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Immunology

Antibodies that neutralize human β interferon biologic activity recognize a linear epitope: Analysis by synthetic peptide mapping

(multiple peptide synthesis/"pepscan"/monoclonal antibodies)

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ABSTRACT **The location of biologically relevant epitopes on recombinant human β interferon in which Ser-17 replaces Cys-17 (rh[Ser^{17}]IFN-β) was evaluated by testing the immunoreactivity of antibodies against 159 sequential, overlapping octamer peptides. Three monoclonal antibodies (mAbs) that neutralize rh[Ser^{17}]IFN-β biologic activity, designated A1, A5, and A7, bound to peptides spanning only residues 39–48, whereas nonneutralizing mAb bound less specifically at multiple sites near the amino terminus. The immunoreactivity of peptides spanning residues 40–47 that contained a series of single amino acid substitutions suggested that residues 41–43 (Pro-Glu-Glu) and 46 (Gln) are important for the binding of neutralizing mAbs. The reactivity of mAbs to larger synthetic peptides containing rh[Ser^{17}]IFN-β sequences from residue 32 through residue 56 was evaluated. All mAbs except A7 reacted with synthetic peptides representing rh[Ser^{17}]IFN-β residues 32–47, 40–56, and 32–56, but only mAbs A1 and A5 bound to the core peptide composed of residues 40–47. Peptide 32–56 effectively blocked the binding of mAbs A1 and A5 to rh[Ser^{17}]IFN-β and markedly inhibited their neutralizing activity. Biologic activity of the peptides was undetectable. Rabbit antisera raised against peptides 32–47 and 40–56 recognized rh[Ser^{17}]IFN-β but did not neutralize its antiviral activity. Thus, structure–function analysis by peptide mapping has permitted the identification of a linear epitope recognized by neutralizing antibody on a biologically active cytokine. We conclude that the region spanning residues 32–56 is of major importance in the expression of the biologic activity of human IFN-β.**

The interferons (IFNs) constitute a family of cytokines that possess multiple activities, including antiviral, antitumor, cell growth regulatory, and immunoregulatory properties (1). The mechanism of action of IFN that allows the expression of these diverse biologic activities is not well understood. One approach to study IFN action is to identify domains and structural properties that are associated with biologic activities. Previous structure–function studies have utilized genetically altered IFN gene products (2–8), peptide mapping of large fragments (9–14), and immunochemical mapping with monoclonal antibodies (mAbs) (15–18). Such analyses of human α and β interferon (hIFN-α and -β) molecules have yielded inconclusive results, implicating large regions near the amino terminus (2, 3, 7, 9, 15), carboxyl terminus (11, 16), or both (4–6, 8, 10), as domains responsible for biologic activity. The screening of short, sequential overlapping peptides for antibody reactivity as described by Geysen *et al.* (19, 20), also known as "pepscan" (21), permits the simultaneous scanning of entire molecules for linear immunoreactive epitopes. This approach has been effective in mapping B- and T-cell epitopes important in the biologic activity of viral and plasmodial proteins (22–26).

To map the location of epitopes identified by a panel of mAbs raised against recombinant Ser-17-substituted hIFN-β (rh[Ser^{17}]IFN-β) (17, 18), we determined their reactivity with sequential, overlapping octamer peptides spanning the entire length of the rh[Ser^{17}]IFN-β molecule. The panel consisted of neutralizing (A) and nonneutralizing (B) mAbs that identify three functionally and immunochemically distinct epitopes, designated sites I, II, and III. Spatially distinct epitopes I and II are recognized by mAbs A1 (or A5) and A7, respectively, whereas site III is recognized by the nonneutralizing B mAbs. Site I- and II-directed mAbs also neutralize the antiproliferative activity of rh[Ser^{17}]IFN-β and block binding to its cell receptors (17, 18). We report here that neutralizing mAbs recognized a unique, linear epitope in the amino-terminal region of hIFN-β spanning residues 39–48; synthetic peptides that include this region inhibited the binding and neutralizing activity of site I-directed mAbs.

