Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 176-184

Partial Purification and Characterization of Masking Protein for β-Type Transforming Growth Factor from Rat Platelets[1]

Toshikazu Nakamura, Toshiki Kitazawa, and Akira Ichihara

Institute for Enzyme Research, School of Medicine,
University of Tokushima, Tokushima 770, Japan

Received October 20, 1986

SUMMARY: β-Transforming growth factor(TGF-β) is stored in platelets and secreted as a high molecular weight latent form associated with a carrier protein of about 440 KD. This carrier protein could be separated from TGF-β in 1 N acetic acid and could again mask the activity of TGF-β under neutral conditions. Therefore, it was named the masking protein of TGF-β. The masking protein was separated from TGF-β by gel filtration on a Sephacryl S-300 column or by anion-exchanger FPLC on a Mono Q column in the presence of 6 M urea. Partially purified masking protein from rat platelets neutralized the activity of TGF-β dose-dependently and was effective at 0.3 μg/ml. This masking protein could also mask the activity of human TGF-β, suggesting that it was not species specific. The masking protein was a heat- and acid-stable protein, but was inactivated by treatment with dithiothreitol. The Physiological role of the masking protein in the mechanisms of wound healing and liver regeneration is discussed.

Many investigators have studied the mechanism of liver regeneration and growth control of mature hepatocytes using primary cultures of adult rat hepatocytes, since these cells can proliferate in vitro when cultured at low cell density in culture medium supplemented with proline, insulin and epidermal growth factor (EGF) [1-10]. We have reported partial purification of a potent growth factor for adult rat hepatocytes, hepatocyte growth factor(HGF), from the serum of rats after partial hepatectomy[11]. Subsequently, we found that rat platelets contained a high concentration of HGF[12-14]. Recently we succeeded in purification of HGF to homogeneity from rat platelets and demonstrated that it is a new growth factor clearly differing from platelet derived growth factor(PDGF) [14].

[1] This work was supported by Grants for Cancer Research from the Ministry of Education, Science and Culture of Japan.
Abbreviations:TGF, transforming growth factor;EGF, epidermal growth factor;HGF, hepatocyte growth factor;PDGF, platelet derived growth factor;PDGI, platelet derived growth inhibitor;PBS, phosphate buffered saline;BSA, bovine serum albumin.

0006-291X/86 $1.50

Moreover, we found that rat platelets contained not only growth factors, but also potent growth inhibitors of adult rat hepatocytes.

Rat platelets contain two types of growth inhibitor, named platelet derived growth inhibitor(PDGI)-α and -β[15]. PDGI-β is a heat- and acid-stable factor with a molecular weight of 25 KD that is secreted from platelets on their treatment with thrombin. Recently, we purified PDGI-β extensively and identified it as TGF-β. PDGI-β(TGF-β) is secreted in a latent form in which its activity is masked, because secreted form becomes active when heated in boiling water for 5 min or treated with 1 N acetic acid.

In this paper we reported partial purification from rat platelets of the masking protein, a carrier protein, of TGF-β that neutralizes TGF-β activity. This masking protein was heat- and acid-stable protein with a molecular weight of about 440 KD.

MATERIALS AND METHODS

Materials --- The materials used for isolation and culture of adult rat hepatocytes were as described[16]. Recombinant human EGF was a gift from Earth Chemical Co., Akoh. Insulin, aprotinin and pure thrombin from bovine plasma were purchased from Sigma Chemical Co. Sephacryl S-300, Mono-S and -Q columns were from the Pharmacia, Uppsala. [Methyl-^{3}H]thymidine(52.4 Ci/mmol) was from New England Nuclear. Human TGF-β was obtained from Biomedical Technology Inc.

Assay of TGF-β activity --- Two independent methods were used to measure TGF-β activity. One was assay of inhibition of DNA synthesis of adult rat hepatocytes in primary culture by TGF-β. Adult rat hepatocytes were isolated and cultured as monolayers as reported before[7, 16]. Insulin(10^{-7}M), EGF(20 ng/ml), TGF-β or test samples were added to the cultures 20 h after plating and 15 h later [^{3}H]thymidine(2.5 μCi/ml, 0.3 μCi/mmol) was added with or without aphidicolin(10 μg/ml). Then 24 h later, DNA synthesis was assayed by measring incorporation of [^{3}H]thymidine as described before[7, 8]. The other method was assay of colony formation of NRK 49F cells in soft agar. NRK 49F cells, obtained from the Americal Type Culture Collection, were maintained in Dulbecco's modified medium containing 5% fetal calf serum. Colony formation in soft agar was assayed in the presence of EGF(2 ng/ml) as described previously[17].

