

The mode of actions of glyceraldehyde-3-phosphate dehydrogenase identified as an immunoglobulin production stimulating factor

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Abstract Immunoglobulin production stimulating factor-II α , which enhances immunoglobulin production of human and mouse hybridomas was purified from cell lysate of human Burkitt's lymphoma, Namalwa cells, and identified as glyceraldehyde-3-phosphate dehydrogenase. The enhancement of immunoglobulin production with this enzyme was not linked with its enzymatic activity. The enzyme enhanced immunoglobulin productivity of transcription-suppressed hybridomas, but did not enhance that of translation-suppressed hybridomas. From these results, it is suggested that this enzyme takes part in the post-translational control or the enhancement of translation activity to stimulate immunoglobulin production of hybridomas.

Key words: Glyceraldehyde-3-phosphate dehydrogenase; Human Burkitt's lymphoma Namalwa cells; Human-human hybridoma; Immunoglobulin production stimulating factor; Serum-free medium

1. Introduction

Monoclonal antibodies (MAbs) have been widely applied to various areas, including diagnosis, therapy, immunohistochemistry, and industrial fields to purify products by immunoaffinity chromatography. For therapeutic application of MAbs, human MAbs are desirable, because only the human MAbs negate the serious problems associated with immunogenic disorders caused by heteroantibodies. Increased demands for human MAbs 'have prompted attempts to' improve the cellular productivity of human-human hybridomas to meet the demand for human MAbs. We have searched for cellular protein factors that enhance immunoglobulin production of hybridomas to accomplish the stimulation of cellular productivity under serum-free conditions. Several factors termed immunoglobulin production stimulating factor (IPSF) were discovered in cell extracts or culture medium. IPSF-I was discovered in culture medium from a human lymphoblastoid cell line HO-323 [1], and IPSF-II was purified from cell lysate of human Burkitt's lymphoma, Namalwa cells [2,3]. The previous data revealed that the Namalwa cell lysate contained two IPSF active substances, IPSF-II α and -II β , respectively. IPSF-II α was completely purified and identified as a 112 kDa protein that has a subunit structure composed of a 40 kDa polypeptide chain and two homogeneous 36 kDa polypeptide chains. The 36 kDa subunit exclusively showed IPSF activity. On the other hand, the 40 kDa protein showed neither IPSF activity nor the synergistic effect of the IPSF activity of the 36 kDa subunit. The sequence of 20 amino acids from the N-terminus of the 36 kDa

subunit is highly homologous to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of various origins, which is a key enzyme in the glycolytic pathway [4]. IPSF-II β was also identified as a 46 kDa protein of which the partial amino acid sequence corresponded with the human enolase α -chain as reported previously [5]. As well as GAPDH, enolase is an enzyme involved in the glycolytic pathway.

GAPDH derived from rabbit muscle enhances IgM production of human-human hybridomas about 10-fold in serum-free medium. IgM production of various human-human and mouse-mouse hybridomas are stimulated by the addition of this enzyme.

Here we report, how GAPDH stimulates immunoglobulin production of hybridomas.

2. Materials and methods

2.1. Cells and cell culture

A human-human hybridoma cell line, HB4C5 was used to assay immunoglobulin production stimulating activity of GAPDH. HB4C5 cells, which produce human lung cancer specific monoclonal IgM, were fusion products of a human lymphocyte and a human fusion partner NAT-30 cells [6]. HB4C5 cells were maintained in ERDF medium (Kyokuto Pharmaceutical, Japan) supplemented with 10 μ g/ml of insulin, 20 μ g/ml of transferrin, 20 μ M ethanolamine and 25 nM sodium selenite (ITES-ERDF) at 37°C under humidified atmosphere of 5% CO₂/95% air [7].

2.2. Assay of IPSF activity

IPSF activity was examined by measuring the amount of immunoglobulin secreted by HB4C5 cells in the cultured medium. HB4C5 cells were inoculated at 1×10^5 cells/ml in a 96-well plate containing 200 μ l of ITES-ERDF medium supplemented with GAPDH derived from rabbit muscle (Boehringer Mannheim, Germany). After incubation in the CO₂-incubator, the amount of IgM in each culture medium was measured by enzyme-linked immunosorbent assay (ELISA) using anti-human IgM antibody as mentioned previously [2].

2.3. Assay of enzymatic activity of GAPDH

GAPDH catalyzes conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, in the presence of NAD⁺ and arsenate. Reaction mixture composed of 0.1 M disodium arsenate, 0.5 mM NAD⁺, 5 mM glyceraldehyde-3-phosphate, 3.5 mM cysteine, 26 mM sodium pyrophosphate (pH 8.4), and 0.1 μ g/ml of GAPDH. The produced NADH by the enzymatic reaction can be quantitatively monitored by spectrophotometrical measurement at 340 nm [8].

