The glycoprotein nature and biosynthetic relationship of two molecularly distinct species of primate T-cell growth factor

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T-cell growth factor (TCGF) produced by the MLA 144 gibbon ape T-lymphosarcoma cell line was biosynthetically radiolabelled with [³⁵S]methionine and isolated by reverse-phase h.p.l.c. [Milstone & Parker (1983) *Biochem. Biophys. Res. Commun.* **115**, 762–768]. Two predominant species, of M_r 16300 and pI 6.8 and of M_r 14300 and pI 7.5, were resolved. Analysis of TCGF labelled with a mixture of [³H]glucosamine and [¹⁴C]methionine demonstrated that only the 16300- M_r protein contained detectable carbohydrate label, approx. 50% of which was present in sialic acid residues, which were largely responsible for the charge difference observed between the two forms of TCGF. The 14300- M_r protein was labelled within 5 min after addition of [³⁵S]methionine and was the predominant intracellular form of TCGF, whereas the 16300- M_r protein was not detected until 25 min later and was the predominant extracellular form of TCGF. Pulse-chase experiments were consistent with the hypothesis that the 14300- M_r protein is an intracellular precursor that is glycosylated to form the 16300- M_r protein, which is then preferentially secreted from the cell.

INTRODUCTION

T-cell growth factor (TCGF, also known as Interleukin 2), first described by Gallo and his colleagues (Morgan *et al.*, 1976), is a mitogenic protein produced by normal T lymphocytes on stimulation by lectin or antigen (Smith *et al.*, 1980). It has been shown that the magnitude of a TCGF-driven proliferative response *in vitro* is dependent in part on the appearance of high-affinity cell-surface bindingsites for TCGF and development of responsiveness to this lymphokine by activated lymphocytes (Bonnard *et al.*, 1979; Coutinho *et al.*, 1979; Smith *et al.*, 1979; Robb *et al.*, 1981; Cantrell & Smith, 1983). In contrast, relatively little is known about the structural basis for the molecular heterogeneity of TCGF or about the control of TCGF biosynthesis and secretion (Gillis *et al.*, 1978; Andersson *et al.*, 1979).

In the current paper we report studies identifying sialic acid-containing oligosaccharide on only one of two molecular forms of TCGF produced by the MLA 144 gibbon ape T-lymphosarcoma cell line. In addition, we demonstrate that the non-glycosylated molecule is post-translationally modified to form the glycosylated molecule, which is the predominant species of TCGF secreted into the culture medium.

MATERIALS AND METHODS

Cell culture

The MLA 144 gibbon ape T-lymphosarcoma cell line was maintained in RPMI 1640 medium (Cancer Research Center, Washington University School of Medicine) supplemented with 10% (v/v) heat-inactivated foetal-bovine serum, 2 mM-glutamine, 1 × non-essential amino acids, 100 units of penicillin/ml and 100 µg of streptomycin/ml. Human peripheral-blood mononuclear-cell blasts were generated essentially as described (Gordon *et*

al., 1980), isolated on Ficoll/Hypaque density gradients and utilized in binding incubations as described in the legend to Fig. 4.

Purification of biosynthetically radiolabelled TCGF

MLA 144 cells were washed three times in labelling medium (the growth medium described above lacking methionine and serum) and resuspended at 2×10^6 cells/ml in 200 ml of labelling medium containing 10 µM unlabelled methionine and 100 µCi of L-[³⁵S]methionine (approx. 1000 Ci/mmol; Amersham)/ml. Alternatively, 0.82 µCi of L-[Me-¹⁴C]methionine (55 mCi/mmol; New England Nuclear)/ml and 16.5 µCi of D-[6-³H(n)]glucosamine hydrochloride (31.3 Ci/mmol; New England Nuclear)/ml were substituted for the unlabelled and ³⁵S-labelled methionine. After 48 h at 37 °C in a CO₂/air (1:19) atmosphere the TCGF was purified by reverse-phase h.p.l.c. by a minor modification of our previously described method (Milstone & Parker, 1983).