Assay of masking protein activity --- Test samples containing masking protein were incubated in 50 μl of phosphate buffered saline(PBS) containing 2.5 mg/ml bovine serum albumin(BSA) and 2 ng/ml rat or human TGF-β(maximum effective dose) at room temperature for 5 min. TGF-β activity in the resulting mixture was assayed by measuring inhibition of DNA synthesis of hepatocytes or stimulation of colony formation of NRK 49F cells. One unit of activity of masking protein was defined as the amount causing 50% recovery of TGF-β activity to inhibit completely DNA synthesis of primary cultured hepatocytes. The specific activity of masking protein was expressed as units per mg protein, protein being measured by the method of Lowry et al.[18].

Preparation of platelet extract --- Adult rat blood was collected in syringes containing 0.1 volume of 0.15 M NaCl-77 mM EDTA(pH 7.4). The blood was centrifuged at 200 x g for 15 min and the resulting supernatant was recentrifuged at 2,500 x g for 15 min to precipitate platelets. The precipitate was washed twice with PBS by centrifugation, yielding a platelet preparation of over 99% purity as judged by microscopic examination. The platelets were suspended in PBS and

treated with 2 U/ml of thrombin for 10 min at room temperature and then aggregates of platelets were precipitated by centrifugation at 2,500 x g for 10 min. The resulting supernatant was used as the starting material for purification of masking protein for TGF-β.

Gel filtration on a Sephacryl S-300 column --- Before gel filtration, the platelet extract was passed through a Mono-S column(1 x 10 cm) which had been equilibrated with 50 mM Tris-HCl buffer(pH 8.5) containing 0.15 M NaCl, 10 mM Hepes and 2 mM $CaCl_2$. The flow-through fractions were pooled and concentrated by ultrafiltration with a PM 10 membrane(Amicon). The concentrated materials (4.3 ml, 1.77 mg protein) was dialyzed against 100 ml of PBS containing 6 M urea overnight to dissociate the masking protein from its complex with TGF-β. The dialysate was applied to a Sephacryl S-300 column(2.6 x 85 cm) previously equilibrated with PBS containing 6 M urea and eluted with the same solution at a flow rate of 26 ml/h. For assays of the activities of TGF-β and its masking protein, 500 µl aliquots of all fractions were supplemented with a solution of BSA(2.5 mg/ml), dialyzed against PBS overnight and sterilized by filtration through a 0.22 µm filter(Millex-GV, Milipore).

Anion-exchanger FPLC on a Mono-Q column --- The extract(1,000 ml) of platelets from 700 rats was concentrated to approximately 200 ml by ultrafiltration on a PM 10 membrane. The concentrated samples(147 mg protein) were equilibrated with 25 mM Tris-HCl buffer(pH 8.5) containing 0.025 M NaCl and 6 M urea by overnight dialysis and applied to a Mono-Q column(1 x 10 cm) previously equilibrated with the same buffer. Elution was carried out at a flow rate of 120 ml/h with three successive gradients of NaCl in the same buffer:0 - 0.25 M for 10 min, 0.25 - 0.55 M for 60 min and 0.55 - 1.0 M for 10 min. Aliquots(100 µl) of all fractions were diluted 5 times with a solution of BSA(2.5 mg/ml) and dialyzed against PBS to remove urea. Then they were sterilized through Millex-GV and their activities of TGF-β and its masking protein were assayed.

Treatments of partially purified masking protein --- Samples of 50 µl(7.4 µg protein) of partially purified masking protein obtained by Mono-Q FPLC were treated with trypsin(10 µg/ml, at 37°C for 2 h), dithiothreitol(50 mM, at 25°C for 2 h), or 1 N acetic acid(at 25°C for 2 h) or heated(in boiling water for 3 min). Digestion of the masking protein with trypsin was stopped by adding phenylmethylsulfonyl fluoride at a final concentration of 1 mM. After these treatments the samples were supplemented with BSA(2.5 mg/ml) and dialyzed against PBS overnight.