2.4. ¹²⁵I-labeling of GAPDH

GAPDH derived from rabbit muscle was labeled with ¹²⁵I-iodine by the immobilized lactoperoxidase: a glucose oxidase method (Radio iodination system; New England Nuclear, USA) as according to manufacturer's directions. One mg of GAPDH was used for the labeling reaction. After the labeling reaction, the reaction mixture was gel filtered with a Sephadex G-25 column (Pharmacia, Sweden) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) to remove free radioisotope.

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2.5. Act-D treatment of hybridomas

Hybridoma, HB4C5 cells were treated with 4 $\mu\text{g}/\text{ml}$ of actinomycin D (Act-D) for 2 h at 37°C under humidified atmosphere of 5% $\text{CO}_2/95\%$ air to suppress their transcription activity of the hybridoma. After Act-D treatment, these cells were washed twice with ERDF medium, and inoculated to examine the IPSF effect of GAPDH on transcription-suppressed hybridomas.

2.6. Cycloheximide treatment of hybridomas

HB4C5 cells were pre-incubated in ITES-ERDF medium supplemented with 10 $\mu\text{g}/\text{ml}$ of cycloheximide for 5 h to suppress their translation activity. Following pre-incubation, cells were washed twice with ERDF medium and cultured in ITES-ERDF medium with or without 10 $\mu\text{g}/\text{ml}$ of cycloheximide to determine the IPSF effect of GAPDH on translation-suppressed hybridomas.

3. Results

3.1. Correlation between enzymatic and IPSF activity of GAPDH

GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate, and a high energy phosphate compound is generated in this oxidation-reduction reaction. The first point for discussion regarding IPSF activity of GAPDH is whether the enzymatic activity of this enzyme takes part in its IPSF activity. Iodine-labeled GAPDH was used to investigate this question, and it completely lost catalytic activity (Fig. 1A). It is supposed that GAPDH is inhibited in its enzymatic activity by the modification of the SH-group of its active center during the iodine-labeling reaction. However, the IPSF activity of this enzyme was completely retained in spite of inactivation of its enzymatic function as shown in Fig. 1B. These results make it clear that the IPSF activity of GAPDH is derived from neither its enzymatic activity nor reaction products. These results also provide an indication that this enzyme

has another function other than enzymatic one in the glycolytic pathway.

3.2. Time-course of enhancement of immunoglobulin production by GAPDH

At first, the time-course effect of GAPDH on IgM production of HB4C5 cells was examined to investigate the mode of action of GAPDH as IPSF-II α . GAPDH was added to ITES-ERDF medium at the concentration of 50 $\mu\text{g}/\text{ml}$, at which GAPDH shows maximal enhancement of activity against HB4C5 cells as shown in Fig. 1B. HB4C5 cells were facilitated in their IgM production by GAPDH immediately after inoculation, and the IPSF effect was maintained for 30 h as shown in Fig. 2. Immunoglobulin production of HB4C5 cells was enhanced 6.6–13.5-fold throughout the cultivation period. Previous work has proved that the IPSF activity of GAPDH continues over 3 days until the culture conditions of the hybridomas deteriorate due to a deficiency of nutrients and an accumulation of metabolites [3]. GAPDH did not stimulate the growth rate of the hybridoma at all. These results reveal that GAPDH enhances immunoglobulin productivity of each hybridoma cell.

3.3. Effect of GAPDH on transcription-suppressed hybridomas

HB4C5 cells treated with 4 $\mu\text{g}/\text{ml}$ of Act-D for 2 h were used to determine the IPSF effect of GAPDH on transcription-suppressed hybridomas. Transcription-suppressed HB4C5 cells were inoculated in ITES-ERDF medium supplemented with 50 $\mu\text{g}/\text{ml}$ GAPDH. The Act-D treated HB4C5 cells cultured without GAPDH were still unable to produce immunoglobulin 6 h after inoculation, as shown in Fig. 3. On the other hand, the IPSF effect of this enzyme on transcription-suppressed HB4C5 cells was observed immediately after inoculation as well as that on non-suppressed cells. The accumulation of IgM secreted in

