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One of two subunits of masking protein in latent TGF-β is a part of pro-TGF-β

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A high molecular mass latent form of transforming growth factor type-β (TGF-β) was purified to homogeneity from rat platelets by a seven-step procedure involving group-specific affinity chromatographies on Red-Toyopearl and zinc chelating-Sepharose. The purified latent TGF-β was a complex of TGF-β (25 kDa) and the binding protein previously named masking protein (~400 kDa) [(1986) Biochem. Biophys. Res. Commun. 141, 176–184]. Analysis of the peptide structure by gel electrophoresis showed that the masking protein consisted of two subunits of 39 kDa and 105–120 kDa linked by disulfide bonds. N-terminal amino-acid sequencing of the 39 kDa subunit indicated that this subunit was identical to the N-terminal part of the TGF-β precursor.

Latent transforming growth factor-β; Transforming growth factor-β precursor; N-terminal sequencing; (Rat)

1. INTRODUCTION

Transforming growth factor type-β (TGF-β), first detected as a tumor growth factor, has been shown to be a potent effector of proliferation and differentiation of many cell types [1,2]. TGF-β is a ubiquitous protein in mammals and the proteins in various species show high homology. cDNA analysis revealed that human and murine TGF-βs are encoded as precursors of 391 amino acid residues, and after processing, 112 residues of the C-terminal part of the precursor form a homodimer linked by a disulfide bridge, i.e. mature TGF-β [3,4].

TGF-β is synthesized in most cells, but in normal cells and certain transformed cells, secreted TGF-β has no biological activity under physiological conditions [5,6]. This latency is due to its formation of a complex with a high-molecular-mass binding protein that prevents its binding to its cellular receptor [7]. The latent complex is dissociated with the appearance of TGF-β activity on drastic in vitro treatments, such as with 1 N acetic acid or 6 M urea [5,6], but the physiological mechanism of activation of latent TGF-β is unknown.

For understanding the activation mechanism, we investigated the binding protein of TGF-β, and previously reported the partial purification of this protein [8]. We also found that the dissociated binding protein reversibly masked TGF-β activity, and so named this protein 'masking protein' (MP). Here we report the purification of MP from rat platelets in the form of a complex with TGF-β, i.e. latent TGF-β, and show that the smaller of the two subunits of MP is identical to the N-terminal part of the TGF-β precursor. After this work was completed, a report of an independent study was published that also showed that one of the two subunits of the binding protein in latent TGF-β purified to homogeneity from human platelets is identical to the N-terminal part of the TGF-β precursor [9].

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Abbreviations: TGF-β, transforming growth factor type-β; MP, masking protein; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

2.1. *Materials*

Sepharose type chromatography resins and pre-packed HR columns were purchased from Pharmacia LKB Biotechnology (Uppsala). Toyopearl was from Tosoh Co. (Tokyo). The materials used for isolation and culture of adult rat hepatocytes were as described in [10]. [^{125}I]Deoxyuridine, [^{125}I]UdR (2200 Ci/mmol), was obtained from Dupont/NEN Research Products (USA). Recombinant human epidermal growth factor (EGF) was a gift from Earth Chemical Co. (Hyogo).

2.2. *Assay of TGF-β activity*

TGF-β activity was assayed by measuring its inhibition of DNA synthesis in primary cultures of adult rat hepatocytes induced by insulin and EGF as described elsewhere using [^{125}I]UdR [11,12]. The latent TGF-β was activated by acidification of preparations with a final concentration of 0.25% (pH 1.75) trifluoroacetic acid. After incubation at room temperature for 20 min, the preparation was neutralized, diluted with phosphate-buffered saline (PBS), and added with insulin and EGF to hepatocytes in primary culture. All test samples for the bio-assay were supplemented with a final concentration of 2.5 mg/ml of bovine serum albumin (BSA) (98–99% purity, from Sigma, USA) to prevent non-specific adhesion.