RESULTS

We showed previously that PDGI-β(TGF-β) is secreted in a latent form from rat platelets on their treatment with thrombin[15]. This latent form of TGF-β could be activated by heating in boiling water for 5 min, or treatment with 1 N acetic acid or 6 M urea. In this work we attempted to isolate and characterize the carrier protein. For this, the materials released from rat platelets by treatment with thrombin were treated with 6 M urea and then applied to Sephacryl-S-300 column which had been equilibrated with 6 M urea. Fig. 1 shows that TGF-β, which inhibits DNA synthesis of hepatocytes, was separated from its carrier protein by this gel filtration. TGF-β was eluted in a position corresponding to a molecular weight of 25 KD, which corresponds to that of human TGF-β, whereas its carrier protein, which was assayed by measuring neutralization of TGF-β activity, was eluted in the same position as ferritin(440 KD). We named this

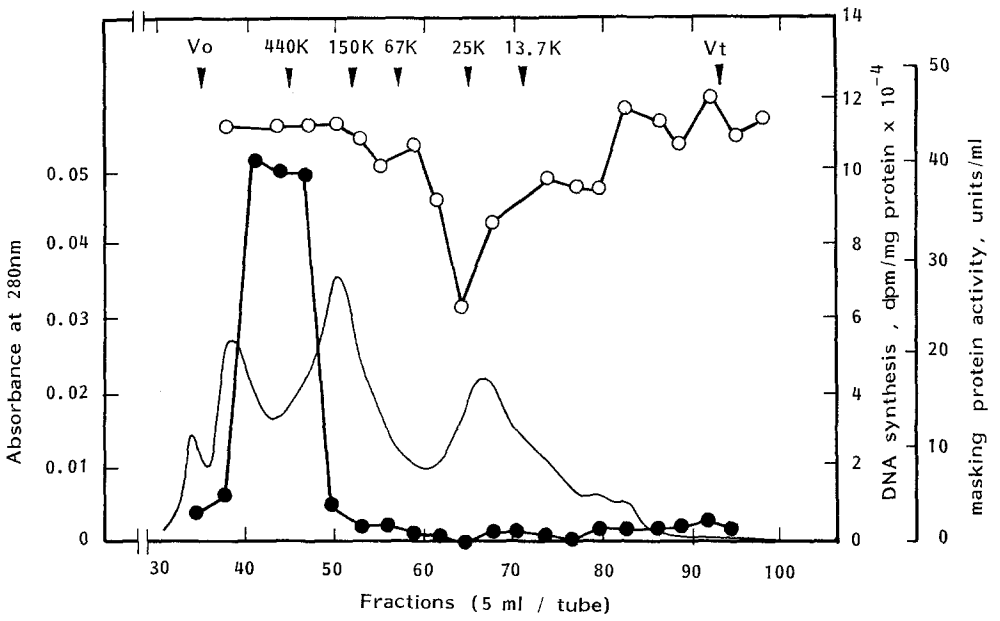

Fig. 1. Gel filtration of platelet extract on a Sephacryl S-300 column in the presence of 6 M urea. As described in MATERIALS AND METHODS, platelet extracts were prepared by passing materials released from platelets through a Mono-S column to remove HGF. The concentrated sample in 6 M urea was applied to a Sephacryl S-300 column. Activities of TGF-β and its masking protein were assayed as described in MATERIALS AND METHODS. The following markers of molecular weight were used:ferritin(440 KD), IgG(150 KD), BSA(67 KD), chymotrypsinogen A(25 KD) and RNase A(13.7 KD). O, TGF-β;●, masking protein;——, A_{280} nm.

carrier protein the masking protein of TGF-β, since it masked the activity of TGF-β within a few minutes when incubated with it in neutral conditions(data not shown).

TGF-β could also be separated from its masking protein by anion-exchanger FPLC on a Mono-Q column. Fig. 2 shows the elution pattern of TGF-β and its masking protein. TGF-β was eluted with 0.25 M NaCl, whereas its masking protein was eluted with 0.4 M NaCl. This step of purification of the masking protein was very effective, increasing its purity to about 70-fold that of the material released from rat platelets. Fig. 3 shows that this purified masking protein was effective at 0.3 μg/ml and was maximally effective at 1.2 μg/ml. At its maximally effective dose, the masking protein can almost completely neutralize the inhibition by TGF-β of DNA synthesis of cultured adult rat hepatocytes stimulated by insulin plus EGF.

