A Long-Acting, Highly Potent Interferon α-2 Conjugate Created Using Site-Specific PEGylation

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Recombinant interferon α-2 (IFN-α2) is used clinically to treat a variety of viral diseases and cancers. IFN-α2 has a short circulating half-life, which necessitates frequent administration to patients. Previous studies showed that it is possible to extend the circulating half-life of IFN-α2 by modifying lysine residues of the protein with amine-reactive poly(ethylene glycol) (PEG) reagents. However, amine-PEGylated IFN-α2 comprises a heterogeneous product mixture with low specific activity due to the large number and critical locations of lysine residues in IFN-α2. In an effort to overcome these problems we determined the feasibility of creating site-specific, mono-PEGylated IFN-α2 analogues by introducing a free (unpaired) cysteine residue into the protein, followed by modification of the added cysteine residue with a maleimide-PEG reagent. IFN-α2 cysteine analogues were expressed in *Escherichia coli* and purified, and their in vitro bioactivities were measured in the human Daudi cell line growth inhibition assay. Several cysteine analogues were identified that do not significantly affect in vitro biological activity of IFN-α2. Certain of the cysteine analogues, but not wild-type IFN-α2, reacted with maleimide-PEG to produce mono-PEGylated proteins. The PEG−Q5C analogue retained high in vitro bioactivity (within 3- to 4-fold of wild-type IFN-α2) even when modified with 20- and 40-kDa PEGs. Pharmacokinetic experiments indicated that the 20-kDa PEG−Q5C and 40-kDa PEG−Q5C proteins have 20-fold and 40-fold longer half-lives, respectively, than IFN-α2 following subcutaneous administration to rats. These studies demonstrate the feasibility of using site-specific PEGylation technology to create a long-acting, mono-PEGylated IFN-α2 protein with high specific activity.

INTRODUCTION

α-Interferons (IFN-α) comprise a family of at least 25 nonallelic genes and pseudogenes that encode proteins with 70% or greater amino acid identity (Henco et al., 1985; Blatt et al., 1996). IFN-α2 is the best-studied member of this family. IFN-α2 is a 19 kDa protein that exhibits antiviral, antiproliferative, and immunomodulatory effects on many cell types (Pestka and Langer, 1987; Gutterman, 1994). Recombinant IFN-α2 has proven useful for the treatment of a variety of human viral diseases and cancers (Gutterman, 1994). Recombinant IFN-α2 has a short circulating half-life and generally is administered to patients by daily or thrice weekly injections. Previous studies showed that it is possible to increase the circulating half-life of IFN-α2 by covalent modification of the protein with amine-reactive poly(ethylene glycol) (PEG) reagents, which typically attach to lysine residues or the N-terminal amino acid (Monkarsh et al., 1997; Wang et al., 2000; Bailon et al., 2001). A limitation of the use of amine-reactive PEGs for modifying IFN-α2 is the fact that IFN-α2 contains 10 (or 11, depending upon the subtype) lysine residues in addition to the N-terminal amino acid. Monkarsh et al. (1997) found that all 11 lysine residues in IFN-α2a are modified to varying extents using amine-reactive PEG reagents. In addition to these 11 positional isomers, amine-PEGylated IFN-α comprises a mixture of mono-, di-, and multiply-PEGylated species. The various positional isomers and mono- and multiply-PEGylated IFN-α2 species have different specific activities (Monkarsh et al., 1997). Amine-PEGylated IFN-α2 also has significantly reduced in vitro bioactivity (4- to 14-fold) compared to IFN-α2 (Monkarsh et al., 1997; Wang et al., 2000; Bailon et al., 2001), which increases the amount of protein required to treat patients. Loss of bioactivity is greatest when IFN-α2 is modified with large PEGs (e.g., 20- and 40-kDa PEGs), which are the most useful PEGs for extending the half-life of the protein, and when the protein is modified with more than one PEG (Monkarsh et al., 1997; Bailon et al., 2001). IFN-α2 contains several lysine residues that are located in regions of the protein believed to be important for receptor binding (Radhakrishnan et al., 1996). It is likely that modification of one or more of the critical lysine residues contributes to the loss of activity upon PEGylation of IFN-α2 with amine-reactive PEG reagents.

In an effort to overcome the heterogeneity and loss of activity problems associated with amine-PEGylation of IFN-α2, we tested the feasibility of using site-specific PEGylation (Goodson and Katre, 1990) to modify IFN-α2. Site-specific PEGylation involves introducing a "free" cysteine residue, that is, a cysteine residue not involved in a disulfide bond, into a target protein using site-directed mutagenesis, followed by covalent modification of the added cysteine residue with a cysteine-specific PEG reagent. A key technical challenge with this technology is the identification of a site(s) in the target protein where

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a cysteine residue can be introduced and PEGylated without significantly affecting in vitro biological activity of the protein. In this report, we demonstrate the utility of this approach for creating a long-acting, highly potent PEGylated cysteine analogue of IFN-α2.

