Volume 306, number 2,3, 262–264 FEBS 11258 July 1992
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Construction and functional analysis of hybrid interleukin-6 variants

Characterization of the role of the C-terminus for species specificity

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Received 12 May 1992

We have constructed several hybrid human interleukin-6 (IL-6) variants in which the carboxyl-terminus, which includes a receptor binding site of IL-6 has been replaced with the C-terminus of various proteins homologous to human IL-6. IL-6 hybrids with the C-terminus of human growth hormone and human granulocyte-colony stimulating factor maintain part of the biological activity of human IL-6. Replacing the C-terminus of human IL-6 with the C-terminus of mouse and rat IL-6 resulted in a normal or increased activity on a mouse cell line; however, this gave a low (to 200-fold less) activity on a human cell line compared to wild-type human IL-6. We therefore conclude that the C-terminus of IL-6 plays an important role in the species specificity of IL-6.

Interleukin-6; Interleukin-6 receptor; Hybrid protein; Species specificity; Growth factor

1. INTRODUCTION

Interleukin-6 is a cytokine with multiple functions, including differentiation of B-cells and T-cells, regulation of the acute phase response and differentiation of hemopoietic progenitor cells [1,2]. cDNAs coding for human, mouse and rat IL-6 have been cloned and the proteins have been characterized [3–5]. Amino acid comparison revealed an identity of the IL-6 proteins for mouse–human, rat–human and mouse–rat of 40%, 58% and 93%, respectively [4,6]. Despite this low identity, human IL-6 exerts activity on rat and mouse cells. However, mouse IL-6 does not have any activity on human cell lines [1].

Although the tertiary structure of human IL-6 is unknown, the secondary structure predictions point towards a growth hormone-like tertiary structure [7]. This includes a bundle of four antiparallel α-helices with a distinctive loop topology. Several regions of the rodent and human IL-6 proteins have a striking high identity, especially in the middle of the protein including the four conserved cysteine residues and the C-terminus, indicating that these regions are important for biological activity [8]. Computer modelling suggests that the C-terminus of IL-6 consists of an amphipathical α-helix, as is found in several homologous proteins [7]. Furthermore, structure-function studies have recently identified an IL-6 receptor binding site in the C-terminus of IL-6 [9].

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In this brief communication we report the construction and analysis of several hyrid IL-6 molecules and demonstrate that the C-terminus of IL-6 may account for the species specificity of interleukin-6.

2. MATERIALS AND METHODS

The construction of the wild-type IL-6 gene has been described previously [10,11]. The IL-6 gene was cloned into the expression vector p340. This vector is used for high-level expression of an IL-6–β-galactosidase fusion protein with small collagenase cleavage site as a linker of the two proteins. This expression vector was constructed from pJG200, in which the λ-promotor was replaced by a P-trc promotor of pKK233-2 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). A synthetic minicistron was inserted downstream of the promotor to increase the level of protein expression [10].

The hybrid IL-6 variants were constructed by replacing 19 residues at the C-terminus of IL-6 with 19 residues of the C-terminus of homologous proteins using oligonucleotide-directed mutagenesis. The expression vector containing the IL-6 gene was used as a template for the polymerase chain reaction (PCR) using two oligonucleotides that flanked the IL-6 gene. One oligonucleotide covered the N-terminal sequence of IL-6 and one was partly hybridized over the C-terminus of IL-6. The sequence of the C-terminal oligonucleotides is:(5′– 3′) CAA GAT CTG ACA TTT GTA GTC TTC CCG GGA AGA CTA AGT TGT CAT GTC CTG CAG CCA CTG. The underlined part of the oligonucleotide is identical to the region coding for Gln[157] to Thr[164]. The non-underlined part serves as a cloning site and contains two *Bbs*I sites and a *Bgl*II site. Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems 380A, Foster City, CA) and purified by reverse- phase high-performance liquid chromatography. Reagents were obtained from American Bionetics (Hayward, CA). PCR was performed according to standard procedures and a fragment of 560 basepairs was obtained [12]. The PCR fragment was cloned into the TA cloning™ vector pCR™ (Invitrogen, San Diego, CA). After cleavage with *Bbs*I we cloned into the IL-6 gene a 61 bp fragment, encoding for the C-terminus of other proteins, that was obtained by annealing two oligonucleotides. This resulted in the reconstruction of the original IL-6 gene; however, it now encodes for a human IL-6