Isoelectric focusing

Radioactively labelled TCGF was made 9 M with respect to urea by the addition of solid urea. Samples were subjected to isoelectric focusing as described by O'Farrell (1975) except that 105 mm × 5 mm (internal diam.) rods and the following ampholyte composition were used: 0.9% (w/v) Ampholines, pH 7–9, 0.9% (w/v) Ampholines, pH 5–7, and 0.2% (w/v) Ampholines, pH 3.5–10. Slices (2 mm) were each added to 1 ml of NCS tissue solubilizer (Amersham)/water (9:1, v/v), heated at 50 °C for 2 h and analysed by liquid-scintillation counting.

Kinetics of TCGF biosynthesis

MLA 144 cells were washed twice in labelling medium, resuspended at 4×10^6 cells/ml and incubated for 5 min at 37 °C. An equal volume of 37 °C labelling medium

Fig. 1. Effect of mild acid hydrolysis on the isoelectric-focusing profile of purified [^{35}S]methionine-labelled TCGF

TCGF [in 34 μl of 33% (v/v) propan-1-ol/0.1% (v/v) trifluoroacetic acid] was made 25 mM with respect to H_2SO_4 , incubated at 80 $^\circ\text{C}$ for 75 min, cooled to room temperature and analysed by isoelectric focusing as described in the Materials and methods section. The 16300- M_r form of TCGF (a and b) and the 14300- M_r form of TCGF (c and d) are illustrated before (a and c) and after (b and d) hydrolysis. The pH gradient was similar to that illustrated in Fig. 2(a).

containing 5–10 mCi of [^{35}S]methionine/ml was added, and 200 μl portions were cultured for various times at 37 $^\circ\text{C}$ in a CO_2 /air (1:19) atmosphere. Culture tubes were then agitated, chilled and centrifuged at 400 g for 3 min at 4 $^\circ\text{C}$. The cell pellets were solubilized with 40 μl of isoelectric focusing lysis buffer (O'Farrell, 1975) and stored at -60°C . Each culture supernatant was added to 4 μg of bovine serum albumin. Trichloroacetic acid

Fig. 2. Isoelectric-focusing analysis of purified [^3H]glucosamine/[^{14}C]methionine-labelled TCGF

(a) 16300- M_r form of TCGF; (b) 14300- M_r form of TCGF. ●—●, ^3H radioactivity. ○—○, ^{14}C radioactivity. The pH gradient illustrated (●—●) was determined by using a gel run in parallel with those shown in (a) and (b).

was then added to a final concentration of 10% (w/v). After incubation for 30 min at 4 $^\circ\text{C}$ and centrifugation at 12000 g for 15 min at 4 $^\circ\text{C}$ the pellet was solubilized in 40 μl of lysis buffer and stored at -60°C . Two-dimensional polyacrylamide-gel electrophoresis was performed, and TCGF was detected and quantified as described below.

Pulse-chase experiments were initiated by twice washing MLA 144 cells in labelling medium and resuspending them at $30 \times 10^6/\text{ml}$. After a 5 min incubation at 37 $^\circ\text{C}$ an equal volume of warm labelling medium containing 10.7 mCi of [^{35}S]methionine/ml was added. After a further 15 min incubation the culture was diluted to 2×10^6 cells/ml by the addition of 13 vol. of warm labelling medium containing 115 μM unlabelled methionine. Then 200 μl portions were cultured for various times, and the cell pellet and culture supernatant were prepared for two-dimensional polyacrylamide-gel electrophoresis and analysed as described above.

Polyacrylamide-gel electrophoresis

Two-dimensional polyacrylamide-gel electrophoresis was performed essentially as described by O'Farrell (1975) except that the more-basic ampholyte composition described above was used. Fluorography was performed as described by Laskey & Mills (1975).