EXPERIMENTAL PROCEDURES

Cloning DNA Sequences Encoding IFN-α2 and IFN-α2 Cysteine Analogues. DNA encoding IFN-α2b (Henco et al., 1985) was amplified using the polymerase chain reaction (PCR) method from human genomic DNA (CLONTECH, Inc., Palo Alto, CA) using primers BB93 (5′-CGCGAATTCGGATATGTAAATAGATACACAGTG-3′) and BB94 (5′-CGCAAGCTTAAAAGATTTAAATCGT-GTCATGGT-3′). The PCR product was digested with *Eco*RI and *Hin*dIII and cloned into similarly digested plasmid pCDNA3.1(+) (Invitrogen Corporation, Carlsbad, CA), creating plasmid pBBT160. The IFN-α2 gene in pBBT160 was modified initially for cytoplasmic expression in *Escherichia coli* by using PCR mutagenesis to add a methionine codon immediately prior to the first residue (C1) of the mature IFN-α2 protein and to add a TAA stop codon following the carboxy-terminal residue, E165. In addition, *Xba*I and *Sal*I sites were added and a *Bgl*II site was eliminated to provide convenient restriction sites for subsequent mutagenesis. None of these alterations changed the amino acid sequence of IFN-α2. Primers used in this PCR reaction were BB99 (5′-CGCAAGCT-TCATATGTGTGATCTGCCTCAAACCCACAGCCTGG-GTTCTAGAAGGACCTTGATGCTC-3′) and BB100 (5′-CGCGAATTCTTATTCCTTACTTCTTAAACTTTCTTG-CAAGTTTGTCGACAAAGAAAAGGATCTCATGAT-3′). The PCR product was digested with *Hin*dIII and *Eco*RI and cloned into plasmid pCDNA3.1(+), creating pBBT164. The *Nde*I−*Eco*RI fragment of pBBT164 containing the met-IFN-α2 sequence was subcloned into pUC18 (Sigma-Aldrich, Inc., St. Louis, MO) to generate plasmid pBBT168.

A secreted form of IFN-α2 was created by fusing DNA encoding the *E. coli* heat-stable enterotoxin gene (STII) signal sequence (Picken et al., 1983) to the coding sequence of mature IFN-α2 via two sequential PCR reactions. The first reaction used forward primer BB101 (5′-GCATCTATGTTCGTTTTCTCTATCGCTACCAAC-GCTTACGCATGTGATCTGCCTCAAACCCACAGC-3′) and reverse primer BB100 with pBBT164 DNA. The PCR product was used as DNA template in a second PCR reaction, which used reverse primer BB100 and forward primer BB11 (5′-CCCCCTCTAGACATATGAAGAAGAA-CATCGCATTCCTGCTGGCATCTATGTTCGTTTTCTC-TATCG-3′). The PCR product was digested with *Nde*I and *Xba*I, and the ∼100 bp *Nde*I−*Xba*I fragment containing the STII leader sequence and amino-terminal 30 bp of IFN-α2 was purified and cloned into similarly digested pBBT168 DNA, creating pBBT177. The STII-IFN-α2 gene was subcloned as an *Nde*I−*Eco*RI fragment into expression vector pCYB1 (New England Biolabs, Beverly, MA), creating pBBT178.

Mutant IFN-α2 genes were constructed by site-directed PCR-based mutagenesis (Higuchi, 1990; Horton et al., 1993), using plasmid pBBT177 as the DNA template. PCR products were digested with appropriate restriction endonucleases and cloned into similarly digested pBBT177 DNA. Correct DNA sequences of all cysteine analogues were confirmed. The STII-IFN-α2 genes encoding the cysteine analogues were excised from the pUC18-based pBBT177 derivatives as *Nde*I−*Eco*RI fragments and subcloned into pCYB1 for expression in *E. coli*.

Expression and Purification of IFN-α2 and IFN-α2 Cysteine Analogues. pCYB1 plasmids encoding STII-IFN-α2 and STII-IFN-α2 cysteine analogues were transformed into *E. coli* strain W3110. Overnight cultures were diluted to an optical density at 600 nm of ∼0.02 in Luria Broth (LB) media, 100 mM MES (pH 5.5), containing 100 μg/mL ampicillin. Typically, cells were grown at 37 °C until the optical densities of the cultures reached 0.3−0.4. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM, and the induced cultures were shifted to 28 °C and incubated for about 16 h. Cultures were subjected to osmotic shock as described by Koshland and Botstein (1980). This procedure was modified for the IFN-α2 cysteine analogues to include 5 mM cystine in the osmotic shock buffers. The osmotic shock supernatant and pellet fractions were processed immediately or stored frozen at −80 °C.

IFN-α2 and certain IFN-α2 cysteine analogues were purified from the osmotic shock supernatants by S-Sepharose and Cu^{2+} immobilized metal affinity chromatography (IMAC) column chromatography (Voss et al., 1994; Khan and Rai, 1990). The osmotic shock supernatants were adjusted to pH 3 and centrifuged, and the supernatants were loaded onto a 5 mL HiTrap S-Sepharose column (Amersham Biosciences Corporation, Piscataway, NJ) equilibrated in 20 mM MES, pH 5.0. The bound proteins were eluted with a linear salt gradient from 0% to 100% 500 mM NaCl, 20 mM MES, 10% ethylene glycol, pH 5.0. Column fractions enriched for the IFN-α2 proteins were identified by nonreducing SDS−PAGE. These fractions were pooled and loaded onto a 1 mL Cu^{2+} IMAC HiTrap column (Amersham Biosciences Corporation) equilibrated in 40 mM sodium phosphate, pH 6.0, 1 M NaCl, 0.1% Tween 20. The IFN-α2 proteins were eluted with a reverse pH gradient from 5.5 to 4.1 in 40 mM sodium phosphate, 1 M NaCl, 0.1% Tween 20. Column fractions were analyzed by nonreducing SDS−PAGE, and fractions containing properly folded IFN-α2 protein and no visible contaminants were pooled and stored at −80 °C.