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variant with 19 amino acids at the C-terminus (165–183) replaced with the C-terminus of proteins homologous to human IL-6. All mutants were analyzed by dideoxy sequencing. Upon *NcoI*/*BglII* digest of the TA cloning™ vector pCR™ the IL-6 hybrid gene was cloned into the expression vector p340. After conformation of a correct insert using PCR, we used these vectors to transform *Escherichia coli* JM101 cells. In all cases dark blue ampicillin resistant colonies were obtained and used for subsequent protein expression. One IL-6 variant with a deletion of amino acids 164–183 was constructed earlier [11].

Purification of our IL-6 variants was done as described previously [10,11]. Upon induction of protein synthesis, high amounts of the IL-6–β-galactosidase fusion protein were obtained and afterwards partly purified. Before quantitation or use in bioassays the fusion protein was cleaved by collagenase (recombinant collagenase, Biosource, Camarillo, CA) to release the IL-6 variant [9,10].

The biological activity of our IL-6 variants was tested on both mouse and human cell lines. Two bioassays were performed: a hybridoma growth factor activity assay using an IL-6 dependent mouse hybridoma cell line 7TD1 and a hepatocyte stimulation activity assay using a human hepatoma cell line HEP3B2. These assays were described previously in detail [9,10]. The activity of the IL-6 variants was calculated as the mean of 2 independent preparations of each variant and expressed as a percentage of wild-type human IL-6.

3. RESULTS AND DISCUSSION

In Fig. 1, the amino acid sequence of the C-terminus of IL-6 is compared with several homologous cytokines, such as mouse and rat IL-6 and other growth factors. As is indicated, several residues in this region are highly conserved not only between the different species but also between IL-6 and the evolutionary related proteins [7]. The C-termini of all of these proteins are predicted to form an amphipathical α-helix as determined by computer modelling [7]. For growth hormone and IL-6 it has been shown that this region of the proteins contains a receptor binding site [9,13]. Based on the similarity of this region between several proteins and the importance of this region for biological activity, we constructed several hybrid IL-6 variants (Fig. 2). We replaced the residues 165–183 of human IL-6 with the corresponding C-terminal residues of several different homologous proteins. The biological activity of these mutants was determined on human and mouse cell lines.

First, the importance of the C-terminus was demonstrated by the inactivity of an IL-6 variant with a deletion of the C-terminus. This mutant had no detectable activity (<10,000 fold less than wild-type human IL-6) in the human and mouse cell line assays, as was reported earlier [11]. Another IL-6 variant with all residues in the C-terminus replaced by conservative amino acid substitutions, was also inactive. Although this variant theoretically has an amphipathical helical structure at the C-terminus, it lost all of its biological activity. The results of both these variants indicate the crucial role of the C-terminus for biological activity.

In order to see whether a naturally occurring amphipathical helix is sufficient for IL-6 receptor recognition and for restoring biological activity, we made a couple of IL-6 hybrids with the C-terminus of human G-CSF and human growth hormone. The IL-6 variant

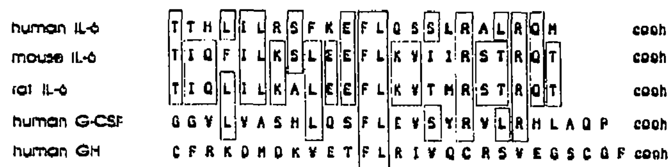

Fig. 1. The C-terminus of interleukin-6 (IL-6) is compared with the C-terminus of proteins that are homologous to human IL-6. Several residues are conserved in IL-6 between different species, but also between IL-6 and other proteins such as human growth hormone (hGH) and human granulocyte-colony stimulating factor (hG-CSF). This figure is based on the secondary structure predictions and computer modelling of these proteins by Bazan [7].

