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SECRETION OF GLYCOSYLATED HUMAN INTERLEUKIN-2 BY RECOMBINANT MAMMALIAN CELL LINES*

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ABSTRACT

The production of glycosylated forms of the human T cell growth factor (interleukin-2, IL-2) has been studied after transfection of a mouse L cell line and a chinese hamster ovary cell line with a plasmid containing the human chromosomal interleukin-2 gene. Both cell lines produced IL-2 constitutively. Based on their behavior in reversed-phase l.c. and their sodium dodecyl sulfate–gel-electrophoresis pattern, human IL-2 protein secreted by L cells showed a similar distribution of glycosylated (M_r 16 500) and nonglycosylated (M_r 14 500) forms as the natural protein secreted by human peripheral lymphocytes, whereas the hamster cell line secreted preponderantly the glycosylated forms. Exoglycosidase digestion of the 16 500 M_r IL-2 protein shifted the gel electrophoretic mobility towards the low-molecular weight form as is true for the natural glycosylated IL-2, which contains the usual tetrasaccharide α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAc (IL-2 N_2) and the trisaccharide α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAc (IL-2 N_1) as the major carbohydrate constituents. These results support the applicability of recombinant DNA technology as a tool for studying glycoprotein biosynthesis in mammalian cells.

INTRODUCTION

In many cases, the preparation of proteins of pharmaceutical value is difficult to achieve since for the most part isolation in desirable amounts from natural sources is hampered by their low concentration. Recombinant DNA technology is now widely used to produce such (secretory)proteins, *e.g.*, interferons, lymphokines, and growth factors in prokaryotes in high amounts. Unfortunately, many of these polypeptides are post-translationally modified, *e.g.*, *N*- or *O*-glycosylated in their natural form, a process which is not performed by prokaryotic organisms.

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Absence of glycosylation may impair the proper folding of these proteins and, hence, may cause severe immunological problems when used in clinical trials.

Manipulation of heterologous mammalian cells with defined DNA fragments can alternatively be used for the production of glycosylated proteins[1]. However, it was not clear whether or not a given mammalian cell line, transfected with a desired DNA, is able to perform a glycosylation identical to that obtained with the naturally occurring secretion product.

Human IL-2 is a 133-amino acid protein that is secreted by T lymphocytes after stimulation with antigen or plant lectins. The factor plays an essential role in the complex network of interactions between soluble signal factors and cells of the immune response[2].

Recently, the carbohydrate structures of natural human IL-2 from human, peripheral-blood lymphocytes have been elucidated[3]. The usual tetrasaccharide, α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-D-GalNAc, and the trisaccharide α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-α-GalNAc, have been determined as the major oligosaccharide constituents of natural human IL-2, being *O*-glycosyl-linked to threonine 3 of the polypeptide chain.

We have used mouse L cells and chinese hamster ovary cells (CHO cells), transfected with the human IL-2 gene under control of the SV 40 early promotor, for the production of human IL-2, and have compared the proteins obtained with the naturally occurring factor.

EXPERIMENTAL

Construction of a recombinant plasmid for expression of human IL-2 in mammalian cells. — The human genomic IL-2 gene has been isolated by recombinant screening of a cosmid library[4]. To enhance human IL-2 expression after gene transfer in mouse and hamster cells, the natural 5′-regulatory sequences of the human interleukin-2 gene were replaced by the SV40 early promotor-enhancer region. A detailed description of the plasmid construction will be published elsewhere.

Cell culture, gene transfer, and selection. — Ltk^- cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% of fetal calf serum. CHO-cells (strain DUKX) were maintained in the same medium supplemented with 10 mg/L of adenosine, deoxyadenosine, and thymidine[5]. Gene transfer was carried out by the $Ca_3(PO_4)_2$ coprecipitate method according to Wigler *et al.*[6]. The molar ratios of IL-2, selector, and carrier DNA was 10:1:10. pAG 60, in which the bacterial neomycine-resistance gene is under the control of the Herpes simplex virus thymidine kinase promotor[8], was used as selector DNA. Selection with G 418 was performed at a concentration of 360–700 μg/mL in DUKX and Ltk^- cells, respectively.

