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Creation of phosphorylation sites in proteins: Construction of a phosphorylatable human interferon α

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ABSTRACT **A phosphorylation site was introduced into human interferon αA (IFN-αA) by site-specific mutation of the coding sequence. Three slightly different phosphorylation sites were created by using the predicted amino acid consensus sequences for phosphorylation by the cAMP-dependent protein kinase. The resultant modified interferons (IFN-αA-P) were expressed in *Escherichia coli* and purified. The purified proteins exhibit antiviral activity on bovine and human cells similar to that of the unmodified IFN-αA. The IFN-αA-P proteins can be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase with [γ-^{32}P]ATP to high specific activity (2000–5000 Ci/mmol; 1 Ci = 37 GBq) with retention of biological activity. The ^{32}P-labeled IFN-αA-P proteins bind to cells and can be covalently bound to the IFN-α/β receptor with a bifunctional reagent as can human IFN-αA. The introduction of phosphorylation sites into proteins provides a procedure to prepare a large variety of radioactive proteins for research and clinical use.**

The study of cell surface receptors for the interferons (IFNs) requires radiolabeled IFNs with high biological activity and high specific radioactivity. IFNs radiolabeled with ^{125}I by various procedures are usually used for this purpose (cited in refs. 1 and 2). The phosphorylation of human and murine IFN-γ by cAMP-dependent protein kinase from bovine heart muscle and [γ-^{32}P]ATP has provided a valuable reagent (3, 4) of high specific radioactivity to study the receptor (5, 6) and to identify the chromosome containing the gene for the human (7, 8) and murine (9) IFN-γ receptors. Several reports identified the phosphorylation sites of human and murine IFN-γ as serine residues at the carboxyl termini (4, 5, 10, 11). However, human IFN-αA and IFN-β cannot be phosphorylated by the cAMP-dependent protein kinase under conditions used for the phosphorylation of IFN-γ (3). To determine the feasibility of constructing human IFN-αA analogues with phosphorylation sites and high biological activity, the study described in this report was undertaken.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Enzymes and Chemicals. *Escherichia coli* RR1 containing the plasmid pRK248*c*Its857 (which encodes the temperature-sensitive repressor of the phage λ P_L promoter) was obtained from Robert Crowl (12). pRK248*c*Its857 was introduced into *E. coli* AR68 provided by Martin Rosenberg of Smith Kline & French Laboratories (13). The catalytic subunit of cAMP-dependent protein kinase from bovine heart muscle, with a specific activity of 20,000 units/ml, was obtained from Sigma. [γ-^{32}P]ATP with specific radioactivity of >5000 Ci/mmol (1 Ci = 37 GBq) was obtained from Amersham; bovine serum albumin, from Miles Laboratories; acrylamide and *N,N'*-methylenebisacrylamide, from International Biotechnologies; sodium dodecyl sulfate (SDS), from Sigma; and disuccinimidyl suberate, from Pierce Chemical.

IFN and Protein Assays. IFN activity was determined by a cytopathic effect inhibition assay with vesicular stomatitis virus and bovine MDBK cells (14). All IFN titers are expressed in reference units/ml calibrated against the reference standard for human leukocyte IFN (G-023-901-527) obtained from the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. Protein concentration was determined by the procedure of Bradford (15) with bovine serum albumin as a standard.

Construction of Modified IFNs. Because it was not possible to phosphorylate human IFN-αA directly with the catalytic subunit of bovine cAMP-dependent protein kinase, introduction of a phosphorylation site was considered. Consensus amino acid sequences recognized by the cAMP-dependent protein kinase have been identified as Arg-Arg-Ala-**Ser**-Val and Arg-Arg-Ala-**Ser**-Leu among others (16, 17). The amino acid sequence of human IFN-αA and its corresponding coding sequence have been reported (18–20). Human IFN-αA as well as other IFNs have been expressed in *E. coli* carrying expression vectors under control of the *trp* and the phage λ P_L promoter (see refs. 1, 19, and 20 for reviews and additional citations). The terminal nucleotides corresponding to the last 10 carboxyl-terminal amino acids of IFN-αA are shown in Fig. 1 as well as sequences corresponding to the modified molecules IFN-αA-P1, -P2, and -P3, which contain putative phosphorylation sites. To construct these molecules, oligodeoxyribonucleotides were synthesized to introduce the insertions and substitutions shown at the carboxyl terminus of IFN-αA (Fig. 1) by site-specific mutagenesis procedures with the appropriate DNA sequences inserted into phage M13mp19 (21–23). Constructions were confirmed by DNA sequencing. The phosphorylation sites in IFN-αA-P1, -P2, and -P3 (Fig. 1) recognized by the cAMP-dependent protein kinase were created by oligodeoxyribonucleotide-directed insertion on the level of DNA as shown in Fig. 2. The expression plasmids pBL281, pBL291, and pBL301, containing the sequences to produce IFN-αA-P1, -P2, and -P3, respectively, under control of the phage λ P_L promoter were constructed as outlined in Fig. 2. *E. coli* AR68 and RR1 containing the compatible plasmid pRK248*c*Its857 were transformed with each of the expression plasmids.