with the C-terminus of G-CSF had low but distinct IL-6 activity on the mouse cell line; however, a very low activity on the human cell line. Although the activity on the mouse cell line was only 3% of wild-type human IL-6, it was at least 2 orders of magnitude more than the IL-6 variant with the C-terminus deleted or the IL-6 variant with the conservative substitutions. As mentioned before, IL-6 is thought to have a similar tertiary structure as growth hormone based on the secondary structure predictions. Also the receptor binding region of both proteins is thought to be in the same location. We constructed an IL-6 variant with the C-terminus replaced by the C-terminus of human growth hormone. Cunningham et al. showed that the affinity of growth hormone to its receptor could be improved four-fold by replacing Glu^{174} by Ala [13]. Therefore we constructed our IL-6-growth hormone hybrid with the Glu residue (at position 172 in the hybrid IL-6) replaced by Ala. The activity of this mutant was low in both human and mouse cell lines, but as was found for the IL-6–G-CSF hybrid, higher than the deletion mutant. The results of both these hybrids indicate that a naturally occurring amphipathical α-helix structure at the C-terminus of IL-6 can maintain partly the function of the IL-6 protein.

Biological activity of hybrid variants of human interleukin-6 with C-terminus of related proteins

		Biological activity 7td1 (%)	Biological activity Hep3b2 (%)
hIL-6 wild-type	ttHLILRSFKEFLQSSLRALRqm	100	100
hIL-6 variant with C-term deleted	tqm	<0.01	<0.01
hIL-6 variant with cons mutations	ttRMVHKTYRDTMNTTMKSMKqm	<0.01	<0.01
hIL-6 variant with C-term hG-CSF	ttVLVASHLQSFLEVSYRVLRqm	2.7	0.033
hIL-6 variant with C-term hGH	ttRKDMDKVATFLRIVQCRSVqm	2.2	4.0
hIL-6 variant with C-term mouse IL-6	ttQFILKSLEEFLKVIIRSTRqm	57	0.45
hIL-6 variant with C-term rat IL-6	ttQLILKALEEFLKVTMRSTRqm	260	12.6

Fig. 2. The biological activity of the various hybrid interleukin-6 (IL-6) variants compared to wild-type human IL-6. The grey bar above the sequences indicates the amino acids that have been changed. The hybrid of human IL-6 with the C-terminus of human growth hormone (hGH) has a substitution at 172 (Glu to Ala) to improve its receptor binding. hG-CSF=human granulocyte-colony stimulating factor.

Upon replacement of the C-terminus of human IL-6 with the corresponding region of mouse IL-6, a hybrid IL-6 variant was obtained that had a near normal biological activity on a mouse cell line. Interestingly, an earlier attempt of Brakenhoff to replace part of the C-terminus of human IL-6 by the last five corresponding residues of mouse IL-6, resulted in an IL-6 variant with no detectable activity on a mouse hybridoma cell line [14]. This clearly indicates that the complete (19 amino acids) helix in the C-terminus of mouse IL-6 is necessary for proper interaction with its own receptor. The activity of this hybrid on human cells was more than 100-fold lower than the activity on the mouse cell line. This indicates that the C-terminus plays an important role in the species specificity of IL-6.

A hybrid of human IL-6 with the C-terminus of rat IL-6, had an activity of 12% compared to wild-type IL-6 on the human cell line. Surprisingly the activity on the mouse cell line was 260% compared to wild-type human IL-6. The 20-fold higher activity of the human–rat IL-6 hybrid on the mouse cell line compared to the human cell line also supports a role of the C-terminus in species specificity of IL-6. The activity of this hybrid on both cell lines is strikingly higher than the activity of the human–mouse hybrid. This might be explained by the fact that other parts of the IL-6 molecule are involved in receptor binding and might form a mutual binding site for the IL-6 receptor. The amino acid sequence identity between human and rat IL-6 is higher than human and mouse. Therefore the rat C-terminus might fit better with other regions of the human IL-6 protein and therefore interact with a higher affinity with the human and mouse IL-5 receptor. This is supported by earlier studies of Brakenhoff et al. who suggested that the IL-6 receptor interaction is not confined to one part of the molecule, but rather mediated by different parts of the molecule. Based on studies using monoclonal antibodies, they suggested that the amino-terminal (residues 21–34) and carboxyl-terminal parts of IL-6 are in close proximity and both involved in receptor binding [15]. However, a human–mouse IL-6 hybrid in which the amino acids 29–34 in the N-terminus of human IL-6 have been replaced by the sequence of mouse IL-6 displayed a similar reduction of biological activity on human and mouse cell lines [16]. Therefore it seems that although this part is important for biological activity, it is not involved in the species specificity of IL-6. More studies using hybrid IL-6 variants substituting different parts of the IL-6 protein might provide more insight in the species specificity of IL-6.