MATERIALS AND METHODS

Reagents, IFN Bioassay, and Radioiodination. Murine mAbs to rh[Ser^{17}]IFN-β were produced, characterized, and purified as described (17, 18). Purified neutralizing [A1 (IgG1), A5 (IgA), and A7 (IgG2a)] and nonneutralizing [B2 (IgG2b) and B7 (IgG2a)] mAbs as well as neutralizing rabbit antisera (Lee Biomolecular Laboratories, San Diego) were used in the epitope analyses. Anti-peptide rabbit antisera were raised by injections of peptide-keyhole limpet hemocyanin conjugates in Freund's adjuvant and purified by ammonium sulfate precipitation. Bioassays for the measurements of antiviral, antiproliferative, and antibody-neutralizing activities have been detailed (17). Radioiodination of rh[Ser^{17}]IFN-β was achieved without loss in antiviral activity (17).

Peptide Synthesis. Duplicate sets of 159 octamer peptides representing the entire sequence of rh[Ser^{17}]IFN-β in a sequential and overlapping manner were synthesized on prederivatized polyethylene pins (Cambridge Research Biochemicals, Valley Stream, NY) arranged with a format and spacing that superimposes the chambers of a 96-well plate as reported (27). An amino acid replacement set analysis was performed on a selected octamer sequence by the synthesis on the pins of octapeptides in which each residue was substituted by the other 19 commonly occurring amino acids, while the remainder of the sequence was kept intact (20). Antibody reactivity toward the solid-phase octapeptides was

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Abbreviations: IFN, interferon; IFN-α and IFN-β, α and β interferons; hIFN-α and -β, human IFN-α and -β; rh[Ser^{17}]IFN-β, recombinant hIFN-β in which Ser-17 replaces Cys-17; mAb, monoclonal antibody.

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determined by an enzyme-linked immunosorbent assay (ELISA). Peptides representing selected sequences of hIFN-β were synthesized using a Biosearch 9500 peptide synthesizer and conventional Merrifield chemistry with *tert*-butyloxycarbonyl/benzyl protection strategy (28). Synthetic peptides were cleaved from the solid support with anhydrous HF and purified to homogeneity by reverse-phase HPLC; residue composition was confirmed by amino acid analysis.

ELISA. The peptide-pin-based ELISA was performed (27) by incubating the peptide pins in phosphate-buffered saline containing 1% bovine serum albumin and 0.05% Tween 20 for 1 hr, followed by incubation with antibody overnight at 4°C. Bound antibody was detected by incubation with an appropriate antibody-horseradish peroxidase conjugate for 1 hr followed by substrate (2,2′-azino-di-[3-ethylbenzthiazoline-sulfonic acid]). An unrelated mAb or enzyme conjugate alone served as controls. The reactivity of polyclonal antibody or protein-A-purified mAb to rh[Ser^{17}]IFN-β was measured in a plate-based ELISA as described (17). Reactivity of antibody to synthetic peptides was determined by ELISA with alkaline phosphatase conjugates (Sigma) specific for mouse IgG or IgA or for rabbit antibody, as required.

RESULTS

Screening of Antibody Reactivity to Octamer Peptides. Overlapping, sequential octamer peptides representing the entire rh[Ser^{17}]IFN-β amino acid sequence were treated with selected mAbs and neutralizing antisera to identify linear epitopes (Fig. 1). The antigenic profiles of the neutralizing mAbs (A1, A5, and A7) were nearly identical. One region composed of octapeptides with initial residues 39, 40, or 41 were all well recognized by these neutralizing mAbs with little or no binding elsewhere. In contrast, nonneutralizing mAbs B2 and B7 demonstrated reactivity throughout the amino terminus. Neutralizing rabbit antibody revealed two major areas of binding with a peak of reactivity near the amino terminus around octapeptides with initial residues 37 and 38.

To analyze neutralizing mAb binding at the single amino acid level, an amino acid replacement-set analysis was performed: peptides spanning residues 40–47 were synthesized to contain single amino acid substitutions and tested for immunoreactivity (Fig. 2). Amino acids substitutions for Pro-41, Glu-42, Glu-43, and Gln-46 led to a loss in reactivity by mAbs A1 and A7, whereas substitutions for the other four residues were generally well tolerated. In contrast, only Glu-43 and Gln-46 were found to be important for the specificity and binding of mAb A5. These data suggest that residues 41–43 and 46 are essential for the binding of neutralizing mAbs. In addition, the evaluation of the binding specificities at the single amino acid level for mAbs A1 and A5 has revealed differences not detected by previous conventional epitope analyses that used neutralization studies, competitive binding assays, and chemical modification of rh[Ser^{17}]IFN-β (17, 18).