Expression and Preparation of IFN-αA-P. *E. coli* RR1(pRK-248*c*Its857) cells harboring pBL281, pBL291, or pBL301 plasmids were grown at 30°C overnight in modified M9CA medium (21) containing thiamine (2 μg/ml), ampicillin (50 μg/ml), and tetracycline (12.5 μg/ml). For expression of proteins, 100 ml of M9CA medium was inoculated with 3–5 ml of an overnight culture. The bacteria were grown at 30°C until the cell density reached an optical density at 600 nm of 0.3–0.5

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Abbreviations: IFN, interferon; IFN-αA-P, human IFN-αA modified so that it contains a site for phosphorylation.

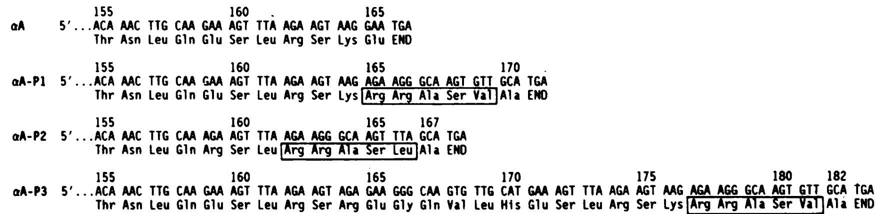

FIG. 1. Nucleotide and amino acid sequences of the carboxyl terminus of human IFN-αA and IFN-αA-P1, -P2, and -P3. The phosphorylation sites recognized by the cAMP-dependent protein kinase created in IFN-αA-P1, -P2, and -P3 are boxed.

(i.e., 2–3 hr), at which time the culture was transferred to 42°C for an additional 2 hr. The bacterial cells were collected by centrifugation and lysed in 8 M guanidine hydrochloride/50 mM Tris·HCl, pH 7.6, at 0°C for 10 min. The supernatant obtained after centrifugation at 14,000 rpm (SA-600 Sorvall rotor) for 30 min was used to assay the antiviral activity or to purify the IFNs. *E. coli* AR68(pRK248*c*Its857) cells harboring plasmids pBL281, pBL291, or pBL301 were grown in LB medium (21) containing ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml) at 32°C overnight. The overnight culture was diluted 5-fold with fresh LB medium containing the same concentration of antibiotics as above and then grown at 32°C for 2–3 hr. Expression of the IFN-αA-P proteins, harvesting of cells, and preparation of supernatants were as described above for *E. coli* RR1 cells.

Purification of IFN-αA-P Proteins. All steps for purification of IFN-αA-P species were carried out at 4–8°C. Ten milliliters of the guanidine hydrochloride supernatant from 100 ml of the expressed culture was diluted 10-fold with cold phosphate-buffered saline (PBS: 0.15 M NaCl/0.01 M sodium phosphate, pH 7.3) and precipitated at 65% saturation of ammonium sulfate at 4°C overnight. The precipitate was