Biological activity of IL-2. — Biological activity was determined as described[3].

Units given in this report refer to the BRMP-Standard Reference Reagent Preparation[7].

Metabolic labelling of IL-2. — For labelling with [^{35}S]methionine, confluent monolayers of mouse L cells or CHO cells where grown for 20 h in medium containing 1/10 of the normal methionine content in the presence of [^{35}S]methionine (14.8 MBq/mL, 29.6 GBq/μmol); Amersham). The medium was harvested, authentic human IL-2 (mixture of glycosylated *N*-form and *M*-form; 500 000 units; 30 μg of protein) were added, and IL-2 was purified as described. Labelling of L cells with ^{3}H-sugar was done in DMEM containing 2mM glucose and 18.5 MBq/mL each of D-[6-^{3}H]glucosamine hydrochloride (1.19 GBq/μmol) and D-[1-^{3}H]galactose (3.85 GBq/μmol). The cells were harvested after 24 h, and the medium diluted with 50 mL of unlabelled, L cell-derived IL-2 medium (64 000 units) and purified as described.

Purification of IL-2. —IL-2-containing medium was concentrated 10-fold by use of an immersible CX-filter (cut-off 10 000, Millipore) and applied to a column (2.5 × 95 cm) of Ultrogel AcA54 equilibrated in 150mM NaCl and 20mM K_2HPO_4 (pH 6.8), and eluted at a flow rate of 20 mL/h. IL-2 activity was eluted as a symmetric peak in the region of the marker myoglobin, and respective fractions were pooled, reduced in the presence of 10mM dithioerythritol at pH 8.0 under an Ar atmosphere, and applied to a Spherisorb S5 hexyl column (0.4 × 25 cm). Elution was performed at a flow rate of 0.4 mL/min with a gradient system as described[3].

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis–autoradiography. —$NaDodSO_4$–PAGE was performed as described[9] on 15% polyacrylamide gels. Protein samples were concentrated by precipitation with ice-cold acetone (6 vol.) and kept at −20° for 2 h. After centrifugation, the protein pellet was dissolved in sample buffer containing 10mM dithioerythritol (5 min at 65°). Autoradiography was performed with Kodak X-Omat Film. ^{3}H-sugar labelled IL-2 was isolated from gel slices as described.

RESULTS AND DISCUSSION

Construction of mammalian cell lines producing human IL-2. — Mouse Ltk$^-$ cells and hamster CHO cells were transfected with a plasmid carrying the human chromosomal IL-2 gene under control of the SV40 early promotor, together with selector and carrier DNA. The SV 40 early promotor is constitutively used in both cell types and the presence of a prosequence should lead to the secretion of the protein product. Pools of more than 100 selected cell clones were grown to mass cultures and screened for secretion of IL-2 into the medium. Levels of constitutively produced IL-2 were determined by use of an IL-2-dependent growing mouse cytotoxic T cell line (CTLL) as target cell[3].

High levels of IL-2 were secreted by confluent growing L cells in the presence of 10% fetal calf serum (1600 U/mL/24 h based on the BRMP standard reference