RESULTS

Purification of TCGF

As described previously (Milstone & Parker, 1983) TCGF from MLA-144 cells can be purified primarily by reverse-phase h.p.l.c., yielding two major species with

Fig. 3. Two-dimensional polyacrylamide-gel electrophoretic analysis of [^{35}S]methionine-labelled MLA 144-cell secreted and cellular proteins

Samples of cellular (a) and secreted (b) proteins from MLA 144 cells cultured with [^{35}S]methionine for 15 min and 60 min respectively were analysed by two-dimensional polyacrylamide-gel electrophoresis and autoradiography. Separate samples of cellular proteins were analysed by two-dimensional polyacrylamide-gel electrophoresis before (c) and after (d) addition of the purified 16300- M_r and 14300- M_r forms of [^{35}S]methionine-labelled TCGF. All second-dimension gels consisted of linear 6–18% gradients of acrylamide, and the positions of M_r standards are indicated. Black arrows indicate the positions of the 16300- M_r and 14300- M_r forms of TCGF, and white arrows identify reference proteins.

apparent M_r values of 16300 and 14300 (in SDS/polyacrylamide-gel electrophoresis). In the present study the effectiveness of the purification was confirmed by autoradiography of SDS/polyacrylamide-gel electrophoretograms and two-dimensional polyacrylamide-gel electrophoresis of [^{35}S]methionine-labelled TCGF (results not shown).

Isoelectric focusing and mild acid hydrolysis

Isoelectric focusing of the purified [^{35}S]methionine-labelled 16300- M_r species revealed a predominant (75% of radioactivity loaded on the gel) protein with apparent pI 6.8 (Fig. 1a). Two minor species, one of slightly larger M_r (results not shown) and pI 6.5 (7%) and one of slightly smaller M_r and pI 7.1 (7%), were also detected. In contrast, isoelectric focusing of the 14300- M_r species revealed only a single (84%) protein of pI 7.5 (Fig. 1c). Its heterogeneous pI suggested that gibbon TCGF might be variably sialylated, as has been proposed for human TCGF (Robb & Smith, 1981). After incubation of the 16300- M_r form of TCGF at pH 1.2 for 75 min at 80 °C, a treatment that has been shown to remove sialic acid residues selectively from glycoproteins (Spiro, 1966), 45% of the ^{35}S radioactivity originally focusing at pI 6.8 (Fig. 1a) was recovered at the more-basic pI 7.5 (Fig. 1b), indistinguishable from that of the 14300- M_r form of TCGF either before (Fig. 1c) or after acid hydrolysis (Fig.

1d). Total recoveries of radioactivity from all regions of the gels were 65–70%.

TCGF labelled with [^3H]glucosamine

When the purified 16300- M_r form of TCGF labelled with [^3H]glucosamine and [^{14}C]methionine was analysed by isoelectric focusing a substantial amount of [^3H]carbohydrate label co-migrated with most of the [^{14}C]protein label of pI 6.8 (Fig. 2a). The $^3\text{H}/^{14}\text{C}$ ratio in this peak is 1.4:1. In contrast, the 14300- M_r form of TCGF contains very little [^3H]carbohydrate label anywhere in the isoelectric-focusing gel (Fig. 2b). Treatment of the 16300- M_r species with neuraminidase before isoelectric focusing resulted in a shift towards more-basic pI values with recovery of a larger absolute amount of [^{14}C]protein label at pI 7.5 (results not shown). The [^3H]carbohydrate/[^{14}C]protein ratio in this species was now reduced to 0.59:1. In contrast, the 14300- M_r species showed no change in its isoelectric-focusing pattern after treatment with neuraminidase.

Identification of TCGF in total MLA 144-cell cellular and secreted proteins resolved by two-dimensional polyacrylamide-gel electrophoresis

Previously described monoclonal antibodies with high binding affinities for human TCGF (Robb *et al.*, 1983; Smith *et al.*, 1983) cannot be used in biosynthetic studies,

Fig. 4. Binding of [^{35}S]methionine-labelled MLA 144-cell-conditioned medium to human peripheral-blood mononuclear cells activated with erythroagglutinating phytohaemagglutinin

Cells were incubated for 60 min at 0 °C with 100 μl of cell-free supernatant obtained by culturing MLA 144 cells for 2 h in the presence of [^{35}S]methionine. Bovine serum albumin and unlabelled methionine were added at final concentrations of 1 mg/ml and 100 μM respectively to this conditioned medium before the binding incubations were performed. After centrifugation at 400 g for 10 min at 4 °C, TCGF was quantified in the supernatants and cell pellets as described in the Materials and methods section. (a) Autoradiographs of two-dimensional polyacrylamide-gel electrophoretograms of supernatants (upper panels) and