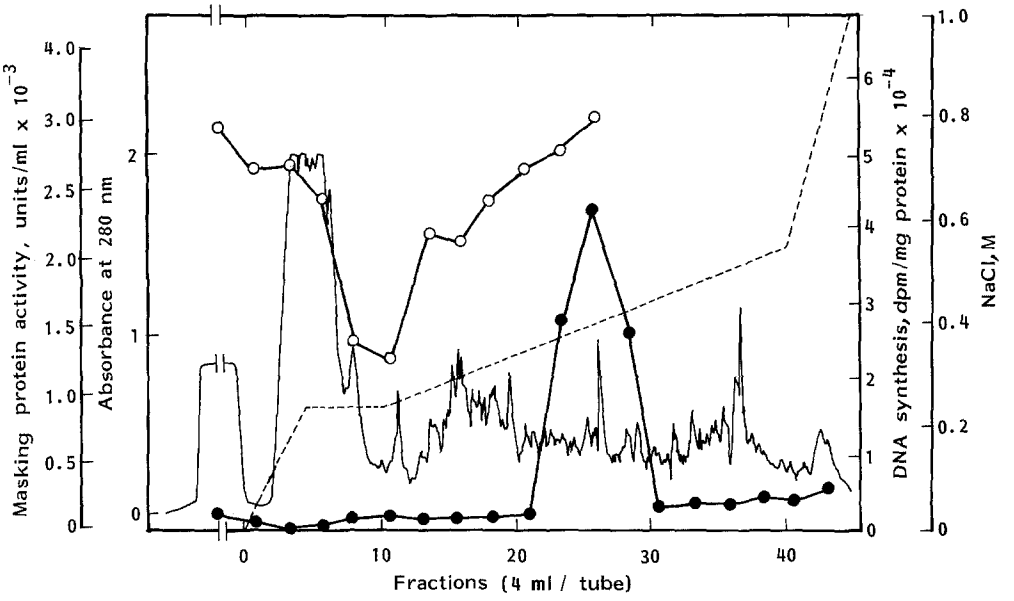

Fig. 2. Anion-exchanger FPLC of platelet extract on a Mono-Q column in the presence of 6 M urea. The concentrated sample in 6 M urea was applied to a Mono-Q column and eluted with three successive gradients of NaCl as described in MATERIALS AND METHODS. O, TGF-β;●, masking protein;—, A_{280} nm;---, NaCl concentration.

Previously we showed that pure human TGF-β strongly inhibited DNA synthesis of adult rat hepatocytes in primary culture[17]. Therefore, we next examined whether partially purified masking protein from rat platelets could mask the

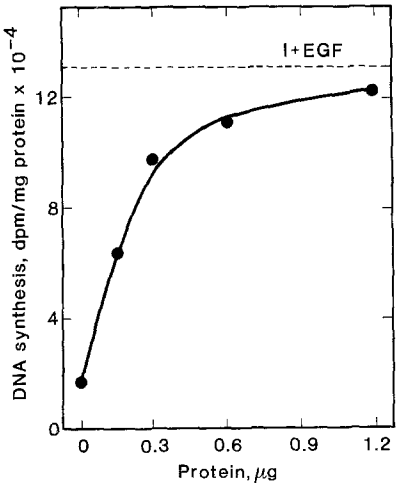

Fig. 3. Dose-dependence of effect of masking protein on TGF-β activity. Partially purified masking protein obtained by Mono-Q FPLC was diluted with BSA solution(2.5 mg/ml) and sterilized. Then masking protein activity was assayed at the indicated concentrations. The dotted line indicates DNA synthesis of hepatocytes by insulin and EGF without addition of TGF-β. Values are means for duplicate dishes.

