Volume 226, number 1, 47–52 FEB 05403 December 1987

Characterization of recombinant glycosylated human interleukin 2 produced by a recombinant plasmid transformed CHO cell line

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Received 2 October 1987; revised version received 2 November 1987

A recombinant plasmid containing expression units for human pre-interleukin 2 (pre-IL-2) and the selectable marker mouse DHFR, was constructed and used to transform $DHFR^-$ CHO cells to the $DHFR^+$ phenotype. Selected colonies were isolated and tested for IL-2 production. Twelve highly IL-2-producing clones were amplified in stepwise increasing concentrations of methotrexate. The IL-2 secreted into the culture medium by one of these clones was purified to homogeneity and partially characterized. N-terminal sequence analysis showed that pre-IL-2 was correctly processed during secretion. SDS gel electrophoresis and chromatofocusing experiments in conjunction with neuraminidase treatment indicated a posttranslational glycosylation of the secreted mature protein similar to that described for the tetrasaccharide structure of the N2 form of natural IL-2. This recombinant IL-2 has a specific activity of 2.5×10^7 U/mg.

Glycoprotein; Interleukin 2; Recombinant lymphokine

1. INTRODUCTION

Interleukin 2 (IL-2) is a lymphokine produced by T-cells after stimulation by mitogens or antigens [1]. This molecule is a key mediator of the growth and functional activity of B- and T-cells and cytotoxic cells including NK and LAK cells [2]. IL-2 has been purified from normal human lymphocyte conditioned medium [3] and from the human lymphoblastoid cell line Jurkat [4]. Several forms were isolated and characterized as being variations in the glycosylation of the same polypeptidic backbone [5]. The importance of these posttranslational modifications in the in vivo activity of the molecule is still unclear [6,7]. Several groups have cloned and expressed the cDNA coding for IL-2 in *E. coli* obtaining large amounts of unglycosylated IL-2 [8–10]. Because of their capacity to glycosylate proteins recombinant eukaryotic systems have also been explored. Infected insect cells that correctly processed pre-IL-2 during secretion, however, were not able to glycosylate the molecule [11]. Recently, the production of rIL-2 by transfected mouse L cells and Chinese hamster ovary (CHO) cells was reported, and in vivo radiolabeling of the rIL-2 produced indicated that those systems could be used as sources of glycosylated rIL-2 [12].

Here, we describe an efficient system for the production of rIL-2 based on transformed CHO cells that correctly secrete large amounts of the matured recombinant protein into the culture medium. Partial characterization of this recombinant protein indicated a glycosylation pattern similar to the tetrasaccharide glycosylated human IL-2 [5]. The availability of large quantities of this rIL-2 may help in understanding the importance of

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

this modification on the structure and biological effects of the molecule.

2. MATERIALS AND METHODS

2.1. *Plasmid construction*

The plasmid pSV 700 was constructed, containing the SV40 early promoter, the coding sequence for human pre-IL-2 isolated from phytohemagglutinin A-stimulated human peripheral lymphocytes and identical to that previously published [13], the second intron of the mouse globin gene [14], and the SV40 early polyadenylation signal. The sequence between *Hin*dIII and *Dde*I was replaced by a synthetic oligonucleotide sequence giving rise to the sequence CCACCATGGCT instead of CCACAATGTAC around the initiator ATG (plasmid pSV 703). A third plasmid containing both the expression units for human IL-2 (from pSV 703) and for the selectable marker mouse DHFR (from pSV2-DHFR [15]) was constructed and termed pSV 720. The constructions are outlined in fig.1.

2.2. *Cell culture, DNA transformation and amplification*

Transient expression assays were performed as in [16]. A DHFR-deficient CHO cell line [17] was propagated in α-medium (Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), 20 μg/ml gentamycin, 60 μg/ml tylocine and 300 μg/ml L-glutamine (non-selective medium).

The DHFR$^-$ CHO cells were transformed to the DHFR$^+$ phenotype with 10 μg plasmid pSV 720 per 10 cm dish, using the calcium phosphate precipitation method [18] and omitting salmon sperm DNA as a carrier. After growth for 3 days in non-selective medium, cells were subcultured at a density of 10^5 cells per 10 cm dish in selective medium: Eagle's minimum essential medium (with Earle's salts, Gibco), containing 10% dialyzed FCS (Gibco), 20 μg/ml gentamycin, 60 μg/ml tylocine (Gibco), 300 μg/ml L-glutamine and 150 μg/ml L-proline. The selective medium was changed every 3 days and after 2 weeks, transformed colonies were isolated.

In order to amplify the IL-2 and DHFR expression units, initial transformants were grown in selective medium containing progressively increasing concentrations of methotrexate [MTX, (+)-amethopterin, Sigma]: 0.02, 0.05, 0.1 and 0.2 μM MTX [17].

