Volume 286, number 1,2, 58–60 FEBS 09912 July 1991

ADONIS 0014579391006212

Site-directed mutagenesis reveals the importance of disulfide bridges and aromatic residues for structure and proliferative activity of human Interleukin-4

Niels Kruse, Thomas Lehrnbecher and Walter Sebald

Physiologisch-Chemisches Institut der Universität Würzburg, Biozentrum, Am Hubland, D-8700 Würzburg, Germany

Received 2 May 1991

Mutant proteins (muteins) of human Interleukin-4 (IL4) were constructed by means of in vitro mutagenesis. The muteins were expressed in *E. coli*, submitted to a renaturation and purification protocol and analysed for biological activity. Exchange of the cysteines at either position 46 or 99 which form one of the three disulfide bridges resulted in a nearly complete loss of biological activity and an unstable protein. The exchange of tyrosine 124 also inactivated the protein, while a mutation of tyrosine 56 left some residual activity. Exchange of the other four cysteines or of the single tryptophane had smaller effects.

Interleukin 4 (human); Recombinant; In vitro mutagenesis; Structure-function

1. INTRODUCTION

Interleukin-4 (IL4) is one of the many cytokines which induce and coordinate the proliferation, maturation, survival and differentiation of lymphoid and myeloid cells (for reviews see [1–3]). In particular, IL4 functions in IgE-mediated immune responses, and promotes directly the proliferation of thymocytes and activated T-cells. A high affinity IL4 receptor protein of M_r 140 000 has been identified, which consists of 800 amino acid residues according to the cDNA sequence [4]. It belongs to a recently described group of receptors, designated as hematopoietin receptor superfamily [5].

The amino acid sequence of mature human IL4 as deduced from the cloned cDNA [6] consists of 129 residues. The cDNA has been expressed in *E. coli* [7,8] and yeast [9]. From these sources recombinant IL4 with high biological activity has been prepared. Circular dichroism and protein-chemical experiments with mouse and human IL4 have been reported that revealed some properties of the aromatic amino acid residues [10,12] as wel as the pairing of the six cysteines in three disulfide bridges [11,13]. In the present study the same amino acid residues of human IL4 were exchanged by in vitro mutagenesis in order to analyse their significance for structure and function of the protein.

Correspondence address: W. Sebald, Physiologisch-Chemisches Institut der Universität Würzburg, Biozentrum, Am Hubland, 8700 Würzburg, Germany. Fax: (49) (931) 888 4113.

2. MATERIALS AND METHODS

An *Eco*RV/*Bam*HI fragment encoding the major part of mature human Interleukin-4 was excised from an engineered cDNA (British Bio-technology Ltd., Oxford, England). This DNA fragment together with synthetic oligonucleotides 5′-CATGCACAAGTGCGAT and 5′-ATCGCACTTGTG, comprising the first 4 amino acid codons of Interleukin-4 plus the codon for methionine, were integrated between the *Nco*I and *Bam*H1 sites of expression vector R^{TS}pRC109 [14]. Oligonucleotide-directed mutagenesis was performed by the gapped duplex DNA approach [14,15]. The final construct of the mutated cDNA was sequenced and after confirmation of the specific change the strain was used for expression of the mutant protein.

Recombinant human Interleukin-4 (rhIL4) and IL4 muteins were isolated essentially as described by Kato et al. [16] with modifications detailed elsewhere [14]. All fractions obtained after CM-Sepharose chromatography and after HPLC were analysed by dodecylsulfate polyacrylamide-gel electrophoresis [17]. Protein concentration was determined by absorbance at 280 nm (1 mg IL4/ml was assumed to yield an absorbance at 280 nm of 1). Proper corrections were made in the case of variants missing tryptophane or tyrosine [18].

The amount of IL4 in the 'inclusion body' fraction was estimated by co-electrophoresis of these fractions and defined amounts of pure rhIL4 and subsequent densitometric determination of bound Coomassie blue stain. Proliferative activities were determined by measuring in duplicate the IL4-dependent incorporation of [^{3}H]thymidine into peripheral blood lymphocytes preactivated for 7 days with phytohemagglutinine [6].

3. RESULTS AND DISCUSSION

The rhIL4 protein is expressed with reasonable efficiency (15–20% of whole cell protein; Fig. 1A) in the constructed *E. coli* strains employing the right promotor of phage lambda and temperature-sensitive lambda repressor together with the ribosome binding site of the *E. coli* atpE cistron [19]. Comparable results are obtained when the same ribosome binding site is

Published by Elsevier Science Publishers B.V.