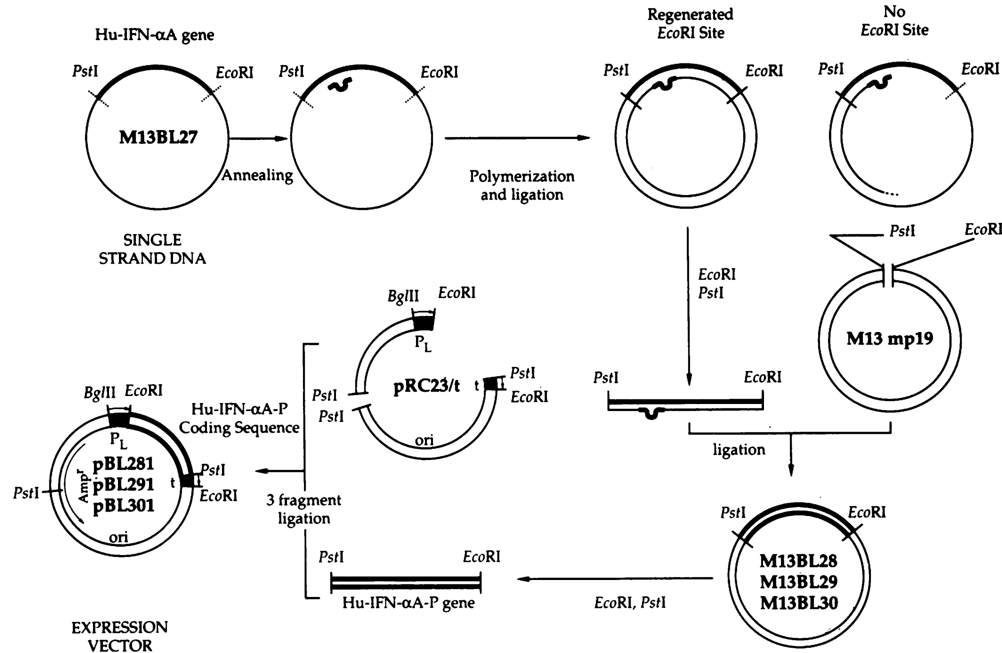

FIG. 2. Construction of expression plasmids for human (Hu-) IFN-αA containing phosphorylation sites. The *Eco*RI–*Pst* I fragment from pIFLrA that contained the coding sequence for human IFN-αA (17, 18) was inserted into the *Eco*RI–*Pst* I site of M13mp19 to form M13BL27, which was used as the template for site-specific alterations as shown. To construct the coding sequences for IFN-αA-P1 and -P2, two oligodeoxynucleotides were synthesized to anneal to M13BL27 with the formation of a loop that would permit the insertion of nucleotides to generate a coding sequence for a phosphorylation site at the carboxyl-terminal end (boxed residues, Fig. 1). The oligonucleotides used to prepare IFN-αA-P1 and -P2, respectively, were AGT-TTA-AGA-AGT-AAG-AGA-AGG-GCA-AGT-GTT-GCA-TGA-AAA-CTG-GTT-CAA and ACA-AAC-TTG-CAA-AGA-AGT-TTA-AGA-AGG-GCA-AGT-TTA-GCA-TGA-AAA-CTG-GTT-CAA. The nucleotides underlined are homologous with the coding sequence of human IFN-αA; the nucleotides not underlined produce a loop for the insertion of additional residues for P1 and P2. A site-specific mutation as well as an insertion was introduced with the P2 oligonucleotide. After annealing of the oligonucleotide to single-stranded DNA from M13BL27, the second strand was synthesized and the double-stranded DNA was cut with restriction endonucleases *Eco*RI and *Pst* I to produce a fragment which was then reinserted into the *Eco*RI–*Pst* I site of phage M13mp19 as shown in the figure. *E. coli* were transformed with the duplex DNA. This excision and religation step was introduced to increase the efficiency of the site-specific mutations, providing an overall yield of about 40% positive clones. Replicative-form (RF) DNAs, prepared from individual phage M13 plaques, were screened for the presence of the inserted *Eco*RI–*Pst* I fragment. Positive clones (i.e., those with insertions; 75–90% of the plaques) were sequenced by the Sanger dideoxynucleotide procedure to identify the proper mutated recombinant and to confirm the sequence. By this procedure, 50% of the transformants sequenced contained the mutated coding sequence with the phosphorylation site. The *Eco*RI–*Pst* I fragments were then excised from the respective RF DNA from the phages (M13BL28, M13BL29, and M13BL30) and religated into the *Eco*RI–*Pst* I site of pRC23t to yield the expression vectors (pBL281, pBL291, and pBL301) as shown. The two *Eco*RI–*Pst* I fragments originating from pRC23t were obtained by restriction endonuclease digestion of an expression plasmid for murine IFN-β that contained the *trp* terminator just downstream from the IFN-β coding sequence. During the construction of the human IFN-αA-P1 expression vector, a clone was isolated with a duplication of the P1 oligonucleotide, a single nucleotide deletion of one adenosine of codon 165 for lysine, and a second deletion of eleven 3′-terminal residues (AA-CTG-GTT-CAA) from the downstream P1 oligonucleotide; this combination of events generated an in-phase coding sequence for a new phosphorylation site on a slightly larger molecule we designated IFN-αA-P3 (Fig. 1). The general recombinant DNA procedures employed have been described (20–22).