We did not perform receptor binding studies with the hybrid IL-6 variants. We performed a competition experiment using the human hepatoma cell line to see if the human–mouse IL-6 hybrid could compete with wild-type IL-6 for receptor binding. The activity of wild-type human IL-6 was not reduced by adding various amounts of the hybrid, and we therefore conclude that the loss of biological activity of this hybrid is caused by the inability to interact with the IL-6 receptor (data not shown). This is in accordance with our earlier studies on the C-terminus of IL-6 and the results of other studies, where the changes in biological activity of IL-6 variants were found to be closely related to its receptor binding ability [9,17].

In conclusion, we have shown that a naturally ocurring amphipathic α-helix of other proteins homologous to IL-6 at the C-terminus can maintain in part the biological activity of IL-6. Earlier studies have already shown the importance of the C-terminus of IL-6 for biological activity and receptor binding. We suggest that the C-terminus of IL-6 is additionally a major determinant for the species specificity of IL-6.

REFERENCES

[1] Van Snick, J. (1990) Annu. Rev. Immunol. 8, 253–278.
[2] Kishimoto, T. (1989) Blood 74, 1–10.
[3] Yasukawa, K., Hirano, T., Watanabe, Y., Muratani, K., Matsuda, T., Nakai, S. and Kishimoto, T. (1987) EMBO J. 6, 2939–2945.
[4] Simpson, R.J., Moritz, R.L., Rubira, M.R. and van Snick, J. (1988) Eur. J. Biochem. 176, 187–197.
[5] Northemann, W., Braciak, T.A., Hattori, M., Lee, F. and Fey, G.H. (1989) J. Biol. Chem. 264, 16072–16082.
[6] Tanabe, O., Akira, S., Kamiya, T., Wong, G.G., Hirano, T. and Kishimoto, T. (1988) J. Immunol. 141, 3875–3881.
[7] Bazan, J.F. (1990) Immunol. Today 11, 350–354.
[8] van Snick, J., Cayphas S., Szikora, J.P., Renauld, J.C., van Roost, E., Boon, T. and Simpson, R.J. (1988) Eur. J. Immunol. 18, 193–197.
[9] Leebeek, F.W.G., Kariya, K., Schwabe, M. and Fowlkes, D.M. (1992) J. Biol. Chem. (in press).
[10] Jambou, R.C., Snouwaert, J.N., Bishop, G.A., Stebbins, J.R., Frelinger, J.A. and Fowlkes, D.M. (1988) Proc. Natl. Acad. Sci. USA 139, 4116–4121.
[11] Snouwaert, J.N., Kariya, K. and Fowlkes, D.M. (1991) J. Immunol. 146, 585–591.
[12] Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higushi, R., Horn, G.T., Mullis, K.B. and Ehrlich, H.A. (1988) Science 239, 487–491.
[13] Cunningham, B.C. and Wells, J.A. Science 244, 1081–1085.
[14] Brakenhoff, J.P.J (1990) Doctoral Thesis, Amsterdam, The Netherlands, p. 84.
[15] Brakenhoff, J.P.J., Hart, M., de Groot, E.R., Di Padova, F. and Aarden, L.A. (1990) J. Immunol. 145, 561–568.
[16] Fontaine,V., Brakenhoff, J., de Wit, L., Arcone, R., Cilliberto, G. and Content, J. (1991) Gene 104, 227–234.
[17] Snouwaert, J.N., Leebeek, F.W.G. and Fowlkes, D.M. (1991) J. Biol. Chem. 266, 23097–23102.