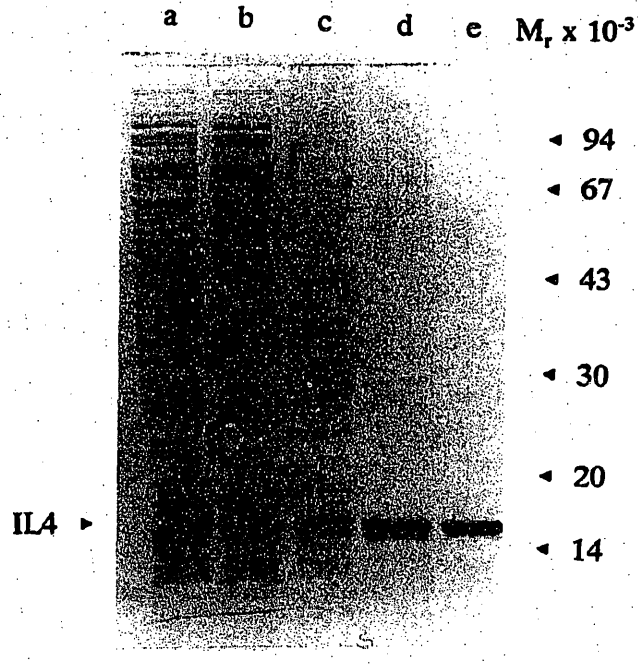

Fig. 1. Purification of recombinant human Interleukin-4 from an overproducing *E. coli* strain. Whole cellular proteins [a], soluble proteins [b], inclusion bodies [c], combined fractions after CM-Sepharose chromatography [d] and after HPLC [e] were submitted to dodecylsulfate-gel electrophoresis and visualized by staining with Coomassie brilliant blue R-250.

used together with a synthetic promotor from phage T5 and the operatur/repressor system from the *lac* operon [20]. As demonstrated in Fig. 1C the recombinant protein is concentrated in the particulate fraction of the *E. coli* homogenate (60–80% of total protein). Compared to sedimented 'inclusion bodies' of other recombinant cytokines, e.g. of IL2 [14], the IL4-containing sediment exhibited a more fluffy and greenish appearance. After renaturation of the 6 M-guanidiniumchloride-dissolved protein the rhIL4 was eluted at 0.35–0.4 M NaCl from the CM-Sepharose column recovering 20–30% of IL4 present in the 'inclusion bodies'. During HPLC over a C4 column the rhIL4 eluted at 40–42% acetonitrile. The final yield was 1–2 mg IL4 per l of original *E. coli* culture with a purity better than 95%. The specific biological activity as measured by the halfmaximal proliferative response of human T-cells amounted to $0.2–0.5 \times 10^6$ U/mg protein.

The six cysteine codons in the IL4 cDNA have been singly replaced by a threonine codon in order to produce muteins C3T, C24T, C46T, C65T, C99T and C127T. The side chain of threonine has a surface area only slightly larger than that of cysteine [21]. Each of the six mutant proteins could be isolated and purified. In the case of C46T very low yields were observed.

Re-analysis of the purified variants by HPLC revealed multiple species with C46T and C99T. Apparently, subsitution of the cysteines at either position 46 or 99 by threonine rendered the protein unstable. Proliferative activity with C46T and C99T was less than 1% of wild-type activity (Fig. 2). Replacement of cysteines in C3T, C24T, C65T and C127T reduced the biological activity to some extent. Specific activities 3–5 times lower than in the normal IL4 were measured. The six cysteine residues in rhIL4 have been assigned to three disulfide bridges [11,13]. Most interestingly, the cysteines at positions 46 and 99 form one disulfide bridge which according to the results above is essential for structure and function. The disulfide bridges formed between cysteines 3 and 127 as well as between cysteines 24 and 65 seem to be less important for structural integrity.

'Inclusion bodies' from IL4 wild-type and mutant strains could be assayed directly for biological activity by diluting solutions of proteins denaturated in guanidiniumchloride or dodecylsulfate buffers into the T-cell culture medium (RPMI/10% fetal calf serum). Specific activities were measured in the order IL4 wild-type $>$ C3T ~ C24T ~ C65T ~ C127T $>$ C46T ~ C99T. These are the same relations as found with the purified proteins (Fig. 2), even though the absolute values were about 20-fold (guanidiniumchloride buffer) or 100-fold (dodecylsulfate buffer) lower.

