Comparative studies of gamma-interferon receptor-like proteins of variola major and variola minor viruses**

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Abstract To study specific properties of the human gamma-interferon (γ-IFN) receptor-like proteins of the highly virulent and low virulent strains of variola (smallpox) virus (VAR) recombinant plasmids determining synthesis of these proteins in *E. coli* cells have been constructed. The recombinant viral γ-IFN receptor-like proteins have been found to have high interferon-neutralising activity with regards to human γ-IFN but not murine γ-IFN and human α-IFN. The variola major and variola minor proteins under study do not differ in the efficiency of human γ-IFN antiviral activity inhibition.

Key words: Smallpox virus; Molecular factor of virulence; Gamma-interferon receptor

1. Introduction

Studies of structure-function genome organisation of different representatives of the Poxviridae family and their comparative analysis allow to predict certain functions of proteins being coded by these or those open reading frames (ORFs) [1]. To reveal genes responsible for overcoming both specific and non-specific protective mechanisms of the host organism, is of special interest.

Computer analysis of the variola virus DNA nucleotide sequence enabled us to reveal in the genome of this virus ORFs determining the homologues of soluble receptors of the key cytokines such as γ-IFN and tumour necrosis factor [1,2]. A hypothesis was put forward that these proteins, together with some other ones, mainly contribute to the manifestation of VAR's pathogenic properties as well as to successful overcoming by the virus of the protective mechanisms of the human organism [3]. Since laboratory animals sensitive to VAR are absent, it is not possible to directly estimate the contribution of the genes revealed to the virulence rate of this virus. Therefore, a necessary step with regard to verification of this hypothesis is to create producers of such viral proteins, isolate these proteins individually and study their specific activity.

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Abbreviations: ORF, open reading frame; VAR, variola virus; γ-IFN, gamma-interferon; α-IFN, alpha-interferon; IND, India-1967; GAR, Garcia-1966; PAGE, polyacrylamide gel electrophoresis.

The objective of this work is to create *E. coli* strains capable of producing a mature form of the VAR γ-IFN receptor-like protein and to study its ability to inhibit the antiviral activity of both human and murine γ-IFN. As a source for the target B9R gene (Fig. 1) [1,2] DNA fragments of the highly virulent India-1967 (IND) strain of VAR (variola major) [4] and low virulent Garcia-1966 (GAR) strain of VAR (variola minor) [5] which were cloned in bacterial plasmids [6] were used.

High level production of the γ-IFN receptor-like protein of VAR has been demonstrated in *E. coli* cells. This recombinant viral protein can effectively inhibit the antiviral activity of human γ-IFN but fails to do so with regard to murine γ-IFN and human α-IFN. The Variola major and Variola minor proteins under study do not differ in the efficiency of human γ-IFN antiviral activity inhibition.

2. Materials and methods

2.1. Bacteria strains and cell cultures

In the work *E. coli* strains were used: JM103 (Δ(*lac-pro*), *thi, strA, endA, sbcB, hsdR*$^-$, *supE, F'*traD36, *proAB, lacIqZ-M15*); JM109 (Δ(*lac-proAB*), *thi, endA1, hsdR17, recA1, gyrA96, supE44,* λ$^-$, *relA1, F'traD36, proAB, lacIqZ-M15*); BL21 (*F*$^-$, *ompT, hsdS*$_B$); VL1201 (*htpR*$^-_{am}$, *supC, strA*); VL1222 (*htpR*$^-_{am}$, *supC, lon*).

The following cell cultures were used: diploid fibroblasts of lung of human embryo L-68 and murine fibroblasts L$_{929}$.