2.3. *Production and purification of recombinant IL-2*

Recombinant IL-2 was obtained in suspension culture in spinner flasks using suspension medium (Gibco) with 5% FCS, 0.2 μM MTX and 150 μg/ml proline. 1 l of the culture supernatant was acidified to pH 4.5 with acetic acid and applied directly to an S-fast flow Sepharose (Pharmacia) column (2.5 × 40.0 cm) previously equilibrated in 0.05 M ammonium acetate (pH 4.5). After loading the sample, the column was washed with 0.05 M ammonium acetate (pH 5.5), 0.05 M NaCl, followed by the same buffer containing 0.5 M NaCl. Biologically active IL-2 containing fractions were loaded on an Altex RP C3 column (1.0 × 25.0 cm) and eluted with a gradient of acetonitrile in water, 0.1% trifluoroacetic acid (TFA), from 5 to 30% in 10 min, from 30 to 50% in 60 min and from 50 to 100% in 10 min at a flow rate of 4 ml/min. rIL-2 was finally chromatographed on an Altex RP C18 column (2.1 × 10 cm). Fractions with IL-2 activity were analysed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) carried out according to Laemmli [20]. Proteins were visualized by silver staining (Bio Rad kit) of the gels.

2.4. *Amino acid composition and sequence analysis*

Samples were dried in borosilicate tubes, hydrolyzed with gaseous HCl and analysed as described [21]. For N-terminal sequence analysis purified unmodified IL-2 was loaded onto a polybrene-coated filter and automatically sequenced with a gas-phase protein sequencer equipped with an on-line phenylthiohydantoin-amino acid separation system (470A and 130A, Applied Biosystems, Foster City, CA).

2.5. *Neuraminidase treatment and chromatofocusing analysis*

rIL-2 was incubated with 1.5 U/ml of *V. cholerae* neuraminidase (Calbiochem) for 4 h at 37°C as in [22], and analysed on a Mono P chromatofocusing column (Pharmacia) equilibrated in 25 mM Tris-HCl, pH 8.5, 0.1% polyethylene glycol and eluted with polybuffer 96

(Pharmacia)/water (1:20), pH 5.5, 0.1% polyethylene glycol at a flow rate of 1 ml/min. Fractions of 1 ml were collected and analysed for IL-2 activity.

2.6. *IL-2 biological assay*

The colorimetric assay of Mosmann [23] was used to determine IL-2 biological activity in the proliferation assay of the IL-2-dependent murine cytotoxic T-lymphocyte line CTLL-2 [24]. IL-2 units were referred to the BRMP-Standard Reference Reagent Preparation [25].

3. RESULTS

3.1. *Isolation of highly productive IL-2 cell lines*

The plasmid pSV 700 directed the synthesis of pre-IL-2 and secretion of IL-2 into the culture medium. To increase the efficiency of this plasmid the sequence surrounding the initiator ATG was changed to conform with the consensus sequence CCACC–ATG–G as described by Kozak [26]. As a result the second amino acid of pre-IL-2, tyrosine, was changed to alanine. This new plasmid, pSV 703, was more efficient than the parental plasmid pSV 700 when tested for transient expression (not shown). The plasmid pSV 720 contains both the expression unit for pre-IL-2 from pSV 703 and that for mouse DHFR.

CHO-DHFR$^-$ cells were transformed with the plasmid pSV 720 (fig.1) and subsequently cultured in selective medium. 81 colonies were isolated and tested for their capacity to secrete IL-2 into the culture medium; 78 of those colonies were found to be positive. 12 of the best producing clones (between 1 and 3 $\times$ 10^4 IL-2 units accumulated per dish, in 5 days, with initially 5 $\times$ 10^5 cells per dish) were subjected to increasing MTX concentrations and several highly productive cell lines were obtained; one of those, clone 32, was used for further studies. Clone 32 (5 $\times$ 10^5 cells), seeded in a 6 cm dish with selective medium containing 0.1 μM MTX and cultured for 5 days, produced 2.5 $\times$ 10^5 U vs 1 $\times$ 10^4 U before amplification.

