A hybrid protein between IFN-γ and IL-2

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The complementary DNAs encoding human interferon-γ (IFN-γ) and human interleukin-2 (IL-2), two different proteins involved in the same immune system, were fused to code a hybrid protein, which was expressed in *E. coli* to investigate the interactions of these two proteins at the molecular level. Through immunoprecipitation analysis, this protein was revealed to be of about 31 kDa, which was expected from nucleotide sequencing, and to have the antigenicities of both IFN-γ and IL-2. The extract from bacteria expressing this hybrid protein showed at least two biological activities: an antiviral activity derived from IFN-γ and the ability to support the growth of natural killer (NK) cells derived from IL-2. Comparing the enhancement of NK cell activity of this hybrid protein with IFN-γ and IL-2, this hybrid protein appears to conserve each activity almost completely without diminishing the other.

complementary DNA *Interferon-γ* *Interleukin-2* *Hybrid protein* *Antigenicity* *Integrated biological activity* *(Human)*

1. INTRODUCTION

Both human interferon-γ (IFN-γ) and human interleukin-2 (IL-2), glycoproteins of approx. 17 and 15 kDa, respectively, are produced by T cells stimulated with mitogens or antigens.

IFN-γ has anti-viral and anti-proliferative activity, enhances the activity of natural killer (NK) cells and modulates the various functions of macrophages [1]. IL-2 has the ability to support the long-term growth of T and NK cells in vitro, and augments the activity of killer cells including cytotoxic T cells, NK cells and lymphokine-activated killer cells [2,3].

Thus, while the various activities of these two glycoproteins seem to collaborate with or antagonize each other, they appear dependently involved in the immune system. IL-2 was reported to regulate the synthesis of IFN-γ by T cells [4], and both IFN-γ and IL-2 are required to induce cytotoxic T cells [3,5]. Furthermore, IL-2-initiated events, such as boosting NK activity, are IFN-γ-dependent [3,5]. Recently, the effects of the two proteins on B cells as well as on T cells were demonstrated independently: IFN-γ directly differentiates resting B cells to immunoglobulin-secreting cells [6] and IL-2 induces the proliferation of *Staphylococcus aureus* Cowan I activated B cells [7].

2. MATERIALS AND METHODS

2.1. *Construction of plasmids*

Complementary DNAs (cDNAs) for both IFN-γ and IL-2 were obtained by cDNA cloning [1,2]. IFN-γ cDNA (1 kbp) cut out from clone pHIT3709 was inserted into the *Cla*I-*Pst*I portion of ptrp771 using *Cla*I adapter. The resulting pHITtrp1101 codes for an IFN-γ with Met-Cys-Tyr at its NH_2-terminus. The 477-bp fragment of IL-2 cDNA prepared from pILOT135-8 was cloned once into ptrp781 using *Eco*RI adapter. Then the 442-bp fragment coding IFN-γ lacking 11 amino acid residues at its COOH-terminus was purified and ligated to the 589-bp fragment prepared from pTF1 using *Eco*RI linker. This ligated DNA