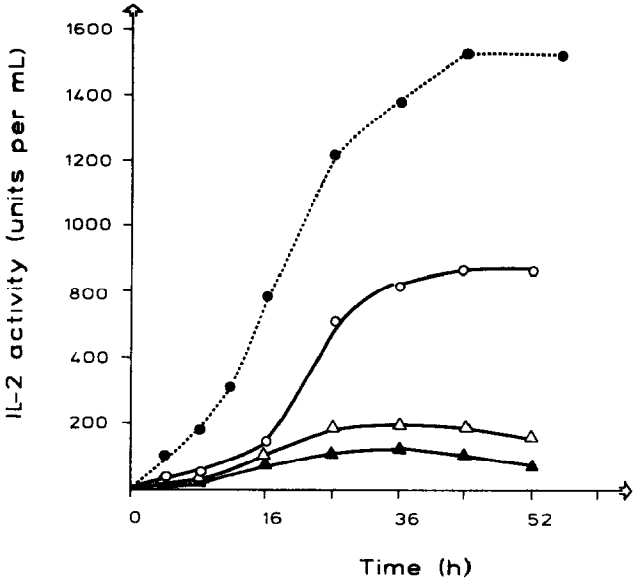

Fig. 1. Secretion of interleukin-2 activity by CHO cells and L cells. Confluent monolayers were grown in DMEM in the presence or absence of 10% fetal calf serum. At the indicated times, aliquots of the medium were tested for IL-2 bioactivity: (●···●···●) IL-2 activity secreted by L cells in the presence and (○—○—○) in the absence of serum; and (△—△—△) IL-2 activity secreted by CHO cells in the presence and (▲—▲—▲) in the absence of serum. Cell densities in the confluent state were 10^6 cells/mL of medium for both cell lines.

reagent). For CHO cells, up to 200 units/mL/24 h were measured. When the calf serum was omitted, IL-2 activity decreased to one half of these values. Secretion rate increased roughly with time over a period of 48 h in the presence of serum and for 30 h without serum (Fig. 1).

Biochemical characterization of IL-2 secreted by CHO cells and mouse L cells. — IL-2 activity produced by CHO cells and L cells was eluted in the same position as natural human IL-2 from peripheral blood lymphocytes when chromatographed on an Ultrogel ACA 54 column. For further analysis of the biochemical properties of IL-2 from supernatants of both cell lines, monolayers were grown in the presence of [^{35}S]methionine and harvested. Authentic human IL-2 (5×10^5 units; mixture of glycosylated IL-2 N and nonglycosylated IL-2 M forms) was added and the protein was purified as described in the Experimental section. The elution profile, after the final reversed-phase l.c., exhibited the typical pattern at A_{280} expected for human IL-2 protein (Fig. 2). The radioactivity profile from IL-2 produced by mouse L cells in the presence of [^{35}S]methionine roughly coincided with the absorbance at 280 nm, suggesting a similar distribution of glycosylated and nonglycosylated IL-2 forms in this material as determined for the protein derived from the natural source[3]. CHO cell-derived radioactive material (as shown in Fig. 2) was preponderantly detected in fractions where the glycosylated forms of authentic human IL-2 are eluted from the hexyl-phase.

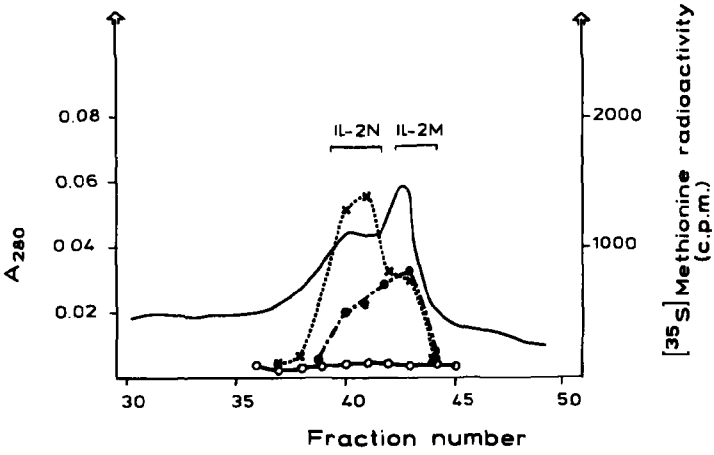

Fig. 2. Elution profiles of [^{35}S]methionine-labeled IL-2 protein from L cells and CHO cells. The supernatants of confluent monolayers were concentrated and gel-filtered as described and subjected to reversed-phase l.c. on hexyl-phase. Aliquots from the fractions were withdrawn for determination of radioactivity: (· X · · X · · X) IL-2 from CHO cells, (· ●— · —●—) IL-2 from L cells, and (○—○—○) radioactivity profile obtained from medium of nontransfected Ltk$^-$ cells which was subjected to an identical purification scheme. For comparison, the absorbance profile at 280 nm obtained for authentic purified human IL-2 is included (——). The bars indicate the positions where the glycosylated IL-2 N forms and the nonglycosylated IL-2 M form were preponderantly detected.