3.2. *Purification and chemical analysis of rIL-2*

Recombinant IL-2 was usually purified from 1 l suspension culture of recombinant CHO clone 32. The rIL-2 was initially concentrated and partially purified by ion-exchange chromatography on an S-

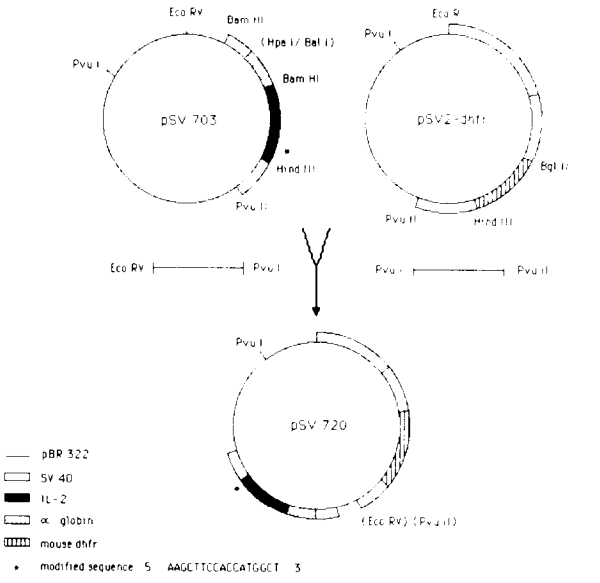

Fig.1. Construction of plasmid pSV 720 using the *Eco*RV-*Pvu*I fragment of pSV 703, containing the expression unit for human pre-IL-2, and the *Pvu*I-*Pvu*II fragment of pSV2-DHFR, containing the selection unit.

fast flow Sepharose column. Biologically active fractions eluted from this column were pooled and finally purified by two HPLC steps. The purity of rIL-2, as assessed from SDS-PAGE, was estimated to be greater than 95% (fig.2A). The overall purification yield was usually better than 40%.

The amino acid composition of rIL-2 is in agreement with that derived from the cDNA and from previously purified natural IL-2 (not shown). Amino acid analysis was the procedure employed for protein quantitation when the specific activity of rIL-2 was determined.

Sequence analysis of the purified material established that the first ten N-terminal residues of the molecule (table 1) are in agreement with the sequence previously reported for the natural product [27]. Alanine is the only residue detected at the amino-terminus, thus showing that pre-IL-2 has been efficiently cleaved at the correct position during secretion. Less than 5% of the expected threonine was recovered in cycle 3 and no other PTH-amino acid was identified in this cycle.

3.3. *Neuraminidase treatment of rIL-2*

Freshly HPLC purified IL-2 shows two major bands on silver-stained SDS-PAGE of 17 and

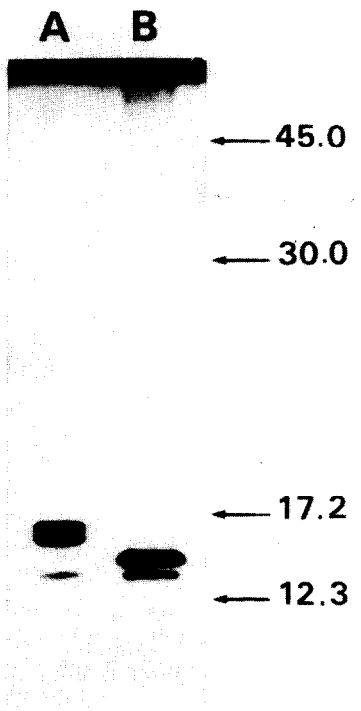

Fig.2. SDS-PAGE analysis of purified rIL-2 before (A) and after (B) neuraminidase treatment. Numbers (kDa) indicate the migration of known proteins.

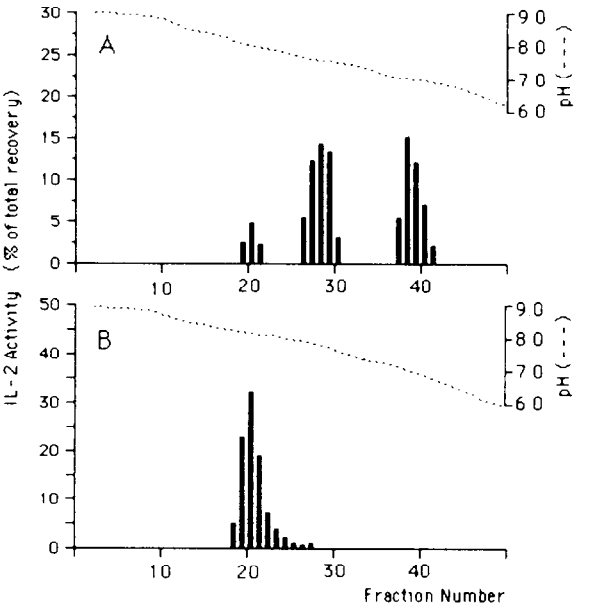

Fig.3. Chromatofocusing analysis of rIL-2 before (A) and after (B) neuraminidase treatment.