collected by centrifugation at 10,000 rpm (Sorvall GSA rotor) at 5°C for 20 min and then dispersed in cold PBS (final volume, 20 ml). After centrifugation at 15,000 rpm (Sorvall SA-600 rotor) for 20 min at 5°C, the supernatant was decanted and saved. The residual pellet was dispersed again in cold PBS (10 ml) and then centrifuged as above. The combined supernatants (30 ml) were mixed with 1 ml of Affi-Gel 10 to which monoclonal antibody LI-8 (against human IFN-α) was linked (24), and the suspension was rocked at 4°C for 1 hr. The immunoabsorbent was loaded into the barrel of a 2-ml disposable syringe and washed with 20 column volumes or more of each of the following cold solutions sequentially (24): PBS; buffer F (0.5 M NaCl/25 mM Tris·HCl, pH 7.5/0.2% Triton X-100); and 0.15 M NaCl. Then the IFN was eluted with buffer H (0.2 M acetic acid/0.15 M NaCl, pH 2.6) and 0.4-ml fractions were collected. The eluted fractions were neutralized with 1 M Tris base to pH 7.0 and the fractions of peak antiviral activity were pooled.

Phosphorylation of Human IFN-γ and IFN-αA-P Proteins and Binding and Crosslinking of [^{32}P]IFN-αA-P to Cells. IFN-αA-P and IFN-γ were labeled with [γ-^{32}P]ATP and the cAMP-dependent protein kinase as described for IFN-γ (3, 6) with minor modifications. Binding of [^{32}P]IFN-αA-P to bovine MDBK cells (kidney cell line) and to human Daudi cells (Burkitt lymphoma line) was performed as described (25, 26). [^{32}P]IFN-αA-P proteins were crosslinked to MDBK cell receptor proteins and analyzed by SDS/polyacrylamide gel electrophoresis as described (6, 7, 27). After electrophoresis, the proteins were stained with silver (28) or with Coomassie brilliant blue. Gels containing radioactive samples were dried under vacuum and autoradiographed.

RESULTS

Expression and Purification of IFN-αA-P Proteins. IFN-αA-P1, -P2, and -P3 were expressed in *E. coli* RR1(pRK-248*c*Its857) and AR68(pRK248*c*Its857) harboring the expression plasmids under control of the λ P_L promoter and *trp* terminator at 42°C. The yields of IFN-αA-P1, -P2, and -P3 in *E. coli* AR68 were 20- to 40-fold higher than in *E. coli* RR1. The products expressed in *E. coli* AR68 at 42°C were used to purify IFN-αA-P1, -P2, and -P3 by immunoaffinity chromatography as described under *Experimental Procedures*. IFN-αA-P1, -P2, and -P3 were purified to specific activities of 1.2 × 10^8, 1.1 × 10^8, and 1.5 × 10^8 units per mg of protein, respectively.

Characterization of IFN-αA-P and Its ^{32}P-Labeled Product. The purified IFN-αA-P1, -P2, and -P3 proteins were analyzed by electrophoresis after reduction with 2-mercaptoethanol (Fig. 3). A single band was observed in the silver-stained gel with IFN-αA-P1 or -P2. IFN-αA-P2 migrated slightly faster than IFN-αA-P1 and a little slower than IFN-αA. IFN-αA-P3 yielded two bands, the slower-migrating band representing the intact molecule.