Immunoreactivity and Biologic Activity of Synthetic Peptides. Since reactivity of short peptides may result from hydrophobic and/or charge interactions (29–31), peptides that contained residues beyond the core hIFN-β immunoreactive sequence (residues 40–47) were synthesized and analyzed for antibody reactivity and biologic activities. These peptides were composed of residues 40–47 (core peptide),

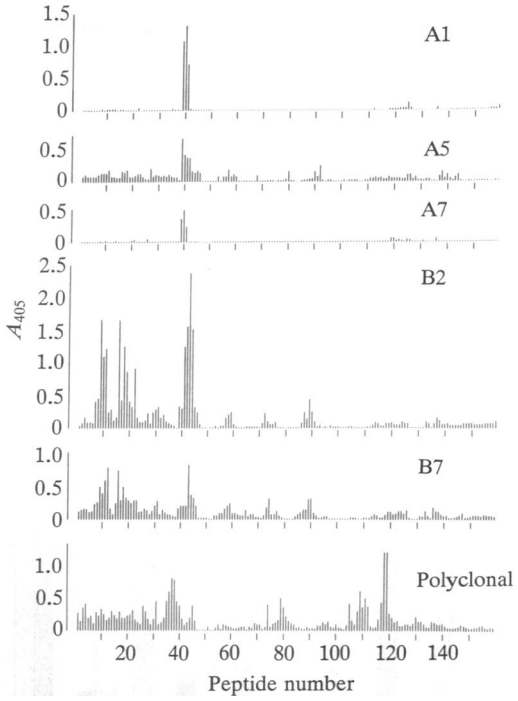

FIG. 1. Antibody reactivity to rh[Ser^{17}]IFN-β octamer peptides. The numerical assignment of the peptides is such that peptide number 1 represents residues 1–8, number 2 represents residues 2–9, etc., to the last peptide number 159 that represents residues 159–166. Binding to octapeptides of neutralizing (A1, A5, and A7) and nonneutralizing (B2 and B7) murine mAbs (0.4–0.5 μg/ml; HPLC-purified) and neutralizing rabbit polyclonal antibody (Lee Biomolecular Laboratories) (1:1000 dilution) was detected in an ELISA. The absorbance is represented as the height of the vertical line for each octapeptide whose amino-terminal residue is designated by the peptide number. Representative scans from duplicate plates are presented.

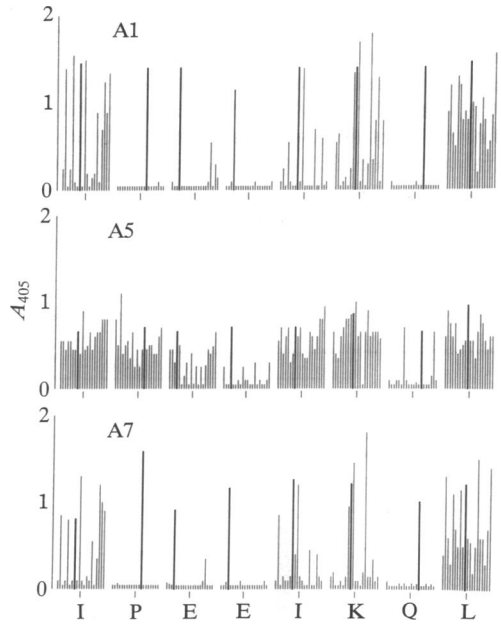

FIG. 2. Replacement set analysis of neutralizing mAb reactivity with the core octapeptide. Each residue of the amino acid sequence Ile-Pro-Glu-Glu-Ile-Lys-Gln-Leu (IPEEIKQL in single-letter code) representing positions 40–47 of hIFN-β (core peptide) was replaced one at a time by alternate amino acids. Each group of 20 lines corresponds to the complete replacement set for one of the eight amino acid positions. Within each group of 20 lines, the line farthest to the left corresponds to the substitution of the original residue by alanine, and successive lines by the remainder of the 19 commonly occurring amino acids in alphabetical order according to the single letter code for the amino acids. The thicker bars represent the replicates of the original sequence in hIFN-β. Results are shown as vertical lines representing the absorbance in the ELISA for each octapeptide. Representative scans from duplicate plates are presented.