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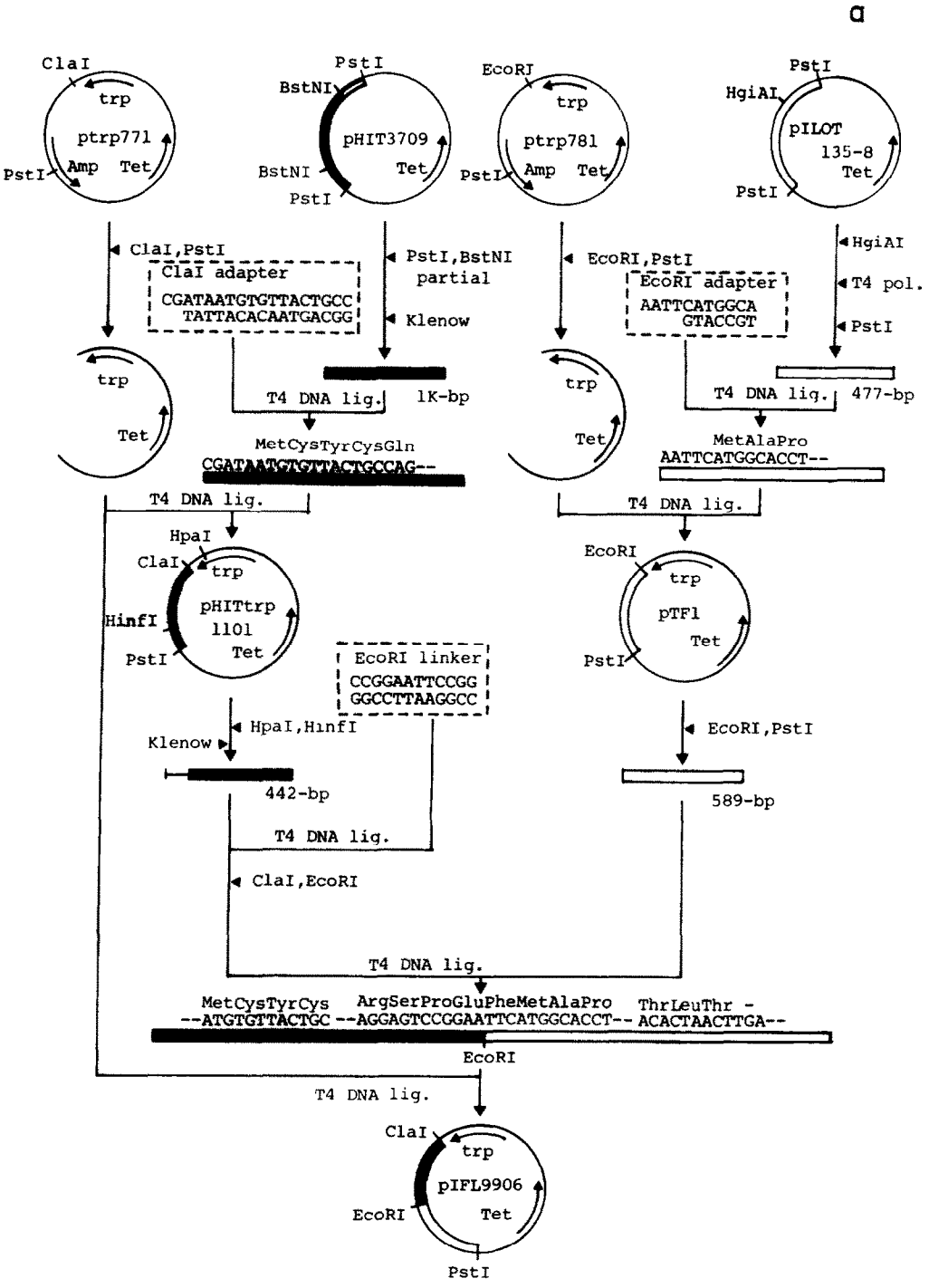
coding for a hybrid protein was inserted into the *ClaI-PstI* portion, downstream of the *trp* promoter, of ptrp771 to construct pIFL9906 (fig.1a).

