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Localization of an antiviral site on the pregnancy recognition hormone, ovine trophoblast protein 1

(interferon α/synthetic peptides)

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ABSTRACT **Ovine trophoblast protein 1 (oTP-1) is the interferon α (IFN-α) variant with potent antiviral activity and low toxicity that is responsible for maternal recognition of pregnancy in sheep. To examine the structure/function basis for the potent antiviral activity of oTP-1, we have exploited the direct approach of synthetic peptide competition with oTP-1 for receptor, using N-terminal oTP-1-(1–37) and C-terminal oTP-1-(139–172) peptides. These peptides possess structures similar to those predicted for the intact molecule on the basis of circular dichroism. oTP-1-(1–37) at 1.5 mM specifically blocked oTP-1 antiviral activity without affecting the antiviral activity of natural ovine IFN-α, recombinant bovine IFN-α, and recombinant human IFN-α. At concentrations as low as 0.15 mM, oTP-1-(139–172) blocked the antiviral activity of oTP-1, as well as that of natural ovine IFN-α, recombinant bovine IFN-α, and recombinant human IFN-α, but not recombinant bovine interferon γ. Further, binding of radiolabeled oTP-1 to endometrial membrane preparations could be effectively inhibited by polyclonal anti-C-terminal and anti-N-terminal antisera, with the anti-C-terminal antiserum being the more effective inhibitor. Consistent with peptide and antiserum functional data, oTP-1 and recombinant bovine IFN-α are predicted to possess similar C-terminal structure but different N-terminal structure by composite surface profile predictions. The findings suggest that the C-terminal regions of IFN-αs bind to a common site on the IFN-α receptor while the N-terminal region binds to a site unique for the particular IFN-α.**

Ovine trophoblast protein 1 (oTP-1) is an antiluteolytic protein that plays an important role in maternal recognition of pregnancy in sheep. Between days 13 and 21 of pregnancy, oTP-1 is the major conceptus secretory product and is responsible for inhibition of pulsatile secretion of uterine prostaglandin $F_{2\alpha}$ (1). This oTP-1-induced inhibition allows for maintenance of the corpus luteum with continued secretion of progesterone. Bovine conceptuses have also been shown to produce antiluteolytic proteins similar to oTP-1, and a human equivalent has been postulated as well (2, 3). oTP-1 exhibits a 45–70% amino acid sequence identity with interferon α (IFN-α) from various species (4). We have previously shown that purified oTP-1 has high specific antiviral activity ($2–3 \times 10^8$ units/mg of protein) and is thus as potent as any known IFN (5). Human peripheral blood lymphocytes have been treated with oTP-1 at doses over 200,000 units/ml without evidence of toxicity (6). Further, oTP-1 is antigenically distinct but does show antigenic relation to both ovine and bovine IFN-α (5). In the present study we use the synthetic peptide approach to examine the structure/function basis for the potent antiviral activity of oTP-1. We have generated peptides corresponding to the N-terminal and C-terminal regions of the oTP-1 molecule as well as polyclonal antisera to these peptides. The ability of these peptides and their antisera to inhibit oTP-1 binding or function has shown that the C-terminal region of the molecule is involved in the antiviral activity of a broad range of IFN-αs, while the N-terminal region may be responsible for the unique properties of oTP-1.

MATERIALS AND METHODS

Reagents. Conceptuses were collected from day 16 (day 0 = first day of estrus) pregnant sheep and cultured *in vitro* in a modified minimal essential medium, and oTP-1 was purified from conceptus culture medium as described previously (7). oTP-1 was homogeneous as assessed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Protein determinations were performed using the bicinchoninic (BCA) assay (Pierce; ref. 8). Recombinant bovine IFN-α (rBoIFN-α) and recombinant bovine interferon γ (rBoIFN-γ) were kindly supplied by Genentech and CIBA–Geigy. The reference preparation of recombinant human IFN-α (rHuIFN-α) was supplied by the National Institutes of Health, and commercial rHuIFN-α was purchased from Lee Biomolecular Laboratories (San Diego). Production of ovine interferons was induced in ovine peripheral blood leukocytes by a 3-day incubation with staphylococcal enterotoxin A (for IFN-γ) (9), or a 24-hr infection with Newcastle disease virus (for IFN-α) (10). All tissue culture media, sera, and IFNs used in this study were negative for endotoxin, as determined by assay with *Limulus* amebocyte lysate (Associates of Cape Cod) at a sensitivity of 0.07 ng/ml.