32–47, 40–56, 32–56, and 118–126 (Fig. 3) and are referred to by the residues they comprise.

All mAbs, except A7, reacted in an ELISA to some degree with peptides 32–56, 32–47, and 40–56 (Fig. 3). Only mAbs A1 and A5 reacted with the core peptide 40–47. Of the nonneutralizing mAbs shown, B2 reacted equally well with peptides 32–56, 32–47, and 40–56, whereas B7 reacted preferentially with peptides 32–56 and 40–56, and minimally with peptide 32–47. No mAb reacted with peptide 118–126. Neutralizing rabbit anti-hIFN-β antisera recognized all five synthetic peptides in the ELISA (not shown).

At concentrations up to 100 μg/ml (33–105 μM), the peptides had no measurable antiviral or antiproliferative activity and had no effect on the antiviral activity of rh[Ser^{17}]IFN-β even when incubated with cells for 24 hr prior to adding IFN. None of these peptides inhibited cell-surface receptor binding of ^{125}I-labeled rh[Ser^{17}]IFN-β to Daudi cells, an IFN-sensitive, lymphoblastoid cell line with rh[Ser^{17}]IFN-β receptors (32).

Blocking of mAb Binding by Peptides. The ability of the peptides to block the binding of mAb to rh[Ser^{17}]IFN-β in an ELISA confirmed the high degree of antibody specificity. Peptide 32–56 inhibited the binding of mAbs A1, A5, and, to a lesser extent, B7 to rh[Ser^{17}]IFN-β (Fig. 4). Peptides 32–47 and 40–56 also inhibited the binding of mAbs A1 and A5 to rh[Ser^{17}]IFN-β but had minimal, if any, effect on the binding of mAb B7. Peptides 40–47 and 118–126 had no effect on antibody binding. The binding of mAb A7 was not inhibited by any of the peptides. The ability of peptides 32–47, 40–56, and 32–56 to block antibody binding was concentration-dependent (Fig. 4). Whereas all three peptides substantially inhibited the binding of mAb A1 to rh[Ser^{17}]IFN-β at a peptide concentration of 10 μg/ml, only the 32–56 peptide inhibited binding to a similar degree at 0.1 μg/ml. The three peptides demonstrated nearly identical dose-dependent inhibition of binding of mAb A5 (not shown). Inhibition of mAb B7 binding by peptide 32–56 was noted only at the highest concentration tested (10 μg/ml), where a 75% reduction in absorbance was observed.

Inhibition of mAb Neutralization Activity by Peptides. To quantitate the efficacy of competition by the peptides for binding to mAb, blocking of the mAb antiviral neutralizing activity was measured over a wide range of peptide concentrations. The inhibition of binding of mAbs A1, A5, and A7 to rh[Ser^{17}]IFN-β by the peptides was measured as a reduction in the mAb neutralization potency relative to control (no added peptide). Peptides 32–56, 32–47, and 40–56 inhibited the neutralization activity of mAb A1 in a dose-dependent fashion (Fig. 5); similar results were observed for mAb A5 (not shown). No effect of any of the peptides was observed on the neutralizing activity of mAb A7. Since the mAbs were raised against rh[Ser^{17}]IFN-β in its native conformation, a large excess of peptide is expected for maximal inhibition of