After NaDodSO$_4$–PAGE of the corresponding fractions and subsequent autoradiography, two major radioactive bands representing the M_r 16 500 and 14 500 forms of IL-2 were detected. As shown in Fig. 3, a 1:1 ratio of the M_r 16 500 and 14 500 forms was detected for L cell-derived IL-2, whereas the radioactive protein from the CHO cells showed preponderantly the M_r 16 500 component (~80% of the total radioactivity).

Treatment with *Vibrio cholerae* neuraminidase and β-D-galactosidase of the

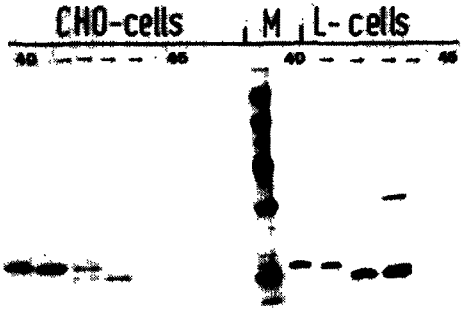

Fig. 3. Autoradiography of [^{35}S]methionine-labelled, l.c.-purified IL-2 protein separated by NaDodSO$_4$–PAGE. Numbers 40–45 refer to the l.c. fractions in Fig. 2. (M) ^{14}C-methylated standard proteins (M_r in parenthesis): phosphorylase b (92 000), bovine serum albumin (67 000), ovalbumin (45 000), carboanhydrase (30 000), and lysozyme (14 500).

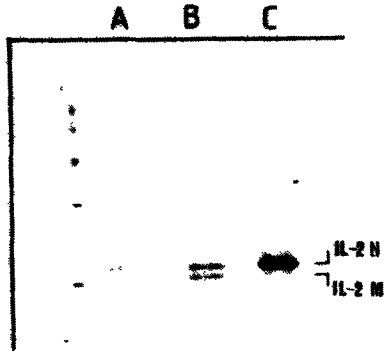

Fig. 4. L.c.-purified, M_r 16 500 IL-2 protein from L cells, labelled with [^{35}S]methionine, was digested with glycosidase(s) and separated by NaDodSO$_4$–PAGE. Radioactivity was detected by autoradiography. At left, standard proteins as in Fig. 3; (A) IL-2 protein treated with β-D-galactosidase; (B) IL-2 protein treated with neuraminidase plus β-D-galactosidase; and (C) untreated protein. Similar autoradiographic patterns were obtained for the CHO cell-derived, M_r 16 500 IL-2 protein. At right, position of the natural glycosylated (IL-2N) and nonglycosylated (IL-2M) forms.

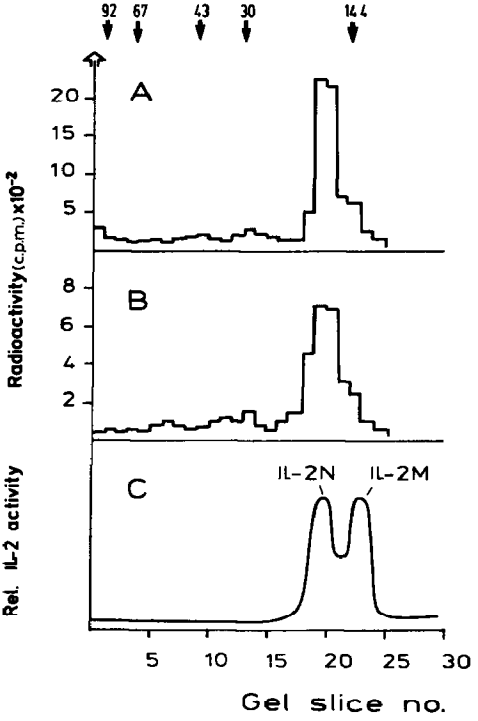

Fig. 5. Distribution of radioactivity obtained from ^{3}H-sugar-labelled IL-2 protein (l.c.-step) secreted by: (A) human peripheral-blood lymphocytes and (B) mouse L cells, after sectioning of a NaDodSO$_4$ gel as indicated; and (C) the biological activity of natural IL-2 eluted from a parallel gel. Arrows indicate the positions of the M_r standards as described in Fig. 3.