2.3. *Purification of latent TGF-β*

Latent TGF-β was obtained from platelets of adult Wistar rats. The platelets were isolated from the blood of 500 rats as described previously [13], and were sonicated in 50 mM Tris-HCl buffer (pH 8.5) supplemented with 0.15 M NaCl, 2 mM $CaCl_2$ and 10 mM Hepes. The preparation was centrifuged at 3000 × *g* for 15 min, and the supernatant was applied to a S-Sepharose Fast Flow equilibrated with the buffer used for sonication. The unadsorbed fraction, which contained latent TGF-β, was collected by the batch method and supplemented with the protease inhibitors described in [14] to prevent degradation of latent TGF-β. The preparation was applied to a Q-Sepharose Fast Flow column (2.6 i.d. × 35 cm) equilibrated with 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl and 1 mM EDTA. The column was washed with the same buffer and then material was eluted on a linear gradient of NaCl in the same buffer. The fraction of eluate from Q-Sepharose containing latent TGF-β was dialyzed against 20 mM phosphate buffer (pH 6.8) containing 0.15 M NaCl, and applied to an AF Red-Toyopearl 650 ml column (2.2 i.d. × 20 cm) equilibrated with the same buffer as for dialysis. The column was washed with the starting buffer and developed with a gradient of 0–10 mM borate buffer (pH 9.2) containing 0.15 M NaCl and 6 M urea. The fraction of eluate containing latent TGF-β was supplemented with a final concentration of 0.5 M NaCl adjusted to pH 8.0, and applied to a Chelating-Sepharose Fast Flow column (1.0 i.d. × 9 cm) to which zinc ion had been bound and which then had been equilibrated with 20 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl. After adsorption of the sample, the column was developed with a gradient from the equilibration buffer to 20 mM citrate-phosphate buffer (pH 4.0) containing 0.5 M NaCl. The eluate from Zn chelating-Sepharose containing latent TGF-β was supplemented with a final concentration of 0.3 M ammonium sulfate and adjusted to pH 8.5. Then it was applied to a phenyl-Sepharose CL-4B column (1.0 i.d. × 6 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.5) containing 0.3 M ammonium sulfate. The column was washed and then developed with a gradient from the equilibration solution to the same solution without ammonium sulfate. Fractions containing latent TGF-β from phenyl-Sepharose were pooled and concentrated to 300 μl by ultracentrifugation in a Centricon-30 (Amicon Co., USA). The concentrated sample was injected into a Superose 12HR 10/30 column equilibrated with PBS at pH 7.4 of a loading rate of 0.3 ml/min. The fractions containing the highly purified latent TGF-β were collected and finally purified on a Mono Q HR 5/5 column. The Mono Q was equilibrated with PBS (pH 7.4) and, after sample application, material was eluted with a gradient of 0.15 M–0.6 M NaCl in the same phosphate buffer.

2.4. *SDS-PAGE*

SDS-PAGE was performed by the method of Laemmli [15]. Samples were heated at 90°C for 5 min in the presence of 1% SDS and 8 M urea at pH 6.8 with or without 2% 2-mercaptoethanol, and then applied to a ready-made gradient slab gel of 4–20% polyacrylamide (Daiichi Pure Chemical Co., Tokyo). Protein on the gel was stained with Coomassie brilliant blue (CBB) R-250 by the method of Burgess [16]. On SDS-PAGE analysis, the molecular mass was calculated from a semilog calibration curve for molecular mass versus distance of

Table 1

Summary of the purification of the latent form of TGF-β

	Total protein[a], mg	TGF-β activity[b], units (yield %)	Specific activity, units/mg (purification fold)
Starting material[c]	1206	25.8×10^6 (100)	2.1×10^4 (1.0)
Q-Sepharose	88	6.0×10^6 (23)	6.8×10^4 (3.2)
Red-Toyopearl	7.2	3.3×10^6 (13)	4.6×10^5 (22)
Zn-Sepharose	0.86	1.5×10^6 (6)	1.7×10^6 (81)
Phenyl-Sepharose	0.34	1.3×10^6 (5)	3.8×10^6 (181)
Superose 12	0.25	1.4×10^6 (5)	5.6×10^6 (267)
Mono Q	0.037	0.27×10^6 (1)	7.3×10^6 (348)