Synthetic Peptides. Peptides corresponding to the N-terminal 37 amino acids, oTP-1-(1–37), and the C-terminal 34 amino acids, oTP-1-(139–172), of oTP-1 were synthesized on a Biosearch 9500AT automated peptide synthesizer using fluorenylmethyloxycarbonyl (Fmoc) chemistry (11). Peptides were cleaved from the resins by using trifluoroacetic acid/ethanedithiol/thioanisole/anisole at a volume ratio of 90:3:5:2. Cleaved peptides were then extracted in diethyl ether and ethyl acetate and subsequently dissolved in water and lyophilized. Reverse-phase HPLC analysis of crude peptides indicated one major peak in each profile. Hence, further purification was not warranted. Amino acid analysis of these peptides showed that the amino acid composition corresponded closely to theoretical. Polyclonal antisera to peptides were produced by hyperimmunization of rabbits. For immunization, peptides were conjugated to keyhole limpet hemocyanin by using glutaraldehyde as the coupling reagent (12). Antisera titers to oTP-1 or peptide were assessed by ELISA. The antigen, oTP-1, was adsorbed to the flat bottoms of plastic tissue culture wells overnight at 600 ng/well and subsequently evaporated to dryness. The plates

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Abbreviations: oTP-1, ovine trophoblast protein 1; IFN-α, interferon α; rBoIFN-α, recombinant bovine IFN-α; rBoIFN-γ, recombinant bovine interferon γ; rHuIFN-α, recombinant human IFN-α.

were blocked with 5% powdered skim milk (Carnation) in phosphate-buffered saline (PBS; Sigma) for 2 hr. They were then washed three times with PBS containing 0.05% Tween 20. Various dilutions of rabbit polyclonal antisera were added and incubated for 3 hr. Binding was detected using goat anti-rabbit IgG (whole molecule) to which horseradish peroxidase had been coupled (Organon Teknika–Cappel). Color development was monitored at 492 nm in an ELISA plate reader after *o*-phenylenediamine and H_2O_2 were added, and the reaction was terminated with 1 M H_2SO_4. The IgG fraction was obtained by affinity purification on a staphylococcal protein A-Sepharose column (12).

Circular Dichroism (CD). CD for selected peptides was determined at room temperature with a JASCO 500C spectropolarimeter. Scans were done with a 0.1-mm pathlength cell at a sensitivity of 1 and a time constant of 8 sec. The wavelength range measured was from 250 nm to 184 nm at a scan rate of 5 nm/min. Scans were carried out on peptides in 25% (vol/vol) trifluoroethanol in water at 0.5–1 mg/ml (13). The CD spectra were expressed in terms of ellipticity, θ, related to the mean residue molecular weight for each peptide. The following formula was used to calculate θ (14):

$$\text{Mean residue ellipticity } [\theta] = \frac{100\cdot[\theta]_{\text{observed}}}{c\cdot l},$$

where $[\theta]_{\text{observed}}$ is expressed in degrees, c equals the mean residue concentration in mol/liter, and l is the pathlength of the cell in cm. $\Delta\varepsilon = \theta/3298$.

Antiviral Assay. Antiviral activity was assessed by using a cytopathic effect assay (15). Briefly, dilutions of oTP-1 or the various IFNs were incubated with Madin–Darby bovine kidney (MDBK) cells for 16–18 hr at 37°C. After incubation, inhibition of viral replication was determined in a cytopathic effect assay using vesicular stomatitis virus as challenge. One antiviral unit caused a 50% reduction in destruction of the monolayer. Specific activities were further evaluated with normal ovine fibroblasts (Shnf), which were supplied by Janet Yamamoto (University of California, Davis), in a plaque inhibition assay (16).