2.2. *Preparation of cell extracts and assays for biological activities*

Bacterial cultures of DH1 cells transformed by



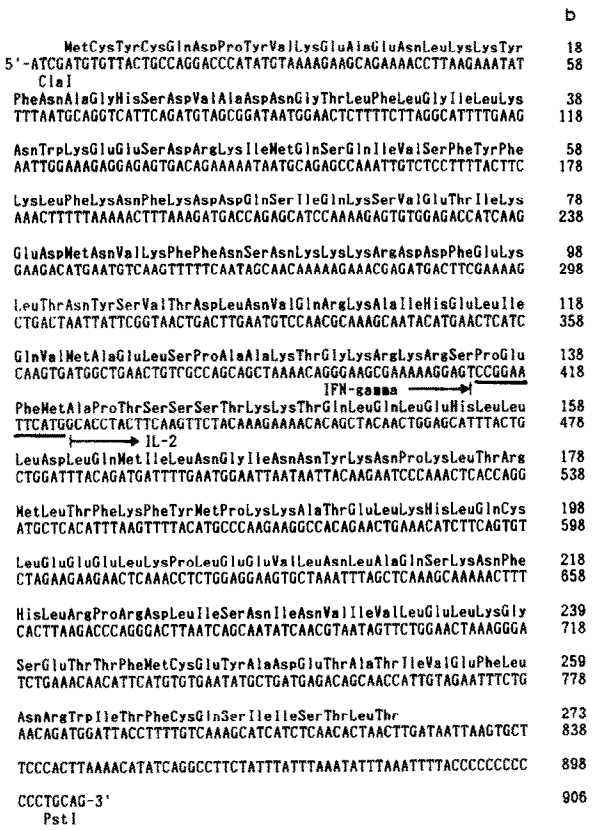


Fig.1.(a) Construction of plasmids that express the hybrid protein. See section 2.1 for details. (b). Nucleotide sequence of fused cDNAs of IFN-γ and IL-2 and predicted amino acid sequence of the hybrid protein. The hybrid protein consists of IFN-γ (lacking 11 amino acid residues from its COOH-terminus) and IL-2 with an intervening sequence of Pro-Glu-Phe-Met (underlined).

different plasmids were grown as described [8]. The cells were harvested, suspended in 1/10 vol. buffer (100 mM Tris-Cl, pH 7.0; 7 M guanidine HCl, 2 mM phenylmethylsulfonyl fluoride), and incubated for 1.5 h at 0°C. After dialysis vs buffer (10 mM Tris-Cl, pH 8.5; 200 mM NaCl, 1 mM EDTA), the cell-free extracts were assayed in duplicate on human FL amnion cells and Sindbis virus system for IFN-γ activity and on an IL-2-dependent mouse cell line, NKC-3, using a [^{3}H]-thymidine incorporation assay [2] for IL-2 activity.

2.3. *Immunoprecipitation analysis*

E. coli containing each plasmid was grown and the cells labelled with [^{35}S]Met [8]. Cell extracts were immunoprecipitated with rabbit antibody, absorbed with staphylococcal absorbent and analyzed using SDS-polyacrylamide gel electrophoresis [9].

2.4. *Cytotoxicity assay*

A natural cell-mediated cytotoxicity assay was performed as in [10]. Human K562 cells in complete minimal essential medium (CMEM) were labelled with $Na_2{}^{51}CrO_4$ and human peripheral blood mononuclear cells (PBMC) used as effector cells. PBMC were cultured in CMEM with each agent. Samples of ^{51}Cr-labelled tumor cells were added to triplicate samples of effector cells (effector to target ratios varied from 100:1 to 12.5:1) in round-bottomed microtiter plates. After appropriate incubation, the supernatants were harvested and counted in a gamma counter.

3. RESULTS AND DISCUSSION

Using cDNA clones, we constructed the plasmid pHITtrp1101 for expressing IFN-γ and pTF1 for IL-2 under the control of the *E. coli trp* promoter (fig.1a). These plasmids encode the entire IFN-γ and IL-2 proteins, both of which have methionines replacing the signal peptides. With these two plasmids we constructed plasmid pIFL9906, which expresses the hybrid protein (fig.1a). The hybrid protein encoded by this plasmid consists of 137 amino acid residues derived from the IFN-γ in its NH_2-terminal side and 134 amino acid residues derived from IL-2 in its COOH-terminal side across 3 amino acid residues derived from a 12-mer *Eco*RI linker (fig.1).

Analysis of [^{35}S]Met-labelled total cell extracts from the *E. coli* cells harbouring pHITtrp1101, pTF1 and pIFL9906 on SDS-polyacrylamide gels showed an induced recombinant IL-2 (rIL-2) of 15 kDa, a recombinant IFN-γ (rIFN-γ) of 17 kDa and the hybrid protein of 31 kDa (fig.2, lanes 4,7,5). Through immunoprecipitation analysis with anti-rIFN-γ antibody and anti-rIL-2 antibody, the hybrid protein was immunoprecipitated with both antibodies while rIFN-γ expressed by pHITtrp1101 and rIL-2 expressed by pTF1 were each immunoprecipitated with only one antibody. Each immunoprecipitated protein is shown as a single band analyzed on the SDS-polyacrylamide gel (fig.2, lanes 1,2,8,10).

