IgG1 was used as control. The samples were analyzed by SDS/8% PAGE under reducing and nonreducing conditions. (*B*) Western blotting of U-266 and H929 cell lysates (5×10^6 cells per lane). IFNaR3 was used as first antibody, and alkaline phosphatase-conjugated goat anti-mouse IgG was used as second antibody.

identification of clones producing antibodies against the different subunits of the IFNα receptor. The three mAbs obtained against the α subunit of the IFNα receptor confirm a relatively high level of IFNα receptor expression on U-266 cells and suggest that this protein is a good immunogen in the mouse. All three mAbs recognize the 130- and 210-kDa ^{125}I-IFNα2/IFNα receptor complexes but fail to precipitate free ^{125}I-IFNα2, indicating that they recognize an epitope present on the IFNα receptor. IFNaR1, IFNaR2, and IFNaR3 are directed against the 130-kDa band detected in crosslinking experiments, which corresponds to the 110-kDa protein that was precipitated in surface iodination experiments and observed on Western blots. No difference was noticed when samples were analyzed by SDS/PAGE under reducing versus nonreducing conditions, implying that the α subunit is not disulfide-linked to any of the other subunits that form the receptor, and no coprecipitation of other proteins was evident under the conditions used for these experiments. These results indicate that the 210-kDa band obtained with crosslinking is a covalently coupled complex, produced by the crosslinker, that contains the α subunit. The complete composition of this complex remains to be demonstrated. Furthermore, IFNaR1, IFNaR2, and IFNaR3 recognize the same or very closely spaced epitopes on the α subunit of the IFNα receptor, as demonstrated by the blocking of the precipitation of ^{125}I-IFNα2/α-subunit complexes by IFNaR1-Sepharose and IFNaR3-Sepharose produced by an excess of soluble IFNaR3 and IFNaR1, respectively.

The pI of the α subunit of the IFNα receptor is 3.5–5.0 as demonstrated by two-dimensional gels in which NEPHGE was run in the first dimension. The same result was obtained by immunoprecipitation after either affinity crosslinking or surface iodination. This broad range for the pI could be due to different degrees of glycosylation of the α subunit, in agreement with the detection of N- and O-linked glycosides on the 130-kDa complex in crosslinking experiments (unpublished results).

To identify other components of the IFNα receptor present in the 210-kDa band, several experiments were performed in which cells were surface-iodinated and then crosslinked using the cleavable (by reducing agents) crosslinker dithio-

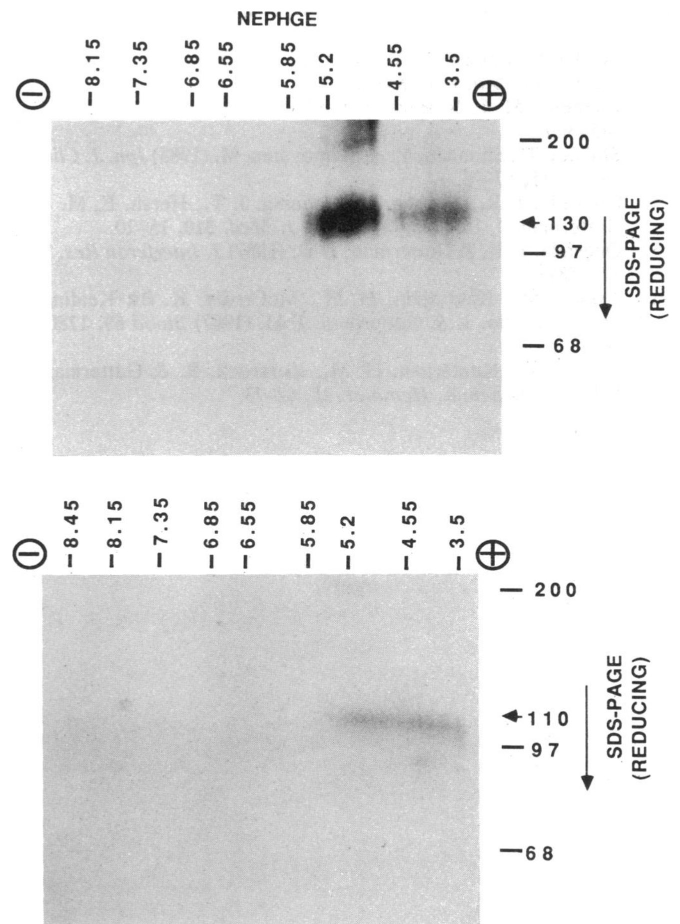

FIG. 4. Two-dimensional electrophoresis. (*A*) Cells were affinity-labeled with ^{125}I-IFNα2, crosslinked, lysed, and immunoprecipitated with IFNaR3. (*B*) Cells were surface-iodinated, lysed, and immunoprecipitated with the IFNaR3. Isoelectrofocusing markers (Pharmacia) were run in parallel and stained with Coomassie blue.

bis(succinimidyl propionate) in the presence or absence of unlabeled IFNα2 (data not shown). No band corresponding to the 210-kDa complex was evident. This could be explained in several ways: (*i*) poor iodination of the other component(s) of the receptor, (*ii*) a low amount of the other components of the IFNα receptors expressed in H929 cells, or (*iii*) a combination of *i* and *ii*. Interestingly, when equivalent numbers of H929 or U-266 cells were used for surface iodination and affinity crosslinking with ^{125}I-IFNα2, a much better signal was always obtained with the second method, suggesting that very few tyrosine residues are exposed on the α subunit of the IFNα receptor.

Uzé *et al.* (27) recently reported the cloning of a gene that codes for a 64-kDa protein with many potential glycosylation sites that corresponds to a subunit of the IFNα receptor (27). We do not know whether that gene corresponds to the α subunit of the IFNα receptor or to the 110-kDa band that is detected in crosslinking experiments and is apparently associated with low-affinity binding of IFNα. That the cloned gene is located on chromosome 21 (27) and that cell hybrids bearing human chromosome 21 express the α subunit of the IFNα region (O.R.C., unpublished data) suggest that they may correspond to the same product.

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