Fig. 5. Kinetic analysis of incorporation of [^{35}S]methionine into TCGF produced by the MLA 144 cell line

Cells were cultured with [^{35}S]methionine for the times indicated, and cell pellets and culture supernatants were subjected to two-dimensional polyacrylamide-gel electrophoresis and autoradiography. Only the areas of the gels that contained the 16300- M_r form (arrows farthest to the right in each panel) and the 14300- M_r form (arrows farthest to the left in each panel) of TCGF are illustrated.

as they recognize less than 10% of the TCGF biological activity secreted by normal human peripheral-blood mononuclear cells (Robb *et al.*, 1983). In addition, attempts in our laboratory to remove MLA 144-cell TCGF activity quantitatively with such reagents, as well as attempts to produce heteroantisera directed against TCGF, have been unsuccessful. Therefore, in order to evaluate their biosynthetic relationship, we identified the two forms of TCGF after high-resolution two-dimensional polyacrylamide-gel electrophoresis and studied their labelling kinetics.

Proteins with mobilities identical with those of the two predominant forms of TCGF were readily detected after two-dimensional polyacrylamide-gel electrophoresis of [^{35}S]methionine-labelled MLA 144-cell-secreted proteins (Fig. 3b, black arrows). In contrast, only the 14300- M_r , pI 7.5 protein is easily seen in the sample of intracellular proteins (Fig. 3a). Purified [^{35}S]methionine-labelled TCGF added to these samples before electrophoresis specifically enriched these same two proteins with radioactivity (Figs. 3c and 3d), confirming their physicochemical identity with authentic TCGF.

In order to determine if all of the radioactivity present in these regions of two-dimensional polyacrylamide-gel electrophoresis represented biologically active TCGF, we studied the binding of unfractionated MLA 144-cell-conditioned medium to cells with surface receptors for

TCGF (Fig. 4). During incubation with phytohaemagglutinin-activated cells, the two proteins of M_r 16300 and pI 6.8 and of M_r 14300 and pI 7.5 were specifically and quantitatively depleted from the unbound supernatant and enriched in the bound cell pellet in a dose-dependent pattern (Fig. 4). Binding to cells that had not been activated with phytohaemagglutinin was approx. 100-fold less efficient. Thus the two-dimensional polyacrylamide-gel-electrophoresis system adequately resolves the major labelled TCGF species from all other labelled proteins present in MLA 144-cell-conditioned medium and cell pellets. In subsequent experiments the relative amount of ^{35}S radioactivity present in the two species of TCGF was determined by quantitative microdensitometry and gravimetric or polarimetric integration (Udey & Parker, 1981).

Production and release of biosynthetically radiolabelled TCGF by MLA 144 cells

When MLA 144 cells were labelled with [^{35}S]methionine for 15 min the 14300- M_r protein was easily detected in the cell pellet, whereas much less radioactivity was present in the region of the 16300- M_r protein (Fig. 5). No label was detected at this time in either the 14300- M_r protein or the 16300- M_r protein in the culture supernatant, even after prolonged autoradiographic exposure. The 16300- M_r protein was first detected in the

cell pellets (lower panels) from binding incubations performed with 5×10^5 total cells that had been cultured without (left) or with (right) erythroagglutinating phytohaemagglutinin. Arrows indicate the positions of the 16300- M_r and pI 6.8 and the 14300- M_r and pI 7.5 forms of TCGF. (b) and (c) Densitometric analysis of the gels shown in (a) and of additional gels from the same experiment. ●, Phytohaemagglutinin-activated cells; ○, unactivated cells. —, Bound material recovered in the cell pellet; —, unbound material recovered in the supernatant. (b) 16300- M_r form of TCGF; (c) 14300- M_r form of TCGF. Results are expressed (relative absorbance) as the area derived by integration of quantitative microdensitometric scans of each species of TCGF in a given experimental condition relative to the area derived from scans of that species in [^{35}S]methionine-labelled conditioned medium that was not exposed to cells during the binding incubation.