IFN-αA-P1, -P2, and -P3 can be phosphorylated by the cAMP-dependent protein kinase with [γ-^{32}P]ATP to a specific radioactivity of 2000–5000 Ci/mmol. After phosphorylation and reduction with 2-mercaptoethanol, [^{32}P]IFN-αA-P1 and -P2 migrate in SDS/polyacrylamide gel as single bands at M_r 19,000–5000 (Fig. 4) corresponding to the same positions as the silver-stained unlabeled bands. The labeled IFN-αA-P3 migrates slower than the P1 or P2 products as expected. Since the faster-migrating P3 band is unlabeled (cf. Figs. 3 and 4), it is likely that the carboxyl-terminal extension of P3 that contains the phosphorylation site is trimmed from the full-length product to yield the faster-migrating form. Unlabeled human IFN-αA and ^{125}I-labeled human IFN-αA were used as controls (Figs. 3 and 4). In a parallel experiment, phosphorylation was found to have little or no effect on the antiviral activity of IFN-αA-P1, -P2, and -P3 measured with both bovine MDBK cells and human WISH cells (data not shown).

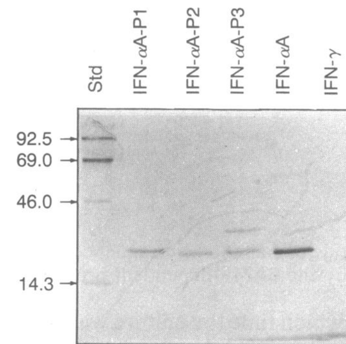

FIG. 3. Analysis of Hu-IFN-αA-P proteins by SDS/polyacrylamide gel electrophoresis. IFN-αA-P1, -P2, and -P3 ($\approx$0.2 μg) were treated with 2-mercaptoethanol and analyzed by electrophoresis in SDS/15% polyacrylamide gels and silver staining. Human IFN-αA and -γ were used as controls. Molecular weight standards (Std) were [^{14}C]methylated proteins from Amersham: from top to bottom, they are phosphorylase *b*, bovine serum albumin, ovalbumin, and lysozyme (M_r × 15^{-3} at left).

Binding of [^{32}P]IFN-αA-P to Cells. [^{32}P]IFN-αA-P1 and -P2 can bind to bovine MDBK and human Daudi cells (Fig. 5) with the specific binding approaching saturation at higher concentrations. Scatchard analysis of the data yielded the following estimates. The bovine MDBK cells have approximately 3800 and 9450 receptors per cell, calculated from the binding of [^{32}P]IFN-αA-P1 and -P2, respectively; Daudi cells have approximately 1650 and 4900 receptors per cell. Dissociation constants (K_d) were calculated to be 1.4 × 10^{-10} M for the binding of [^{32}P]IFN-αA-P1 to both the human and the bovine cells; and 3.5 × 10^{-10} M and 2.2 × 10^{-10} M for the binding of [^{32}P]IFN-αA-P2 to human and bovine cells, respectively. Similar results were obtained with IFN-αA-P3 (data not shown). All these phosphorylated IFN-αA-P derivatives bind to the human IFN-α/β receptor, since their binding to Daudi cells was competitively blocked by human IFN-αA and IFN-β, but not by human IFN-γ (data not shown).

Covalent Crosslinking of [^{32}P]IFN-αA-P to the Receptor. [^{32}P]IFN-αA-P1, -P2, and -P3 can be covalently crosslinked to the receptors after binding to cells (Fig. 6). When analyzed by SDS/polyacrylamide gel electrophoresis, the radioactive complexes from the Daudi cells migrate as several bands at M_r 100,000–200,000, and those from the MDBK cells migrate as a broad band at M_r 150,000. The crosslinked complexes of the receptors on the cells with [^{32}P]IFN-αA-P1, -P2, and -P3 appear to be the same by SDS/polyacrylamide gel electro-

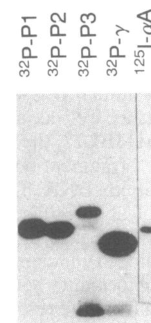

FIG. 4. SDS/polyacrylamide gel electrophoresis of phosphorylated proteins. The gel was dried after staining with Coomassie brilliant blue and autoradiographed for 1 hr with Kodak X-Omat film at room temperature. Molecular weight markers were not included, since the labeled human IFN-αA and IFN-γ served as internal controls (cf. Fig. 3).