Recently, biophysical and protein-chemical studies on the aromatic side chains of human IL4 have been reported [10,12]. We have singly replaced the two tyrosines by aspartic acid and the tryptophane by arginine, yielding the muteins Y56D, 124D and W91R. Despite these drastic changes affecting size, polarity and charge of the particular side chains, these three muteins could be purified in yields comparable to wild-type IL4. Replacing the aromatic side chains lead to the expected changes of the absorbance spectrum in the near UV. During re-analysis by HPLC all three muteins were eluted as single peaks at an unchanged acetonitrile concentration. This suggests that the structure was not impaired to a major extent by these amino acid exchanges. Interestingly, W91R showed an unchanged or even moderately enhanced specific proliferative activity (Fig. 2). According to circular dichroism measurements the tryptophane is largely accessible to the solvent [12]. It is also easily modified by *N*-bromosuccinimide [10]. In mouse IL4 the position of tryptophane 91 appears to be also occupied by an arginine [11]. Thus it is conceivable that the human IL4 mutein W91R exhibits full biological activity. It has to be noted, however, that chemical modification reduces biological activity [10]. The Y124D mutein was completely inactive, while Y56D retained 5–10% of the wild-type activity. Tyrosine 124 but not tyrosine 56 is easily modified by tetranitromethane [10]. Accordingly, tyrosine 124 is probably exposed at the protein surface. The activity is only marginally reduced by this chemical modification. Both tyrosines are conserved between human and mouse IL4. Mutein Y124D where tyrosine 124 is

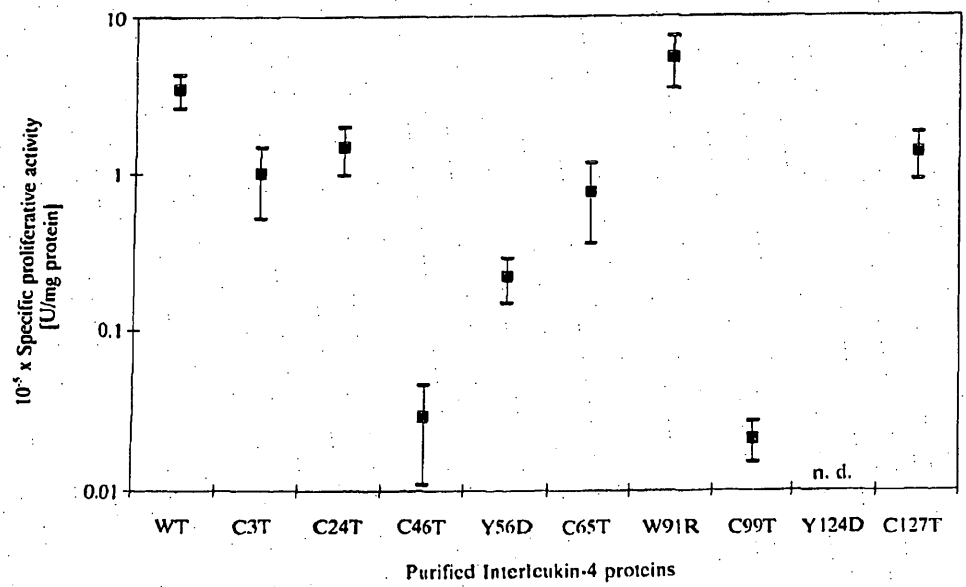

Fig. 2. Specific proliferative activities (U/mg protein) of purified Interleukin-4-wild-type (WT) and mutant proteins. Three independent samples of each protein were assayed in duplicate. Standard deviations are indicated by bars. The abbreviations of the purified IL4 protein-wild type (WT) and muteins are defined in the text. The activity of the Y124D protein could not be determined (n.d.); even at 1 μg/ml no [^{3}H]thymidine incorporation could be measured.

substituted by an acidic side chain seems to be structurally intact as it eluted as a single peak during HPLC. Thus, the functional impairment observed after mutation of this side chain suggests a direct functional role of tyrosine 124 in receptor binding. The chemically inactive and therefore possibly internal residue tyrosine 56 is less likely to be involved in receptor binding. Thus, its substitution by aspartic acid possibly disturbed the folding of the protein with a concomitant loss in biological activity. This of course has to be established by a more thorough comparison of the conformation of wild-type and mutant proteins.