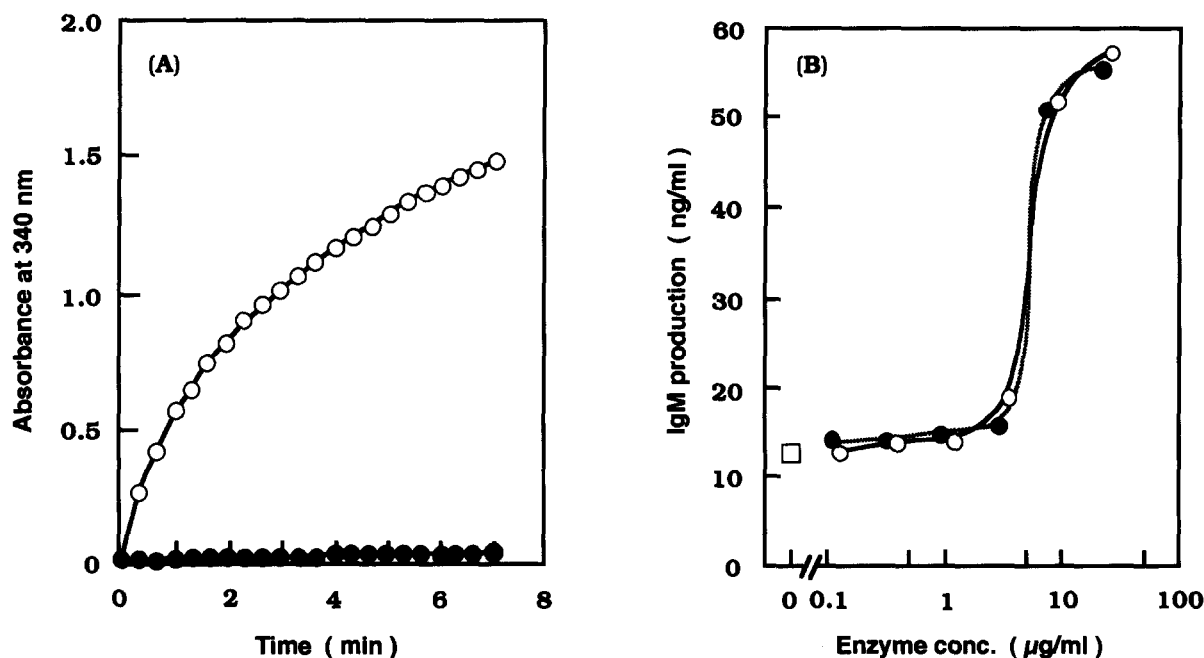


Fig. 1. Enzymatic and IPSF activities of iodine-labeled GAPDH. GAPDH catalyzes conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the presence of NAD^+ and arsenate. The enzymatic reaction of 25 $\mu\text{g}/\text{ml}$ of iodine-labeled (●) and native GAPDH (○) can be quantitatively monitored by spectrophotometrically measuring the quantity of produced NADH at 340 nm at room temperature (section A). IPSF activity of various concentrations of iodine-labeled (●) and native enzyme (○) were measured as described in section 2 (section B). The open square represents IgM productivity of HB4C5 cells in ITES-ERDF medium without GAPDH. Results are expressed as the mean of two experiments.

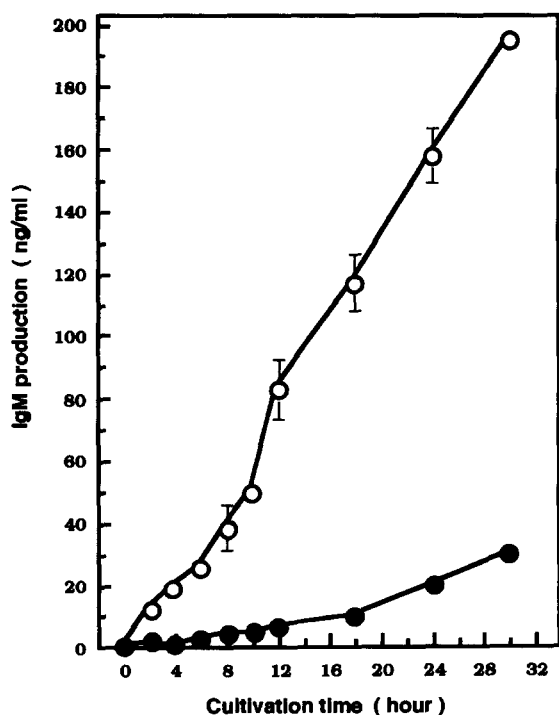


Fig. 2. Time-course of IPSF activity of GAPDH on IgM production of human-human hybridoma HB4C5 cells. IgM production of HB4C5 cells was measured in the presence of 50 $\mu\text{g/ml}$ of GAPDH derived from rabbit muscle (○) on each sampling time. Secreted and accumulated IgM amount was measured by ELISA. The closed circle (●) represents IgM production of the hybridoma cultured in ITES-ERDF medium without GAPDH. Results represent the mean \pm S.D. of three independent measurements.

the medium linearly increased for 6 h. GAPDH finally enhanced immunoglobulin productivity of Act-D treated HB4C5 cells by 8-fold. Transcriptional suppression of the hybridoma did not influence the IPSF effect of GAPDH at all. If GAPDH acted on pre-transcriptional and/or transcriptional processes, the IPSF effect of this enzyme would not be observed. From these results, it is supposed that GAPDH acts on the post-transcriptional process of protein synthesis to enhance immunoglobulin productivity of the hybridoma.