Inhibition of oTP-1 antiviral activity by oTP-1 synthetic peptides was assessed by incubating cells with various concentrations of peptides at 37°C for 20 min prior to the addition of oTP-1. The peptides were examined for their ability to block the antiviral activity of oTP-1, natural ovine IFN-α, rBoIFN-α, rHuIFN-α, and rHuIFN-γ. Neutralization of oTP-1 antiviral activity by polyclonal antisera was determined by incubation of oTP-1 with antibodies for 20 min prior to addition to cells. Residual oTP-1 antiviral activity was determined as described above.

oTP-1 Binding. Five micrograms of oTP-1 was iodinated with 1 mCi of $Na^{125}I$ (Amersham; 1 Ci = 37 GBq) using Iodo-Beads (Pierce). Radioiodinated oTP-1 (^{125}I-oTP-1) was separated from free iodine on a 1 × 10 cm column of Sephadex G-10. Labeled oTP-1 had a specific activity of 42 μCi/μg. Membranes were prepared from uterine endometrium collected on day 12 of the estrous cycle as described (17). Binding of ^{125}I-oTP-1 was assessed by using our modification of an endometrial receptor binding assay (17); 1 nM ^{125}I-oTP-1 was incubated for 24 hr at room temperature with 50 μl of endometrial membranes at 1 g/ml in the presence of various concentrations of unlabeled oTP-1 or oTP-1 peptides. Inhibition of binding by anti-peptide antibodies was assessed by incubation of antibodies with ^{125}I-oTP-1 for 20 min prior to the addition of membranes.

RESULTS AND DISCUSSION

Those regions of oTP-1 expected to be antigenic or important in function are likely to be located on or exposed to the surface of the molecule. The amino acid sequence of one of the isoelectric variants of oTP-1 has been predicted from the cDNA (4). Using this sequence, we derived a surface profile for oTP-1 from a computer program that employs a composite of three parameters: (*i*) HPLC hydrophilicity, (*ii*) accessibility, and (*iii*) segmental mobility (*B* value) (18). Surface profiles generated by using this program of composite parameters show a high agreement with surface-accessible residues as determined by x-ray crystallography data for several proteins. The composite surface plot of the predicted surface profile for oTP-1 is presented in Fig. 1*A*. The higher the surface value the more likely the sequence or segment will be located on the surface of the molecule. Those regions that are predicted to be surface accessible are a long stretch of residues in the N terminus from approximately 18 to 53, as well as 68–76, 88–114, 130–138, and the C-terminal residues 159–172. It is of interest that C-terminal regions of oTP-1 and rBoIFN-α_{II} exhibit both significant sequence identity (54%) and similar composite surface profiles (compare Fig. 1 *A* and *B*).

As a correlate to surface profiles, secondary structure predictions based on the amino acid sequence can be made. Fig. 2 represents a secondary structure prediction for oTP-1 by the method of Chou and Fasman (20). This algorithm suggests a protein that is generally globular with protruding β-turns and possibly ω loops (21). The predicted loop/bend regions are of particular interest in that the sequence identity between human and murine IFN-αs and oTP-1 in these areas approaches 70%. Further, the loop/bend regions coincide with segments predicted to be on the surface of the molecule by the composite profile. Therefore, the β-turns and ω loops may play a role in the elicitation of various functions, while less conserved regions may serve to stabilize secondary structure.

On the basis of these predictive methods we have synthesized two peptides, corresponding to the N-terminal 37 amino acids, oTP-1-(1–37), and the highly hydrophilic C-terminal 34 amino acids, oTP-1-(139–172), the sequences of which are presented in Fig. 3. We examined the ability of our peptides

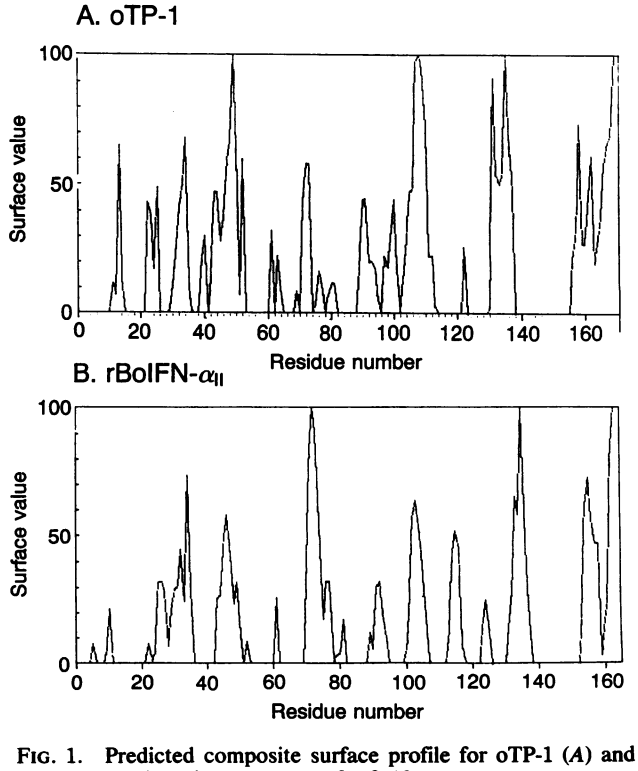

FIG. 1. Predicted composite surface profile for oTP-1 (*A*) and rBoIFN-α_{II} (*B*) based on program of ref. 18.