Table I. Neutralization of activity of human and rat TGF-β by masking protein from rat platelets

Additions	DNA synthesis of hepatocytes (dpm/mg protein x 10^{-4})	Colony formation of NRK cells (colonies/dish)
None	0.58 ± 0.07	0
Insulin + EGF	5.50 ± 0.47	8 ± 14
Insulin + EGF + rat TGF-β	0.96 ± 0.19	557 ± 84
Insulin + EGF + rat TGF-β + masking protein	5.99 ± 0.96	0
Insulin + EGF + human TGF-β	1.05 ± 0.13	651 ± 150
Insulin + EGF + human TGF-β + masking protein	5.99 ± 1.13	4 ± 7

Concentrations of compounds: insulin (10^{-7}M), EGF (20 ng/ml), TGF-β (1 ng/ml) and masking protein (1 μg/ml). Values are means ± SD for triplicate dishes.

activity of human TGF-β. Table I shows that the masking protein from rat platelets also neutralized the activity of human TGF-β. Therefore, neither TGF-β nor its masking protein shows species-specificity. It is noteworthy that the activities of TGF-β for inhibition of DNA synthesis of hepatocytes and stimulation of colony formation of NRK cells in soft agar were neutralized equally by this masking protein.

Table II shows the effects of various treatments on the activity of masking protein purified by Mono-Q FPLC. The masking protein was heat- and acid-stable, but its activity was completely lost on its treatment with 50 mM dithiothreitol, suggesting that disulfide bridges are required for its activity. It was also inactivated by digestion with trypsin.

DISCUSSION

TGF-β is known to stimulate anchorage-independent growth of NRK 49F fibroblasts and AKR-2B cells in soft agar in the presence of TGF-α or EGF[19, 20], and also to inhibit anchorage-dependent growth of many cell lines in monolayer cultures[21, 22]. It also inhibits growth in primary cultures of adult rat hepa-

Table II. Effects of various treatments on activity of masking protein

Treatment of masking protein	DNA synthesis of hepatocytes (dpm/mg protein x 10^{-4})	Remaining activity (%)
No addition	1.93 ± 0.31	
Insulin(Ins) + EGF	13.26 ± 0.41	
Ins + EGF + rat TGF-β(TGF)	1.71 ± 0.05	
Ins + EGF + TGF + masking protein(untreated)	10.70 ± 0.79	100
Ins + EGF + TGF + masking protein(100°C for 3 min)	11.08 ± 0.64	100
Ins + EGF + TGF + masking protein(1 M acetic acid at 25°C for 2 h)	8.89 ± 0.45	80
Ins + EGF + TGF + masking protein(50 mM dithiothreitol for 2 h)	1.74 ± 0.11	0
Ins + EGF + TGF + masking protein(10 μg trypsin for 2 h at 37°C)	1.97 ± 0.71	0

Concentrations of all hormones used were as described in Table I. Values are means ± SD for triplicate dishes.

tocytes, which retain many liver specific functions in vivo[17, 23]. Previously we found that rat platelets contain two types of inhibitor of growth of adult rat hepatocytes in primary culture[15]:PDGI-α, which is a heat- and acid-labile protein, and PDGI-β, which is a heat- and acid-stable factor with a molecular weight of 25 KD. Recently, Brown and Clemmons[24] also reported that human platelets contain a heat-labile growth inhibitor of endothelial cells with a molecular weight of 35-40 KD.

We found that PDGI-β is released from platelets in a latent form, in which its activity is masked[15]. More recently, we purified PDGI-β to homogeneity and identified it as TGF-β, judging by its amino acid composition and N-terminal sequence(to be published). Pircher et al.[25] also reported that TGF-β is stored in human platelets as a latent high molecular weight complex.

In the present work, we separated a protein, that is bound to TGF-β and masks its activity. We then purified this masking protein and found that it was a heat- and acid-stable protein with a molecular weight of about 440 KD. Recently

we found that this masking protein showed high affinity to concanavalin A and was dissociated by addition of α-methylmannoside, indicating that the masking protein is a glycoprotein(to be published). This masking protein could reassociate with TGF-β in neutral conditions and again mask the activity of TGF-β. This protein showed dose-dependent masking activity;it was effective at 0.3 μg/ml and maximally effective at about 1.2 μg/ml.