The osmotic shock pellets containing insoluble IFN-α2 cysteine analogues were treated with B-PER bacterial protein extraction reagent (Pierce Chemical Company, Rockford, IL) according to the manufacturer's directions. Insoluble material was recovered by centrifugation, washed with water, and solubilized in 5 mL of 6 M guanidine, 50 mM cysteine in 20 mM Tris base. The mixture was stirred for 30 min and then dialyzed overnight at 4 °C against 400 mL of 40 mM sodium phosphate, 150 mM NaCl, pH 8.0. The refold mixture was then adjusted to pH 3.0 and centrifuged, and refolded IFN-α2 proteins were purified from the supernatants by S-Sepharose and Cu^{2+} IMAC column chromatography as described above.

For larger scale production of the Q5C analogue, the osmotic shock step was omitted, and the protein was refolded from whole cells as follows. Frozen cells from 400 mL cultures were thawed and resuspended in 10 mL of 8M guanidine, 20 mM cysteine, 2% Tween 20, 20 mM MES, pH 3. After mixing for 3 h, the solution was diluted 20-fold, the guanidine concentration was adjusted to 0.6 M, urea and copper sulfate were added to final concentrations of 1 M and 40 μM, respectively, and the solution was adjusted to pH 6. The refold mixture was incubated for 1−2 days, adjusted to pH 3, and clarified by centrifugation. The refold mixture was diluted 4-fold with H_2O, and refolded Q5C protein was purified by S-Sepharose and Cu^{2+} IMAC column chromatography as described above.

PEGylation of IFN-α2 Cysteine Analogues. IFN-α2 proteins were incubated at room temperature with a 10- to 15-fold molar excess of Tris[2-carboxyethylphosphine] hydrochloride (TCEP, Pierce Chemical Company) and a 15- to 20-fold molar excess of 5 kDA maleimide PEG, 10 kDa maleimide PEG, 20 kDa maleimide PEG or 40 kDa branched maleimide PEGs (Nektar, Inc., Huntsville, AL) at pH 8.5 for 60 min. The PEGylation reactions were then diluted 10-fold with 20 mM MES, pH 5.0, adjusted to pH 3.0, and subjected to S-Sepharose column chromatography using the conditions described above.

In Vitro Bioactivity Measurements. The human Daudi B cell line (Horoszewicz et al., 1979; Evinger and Pestka, 1981) was obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (assay media). For bioassays, washed cells were resuspended at a concentration of 4×10^5 cells/mL in assay media, and 50 μL (2×10^4 cells) of the cell suspension was aliquotted per well of a flat bottom 96 well tissue culture plate. Serial 3-fold dilutions of the protein samples were prepared in assay media, and 50 μL of the diluted protein samples were added to the test wells. Protein samples were assayed in triplicate wells. Control wells contained media but no cells. A commercial wild-type IFN-α2 (Endogen, Inc.), and wild-type IFN-α2 prepared by us were analyzed in parallel to control for interday variability in the assay. Plates were incubated for 4 days at 37 °C in a humidified 5% CO_2 tissue culture incubator. Following the incubation period, 20 μL of CellTiter 96 AQueous One Solution Reagent (Promega Corporation, Madison, WI) was added to each well, and the plates were incubated at 37 °C in the tissue culture incubator for 1−4 h. Absorbance was read at 490 nm using a microplate reader.

Pharmacokinetic Experiments in Rats. Animal experiments were performed with the approval of BolderPATH's Institutional Animal Care and Use Committee. Groups of three male Sprague Dawley rats, weighing approximately 350 g each, received a single intravenous injection (lateral tail vein) of Roferon (recombinant *E. coli*-derived IFN-α2a, Roche), 10-kDa PEG−IFN-α2 Q5C, 20-kDa PEG−IFN-α2 Q5C, or 40-kDa PEG−IFN-α2 Q5C, each at a dose of 100 μg of protein/kg of body weight. At selected time points, blood samples (0.4 mL) were drawn from the rats into EDTA anticoagulant tubes. The blood samples were centrifuged, and the plasma samples were stored at −80 °C. A predose sample (0 h) was drawn 1 day prior to injection of the test compounds. Plasma levels of the test proteins were quantitated using human IFN-α2 ELISA kits (Pierce Chemical Company.). Serial dilutions of plasma samples from one animal in each test group were analyzed in the in vitro bioassay to identify dilutions that fell within the linear range of the ELISA (25.6−1000 pg/mL). Duplicate samples of appropriate dilutions of plasma samples from all rats were then analyzed in the ELISA. A subcutaneous pharmacokinetic experiment was performed in the same manner except that the protein samples were injected subcutaneously into the lateral sides of the rats. Pharmacokinetic parameters were analyzed using the WinNonlin software package (Pharsight, Inc., Mountain View, CA) using noncompartmental methods.

Biochemical Methods. Protein samples were analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) on precast Tris−glycine polyacrylamide gels (Invitrogen Corporation, Carlsbad, CA) and stained with Coomassie Blue. 2-Mercaptoethanol was added to a final concentration of 1−5% (v/v) to reduce the proteins. Protein concentrations were determined using a Bradford protein assay kit (Bio-Rad Laboratories, Richmond, CA), using bovine serum albumin as the protein standard.

RESULTS

***E. coli* Expression, Purification, and Bioactivity of IFN-α2 and IFN-α2 Cysteine Analogues.** Human IFN-α2 has a compact globular structure, comprising five α helices joined by loops (Radhakrishnan et al., 1996; Klaus et al., 1997). The five α helices are termed A−E beginning from the N-terminus of the protein. The loop regions are referred to by the helices they join, for example, the A−B loop joins helices A and B. We constructed seven cysteine analogues in various nonhelical regions of IFN-α2. We constructed one cysteine substitution analogue in the amino-terminal region preceding helix A (Q5C), three cysteine substitution analogues in the CD loop (Q101C, T106C, E107C), and one cysteine substitution analogue in the carboxy-terminal region following helix E (S163C). We also constructed one cysteine addition analogue, *166C, that adds a cysteine residue following the carboxy-terminal residue, E165. In addition, we constructed the C98S analogue, which eliminates the nonessential C1−C98 disulfide (Morehead et al., 1984) and creates an unpaired cysteine (C1) in the region preceding helix A.