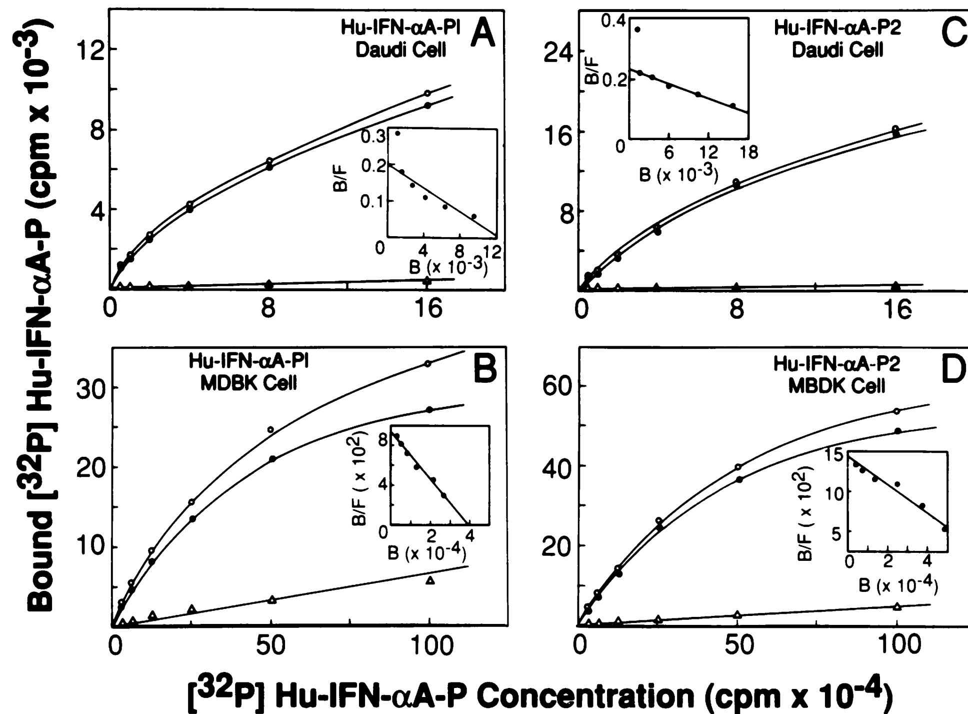

FIG. 5. Binding of ^{32}P-labeled human (Hu-) IFN-αA-P proteins to cells. Each [^{32}P]IFN-αA-P was added to cells at the indicated concentrations in the absence (total binding, ○) or presence (nonspecific binding, △) of excess nonradioactive human IFN-αA to measure the total and nonspecific binding at room temperature (24°C) for 1 hr. Specific binding (●) represents the difference between the total and nonspecific binding. (*Inset*) Scatchard analysis of the specific binding data. B, radioactivity of ligands bound to cells; F, radioactivity of free or unbound ligands. (*A* and *B*) Binding of [^{32}P]IFN-αA-P1 to Daudi and MDBK cells, respectively. (*C* and *D*) Binding of [^{32}P]IFN-αA-P2 to Daudi and MDBK cells, respectively.

phoresis but differ from the crosslinked complexes of [^{32}P]-IFN-γ formed with human Daudi cells. Neither the crosslinked complexes nor the free [^{32}P]IFN-αA-P1 and -P2 are seen if excess nonradioactive human IFN-αA is included during the binding reaction (Figs. 5 and 6).

DISCUSSION

To carry out biological functions, IFNs must first interact with cell surface receptors. Generally, IFNs labeled with ^{125}I have been used for binding and crosslinking studies (1, 26, 29–33). Several years ago, it was found that IFN-γ can be phosphorylated to very high specific radioactivity while retaining biological activity (3, 4). Accordingly, ^{32}P-labeled human and murine IFN-γ preparations were used to study the human and