Fig. 6. Pulse-chase analysis of incorporation of [^{35}S]methionine into TCGF produced by the MLA 144 cell line

Cells were pulse-labelled by culturing with [^{35}S]methionine for 15 min, chased until the times indicated by dilution with medium containing unlabelled methionine, and TCGF was quantified in the cell pellets and culture supernatants as described in the text. ●, 14300- M_r form of TCGF; □, 16300- M_r form of TCGF. —, Cell pellet; ---, culture supernatant. Results are expressed (relative absorbance) as the area derived by integration of densitometric scans of each species of TCGF in a given experimental condition relative to the areas derived from scans of the 14300- M_r species in the cell pellet after 0 min of chase (—) or the sum of the 16300- M_r and 14300- M_r species in the culture supernatant after 180 min of chase (---). The 16300- M_r species in the cell pellet could not be reliably quantified owing to interference from adjacent labelled species.

culture supernatant after 30 min, and radioactivity continued to accumulate in this species for up to 4 h. Labelling of the 14300- M_r protein in the culture supernatant followed a similar time course, but this species contained only approx. 20% as much radioactivity as was present at the same times in the 16300- M_r species.

In pulse-chase experiments (Fig. 6) the amount of radioactivity present in the 14300- M_r protein in the cell pellet immediately began to decline. After a chase for 90–120 min greater than 90% of the label originally present in this species was no longer detectable. Label accumulated simultaneously in the 16300- M_r and 14300- M_r species in the culture supernatant, although, as observed previously, the latter contained much smaller absolute amounts of radioactivity. The total amount of label present in the 16300- M_r and 14300- M_r proteins in the culture supernatant after 180 min of chase is approx. 20% of that initially present in the 14300- M_r protein in the cell pellet immediately after the pulse-labelling period.

DISCUSSION

In this study we demonstrate that most of the TCGF produced by the primate cell line MLA 144 exists as two molecular species that differ markedly in their degree of

glycosylation. A 16300- M_r pI 6.8 species contains sialic acid and incorporates considerable radioactivity from [^3H]glucosamine, whereas a 14300- M_r pI 7.5 species contains no detectable sialic acid and labels very poorly or not at all with [^3H]glucosamine. The data also support a precursor-product relationship, involving intracellular glycosylation and selective secretion of the carbohydrate-rich species. This model is consistent with reports that the human genome contains only a single gene for TCGF (Taniguchi *et al.*, 1983).

Although primate TCGF has previously been shown to be molecularly heterogeneous (Robb & Smith, 1981; Welte *et al.*, 1982; Milstone & Parker, 1983; Henderson *et al.*, 1983), the structural basis for this heterogeneity has not been clear. Although it was originally suggested (Mier & Gallo, 1982) that human TCGF was not glycosylated, a subsequent study (Robb & Smith, 1981), which relied on enzymic degradation of uncontrolled 2-deoxy-D-glucose inhibition experiments, implied the presence of sialylated and N-linked carbohydrate on the protein. This is inconsistent with the lack of a conventional N-linked glycosylation site in the amino acid sequence predicted from cloned copies of the gene and mRNA for human TCGF (Taniguchi *et al.*, 1983). In contrast, our data provide direct evidence demonstrating that only one of the two forms of purified TCGF derived from the MLA 144 cell line contains detectable carbohydrate and that variable sialylation is largely responsible for the different pI values observed for the two forms.

Approx. 20% of the ^{35}S radioactivity initially present in the intracellular 14300- M_r protein was eventually recovered in the extracellular 16300- M_r and 14300- M_r proteins. The fate of the additional intracellular 14300- M_r protein is unclear. The extracellular stability of the 14300- M_r protein, as well as additional control experiments (D. S. Milstone & C. W. Parker, unpublished work), argue against its rapid degradation in the medium subsequent to secretion. We have not detected major differences between the two forms of TCGF in any of the three different T-cell proliferation systems used to date for the detection of primate TCGF in our laboratory. Nonetheless, the paucity of carbohydrate in the 14300- M_r species may effect its stability *in vivo*, distribution or biological half-life.

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