Wild-type IFN-α2 and IFN-α2 cysteine analogues were expressed as periplasmic secreted proteins in *E. coli* strain W3110 using the *E. coli* STII leader sequence, as described in Experimental Procedures. Voss et al. (1994) reported that correct processing of the STII secretion signal from IFNα2 was pH-dependent, being enhanced when culture pH was maintained at or below 6.7. Similarly, we found that Luria Broth medium buffered with 100 mM MES to a pH of 5.5 gave optimum yields of correctly processed IFN-α2 and IFN-α2 cysteine analogues (data not shown), and it was used routinely for expression of the proteins. Induced cell cultures were subjected to osmotic shock (Koshland and Botstein, 1980) to release the soluble contents of the periplasm. Approximately 25−50% of the correctly processed wild-type IFN-α2 protein was recovered in the supernatant fraction following centrifugation of the osmotic shock-treated cells. Wild-type IFN-α2 was purified from the osmotic shock supernatant by S-Sepharose column chromatography followed by affinity chromatography using a Cu^{2+} IMAC column (Figure 1). Purified wild-type IFN-α2 had a similar mean IC_{50} to a commercial IFN-α2 standard in the human Daudi B cell line growth inhibition assay (Figure 2 and Table 1). Relative IC_{50}'s for these proteins were consistent between experiments, although their absolute IC_{50} values varied by up to 2- to 3-fold in assays performed on different days; therefore comparisons between proteins were made on samples analyzed on the same days.

In contrast to wild-type IFN-α2, most IFN-α2 cysteine analogues were present in the osmotic shock supernatants at significantly reduced levels relative to wild-type IFN-α2 (data not shown). The cysteine analogues partitioned primarily into the osmotic shock pellets. Only the Q5C and S163C proteins were present at appreciable levels in the osmotic shock supernatants. These analogues were purified from the osmotic shock supernatants by S-Sepharose and Cu^{2+} IMAC chromatography as described for wild-type IFN-α2, with the exception that 5 mM cystine was added to the osmotic shock buffers. In

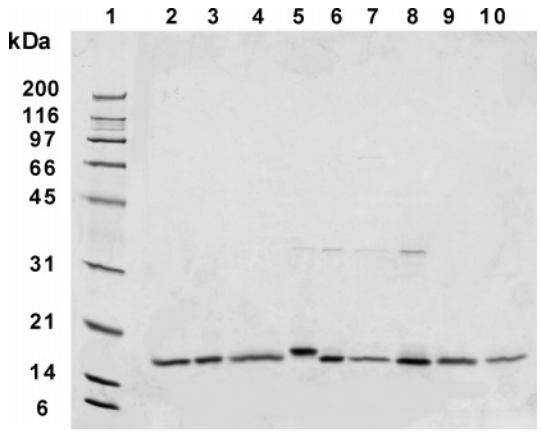

Figure 1. Nonreduced SDS−PAGE analysis of column pools of purified recombinant wild-type IFN-α2 and IFN-α2 cysteine analogues: lane 1, molecular weight markers; lane 2, wild-type IFN-α2 prepared by us; lane 3, Q5C soluble, periplasmically expressed; lane 4, Q5C, refolded; lane 5, C98S; lane 6, Q101C; lane 7, T106C; lane 8, E107C; lane 9, S163C; lane 10, *166C.

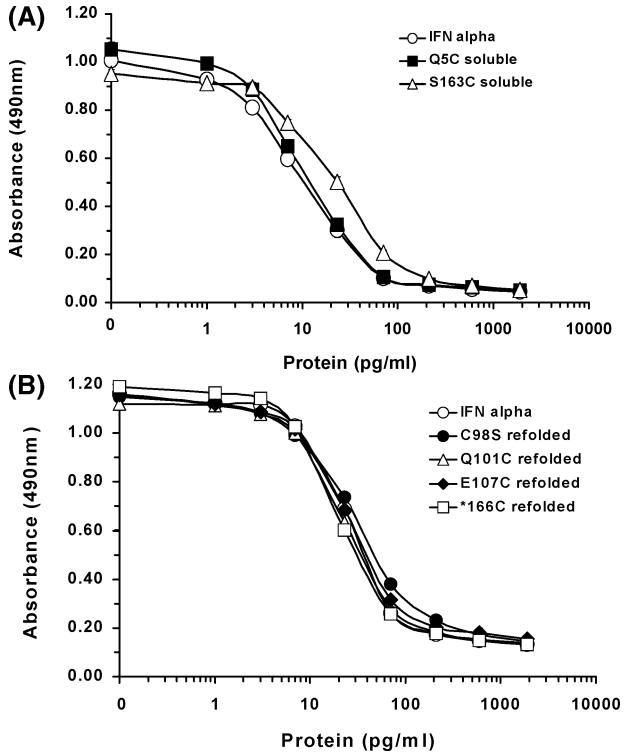

Figure 2. Dose response curves of IFN-α2 cysteine analogues and wild-type IFN-α2 for inhibiting proliferation of Daudi cells. Data are means of triplicate wells ± SD from representative experiments. SDs were less than 10% of the means. Proteins shown in the same panel were analyzed on the same days. Wild-type IFN-α2 used in the bioassay shown in panel A was prepared by us. Wild-type IFN-α2 used in the bioassay shown in panel B was purchased from Endogen, Inc. The Q5C and S163C proteins analyzed in panel A were purified from the soluble (supernatant) fraction of osmotic shock lysates; the C98S, Q101C, E107C, and *166C proteins analyzed in panel B were refolded from the insoluble (pellet) fraction of the osmotic shock lysates (see Experimental Procedures).

the absence of cystine, the cysteine analogues eluted in multiple fractions throughout the column gradients rather than as single peaks (data not shown).