TABLE I

GLYCOSIDASE TREATMENT OF ^{3}H-SUGAR-LABELLED INTERLEUKIN-2 SECRETED BY MOUSE L CELLS

Enzyme treatment[a]	*Residual protein-associated radioactivity (%)*
None	100
β-D-Galactosidase	98.8
Neuraminidase[b]	69.4
Neuraminidase plus β-D-galactosidase	53.0

[a]The ^{3}H-sugar-labeled IL-2 was purified from medium of mouse L cells by l.c. and subjected to NaDodSO$_4$–PAGE. The region corresponding to the position of natural glycosylated IL-2 N forms was cut out and radioactive protein was eluted with 75mM Na_2HPO buffer (pH 6.5) containing 0.1% of sodium dodecyl sulfate, 0.5 mg/mL of bovine serum albumin, and 3mM phenylmethanesulfonyl fluoride. Recovered protein was gel-filtered through a small column of Sephadex G-25, precipitated with ice-cold acetone (6 vol.), and collected by centrifugation. For digestion with glycosidase, the protein was resolubilized in the appropriate buffer. After incubation with enzyme(s), the residual radioactive protein was precipitated with acetone and the radioactivity counted after resolubilization. [b]Comparable values were obtained when the IL-2 protein was subjected to mild acid treatment (37.5 mM H_2SO_4, 1 h, 80°)[11]. The radioactive material released by either method was identified as *N*-acetylneuraminic acid upon t.l.c. as described in ref. 14.

l.c.-purified M_r 16 500 protein from either cell line, followed by NaDodSO$_4$–PAGE and autoradiography, led to the appearance of the M_r 14 500 form, though some radioactive material was still detected in the high-molecular-weight band (see Fig. 4). β-D-Galactosidase treatment alone did not alter the NaDodSO$_4$–PAGE pattern of the radiolabelled protein.

Since the GalNAc derivative of IL-2 (which is the major glycosylated IL-2 form derived from the human leukemic T cell line Jurkat[12]) migrates in the region of the nonglycosylated IL-2 form upon NaDodSO$_4$–PAGE, we suggest that the M_r 16 500 protein-band secreted by L cells and CHO cells comprises indeed higher-glycosylated forms similar to those detected in the natural protein.

Purification of IL-2 from L cells grown in the presence of D-[^{3}H]galactose–D-[^{3}H]glucosamine enabled us to further support this suggestion. The ^{3}H radioactivity was almost exclusively found in l.c. fractions corresponding to the naturally glycosylated IL-2 N_1 and IL-2 N_2 forms (data not shown). NaDodSO$_4$–PAGE analysis of the L cell-derived, ^{3}H-sugar-labelled IL-2 revealed that radioactivity migrated as a M_r 16 500 form (see Fig. 5). This material was eluted from the gel and further subjected to glycosidase digestion. Neuraminidase treatment released 30% of the total radioactivity (*cf.* Table I), and subsequent β-D-galactosidase treatment again liberated 20% of the remainder.

Therefore, we conclude that, after transfection with the chromosomal human IL-2 gene, mouse L cells and CHO cells secrete glycosylated IL-2 form(s) that exhibit the same chromatographic properties, in reversed-phase l.c., and have the same mobility in NaDodSO$_4$–PAGE as the glycosylated forms found in natural IL-2 (ref. 3). After digestion with neuraminidase–β-D-galactosidase, part of the 16 500 M_r form decreased to the 14 500 M_r form, as would be expected for mono-

or di-sialylated Gal-GalNAc structures present on the recombinant protein and similar to those found in natural IL-2. Additionally, differently glycosylated forms seem to be present, since the 16 500 M_r protein was not shifted quantitatively into the 14 500 M_r band (*cf.* Fig. 4). Similarly, it has been shown[3] that small amounts of L-Fuc- and GlcNAc-containing carbohydrate structures may also be present in natural human IL-2.

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