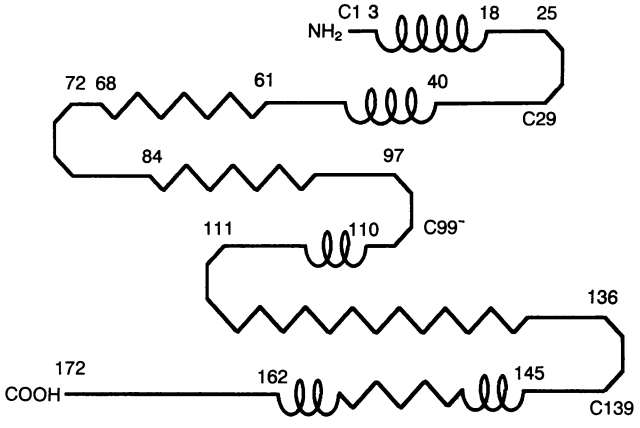

FIG. 2. Diagram of the secondary structure for oTP-1. As in IFN-α (19), disulfide bonds are formed between cysteines (C) at positions 1 and 99 and 29 and 139. Regions of oTP-1 predicted to attain α-helical secondary structure in the native conformation of the molecule are represented as coils. The number of turns in the coil is not intended to represent the actual topography of the helix as it exists in oTP-1. β-Sheet regions are represented by zigzags and β-turn regions are represented by half-trapezoid shapes. Regions for which no stable secondary structure is predicted are represented by straight lines. Lengths of the various segments are drawn approximately to scale; however, spatial relationships between various elements are arbitrary and are not intended to imply a predicted tertiary structure.

to compete with oTP-1 in a functional assay (Fig. 4). The N-terminal peptide at 1.5 mM blocked the antiviral activity of oTP-1 by 90% while having no effect on the antiviral activity of natural ovine IFN-α, rBoIFN-α, or rBoIFN-γ. In contrast, the C-terminal peptide at concentrations as low as 0.15 mM blocked the antiviral activity of oTP-1 and both IFN-αs, but it had no effect on IFN-γ. An irrelevant peptide of similar size (33-mer) corresponding to the N-terminal extracellular arm of the mouse β-adrenergic receptor did not diminish oTP-1 antiviral activity (data not shown). Thus, the inhibition of IFN function by the synthetic peptides appeared to be specific. The peptides displayed no evidence of toxicity when added to cells in the absence of virus. Similar results have been obtained with the ovine Shnf cell line (data not shown). Thus we have identified structural elements important for antiviral activity of oTP-1 and IFN-αs.

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oTP-1-(1-37)

CYS TYR LEU SER ARG LYS LEU MET LEU ASP
ALA ARG GLU ASN LEU LYS LEU LEU ASP ARG
MET ASN ARG LEU SER PRO HIS SER CYS LEU
GLN ASP ARG LYS ASP PHE GLY


oTP-1-(139-172)

CYS ALA TRP GLU ILE VAL ARG VAL GLU MET
MET ARG ALA LEU THR VAL SER THR THR LEU
GLN LYS ARG LEU THR LYS MET GLY GLY ASP
LEU ASN SER PRO
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FIG. 3. Primary amino acid sequence of oTP-1-(1–37) and oTP-1-(139–172).

oTP-1-(1–37) contains a large segment of the predicted first loop region as well as a β-turn. The degree to which these peptides inhibit any oTP-1 activity should be roughly proportional to the extent to which their conformation resembles that of the molecule itself. Therefore, we directly assessed the secondary structure of synthetic peptides of oTP-1 by using CD spectroscopy, which is a useful tool for the measurement of secondary structures of proteins and synthetic peptides (22). CD spectra of the N-terminal and C-terminal peptides of oTP-1 were generated into the vacuum UV range (Fig. 5). These peptides contain different secondary structures as exhibited by their different CD profiles. oTP-1-(1–37) showed more α-helical structure than oTP-1-(139–172), as seen by a depression in the curve at approximately 222 nm with a corresponding peak at around 190 nm. These results are consistent with the predicted secondary structure shown in Fig. 2. The fact that oTP-1 and rBoIFN-α_{II} display similar C-terminal composite surface profiles is consistent with the inhibition of antiviral activity of oTP-1 and the IFN-αs by the C-terminal peptide. The specific inhibition of oTP-1 function by the N-terminal peptide could indicate that this region of the molecule is responsible for its unique properties of pregnancy recognition and associated low toxicity for cells.