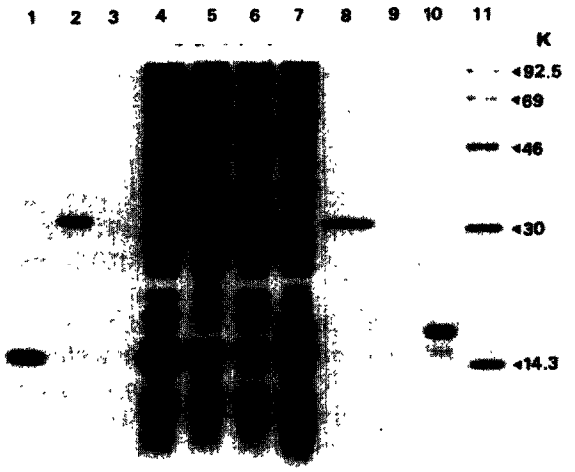


Fig.2. Autoradiogram of SDS-polyacrylamide gel. Cell extracts, immunoprecipitated with rabbit antibody and absorbed with staphylococcal absorbent, labelled with [^{35}S]Met were analyzed on 17.25% SDS-polyacrylamide gel electrophoresis. Lanes: 1–3, protein immunoprecipitated with anti-rIL-2 antibody; 1, pTF1; 2, pIFL9906; 3, ptrp771; 4–7, total cell extracts; 4, pTF1; 5, pIFL9906; 6, ptrp771; 7, pHITtrp1101; 8–10, proteins immunoprecipitated with anti-rIFN-γ antibody: 8, pIFL9906; 9, ptrp771; 10, pHITtrp1101; 11, molecular mass markers (lysozyme, 14.3 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 46 kDa; bovine serum albumin 69 kDa; phosphorylase *b*, 92.5 kDa).

These extracts from *E. coli* were assayed for IL-2 and IFN-γ activity. The extract from pIFL9906 contained about 10^6 IU IFN-γ activity/l bacterial culture and 10^4 U IL-2 activity in equivalent culture, while that from pHITtrp1101 contained 3×10^8 IU and that from pTF1 10^5 U/l bacterial culture (table 1).

The hybrid protein expressed by pIFL9906 has not only the antigenicities of IFN-γ and IL-2 but also the activities of both.

We have purified rIFN-γ and the rIL-2 using pHITtrp1101 and pTF1 or some other derivatives, and concluded that 1 mg IFN-γ has about 4×10^6 IU and 1 mg IL-2 about 4×10^4 U. From these values the ratio of the activities between equimolar IFN-γ and IL-2 is calculated as

(IFN-γ activity/IL-2 activity) =
$6.8\times10^{13}/6\times10^{11}$ = 113

Table 1

Biological activities of the hybrid protein produced in *E. coli*

Clone	IFN-γ activity (IU/l broth)	IL-2 activity (U/l broth)
pIFL9906	1.2×10^6	1.1×10^4
pHITtrp1101	300.0×10^6	0
pTF1	0	12.0×10^4

Cell-free extracts were assayed in duplicate on human FL amnion cells and Sindbis virus system for IFN-γ activity and on an IL-2-dependent mouse cell line, NKC-3, using a [^{3}H]thymidine incorporation assay [2] for IL-2 activity

the molecular mass of IFN-γ and IL-2 being 17 and 15 kDa, respectively. This facilitates estimation of the ratio of activities in a molecule of the hybrid protein. From table 1 the ratio is calculated as

(IFN-γ activity/IL-2 activity)$_{hybrid}$ =
$1.2\times10^6/1.1\times10^4$ = 109

This value is almost equal to that calculated above, which probably means that this hybrid protein totally conserves the activities of both IFN-γ and IL-2.

This estimation was also supported by the enhancement of NK cell activity (fig.3). The enhancement was almost equal to that of rIFN-γ and rIL-2 together; this reflects the derived values, 113 and 109.

We made the 11 deleted amino acid residues from the COOH-terminus of IFN-γ to design this hybrid protein for the convenience of ligating the DNA fragments. We knew that this deletion did not affect the IFN-γ activity (not shown). Based on data already presented, the influence on the activities and antigenicities of the hybrid protein of the intervening sequence of 4 amino acid residues, Pro-Glu-Phe-Met, derived from the linker and adaptor (fig.1a), appeared negligible. Whether these bifunctional activities and antigenicities would be maintained in other hybrid proteins of the NH_2-[IL-2]-[IFN-γ]-COOH type including the intervening sequence is unknown.