2.2. Enzymes and chemicals

Enzymes and chemicals that were used were: restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase produced either by SibEnzyme (Novosibirsk, Russia) or NPO Fermentas (Vilnius, Lithuania), mitomycin C, human recombinant γ-IFN and murine recombinant γ-IFN (Sigma, USA), [γ-^{32}P]ATP (1,000–4,000 Ci/mmol) produced by Isotope (Tashkent, Usbekistan), oligonucleotides produced by Vector-BioProduct (Koltsovo, Novosibirsk Region, Russia), human recombinant α-IFN produced by SRC VB Vector (Koltsovo, Novosibirsk Region, Russia).

2.3. Plasmid construction

Preparation of media, enzymatic reactions, polyacrylamide gel electrophoresis (PAGE), and *E. coli* cells transformation were performed as described [7]. The DNA fragments with genes of the target proteins were obtained from recombinant plasmids bearing extended VAR genome segments which included the B9R ORF. As regards the IND strain, the *Asu*II-*Sal*GI fragment with the target ORF was isolated; as regards the GAR strain, the *Asu*II–*Xho*I fragment with the corresponding ORF was isolated.

As expression vector, a pRTU1 plasmid, constructed by us, was used. To construct the recombinant pRIRgT and pRIRinT plasmids, the *E. coli* JM109 cells were transformed by a mixture consisting of 0.4 pmol pRTU1/*Eco*RI–*Sal*GI, 4 pmol of the corresponding *Asu*II–*Sal*GI (for IND) or *Asu*II–*Xho*I (for GAR) fragment with the target viral ORF and 12 pmol of the phosphorylated synthetic *Eco*RI–*Asu*II DNA duplex after ligase reaction (Fig. 2). The clones obtained were analysed by hybridisation with the ^{32}P-labelled oligonucleotide, which was used as probe, of the synthetic duplex' upper chain and by re-

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striction enzyme hydrolysis. From 10 clones (5 for each ORF) which met all requirements, plasmids were isolated and the *E. coli* JM103 cells were transformed. The $pRIR_gT$ and $pRIR_{in}T$ plasmids that provided for the highest viral proteins production rate in the *E. coli* cells were selected by electrophoretic analysis. The plasmids of those clones were isolated and analysed by restriction enzymes hydrolysis, and then used for transformation of the other strains of *E. coli* in order to achieve the maximum efficient viral proteins expression.

2.4. Recombinant proteins isolation

The producer strains were cultured and the target recombinant proteins synthesis induced as described earlier [8].

The recombinant proteins were isolated from the cellular biomass as follows. The cells were destroyed in an ultrasonic disintegrator and thus a fraction of non-soluble inclusion bodies was obtained as described [8]. Inclusion bodies were consecutively washed with 1% solution of Triton X-100, 2 M and 4 M carbomide prepared in PBS buffer [7] while the precipitate was collected by centrifugation. The precipi-