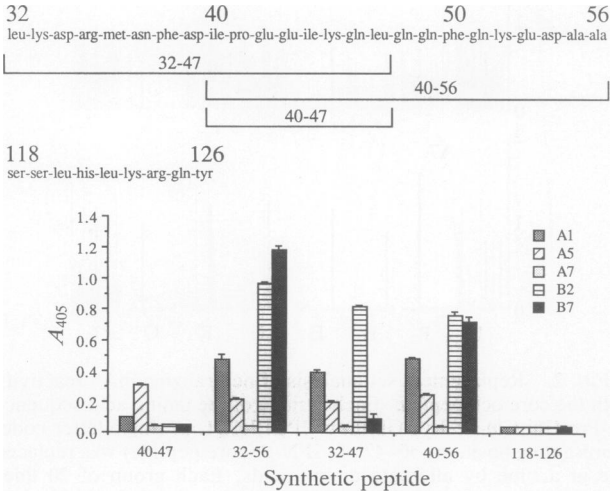

FIG. 3. Derivative hIFN-β synthetic peptides and their reactivity with mAb. (*Upper*) The amino acid sequences of the four synthetic peptides. (*Lower*) Reactivity of anti-rh[Ser^{17}]IFN-β mAbs with synthetic peptides. Selected mAbs (10 μg/ml) were incubated in Corning ELISA plates coated with peptides (500 ng per well) followed by an overnight incubation at 4°C with conjugate. Absorbance was measured after substrate addition and color development. Data are expressed as the mean absorbance ± SD of quadruplicate determinations.

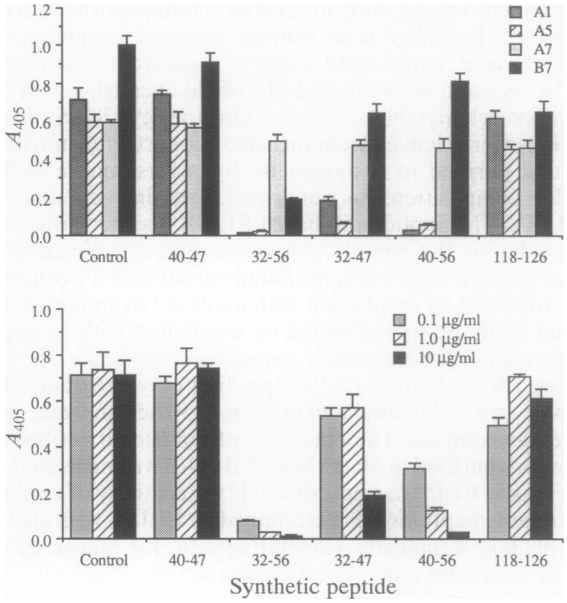

FIG. 4. Effect of synthetic peptides on the binding of mAbs to rh[Ser^{17}]IFN-β. (*Upper*) Peptide inhibition of mAb binding to IFN. Peptides (10 μg/ml; 3.3–10.5 μM) were incubated with selected mAbs (100 ng/ml; 0.6 nM for IgG and monomeric IgA mAbs) for 15 min at 20°C, and the mixtures were added to rh[Ser^{17}]IFN-β-coated ELISA plates. Bound mAb was detected after an overnight incubation of conjugate followed by substrate. (*Lower*) Dose-dependent peptide inhibition of mAb A1 binding. Different concentrations of peptides were incubated with mAb A1 (100 ng/ml; 0.6 nM) prior to the ELISA. At 10 μg/ml, the molar concentration of peptides ranged from 3.3 to 10.5 μM. Controls for both experiments did not contain peptide. Data are expressed as the mean absorbance ± SD of quadruplicate determinations.

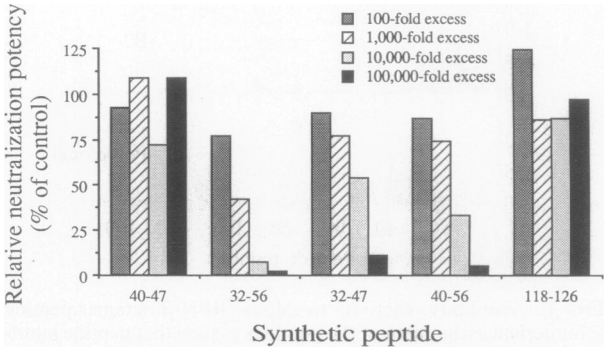

FIG. 5. Effect of synthetic peptides on the neutralizing activity of mAb A1. Individual peptides (100- to 100,000-fold molar excess over subsequently added IFN) were incubated with dilutions of mAb A1 for 1 hr at 37°C prior to the addition of 10 laboratory units of rh[Ser^{17}]IFN-β per ml. After further incubation for 1 hr at 37°C, residual mAb neutralizing activity was measured. Data from a representative experiment are expressed as the mAb neutralization potency relative to control without added peptide.

mAb A1 and A5 neutralizing activity. Similar findings were observed in competition studies of peptides of sperm whale myoglobin (33).