The significance of such a multifunctional hybrid protein is as follows. Cells that have IL-2 (IFN-γ) receptor but no IFN-γ (IL-2) receptor can accept

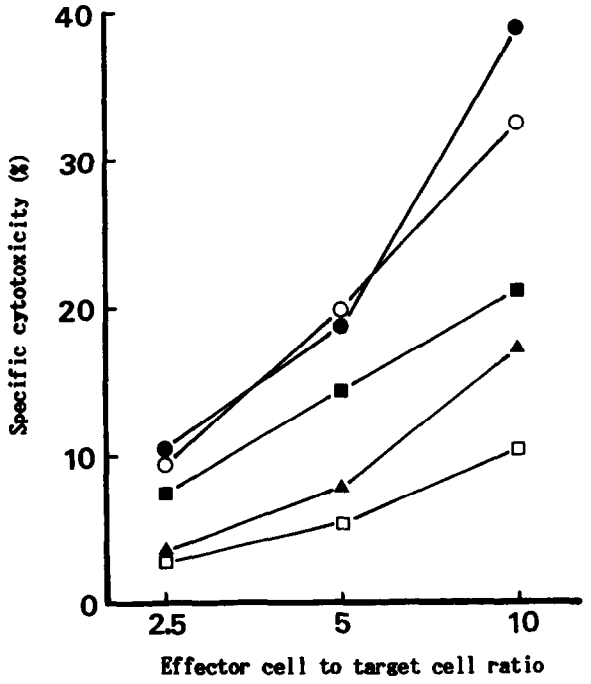


Fig.3. Enhancement of NK cell activity. Human K562 cells were labelled with $Na_2{}^{51}CrO_4$ and human PBMC were used as effector cells. The PBMC were cultured in CMEM with 0.1 U rIL-2 from *E. coli* containing pTF1 (■), 10 IU rIFN-γ from *E. coli* containing pHITtrp1101 (▲), hybrid protein equivalent to each activity of rIL-2 and rIFN-γ from *E. coli* containing pIFL9906 (○), both 0.1 U rIL-2 and 10 IU rIFN-γ (●), and no agents (□). The percent specific cytotoxicity was calculated as [{(cpm experimental ^{51}Cr release) − (cpm spontaneous ^{51}Cr release)}/{(cpm maximum ^{51}Cr release) − (cpm spontaneous ^{51}Cr release)}] × 100.

IL-2 (IFN-γ) molecules and response reflecting some effects by IL-2 (IFN-γ) activity but cannot accept IFN-γ (IL-2) lacking its receptor. These cells will accept the hybrid molecule exhibiting some responses as the results of both IL-2 and IFN-γ activity. The mechanisms of the reaction between the factors and their receptors and of the post-reaction processing are still obscure, however, the hybrid protein can be used to investigate lymphokine-receptor mechanisms. Furthermore, this kind of hybrid molecule may have clinical application.

The possibility of constructing multifunctional proteins is also interesting from the stereochemical point of view. The hybrid protein may be considered to be a protein consisting of two active domains [11] derived from IFN-γ and IL-2 because its predicted amino acid sequence (fig.1b) is complete enough to divide it into two domains, one for each active site. Although it is probable that this hybrid protein is a bifunctional protein with two active domains, it might be possible to construct a bifunctional protein with two active sites in one domain, i.e. a hybrid molecule consisting of one skeletal structure derived from either IFN-γ or IL-2 with two activities from the two immunological factors. This type of trial, which is in progress in our laboratory, will minimize the M_r and thus make more active proteins in vivo avoiding immunological attack. There are successful reports of protein engineering by 'site-directed mutagenesis', which involves replacing the amino acid composition of a protein to obtain more powerful activities and/or stabilities or different activities [12,13]. Our way of creating the hybrid protein, by genetically fusing two proteins, is another way of engineering new useful proteins that are multifunctionally active. Creating such hybrid proteins is expected to be a powerful strategy for protein engineering in the future.

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