[a] Protein was measured by analysis with dye-reagent (Bio-Rad Lab., USA) except in fractions from Superose 12 and Mono Q, in which it was estimated from its absorption using the approximate formula: protein concentration (mg/ml) = $1.45 \times A_{280} - 0.74 \times A_{260}$ [19]

[b] TGF-β activity was assayed after acid-activation

[c] The starting material was an unadsorbed fraction to S-Sepharose of lysate of platelets from 500 rats

migration obtained with the following marker proteins: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa).

2.5. *N-terminal amino acid sequencing*

Purified latent TGF-β was subjected to SDS-PAGE under reducing conditions, and then transferred to a polyvinylidene difluoride membrane by the method of Matsudaira [17]. After Western blotting, proteins on the membrane were detected with CBB R-250. Stained spots were cut out and their amino acid sequences were determined in an Applied Biosystems model 470A protein/peptide sequencer equipped with an on-line detection system, an Applied Biosystems model 120A phenylthiohydantoin (PTH) analyzer.

3. RESULTS

3.1. *Purification of latent TGF-β*

Previously, we reported purification from rat platelets of the binding protein of TGF-β, named masking protein, in a form dissociated from its complex with TGF-β by treatment with 6 M urea [8]. In the present study, we purified MP as a complex with TGF-β to obtain information about latent TGF-β.

Hepatocyte growth factor (HGF) is present in a lysate of whole platelets [18]. Therefore, the lysate was first subjected to cation-exchange S-Sepharose chromatography to separate latent TGF-β from HGF, and the unadsorbed fraction was used as starting material for purification of latent TGF-β. This material was subjected to the six successive column chromatographies described in section 2 (elution profiles are not shown). Purification of latent TGF-β was followed by monitoring growth inhibitory activity on hepatocytes after activation of TGF-β by acidification of the fractions. At all steps, TGF-β activity was detected after acidification, but not without acidification, indicating that TGF-β was present in the latent form (not shown).

The purification of latent TGF-β from platelets of 500 rats is summarized in table 1. The final yield of purified latent TGF-β was 37 μg.

3.2. *Analysis of subunit structure of latent TGF-β by SDS-PAGE*

The protein components of purified latent TGF-β obtained from Mono Q HR 5/5 were analyzed by SDS-PAGE with CBB R-250 staining (fig.1). Under non-reducing conditions, a band of TGF-β