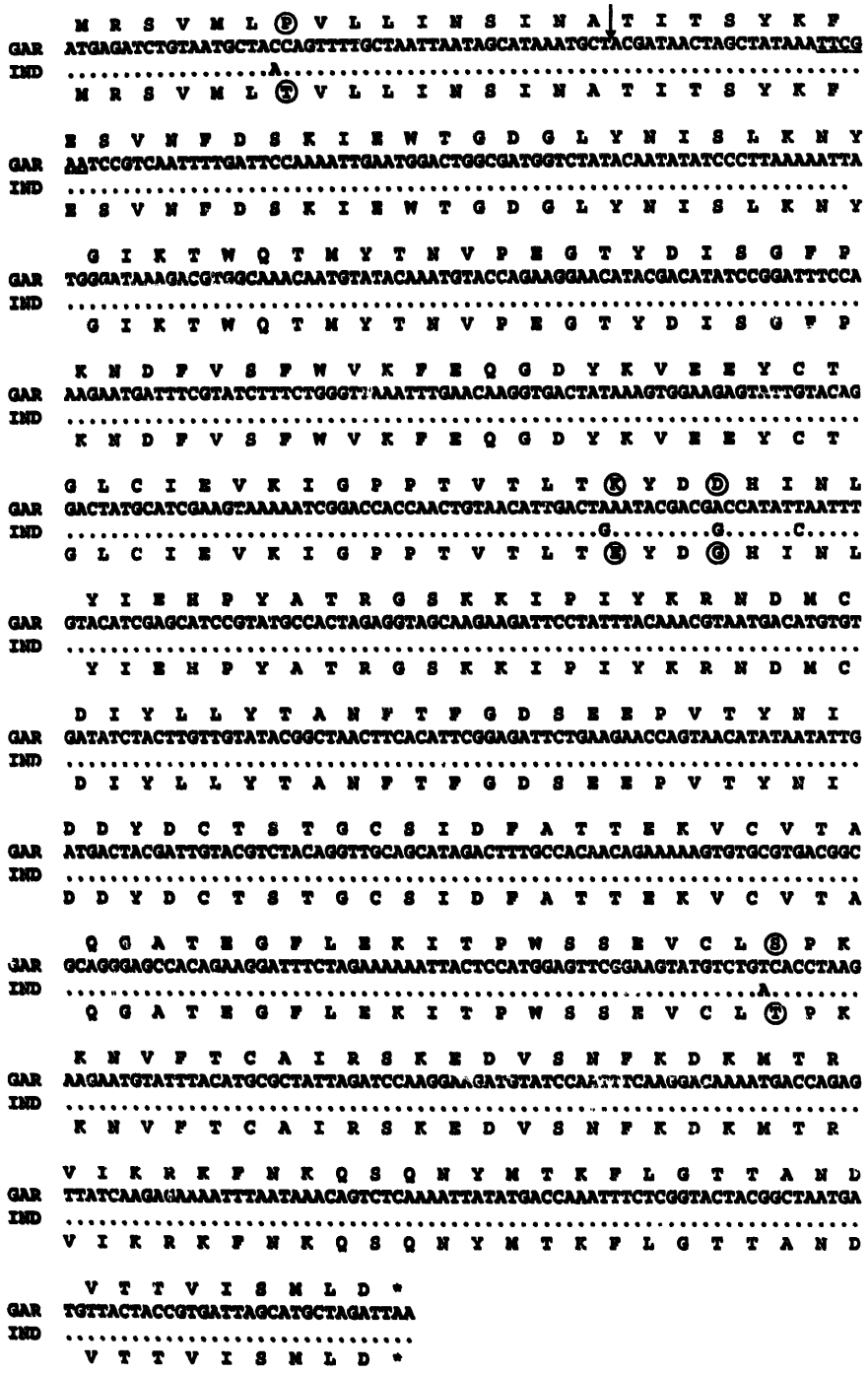

Fig. 1. Alignment of nucleotide and amino acid sequences of the B9R gene of the India-1967 (IND) and Garcia-1966 (GAR) strains of VAR. In the sequence of IND, nucleotides identical to those of GAR are marked with dots. In sequences of the B9R protein, amino acid residues which are different in IND and GAR are marked with circles. Horizontal line marks the site of DNA hydrolysis by the *Asu*II restriction enzyme. Vertical arrow shows the presumed location of the site of pre-protein's signal peptide splitting off.

tate obtained was dissolved in 6 M carbomide, centrifuged, and the supernatant was applied on a column with Sephacryl S-200. Fractions containing the target protein and the rate of its being pure were analysed electrophoretically [9]. The fractions containing the target protein were combined, diluted 5 times with cold PBS buffer, dialysed against the same buffer for 15 to 18 h, centrifuged, and the supernatant was filtrated through a sterilising filter. The protein's concentration was determined by the method described in [10] while using a kit by Bio-Rad Laboratories Co. (USA).