Acknowledgements: This work was supported by the Bundesministerium für Forschung und Technologie, Vorhaben 0319015B, and by the Fonds der Chemischen Industrie. We are grateful to Biotest, Dreieich and to Dr. U. Schwuléra for continuous support. The skillful technical assistance of Ch. Söder and Ch. Harmening is acknowledged.

REFERENCES

[1] Arai, K.I., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990) Annu. Rev. Biochem. 59, 783-836.
[2] Finkelman, F.D., Holmes, J., Katona, I.M., Urban, J.F., Beckmann, M.P., Park, L.S., Schooley, K.A., Coffman, R.L., Mosmann, T.R. and Paul, W.E. (1990) Annu. Rev. Immunol. 8, 303-333.
[3] Yokota, T., Arai, N., De Vries, J., Spits, H., Banchereau, J., Zlotnik, A., Rennick, D., Howard, M., Takebe, Y., Miyatake, S., Lee, F. and Arai, K.I. (1988) Immunol. Rev. 102, 137-187.
[4] Idzerda, R.L., March, C.J., Mosley, B., Lyman, S.D., Vanden Bos, T., Gimpel, S.D., Din, W.S., Grabstein, K.H., Widmer, M.B., Park, L.S., Cosman, D. and Beckmann, M.P. (1990) J. Exp. Med. 171, 861-873.
[5] Cosman, D., Lyman, S.D., Idzerda, R.L., Beckmann, M.P., Park, L.S., Goodwin, R.G. and March, C.J. (1990) Trends Biochem. Sci. 15, 265-270.
[6] Yokota, T., Otsuka, T., Mosmann, T., Banchereau, J., DeFrance, T., Blanchard, D., De Vries, J.E., Lee, F. and Arai, K.I. (1986) Proc. Natl. Acad. Sci. USA 83, 5894-5898.
[7] Van Kimmenade, A., Bond, M.W., Schumacher, J.H., Laquoi, C. and Kastelein, R.A. (1988) Eur. J. Biochem. 173, 109-114.
[8] Jayaram, B., Devos, R., Guisez, Y. and Fiers, W. (1989) Gene 79, 345-354.
[9] Solari, R., Quint, D., Obray, H., McNamee, A., Bolton, E., Hissey, P., Champion, B., Zanders, E., Chaplin, A., Coomber, B., Watson, M., Roberts, B. and Weir, M. (1989) Biochem. J. 262, 897-908.
[10] Windsor, W.T., Syto, R., Durkin, J., Le, H.V., Tindall, S. and Trotta, P.P. (1990) Biophys. J. 57, 423a.
[11] Carr, C., Aykent, S., Kimack, N.M. and Levine, A.D. (1991) Biochemistry 30, 1515-1523.
[12] Windsor, W.T., Syto, R., Le, H.V. and Trotta, P.P. (1991) Biochemistry 30, 1259-1264.
[13] Windsor, W.T., Syto, R., Durkin, J., Das, P., Reichert, P., Pramanik, B., Tindall, S., Le, H.V., Labdon, J., Nagabhushan, T.L. and Trotta, P.P. (1990) Biophys. J. 57, 423a.
[14] Weigel, U., Meyer, M. and Sebald, W. (1989) Eur. J. Biochem. 180, 295-300.
[15] Kramer, W., Drutsa, V., Jansen, H.-W., Kramer, B., Pflugfelder, M. and Fritz, H.-J. (1984) Nucleic Acids Res. 12, 9441-9456.
[16] Kato, K., Yamada, T., Kawahara, K., Onda, H., Asano, T., Sugino, H. and Kakinuma, A. (1985) Biochem. Biophys. Res. Comm. 130, 692-699.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, New York.
[18] Fasman, G.D. (1976) CRC Handbook of Biochemistry and Molecular Biology, CRC Press, pp. 187-188, Boca Raton, FL.
[19] McCarthy, J.E.G., Schairer, H.U. and Sebald, W. (1985) EMBO J. 4, 519-526.
[20] Stüber, D., Ibrahimi, I., Cutler, D., Dobberstein, B. and Bujard, H. (1984) EMBO J. 3, 3143-3148.
[21] Rose, G.D. and Dworkin, J.E. (1989) in: Prediction of Protein Structure and the Principles of Protein Conformation (Fasman, G.D., ed) Plenum, New York.