Anti-Peptide Antisera. Rabbit antiserum against peptide 32–47 or 40–56 recognized its antigen and rh[Ser^{17}]IFN-β in an ELISA but failed to recognize either the other peptide, peptide 40–47, or peptide 118–126 (not shown). Neither of the two anti-peptide antisera at a 1:10 dilution neutralized rh[Ser^{17}]IFN-β antiviral activity.

DISCUSSION

By scanning the entire sequence of rh[Ser^{17}]IFN-β for immunoreactivity of sequential, overlapping octapeptides, we have identified a short, immunodominant linear epitope spanning residues 39–48 that was recognized by different neutralizing mAbs (A1, A5, and A7). Furthermore, a replacement set analysis revealed that Pro-41, Glu-42, Glu-43, and Gln-46 were essential for the binding of these neutralizing mAbs; however, only Glu-43 and Gln-46 were equally important for all three mAbs. In addition, the immunoreactive profile of neutralizing polyclonal antisera demonstrated a peak of reactivity with octapeptides that contained these essential residues.

Our study supports the work of others emphasizing the importance of amino-terminal regions in the biologic activity of hIFN-β (7, 8). Creation of IFN-β variants, genetically engineered such that the amino-terminal sequences of hIFN-β (residues 1–81) were replaced with corresponding sequences from hIFN-α_1, resulted in significant effects on biologic activity in nearly all of the IFNs evaluated, while carboxyl-terminal replacements had no significant effects (7). Further, after chemical mutagenesis of the hIFN-β gene, amino acid residues 42 and 43, among others, were found to be critically important for the retention of biologic activity (8). The domain in the amino-terminal portion of hIFN-αJ1 spanning residues 10–44 was found to be essential for biologic activity, with residues 42–44 (Glu-Phe-Asp) contributing the most to activity (3). Additionally, the hIFN-β domain spanning residues 28–43 is highly conserved among mammalian IFN-α and -β proteins. Indeed, the glutamic acid residue at position 43 in IFN-β that is equivalent in terms of homology to position 41 in IFN-α is absolutely conserved in all IFN-α and -β species sequenced to date (8, 34–36). Despite the relative homology of hIFN-α and hIFN-β proteins, mAbs A1, A5, and A7 do not bind or neutralize hIFN-α preparations (17). The observation that single amino acid substitutions will abrogate binding by these mAbs, as shown in Fig. 2, illustrates the exquisite specificity of mAbs, which may explain their lack of reactivity with IFN-α.

The binding specificity of neutralizing mAbs A1 and A5 to an epitope contained, at least in part, within the domain spanning residues 32–56 was confirmed by (*i*) mAb reactivity to peptide fragments in an ELISA and (*ii*) inhibition of mAb binding and neutralizing activity by the peptides. Previous epitope analyses have suggested that mAbs A1 and A5 bind to or near the domain(s) responsible for biologic activity (18). In addition, these mAbs prevent binding of rh[Ser^{17}]IFN-β to its cell surface receptor (18). Thus, residues 32–56 may be part of, or are located near, the receptor-binding domain on hIFN-β. Alternatively, the mechanism of neutralization by these mAbs may depend on inhibition of receptor binding by steric hindrance (37) or conformational perturbations of the active site of the molecule induced by mAb binding (38, 39) rather than direct inhibition of receptor binding. The evaluation of Fab fragments may allow a more precise determination of the role of steric hindrance.