SDS−PAGE analyses revealed that the C98S, Q101C, T106C, E107C, and *166C cysteine analogues expressed well but accumulated primarily in the osmotic shock pellets, suggesting that they were insoluble. When denatured and reduced these analogues comigrated with wild-type IFN-α2, indicating that the STII leader had been removed. Insoluble IFN-α2 cysteine analogues were refolded from the osmotic shock pellets and purified by S-Sepharose and Cu^{2+} IMAC column chromatography. The Q5C protein was recovered in both the pellet and the supernatant of the osmotic shock lysate and was purified from both sources. Nonreducing SDS−PAGE analysis of the purified Q5C, C98S, Q101C, T106C, E107C, S163C, and *166C proteins is shown in Figure 1. The cysteine analogues were recovered predominantly as monomers that comigrated with wild-type IFN-α2 under reducing and nonreducing SDS−PAGE conditions. The C98S analogue migrated with a slightly greater apparent molecular mass than the other INF-α2 analogues under nonreducing conditions, presumably due to the absence of the native C1−C98 disulfide bond (Morehead et al., 1984). Certain of the purified IFN-α2 analogues (C98S, Q101C, T106C, E107C) contained small amounts of disulfide-linked IFN-α2 dimers, which were apparent when the proteins were analyzed by nonreducing SDS−PAGE (Figure 1).

Table 1. In Vitro Bioactivities of IFN-α2 Cysteine Analogs

IFN-α2 protein	free cysteine location[a]	form assayed[b]	IC_{50} (pg/mL)[c]
IFN-α2[d]			13 ± 5
IFN-α2[e]		soluble	12 ± 4
Q5C	preceding helix A	soluble	13 ± 4
Q5C	preceding helix A	refolded	17 ± 3
C98S	preceding helix A[f]	refolded	28 ± 5
Q101C	C−D loop	refolded	18 ± 7
T106C	C−D loop	refolded	18 ± 0
E107C	C−D loop	refolded	18 ± 9
S163C	following helix E	soluble	27 ± 8
*166C	C-terminus	refolded	15 ± 6

[a] Based upon the IFN-α2 structure described by Radhakrishnan et al. (1996). [b] Soluble and refolded forms purified from the osmotic shock supernatant and pellet fractions, respectively. [c] Means ± SD for at least three assays for each protein except for the T106C analogue, which was assayed twice. [d] Wild-type IFN-α2 standard (Endogen, Inc.). [e] Wild-type IFN-α2 prepared by us. [f] Mutation creates a free cysteine (C1) in the region preceding helix A.

The Q5C, Q101C, T106C, E107C, and *166C proteins had in vitro bioactivities (IC_{50}'s) that were indistinguishable from that of wild-type IFN-α in the Daudi cell assay. In contrast, IC_{50}'s for the C98S and S163C proteins were consistently reduced about 2-fold relative to the IC_{50} of wild-type IFN-α2. Q5C recovered from the soluble periplasmic fraction and Q5C recovered from the insoluble fraction and refolded had IC_{50}'s equivalent to each other and to wild-type IFN-α2. These data are summarized in Table 1. Dose response curves for representative cysteine analogues are shown in Figure 2.

Preparation and in Vitro Bioactivity of PEG−Q5C. For PEGylation, the purified IFN-α2 cysteine analogues were reacted for 1 h at room temperature with TCEP and 5 kDa maleimide PEG at pH 8.5, as described in Experimental Procedures. Under these conditions, the Q5C analogue showed the greatest reactivity with the PEG reagent, yielding about 60% mono-PEGylated Q5C protein without detectable di- or tri-PEGylated protein (Figure 3). Control experiments (not shown) demonstrated that inclusion of a reducing agent such as TCEP in the PEGylation reaction was required for efficient PEGylation of Q5C and other IFN-α2 cysteine analogues. Wild-type IFN-α2 showed no detectable PEGylation under identical conditions, indicating that the native disulfide bonds of the protein are not disrupted by the

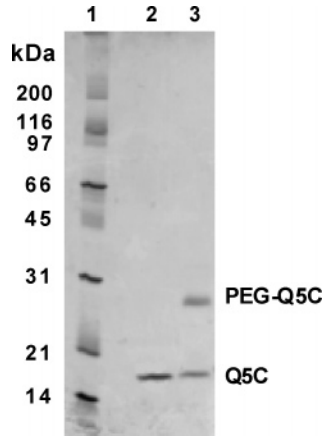

Figure 3. Nonreducing SDS−PAGE of Q5C and wild-type IFN-α2 PEGylation reaction products: lane 1, molecular weight markers; lane 2, wild-type IFN-α2 PEGylation reaction products; lane 3, Q5C PEGylation reaction products. Reaction conditions were as described in Experimental Procedures.

reaction conditions (Figure 3). The T106C, E107C, and S163C analogues also generated mono-PEGylated proteins using these PEGylation conditions, but the extent of PEGylation was less than that observed for the Q5C analogue (data not shown). The C98S, Q101C, and *166C proteins reacted only weakly with the PEG reagent under these conditions (data not shown).