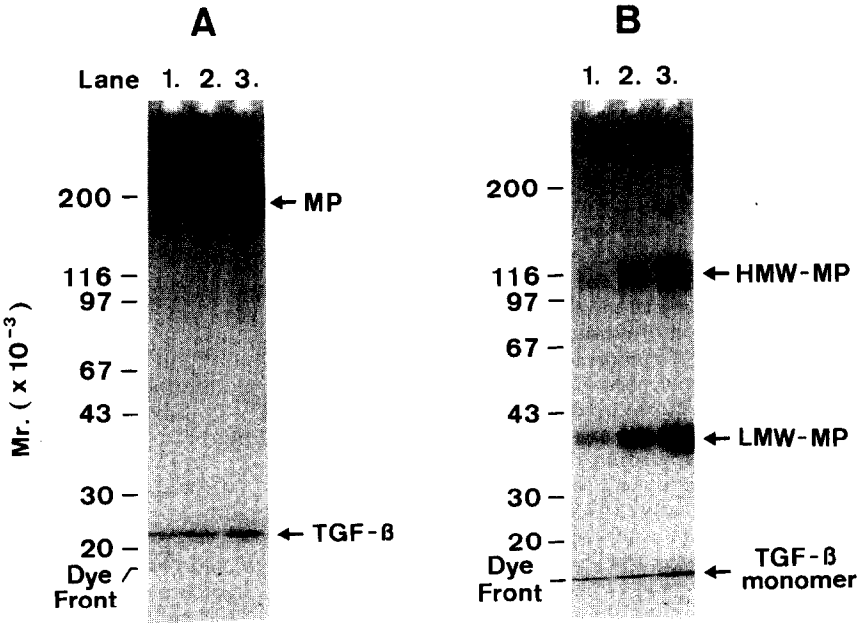

Fig.1. SDS-PAGE analysis of latent TGF-β. Various doses of latent TGF-β were electrophoresed on gradient gel and then analyzed by CBB R-250 staining. Lanes 1–3 were loaded with 1, 2 and 4 μg protein, respectively. (A) Unreduced sample on 4–20% polyacrylamide gel; (B) reduced sample on the same gel. The values on the left indicate positions of marker proteins. HMW and LMW mean high and low molecular mass, respectively, and MP means masking protein.

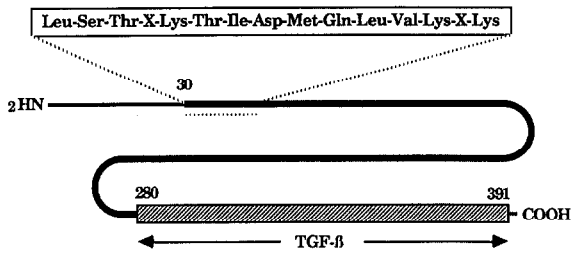

Fig.2. N-terminal amino acid sequence of the 39 kDa subunit of MP. The sequence determined is boxed. The position of this sequence in the precursor of TGF-β is shown diagrammatically. The hatched box represents mature TGF-β after processing.

of 25 kDa was observed, and another large component of 180–210 kDa, which was thought to be a MP, was detected as a slightly broad band (fig.1A). We extracted the 180–210 kDa band from the gel and confirmed that it had a masking activity against TGF-β (not shown). Under reducing conditions, TGF-β was converted to a 12.5 kDa monomer and migrated to the same position as the dye front. Furthermore, a high-molecular-mass band of MP was converted to 105–120 kDa and 39 kDa (fig.1B). These findings indicated that the latent TGF-β consisted of three components: TGF-β of 12.5 kDa, and MP subunits of 105–210 kDa and 39 kDa.

3.3. *N-terminal sequencing of components of latent TGF-β*

The N-terminal amino acid sequence of components of the latent TGF-β was analyzed after Western blotting of a reduced sample. The N-terminal sequence of the 12.5 kDa component was Ala-Leu-Asp-Thr-Asn-Tyr-X-Phe. This sequence was identical to the N-terminus of TGF-β type 1, from murine platelets [4]. The N-terminal sequence of the 39 kDa subunit of MP was Leu-Ser-Thr-X-Lys-Thr-Ile-Asp-Met-Gln-Leu-Val-Lys-X-Lys, which was identical to the N-terminal part of the TGF-β precursor starting 30 residues from the initiation site of the transcribed precursor (fig.2). No PTH-amino acid was detected in the 105–120 kDa subunit of MP, indicating that the N-terminus of this component was blocked.

4. DISCUSSION

Previously, we reported the partial purification of MP dissociated from TGF-β from murine platelets [8]. This partially purified MP caused dose-dependent neutralization of the activity of rat or human TGF-β and was inactivated by reduction with dithiothreitol, but not by heat or acid treatment. In this work, we purified MP to homogeneity as a complex with TGF-β and analyzed its structure. SDS-PAGE analysis under reducing conditions showed that the latent TGF-β was composed of at least three components: TGF-β monomer of 12.5 kDa, and MP subunits of 39 kDa and 105–120 kDa. These two MP subunits formed a complex of 180–210 kDa in which 39 kDa subunits were linked with the 105–120 kDa component by disulfide bonds. The 180–210 kDa protein extracted from the polyacrylamide gel after electrophoresis under non-reducing conditions had a masking activity, but the 39 kDa and 105–120 kDa components in the reduced sample had no activity (not shown). The 180–210 kDa component is thought to be the minimum active unit for masking of TGF-β. Native MP was estimated by gel-filtration chromatography to be a protein of 400–500 kDa [6,8]. The 180–210 kDa component separated by SDS-PAGE in non-reducing conditions is thought to form a dimer with a non-covalent bond(s).