Antibody A7, which neutralizes recombinant hIFN-β (containing the native sequence) with an efficacy equal to that of mAb A1, is distinguished from mAbs A1 and A5 in that it recognizes a spatially distinct epitope on rh[Ser^{17}]IFN-β and does not bind or neutralize natural (glycosylated) hIFN-β (17, 18). A7 also failed to bind to the peptide fragments adsorbed onto plastic in an ELISA. Therefore, the reactivity of mAb A7 in the epitope analysis by peptide-pin ELISA must be interpreted with caution. Nonspecific hydrophobic and charge interactions have been reported for small peptide fragments, emphasizing the need to confirm the biologic specificity of antibody binding by analyses with larger peptides (29–31). On the other hand, the binding of mAb A7 may require certain structural features or orientations not present in the peptides either adsorbed to plastic or free in solution compared to the peptides covalently attached to a peptide-like spacer on polyethylene rods (20, 33). Indeed, others have suggested that reactivity of antibodies to peptides is more reliably detected by the peptide-pin approach than the conventional ELISA (20). Nonetheless, the lack of reactivity of mAb A7 with the peptides adsorbed onto plastic suggests that epitopes outside the 32–56 domain may also be associated with biologic activity. This possibility is further suggested by the observation that mutations of single amino acids in the carboxyl-terminal region of recombinant hIFN-β, specifically residues 141, 142, 149, and 152, alter biologic activity (8).

An additional perspective is provided by examination of a predicted tertiary structure of hIFN-β proposed by Carter *et al.* (40) (Fig. 6). In the model, the amino-terminal portion of the 32–56 domain is positioned in spatial proximity to residues 141–152. Alterations of this carboxyl-terminal region could interfere with the tertiary structure of the 32–56 region and thereby alter biologic activity.

Antisera raised against two of the synthetic peptides (32–47 and 40–56) demonstrated specific binding to its respective peptide and the intact rh[Ser^{17}]IFN-β molecule; however, these antisera lacked demonstrable neutralizing activity. Similarly, antibody raised against peptides 1–21 and 18–45 of hIFN-β was reported to lack neutralization activity (14). The development of antisera against the larger 32–56 peptide may

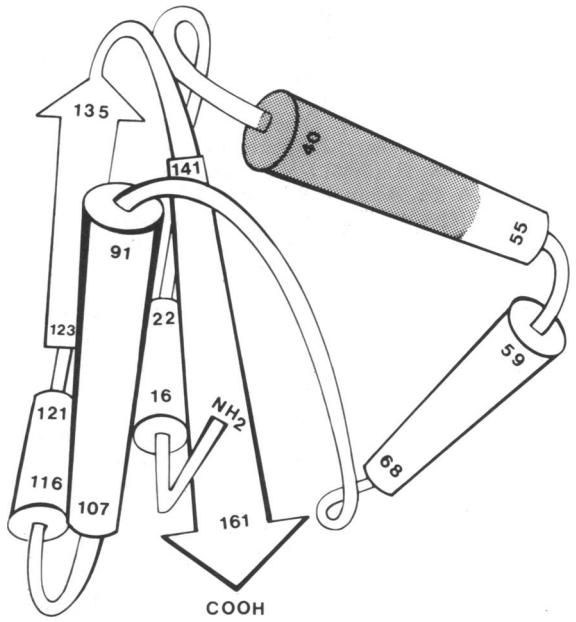

FIG. 6. Chou–Fasman-predicted tertiary structure of hIFN-β as proposed by Carter *et al.* (40). The structural conformation of hIFN-β is depicted with cylinders for α-helices and arrows for β-pleated sheets. The shaded area corresponds to the span of amino acids, residues 39–48, recognized by neutralizing mAbs in the peptide-pin epitope analysis. [Reproduced with permission from ref. 40 (copyright Raven Press, New York).]

help to determine the importance of this domain in hIFN-β activity.

The finding that a nonapeptide of human interleukin-1β is biologically active (41) suggests that short peptide fragments of other cytokines may be found that retain or are associated with biologic activity (42–45). However, the peptides in our study did not possess measurable biologic activity. hIFN-α peptides representing portions of amino- or carboxyl-terminal regions have also failed to exhibit biologic activity (12, 13, 46–48). A large proteolytic fragment (residues 1–110) of hIFN-α_2 has been reported to possess residual biologic activity (9), but others have raised the concern that intact IFN may have contaminated the fragment preparation (3).

Without x-ray crystallographic data, the precise role of the 32–56 domain in hIFN-β structure and function cannot be resolved with certainty. Nonetheless, our data along with studies of recombinant mutant hIFN-β molecules and analyses of highly conserved amino acid residues in hIFN proteins suggest that this domain has a crucial role in hIFN-β function.

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