The Q5C protein was modified at a larger scale with 5-, 10-, 20-, and 40-kDa maleimide-PEGs. PEG−Q5C was separated from unreacted Q5C protein by S-Sepharose column chromatography. A representative column elution profile for the 5-kDa PEG−Q5C PEGylation reaction mixture is shown in Figure 4A. The S−Sepharose column elution profile showed two major peaks. The early-eluting peak was determined to be mono-PEGylated Q5C by nonreducing SDS−PAGE (Figure 4B). The late-eluting peak was determined to be unreacted Q5C protein. Fractions containing PEG−Q5C and no visible unmodified protein were pooled and used for bioactivity measurements. Similar procedures were used to prepare Q5C modified with 10-, 20- and 40-kDa PEGs. Nonreducing SDS−PAGE of the purified PEG−Q5C proteins is shown in Figure 5.

When tested in the in vitro Daudi cell growth inhibition assay, bioactivity of the 5-kDa PEG−Q5C protein was reduced approximately 2-fold (IC_{50} of 22 ± 7 pg/mL; N = 3) compared to bioactivities of wild-type IFN-α2 (IC_{50} of 13 ± 6 pg/mL) and unmodified Q5C protein (IC_{50} of 13 ± 5 pg/mL) analyzed in the same assays (Figure 6A). Bioactitvities of the 10-, 20- and 40-kDa PEG−Q5C proteins were reduced approximately 3- to 4-fold relative to bioactivities of wild-type IFN-α and unmodified Q5C proteins analyzed in the same assays (Figure 6B and Table 2).

Pharmacokinetic Studies of PEG−Q5C Proteins. Circulating half-lives of the PEG−Q5C proteins were compared to that of wild-type IFN-α2 (Roferon, Roche) following intravenous and subcutaneous injection in rats. Circulating levels of the proteins were measured by ELISA. Following intravenous injection (Figure 7A), wild-type IFN-α2 cleared rapidly from the rats with a terminal half-life of 0.5 h, similar to what is reported in the literature (Bailon et al., 2001). In contrast, terminal half-lives for the 10-, 20-, and 40-kDa PEG−Q5C proteins were 22, 24, and 32 h, respectively. The initial distribution phase also appeared to be lengthened in the PEG−Q5C proteins relative to wild-type IFN-α2; however, not

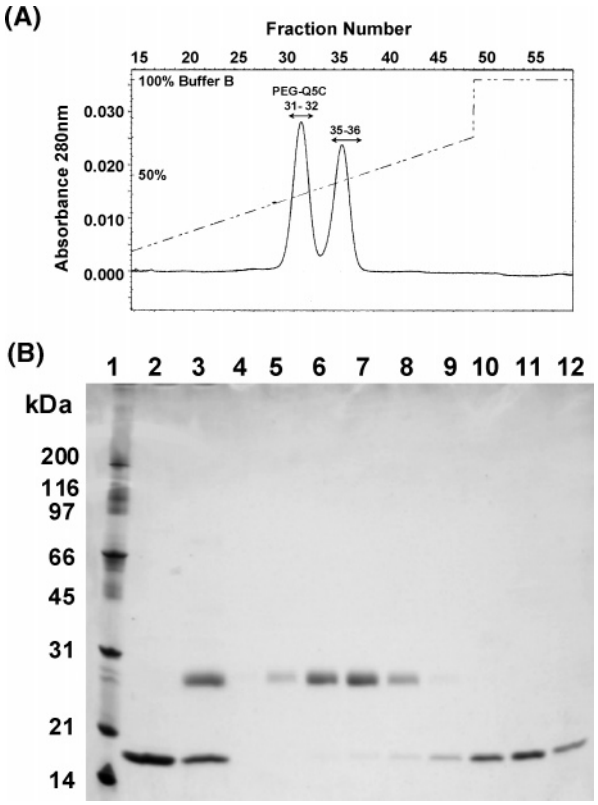

Figure 4. Purification of 5-kDa PEG−Q5C by S-Sepharose column chromatography: (A) the elution profile for the PEG reaction mixture applied to an S-Sepharose column; (B) nonreducing SDS−PAGE analysis of Q5C (lane 2), the Q5C PEGylation reaction products (lane 3), and fractions 29−37 recovered from the column (lanes 4−12). Fractions 31 and 32 (lanes 6−7) were pooled. Fractions 34−37 (lanes 9−12) contain predominantly unreacted Q5C. Molecular weight markers are shown in lane 1.

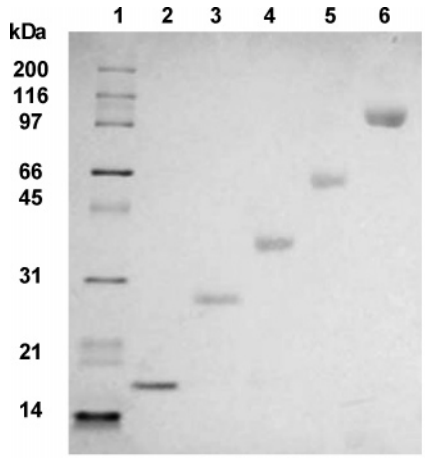

Figure 5. Nonreducing SDS−PAGE analysis of Q5C modified with different molecular weight PEGs: lane 1, molecular weight markers; lane 2, wild-type IFN-α2 prepared by us; lane 3, 5-kDa PEG−Q5C; lane 4, 10-kDa PEG−Q5C; lane 5, 20-kDa PEG−Q5C; lane 6, 40-kDa PEG−Q5C.

enough early time points were sampled to obtain accurate values for the distribution phase.