N-terminal sequencing showed that the 39 kDa subunit was identical with the N-terminal part of the TGF-β precursor starting at leucine, residue 30 from the transcription initiation site. The peptide from residue 1–29 of the precursor seems to be a hydrophobic signal peptide that is cut off during processing. The 39 kDa subunit is a little larger than the precursor without the signal peptide and mature TGF-β, which is calculated to be 28 kDa from its amino acid sequence. Because this N-terminal part of the precursor has three *N*-glycosylation sites, the 39 kDa subunit is probably nearly the same as the N-terminal glycoprotein of processed TGF-β. The 39 kDa subunit (N-terminal part) and TGF-β monomer (C-terminal part) are probably bound in a 1:1 molar ratio in latent TGF-β. Therefore, the 39 kDa subunit is thought to form a dimer as well as mature TGF-β. As described above, SDS-PAGE analysis indicated that a dimer of 39 kDa was bound to the 105–120 kDa subunit of MP by a disulfide bond(s). Thus, we propose that one molecule of native latent TGF-β contains two molecules of mature TGF-β and a dimer of the 180–210 kDa

masking protein, which is composed of two 39 kDa subunits and a 105–120 kDa subunit.

Analysis of the partial amino acid sequence of the other MP subunit, 105–120 kDa protein, is still incomplete, but the role of this component in masking and releasing TGF-β is very interesting. We are now studying the primary structure of the 105–120 kDa subunit of MP by the molecular cloning technique. Further structural analysis of MP as a regulatory protein for TGF-β should provide information on the physiological mechanism of activation of latent TGF-β.

REFERENCES

[1] Sporn, M.B., Roberts, A.B., Wakefield, L.M. and De Crombrugghe, B. (1987) J. Cell Biol. 105, 1039–1045.
[2] Massaqué, J. (1987) Cell 49, 437–438.
[3] Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B. and Goeddel, D.V. (1985) Nature 316, 701–705.
[4] Derynck, R., Jarrett, J.A., Chen, E.Y. and Goeddel, D.V. (1986) J. Biol. Chem. 261, 4377–4379.
[5] Pircher, R., Lawrence, D.A. and Jullien, P. (1984) Cancer Res. 44, 5538–5543.
[6] Lawrence, D.A., Pircher, R. and Jullien, P. (1985) Biochem. Biophys. Res. Commun. 133, 1026–1034.
[7] Wakefield, L.M., Smith, D.M., Masui, T., Harris, C.C. and Sporn, M.B. (1987) J. Cell Biochem. 105, 965–975.
[8] Nakamura, T., Kitazawa, T. and Ichihara, A. (1986) Biochem. Biophys. Res. Commun. 141, 176–184.
[9] Miyazono, K., Hellman, U., Wernstedt, C. and Heldin, C.H. (1988) J. Biol. Chem. 263, 6407–6415.
[10] Tanaka, K., Sato, M., Tomita, Y. and Ichihara, A. (1978) J. Biochem. 84, 937–946.
[11] Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K. and Ichihara, A. (1985) Biochem. Biophys. Res. Commun. 133, 1042–1050.
[12] Nakamura, T., Arakaki, R. and Ichihara, A. (1988) Exp. Cell Res., in press.
[13] Nakamura, T., Teramoto, H. and Ichihara, A. (1986) Proc. Natl. Acad. Sci. USA 83, 6489–6493.
[14] Massagué, J. (1985) J. Biol. Chem. 260, 7059–7066.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Burgess, R.R. (1969) J. Biol. Chem. 244, 6168–6176.
[17] Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.
[18] Nakamura, T., Nawa, K., Ichihara, A., Kaise, N. and Nishino, T. (1987) FEBS Lett. 224, 311–316.
[19] Kalckar, H.M. (1947) J. Biol. Chem. 167, 461–475.