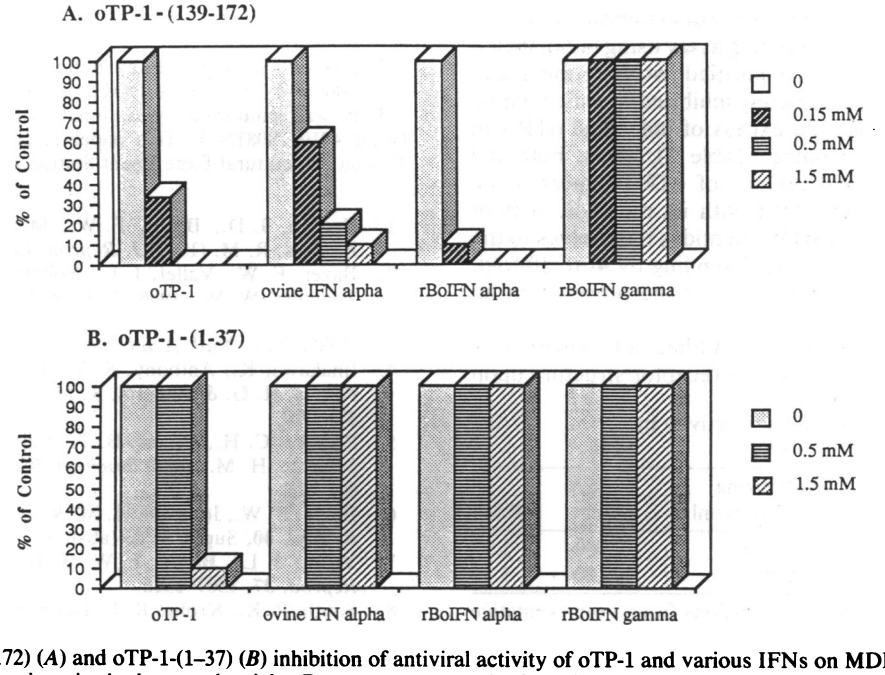

FIG. 4. oTP-1-(139–172) (*A*) and oTP-1-(1–37) (*B*) inhibition of antiviral activity of oTP-1 and various IFNs on MDBK cells. Peptides were present at concentrations given in the key on the right. Data represent results from four experiments.

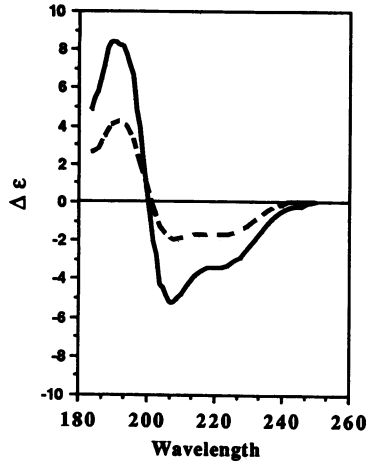

FIG. 5. CD spectra of oTP-1-(1–37) (—) and oTP-1-(139–172) (---). Scans were carried out on peptides in 25% trifluoroethanol in water at 0.622 mg/ml for oTP-1-(1–37) and 0.920 mg/ml for oTP-1-(139–172). The scan rate was 5 nm/min over a wavelength range from 250 to 184 nm. Scans were done with a 0.1-mm pathlength cell at a sensitivity of 1 and a time constant of 8 sec. Spectra are expressed as $\Delta\varepsilon$, which is equal to the ellipticity $[\theta]$ divided by 3298.

It would be of interest, however, to synthesize longer N-terminal peptides to determine whether they become more effective in blocking oTP-1 function, as well as the function of other IFN-αs.