16.5 kDa, and a minor one of 15 kDa (fig.2A). Neuraminidase treatment of rIL-2 rapidly increased formation of the 15 kDa component (fig.2B) suggesting that covalently linked sialic acids are present in rIL-2 and responsible for the 17 and 16.5 kDa forms. Complementary information was obtained by chromatofocusing experiments. The purified material shows two main forms of p*I* 7.0 and 7.6. Biological activity in neuraminidase-treated material eluted from the column mainly with a p*I* value of 8.0 (fig.3).

Table 1

N-terminal sequence of CHO-derived IL-2

Cycle	PTH-amino acid		Yield (pmol)
	Expected	Identified	
1	Ala	Ala	288.8
2	Pro	Pro	235.0
3	Thr	X	–
4	Ser	Ser	47.7
5	Ser	Ser	50.7
6	Ser	Ser	42.4
7	Thr	Thr	62.5
8	Lys	Lys	103.4
9	Lys	Lys	124.3
10	Thr	Thr	60.9

Initial yield, 58%; X, no PTH-amino acid identified

4. DISCUSSION

The isolation of CHO cell lines that are highly productive for human IL-2 allows an almost unlimited supply of this lymphokine with a structure close to, or identical to, natural human IL-2 to be made available. Production levels are at least two orders of magnitude higher than those observed after stimulation of human peripheral lymphocytes and one order of magnitude greater than those obtained with highly productive cell lines derived from Jurkat [28]. The advantage of the CHO cell line described here is that the production of IL-2 is continuous and no stimulation is required. The IL-2 production level obtained in this study, up to 5×10^4 U/ml, compares favorably with those previously described, i.e. 2×10^2 U/ml

for transformed CHO cells and 1.5×10^3 U/ml for transformed L cells [12].

rIL-2 was purified from culture medium via a simple and rapid procedure that combined cation-exchange chromatography and reverse-phase HPLC; SDS-PAGE analysis of purified rIL-2 showed two major components of 17 and 16.5 kDa, and a minor one of 15 kDa. The amino acid composition of rIL-2, consistent with that deduced from the cDNA, was used to calculate the concentration of purified material used for IL-2 biological activity assay in a murine IL-2-dependent cytotoxic T-lymphocyte line. The specific activity of this rIL-2, related to the IL-2 Reference Reagent Preparation obtained from the Biological Response Modifier's Program, was 2.5×10^7 U/mg. In a comparative study of six human lymphoid and six recombinant IL-2 preparations, 11 showed specific activities lower than 2.5×10^7 U/mg and one a specific activity greater than this value [29]. Other published specific activities for recombinant *E. coli* derived IL-2 are: 1.0×10^7 U/mg [30] and 0.4×10^7 U/mg [7].

N-terminal sequence analysis showed that pre-IL-2 was correctly processed during secretion. The first 10 Edman degradation cycles yielded a single sequence identical to that expected from the cDNA except for cycle three, where the threonine gave non-quantitative recovery, suggesting post-transcriptional modification at this residue. This threonine has been described as the site of glycosylation in natural and Jurkat IL-2 [5,27]. Further information was obtained from chromatofocusing analysis of rIL-2 where two major forms eluted at p*I* values of 7.0 and 7.6. Treatment of rIL-2 with neuraminidase shifted the biological activity from p*I* 7.0 and 7.6 to p*I* 8.0, and from 17 and 16.5 kDa to 15 kDa, indicating that heterogeneity is related to the presence of sialic acid in the molecule.

These results show that this transformed CHO cell line is able to produce large quantities of an *O*-glycosylated IL-2 of 17 kDa and p*I* 7.0. Similar properties have been described for one form of natural IL-2, N2, whose carbohydrate structure corresponds to o-NeuAc-(2–3)-β-D-Galp-(1–3)-[o-NeuAc-(2–6)]-D-GalNAc [5]. The rIL-2 of 16 kDa and p*I* 7.6 is assigned to a form similar to the natural monosialylated IL-2 N1 [5]. This form may not be produced by the cells but, rather, may result from desialylation of the 17 kDa rIL-2 either in the culture medium and/or during purification, since storage of the culture medium before purification or storage of the purified IL-2 at 4°C in the acidic HPLC eluting buffer resulted in an increased proportion of the 16 kDa form and occasionally also in the production of a 15 kDa form (not shown).

This study shows that large amounts of IL-2 can be obtained by recombinant plasmid transformed CHO cells without obvious alteration of the natural glycosylation pattern. Recently, Pawelec et al. [7] stressed the importance of the glycosylation based on the observation that natural IL-2, a mixture of three different glycosylated molecules, was more effective than *E. coli*-derived IL-2 in the support of the clonal outgrowth of human T-cells. Current work in our laboratory is directed at the full characterization of this CHO-derived rIL-2 and at exploring in detail the biological role of *O*-glycosylation in the half-life and in vivo effects of the molecule.

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