2.5. Analysis of recombinant proteins specific activity

The recombinant viral proteins were studied for specific activity by the modified method of interferon-neutralising antibodies evaluation [11] where plate wells with the L-68 cell culture got introduced serial dilutions of samples under study (previously standardised considering the recombinant protein's concentrations) in Eagle's minimum essential medium (Eagle's MEM), and human γ-IFN was added up to final concentration 10 IU/ml. After 24 h of incubation at 37°C, the culture medium was removed from the wells, the cell monolayer was washed three times with Eagle's MEM, and a challenge virus (murine encephalomyocarditis virus) was introduced as described [11]. After 24 h, results were evaluated and interferon-neutralising activity of recombinant viral proteins was determined as described [11]. Simultaneously, biological activity of γ-IFN used was monitored as described above.

3. Results and discussion

Amino acid sequences of γ-IFN receptor-like proteins of the two strains under study, variola major and variola minor viruses, show high homology. The protein from the IND strain bears Thr, Glu, Gly and Thr in positions 7, 110, 113, and 208, respectively, while the corresponding protein from the GAR strain has in these positions Pro, Lys, Asp, Ser, respectively (Fig. 1). To answer the question whether point substitutions are able to considerably influence the presumed specific activity of the viral proteins, one has to isolate the corresponding proteins individually. At present there is only one method available as to how to obtain the VAR proteins under study: construction of genetically engineered producer strains of the target proteins.

To achieve expression of the target viral ORFs, hybrid plasmids $pRIR_gT$ and $pRIR_{in}T$ were constructed (Fig. 2). Those plasmids were created by cloning in the pRTU1 expression vector a corresponding fragment of the viral DNA with the target ORF ligated with the phosphorylated synthetic DNA duplex containing a translation initiating triplet (ATG) (Fig. 2).

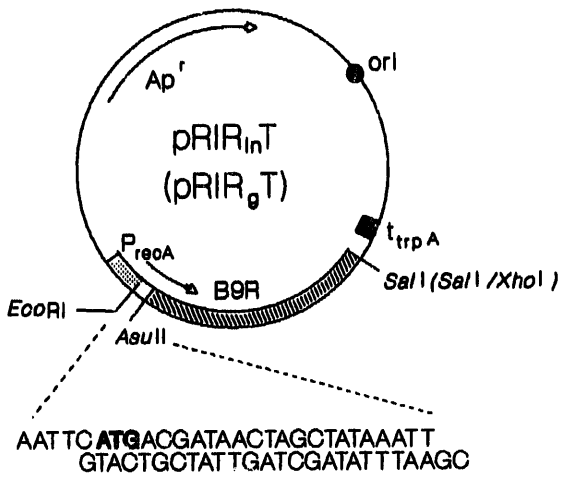

Fig. 2. Scheme of hybrid plasmids $pRIR_{in}T \times pRIR_gT$ containing target gene of IND and GAR, respectively. Sequence of synthetic duplex providing joining of the recA promoter fragment to the structural part of the gene of the γ-IFN receptor-like protein mature form (Fig. 1), is shown at the bottom. Translation initiating triplet ATG is given in bold type.

The structure of the hybrid plasmids is so that the target gene transcription is initiated at the inducible promoter of the *recA Proteus mirabilis* gene [8] and stopped at the ρ-independent terminator of the *E. coli trpA* gene (Fig. 2). The coding sequence of the inserted target viral ORF determines the mature form of the γ-IFN receptor-like protein without 16 N-terminal amino acid residues of the signal peptide (Figs. 1 and 2). The hybrid plasmids $pRIR_gT$ and $pRIR_{in}T$ were used to transform cells of the *E. coli* strains JM103, BL21, VL1201, VL1222, and production of the recombinant proteins was analysed. The target protein accumulated in bacterial cells in the inclusion bodies and then was dissolved in 6 M carbomide (Fig. 3a). This allowed to rather simply isolate it from the cellular biomass by using consecutive washing the inclusion bodies, dissolving the target protein in 6 M carbomide, gel filtration, and dialysis (Fig. 3b). Molecular weight of the expression product (approximately 30 kDa) was close to the predicted one. The *E. coli* strains VL1201, VL1222 and BL21 should be noted to show, though they have a decreased ability of intracellular proteolysis, no considerable differences from the JM103 strain with regard to the recombinant viral protein production level determined by densitometry: 12% to 15% of the total cellular protein as regards the two former strains, and 10% to 12% of the total cellular protein as regards the two latter strains.