An important problem is why in the process of wound-healing, including liver regeneration, cell proliferation stops when the injury is repaired. When liver regeneration is complete, TGF-β may be involved in the mechanism of stopping hepatocyte growth, besides due to cell-cell contact that we suggested before [8, 26, 27]. However, on activation of platelets, TGF-β is secreted in a latent form that is associated with masking protein, together with growth factors such as PDGF and HGF. The inactive TGF-β-masking protein complex may be activated in wound tissue after cell proliferation is complete and the active TGF-β released from its complex may stop cell growth to prevent tissue overgrowth during wound healing. That is, the masking protein may be a regulator of the activity of TGF-β:TGF-β may become active on its release from the masking protein whether by limited proteolysis or acidification in the micro-environment in the injury tissue. Further purification and characterization of this protein should provide information on the mechanism of wound-healing and liver regeneration.

REFERENCES

1. Koch, K.S. & Leffert, H.L.(1980) Ann. N.Y. Acad. Sci. 349, 111-127.
2. Tomita, Y., Nakamura, T. & Ichihara, A.(1981) Exp. Cell Res. 135, 363-371.
3. McGowan, J.A., Strain, A.J. & Bucher, N.L.R.(1981) J. Cell Physiol. 108, 353-363.
4. Michalopoulos, G., Cianciulli, H.D., Novotony, A.R., Kligerman, A.D., Strom, S.C. & Jirtle, R.L.(1982) Cancer Res. 42, 4673-4682.
5. Hasegawa, K., Watanabe, K. & Koga, M.(1982) Biochem. Biophys. Res. Commun. 104, 259-265.
6. Strain, A.J., McGowan, J.A. & Bucher, N.L.R.(1982) In Vitro 18, 108-116.
7. Nakamura, T., Tomita, Y. & Ichihara, A.(1983) J. Biochem. 94, 1029-1035.
8. Namamura, T., Yoshimoto, K., Nakayama, Y., Tomita, Y. & Ichihara, A.(1983) Proc. Natl. Acad. Sci. U.S.A. 80, 7229-7233.
9. Nakamura, T., Teramoto, H., Tomita, Y. & Ichihara, A.(1984) Biochem. Biophys. Res. Commun. 122, 884-891.
10. Nakamura, T. & Ichihara, A.(1985) Cell Struct. Funct. 10, 1-16.
11. Nakamura, T., Nawa, K. & Ichihara, A.(1984) Biochem. Biophys. Res. Commun. 122, 1450-1459.
12. Russell, W.E., McGowan, J.A. & Bucher, N.L.R.(1984) J. Cell. Physiol. 119, 183-192.
13. Paul, D. & Piasecki, A.(1984) Exp. Cell Res. 154, 95-100.

14. Nakamura, T., Teramoto, H. & Ichihara, A.(1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6489-6493.
15. Nakamura, T., Teramoto, H., Tomita, Y. & Ichihara, A.(1986) Biochem. Biophys. Res. Commun. 134, 755-763.
16. Tanaka, K., Sato, M., Tomita, Y.& Ichihara,A.(1978)J. Biochem. 84, 937-946.
17. Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K. & Ichihara, A. (1985) Biochem. Biophys. Res. Commun. 133, 1042-1050.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J.(1951) J. Biol. Chem. 193, 265-275.
19. Roberts, A.B., Frolik, C.A., Anzano, M.A. & Sporn, M.B.(1983) Fed. Proc. 42, 2621-2626.
20. Assoian, R.K., Komoriya,A., Meyers, C.A., Miller, D.M. & Sporn, M.B.(1983) J. Biol. Chem. 258, 7155-7160.
21. Tucker, R.F., Shipley, G.D., Moses, H.L. & Holley, R.W.(1984) Science 226, 705-707.
22. Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F. & Sporn, M.B.(1985) Proc. Natl. Acad. Sci. U.S.A. 82, 119-123.
23. Hayashi, I. & Carl, B.I.(1985) J. Cell. Physiol. 125, 82-91.
24. Brown, M.T. & Clemmons, D.R.(1986) Proc. Natl. Acad. Sci. U.S.A. 83, 3321-3325.
25. Pircher, R., Jullien, P. & Lawrence, D.A.(1986) Biochem. Biophys. Res. Commun. 136, 30-37.
26. Nakamura, T., Nakayama, Y. & Ichihara, A.(1984) J. Biol. Chem. 259, 8056-8058.
27. Nakamura, T., Nakayama, Y., Teramoto, H., Nawa, K. & Ichihara, A.(1984) Proc. Natl. Acad. Sci. U.S.A. 81, 6398-6402.