Following subcutaneous administration (Figure 7B), plasma concentrations of wild-type IFN-α2 peaked 1 h after administration (10.2 ng/mL) and decreased to undetectable levels by 24 h postinjection. The terminal half-life was 1.2 h. In contrast, maximum plasma levels of the PEG−Q5C proteins were not attained until 10 h

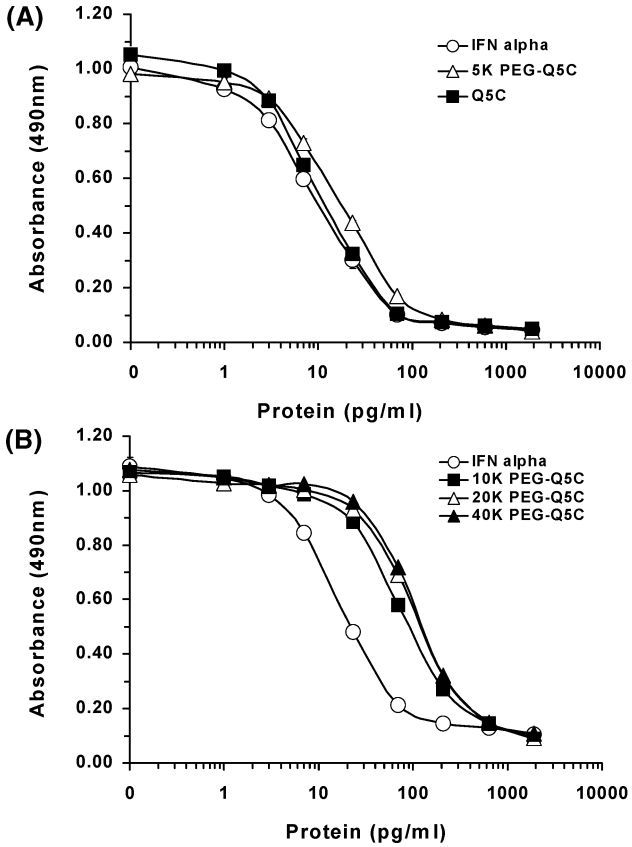

Figure 6. Dose response curves for PEG−Q5C proteins, unmodified Q5C, and wild-type IFN-α2 for inhibiting proliferation of Daudi cells: (A) comparison of the activities of commercial wild-type IFN-α2, Q5C, and 5-kDa PEG−Q5C; (B) comparison of the activities of commercial wild-type IFN-α2, 10-kDa PEG−Q5C, 20-kDa PEG−Q5C, and 40-kDa PEG−Q5C. Data are means of triplicate wells ± SD from representative experiments. SD were less than 10% of the means.

Table 2. In Vitro Bioactivities of PEG−Q5C Proteins

protein	IC_{50} (pg/mL)[a]
IFN-α2[b]	24 ± 5
IFN-α2[c]	29 ± 1
Q5C[d]	31 ± 2
10-kDa PEG-Q5C[d]	70 ± 0
20-kDa PEG-Q5C[d]	100 ± 7
40-kDa PEG-Q5C[d]	108 ± 11

[a] Means ± SD for at least three assays for each protein, except for the 10-kDa PEG−Q5C protein and our wild-type IFN-a2 protein, which were assayed twice. [b] Wild-type IFN-α2 standard (Endogen, Inc.). [c] Wild-type IFN-α2 prepared by us. [d] Q5C protein refolded from whole cells.

(10-kDa PEG−Q5C, 15.9 ng/mL), 24 h (20-kDa PEG−Q5C, 20.7 ng/mL), and 48 h (40-kDa PEG−Q5C, 33.3 ng/mL) postinjection (Figure 7B), and the PEG−Q5C proteins were cleared much more slowly than IFN-α. Terminal half-lives for the 10-, 20-, and 40-kDa PEG−Q5C proteins were 17, 27, and 48 h, respectively.

DISCUSSION

Our goal was to identify a site(s) in IFN-α2 where a cysteine residue can be introduced and PEGylated without significant loss of in vitro bioactivity. We focused mutagenesis efforts away from the presumptive receptor binding sites in IFN-α2, which have been localized to the N-terminal end of the A−B loop, helices C and D and the D−E loop (Uze et al., 1995; Rhadhakrishnan et al., 1996; Klaus et al., 1997; Roisman et al., 2001). The three

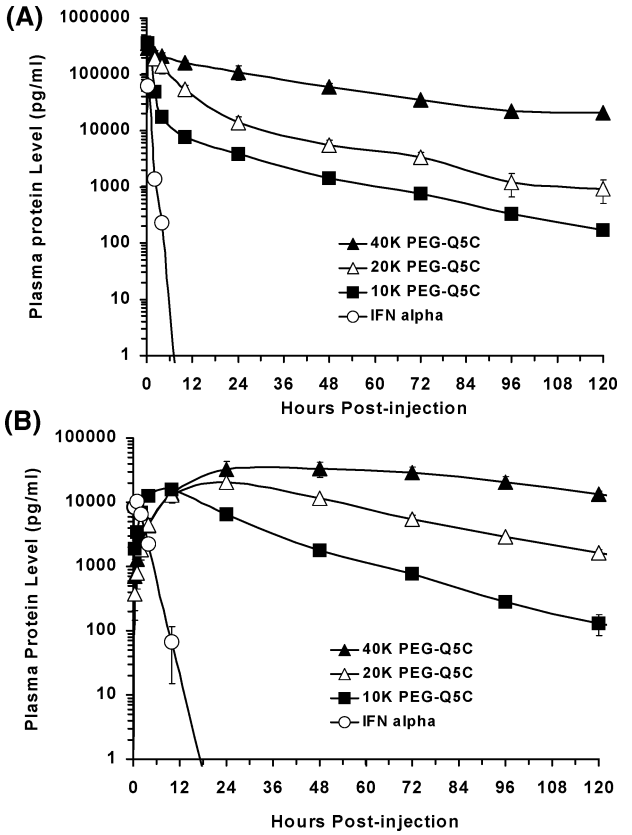

Figure 7. Changes in plasma protein levels following (A) intravenous and (B) subcutaneous administration of IFN-α2 (Roferon) and Q5C modified with 10-, 20-, and 40-kDa maleimide PEGs to rats. Protein levels were measured using human IFN-α2 ELISA kits. Data are means ± SD for three rats per group. SDs were generally less than 25% of the means.