Confirmation of the importance of the C-terminal region of oTP-1 in elicitation of antiviral activity was provided by antibody neutralization experiments. The ability of anti-N-terminal antiserum to neutralize oTP-1 was approximately 1/4th of that of anti-C-terminal antiserum when the data were normalized for antisera titer as assessed by ELISA (Table 1). In addition to neutralizing oTP-1, anti-C-terminal peptide antiserum also inhibited antiviral activity of rBoIFN-α and rHuIFN-α to the same extent as oTP-1, but not that of rBoIFN-γ (data not shown), which is further evidence of a common structure in the C-terminal regions of oTP-1 and the IFN-αs. Thus, immunochemical experiments corroborate involvement of both regions of oTP-1, but predominantly the C-terminus in antiviral activity, as suggested by peptide data. The mechanism of anti-peptide antiserum inhibition of oTP-1 activity was investigated in a binding assay using radiolabeled oTP-1. At 250 μg/ml, protein A-purified anti-N-terminal and anti-C-terminal antisera completely inhibited specific binding when compared to a 100-fold excess of unlabeled oTP-1 in blockage of ^{125}I-oTP-1 binding (Table 2). Thus, both the C-terminal and N-terminal regions of oTP-1 appear to be involved in interaction of oTP-1 with its receptor. It is of interest that addition of the oTP-1 peptides themselves to the binding assay enhanced ^{125}I-oTP-1 binding by 4- to 10-fold, and this enhancement did not occur in the presence of an irrelevant peptide corresponding to the N-terminal 27 amino acids of staphylococcal enterotoxin A (data not shown). This may be indicative of modification of receptor structure upon interaction with the peptide, such that oTP-1 binding is facilitated but function is inhibited.

Table 1. Inhibition of oTP-1 antiviral activity by anti-peptide antisera

Antiserum	Neutralizing activity, units/ml	ELISA titer	Ratio
Anti-oTP-1-(1–37)	1,200	1:1,000	1.2
Anti-oTP-1-(139–172)	50,000	1:10,000	5

Results are from duplicate determinations from a representative experiment that was performed three times. ELISA titers were determined with oTP-1 as the antigen. Ratio = neutralizing activity/ELISA titer.

Table 2. Inhibition of ^{125}I-oTP-1 binding to endometrial membrane preparation by anti-peptide antisera

Additions	Binding, cpm
1 nM ^{125}I-oTP-1 alone	18,727 ± 1,523
1 nM ^{125}I-oTP-1 + 100 nM oTP-1	10,237 ± 492
1 nM ^{125}I-oTP-1 + NRS at 250 μg/ml	13,342 ± 720
1 nM ^{125}I-oTP-1 + anti-N-terminal antiserum at 250 μg/ml	9,421 ± 1,128
1 nM ^{125}I-oTP-1 + anti-C-terminal antiserum at 250 μg/ml	9,191 ± 1,657

Unlabeled oTP-1, normal rabbit serum (NRS), or anti-peptide antisera were preincubated with ^{125}I-oTP-1 for 20 min. This mixture was incubated for 24 hr at room temperature with endometrial membranes at 1 g/ml. The results are presented as mean ± SD from a representative experiment that was performed three times.

It should be emphasized that oTP-1 is a variant of IFN-α (4, 5). In this regard, it has been shown that the 110-residue N-terminal fragment of rHuIFN-α_{II} that was generated by treatment of rHuIFN-α_{II} with the protease thermolysin retained antiviral activity (23). Further, site-directed mutagenesis within the coding region for the N-terminal 44 residues suggested that at least part of the 10–44 domain of IFN-α is involved in recognition of the receptor (19). The importance of the N-terminal region of oTP-1 in function is confirmed by our observation that the peptide oTP-1-(1–37) specifically blocked the antiviral activity of oTP-1. That the C-terminal region of IFN-αs is also involved in antiviral activity has been suggested by the recent observation that monoclonal antibodies directed against a synthetic peptide representing amino acids 133–147 of HuIFN-α neutralized the antiviral effect of human leukocyte IFN (24). In our study, the C-terminal oTP-1 peptide inhibited oTP-1, ovine IFN-α, rBoIFN-α_{II}, and rHuIFN-α antiviral activities to essentially the same extent in a dose–response study, but only oTP-1 function was inhibited by the N-terminal peptide. This suggests that the C-terminal regions of IFN-αs bind to a common site on the IFN-α receptor, while the N-terminal region binds to a site unique for the particular IFN-α. This would reconcile the common receptor for IFN-αs apparent in competition studies with the unique functions of the different subtypes of IFN-αs.

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