In the course of three independent analyses of the purified recombinant viral proteins specific activity, the interferon-neutralising activity of the IND strain's recombinant viral protein was determined as $(4.0 \pm 2.0) \times 10^5$ U/mg, and that of the GAR strain $(2.5 \pm 1.5) \times 10^5$ U/mg, i.e. these magnitudes of the proteins compared practically do not differ. Considering that antiviral activity of the recombinant human γ-IFN (Sigma) was 10^7 IU/mg, and as 1 activity unit of the recombinant viral protein is a minimum of its amount was taken which caused a complete antiviral activity inhibition of 1 IU of γ-IFN, one can conclude that the recombinant viral proteins homologous to the human γ-IFN receptor show a relatively high interferon-neutralising activity.

The results obtained suggest that glycosylation of the viral proteins homologous to the γ-IFN receptor does not considerably influence the manifestation of their specific activity. Antiviral activity inhibition was not observed when recombinant human α-IFN (10 IU/ml) was used instead of γ-IFN: with recombinant viral proteins concentration of 1 μg/ml, α-IFN revealed 100% antiviral activity. This testifies to strict specificity of the recombinant viral proteins activity with regard to γ-IFN, one of the most important cytokines participating in formation of the organism's protective mechanisms against infectious agents [3,12–14].

It should be noted that activity of the highly virulent IND strain's recombinant protein of VAR does not differ from that of the low-virulent GAR strain. These data allow us to conclude that differences in virulence rate of different VAR strains are determined by other viral genes or complex of genes.

Variola virus restricted, in the course of evolution, the range of sensitive animals with only one species: *Homo sapiens* [15]. That would suggest that mechanisms of modulation