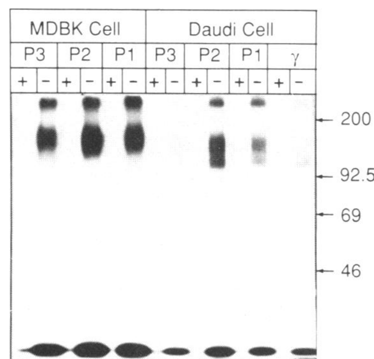

FIG. 6. Electrophoretic analysis of crosslinked [^{32}P]IFN-αA-P–receptor complexes. ^{32}P-labeled IFN-αA-P1, -P2, or -P3 or IFN-γ was added to cells in the absence (−) or presence (+) of excess nonradioactive human IFN-αA (for P1, P2, and P3) or IFN-γ (for γ) and crosslinking and analysis in 8% polyacrylamide gels was carried out as described in *Experimental Procedures*. Positions of standards ($M_r \times 10^{-3}$) are at right.

murine IFN-γ receptors (5–9). Because IFN-α and -β species, however, cannot be phosphorylated under these conditions (3), we designed modified human IFN-αA molecules with phosphorylation sites to provide convenient reagents for studying the IFNs and their receptors. IFN-αA was chosen as the molecule to modify because its binding to cells was well characterized (1, 2, 26, 29, 33–38). The phosphorylation of IFN-αA-P conveniently provides molecules with high specific radioactivity (2000–5000 Ci/mmol) with retention of biological activity. Thus, the phosphorylation site added to human IFN-αA at the carboxyl terminus does not affect the biological activity (antiviral activity) and can be efficiently recognized by the cAMP-dependent protein kinase. Although IFN-αA-P1 and -P2 are stable during purification and phosphorylation, larger insertions may affect the stability of the modified protein. One such protein, IFN-αA-P3, with an additional heptadecapeptide at the carboxyl terminus was degraded into at least two fragments. This result is reminiscent of the degradation and short half-life of a genetically engineered human IFN-αA with a modified carboxyl terminus (39).

From the binding of [^{32}P]IFN-αA-P1 and -P2 to cells, some parameters can be calculated. The dissociation constants (K_d) and receptor numbers determined were similar to those determined with ^{125}I-labeled human IFN-αA or -α2 (18, 33, 34–36). With both cell lines, the calculated values for the K_d and number of binding sites per cell are systematically higher by a factor of 1.6–3.0 for IFN-αA-P2 than for IFN-αA-P1. The constancy of this trend likely reflects errors in the computed specific radioactivity. Because the specific aim of this report was to demonstrate the usefulness of this new reagent, we did not focus on methods to obtain more accurate specific activities.

[^{32}P]IFN-αA-P1, -P2, and -P3 crosslinked to cells exhibit one complex of M_r ≈150 kDa with bovine MDBK cells and several complexes of apparent molecular mass 140–155 kDa (the main band), 115–125 kDa, and >200 kDa with human

Daudi cells. These size differences of the complexes with the two cell lines could primarily reflect differences in sizes of the receptor polypeptides or in the degree of posttranslational modification of the proteins. The pattern of crosslinking on Daudi cells is identical whether [^{32}P]IFN-αA-P or ^{125}I-labeled IFN-αA are used (data not shown). Joshi *et al.* (37) reported that crosslinking ^{125}I-labeled human IFN-α2 to four different cell lines including human Daudi cells yielded a similar complex of 150 kDa with all four cell lines; a "300-kDa" complex containing the 150-kDa complex was also reported (38) as observed here.

The introduction of phosphorylation sites into IFNs and other proteins provides a convenient and economical method to label the proteins to high specific radioactivities. The phosphorylation of antibodies targeted to tumor-specific antigens could provide a tool for the diagnosis and therapy of malignancies. The relatively low energy and high tissue absorption efficiency of the β particle compared to high-energy γ radiation may make introduced phosphorylation sites extremely useful. By introduction of multiple phosphorylation sites into proteins, even higher specific radioactivities can be achieved, particularly with large proteins. The method provides a relatively gentle way to radiolabel ligands compared to chemical methods and many iodination procedures that destroy biological activity to one extent or another. With the use of phosphate analogues, other isotopes such as ^{35}S can also be introduced into the proteins by the same enzymic procedure. The introduction of phosphorylation sites into proteins should prove to have a large variety of uses. Because of their high radiological and biological activities, ^{32}P-labeled IFN derivatives should be useful reagents for the characterization and assay of the IFN receptors.

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