regions chosen for mutational analysis, the region preceding helix A, the C−D loop, and the region following helix E, exhibit significant flexibility and high solvent exposure (Klaus et al., 1997). We found that the cysteine analogues analyzed in these regions retained complete (Q5C, Q101C, T106C, E107C, and *166C) or near complete (C98S, S163C) in vitro biological activity. Our data for the C98S analogue confirm previous studies indicating that the C1−C98 disulfide is not absolutely required for IFN-α2 bioactivity (Morehead et al., 1984; DeChiara et al., 1986). This is in contrast to the C29−C138 disulfide bond, which is required for IFN-α2 bioactivity (Morehead et al., 1984). We found that addition of cystine to the osmotic shock buffers and use of cysteine in the refold buffers was beneficial in improving recoveries of the IFN-α2 cysteine analogues from *E. coli*. We postulate that cystine/cysteine react with the free cysteine residues in the analogues to form a stable mixed disulfide that prevents or minimizes disulfide shuffling and aggregation of the proteins during subsequent column purification steps. This hypothesis is consistent with the finding that the purified cysteine analogues must be exposed to a reducing agent such as TCEP (presumably to reduce the mixed disulfide) for optimal reactivity with maleimide PEGs.

The Q5C protein was chosen for further study because it expressed well, displayed wild-type in vitro biological activity, and PEGylated efficiently. The mono-PEGylated Q5C protein could be purified readily from non-PEGylated protein by ion-exchange chromatography. The PEG−Q5C protein eluted as a single symmetrical peak, consistent with the PEG−protein having a homogeneous

structure. In contrast, amine-PEGylated IFN-α2 elutes as multiple peaks from ion-exchange columns due to heterogeneity resulting from the large number of positional isomers and multiply-PEGylated species (Monkarsh et al., 1997). In vitro bioactivity of the purified 5-kDa PEG−Q5C protein was within 2-fold of wild-type IFN-α2, whereas in vitro bioactivities of the Q5C protein modified with 10-, 20-, and 40-kDa PEGs were within 3- to 4-fold of wild-type IFN-α2. By contrast, in vitro bioactivity of IFN-α2 is reduced 4- to 5-fold when modified with 5-kDa amine-reactive PEGs and at least 14-fold when modified with 40-kDa amine-reactive PEGs (Monkarsh et al., 1997; Wang et al., 2000; Bailon et al., 2001). Thus, targeted attachment of PEG to IFN-α2 using site-specific PEGylation not only provides a structurally homogeneous product but also appears to yield a PEGylated IFN-α2 protein with higher specific activity than comparable amine-PEGylated IFN-α2 proteins. Pharmacokinetic studies showed that the circulating half-life of the Q5C protein varied depending upon the size of the attached PEG moiety, larger PEGs extending the half-life more than smaller PEGs. Attachment of a single 40-kDa PEG to the Q5C protein increased its half-life up to 40-fold relative to wild-type IFN-α2 following subcutaneous administration to rats. Modification of the Q5C protein with PEG also significantly slowed absorption of the protein from the injection site following subcutaneous administration, larger PEGs slowing absorption more than smaller PEGs. The half-life of the 40-kDa PEG−Q5C protein following subcutaneous administration to rats (48 h) is similar to that reported for IFN-α2a modified with a 40-kDa amine-reactive PEG following subcutaneous administration to rats (51 h; Bailon et al., 2001).

Two amine-PEGylated IFN-α2 products, PEG-Intron (Schering-Plough) and PEGASYS (Roche), are approved for use in humans. Both products have longer half-lives than IFN-α2, can be administered less frequently than IFN-α2 (once per week), and are more efficacious than IFN-α2 at treating hepatitis C (Craxi and Licata, 2003; Foster, 2003; Pedder, 2003). Clinical trials of these PEGylated IFN-α2 products in cancer patients are in progress (Choueiri et al., 2003). Although both products are prepared using amine-PEGylation technologies, the PEGylated proteins have quite different physical properties due to the PEGs used to prepare the proteins. PEG-Intron is modified with a 12 kDa linear PEG, whereas PEGASYS is modified with a 40 kDa branched PEG (Pedder, 2003). PEGASYS has lower activity than PEG-Intron in in vitro bioassays but a longer half-life than PEG-Intron in vivo (Gilbert and Park-Cho, 1999; Bailon et al., 2001; Pedder, 2003). The site-specific PEGylated Q5C protein described here is distinguished from PEG-Intron and PEGASYS by possessing both high in vitro bioactivity and a long half-life. Whether these unique features of PEG−Q5C will lead to a more potent or efficacious PEG-IFN-α2 product in vivo remains to be determined.

In conclusion, these studies demonstrate the feasibility of using site-specific PEGylation to create a long-acting IFN-α2 conjugate with high specific activity. Because of the shared structural features of members of the IFN gene family (IFN-α, IFN-β, IFN-γ, IL-10) information gained from these studies may prove useful for creating long-acting, site-specific PEGylated forms of other interferon species.

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