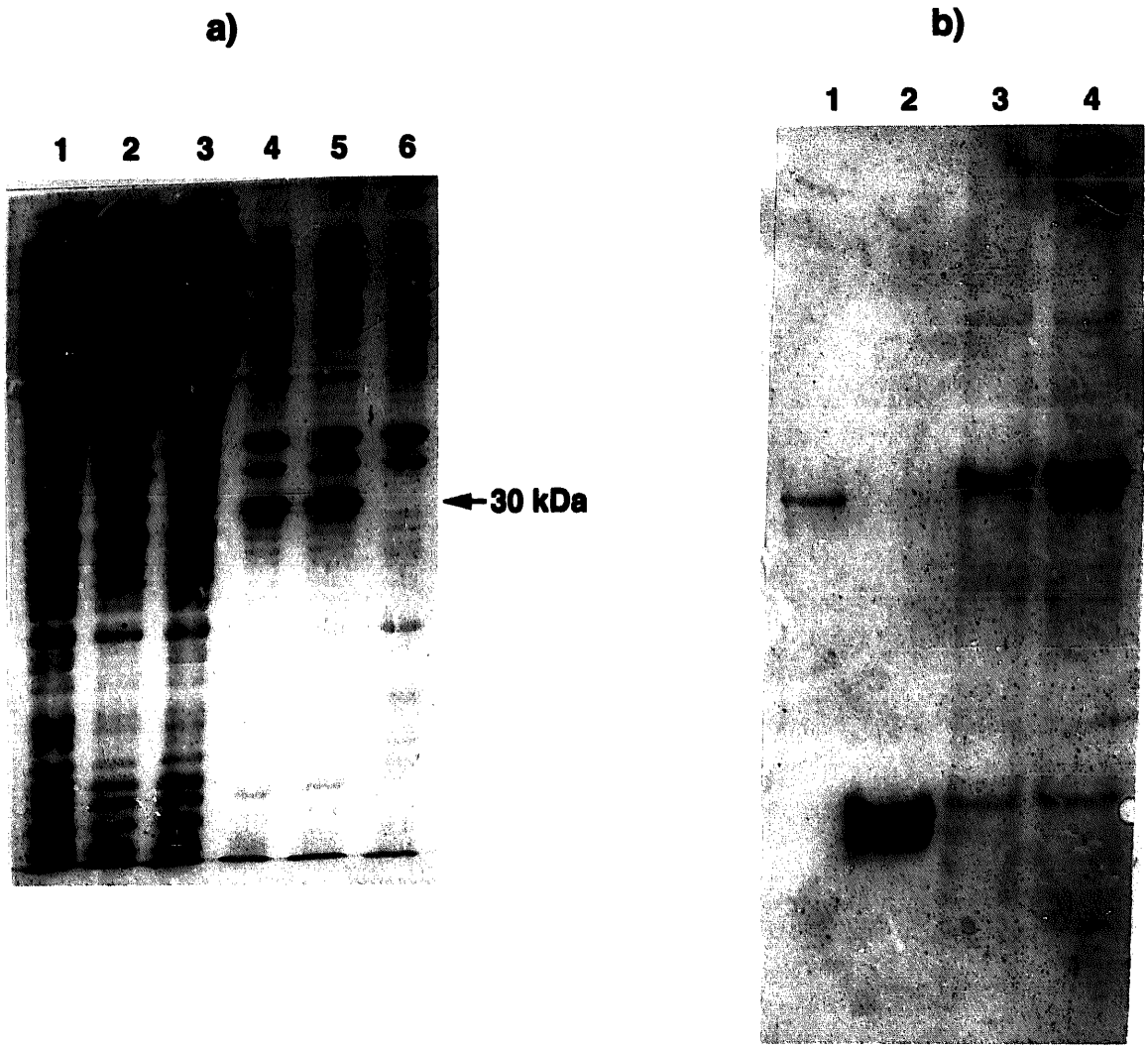

Fig. 3. Production of the recombinant viral proteins homologous to the human γ-IFN receptor in the *E. coli* VL1222 cells. Analysis of samples by SDS-PAGE (15% separation gel) and Coomassie blue staining of the gel. a: localisation of recombinant proteins in *E. coli* cells. Lanes 1 to 3, soluble cellular proteins; lanes 4 to 6, insoluble cellular proteins (inclusion bodies). Lanes 1 and 4, correspond to $pRIR_gT$-bearing cells, induced by mitomycin C, lanes 2 and 5 to those with plasmid $pRIR_{in}T$, and lanes 3 and 6 to those without plasmid; b: recombinant viral proteins, isolated from the inclusion bodies and then purified, being coded by plasmid $pRIR_{in}T$ or $pRIR_gT$ (lanes 3 and 4, respectively). Lanes 1 and 2, marker proteins with molecular weight of 29 and 14 kDa, respectively.

of the host organism protective responses by variola virus adapted themselves evolutionarily and specifically to the corresponding systems of humans. To verify this presumption, besides human γ-IFN, murine γ-IFN was also analysed.

We used a dose of VAR recombinant proteins homologous to γ-IFN receptor which completely inhibited antiviral activity of the recombinant human γ-IFN in a concentration of 250 IU/ml in the L-68 cell culture. After that, those samples of VAR proteins were studied for their ability to suppress the recombinant murine γ-IFN antiviral activity in the murine fibroblasts L_{929} cell culture. The recombinant viral proteins were established to be unable, in the chosen dose, to inhibit the murine γ-IFN antiviral activity even when the latter had a concentration of 4 IU/ml. The data obtained show that the VAR proteins under study can actively interact with human γ-IFN but not with murine γ-IFN. Similar data have been reported recently for the γ-IFN-binding protein of vaccinia virus [15].

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