3.4. Effect of GAPDH on translation-suppressed hybridomas

The IPSF effect of GAPDH on translation-suppressed HB4C5 cells was examined to confirm whether the enzyme acts on post-transcriptional process. Suppression of the translation activities of HB4C5 cells was accomplished by cycloheximide treatment. After pre-incubation of the hybridoma with 10 $\mu\text{g/ml}$ cycloheximide, assay of the IPSF activity was performed in ITES-ERDF medium containing cycloheximide, to investigate the effect of GAPDH under translation-suppressed conditions as shown in Fig. 4A. The inhibition of the translation activity of HB4C5 cells caused a loss of the IPSF effect of GAPDH. On the other hand, the IPSF assay performed without cycloheximide showed that HB4C5 cells re-activated their translation activities and were clearly accelerated in their immunoglobulin productivity by the addition of GAPDH (Fig. 4B). There was no evidence for the loss of IPSF activity of GAPDH. If this enzyme acted on the post-translational process, such as

secretion, the IPSF activity should be observed under translation-suppressed conditions. These results provide evidence that GAPDH participates in the post-transcriptional process or the translational control of protein synthesis to enhance IgM productivity.

4. Discussion

We have previously reported that immunoglobulin production stimulating factor-II α (IPSF-II α) was purified from extracts of human Burkitt's lymphoma, Namalwa cells [3]. IPSF-II α was a 112 kDa protein composed of two homogeneous 36 kDa polypeptides and a 40 kDa polypeptide. The 36 kDa subunit that exclusively retained IPSF activity was identified as GAPDH, a key enzyme in the glycolytic pathway, on the basis of its N-terminal amino acid sequence and enzymatic activity [4]. The enzymes from other origins, such as rabbit muscle, human erythrocyte, and *Bacillus stearothermophilus* stimulated IgM production of human-human and mouse-mouse hybridomas under serum-free conditions.

Then, the mode of actions of GAPDH on enhancement of immunoglobulin production was investigated. The enzyme completely lost its enzymatic activity during the iodine labeling reaction. However, iodine-labeled GAPDH still had the same stimulatory activity for immunoglobulin production as native GAPDH. It was proved from these results that the IPSF activity of this enzyme was irrelevant to its enzymatic function or reaction products in the glycolysis. This also indicates that GAPDH has another biological function besides its enzymatic activity.

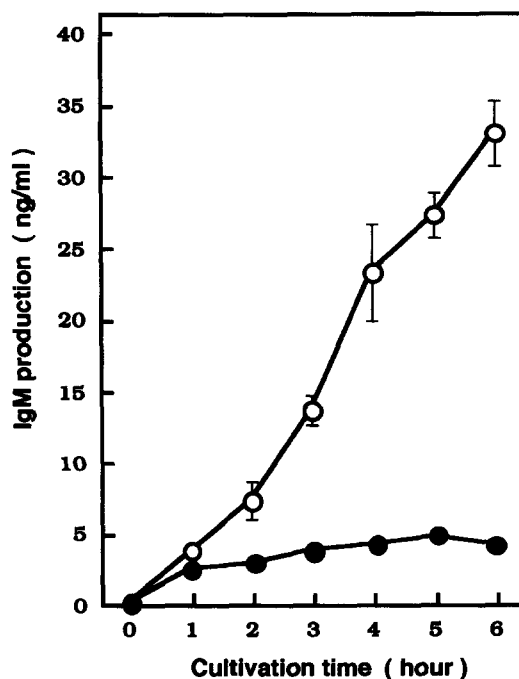


Fig. 3. IPSF effect of GAPDH on transcription-suppressed HB4C5 cells. HB4C5 cells treated with 40 $\mu\text{g/ml}$ of actinomycin D for 2 h were cultured with 50 $\mu\text{g/ml}$ of GAPDH derived from rabbit muscle in ITES-ERDF medium and IgM concentration of the cultured medium was quantified. Each symbol shows IgM accumulation in the medium containing GAPDH (○) or not (●). Results are expressed as the mean \pm S.D. of three independent measurements.

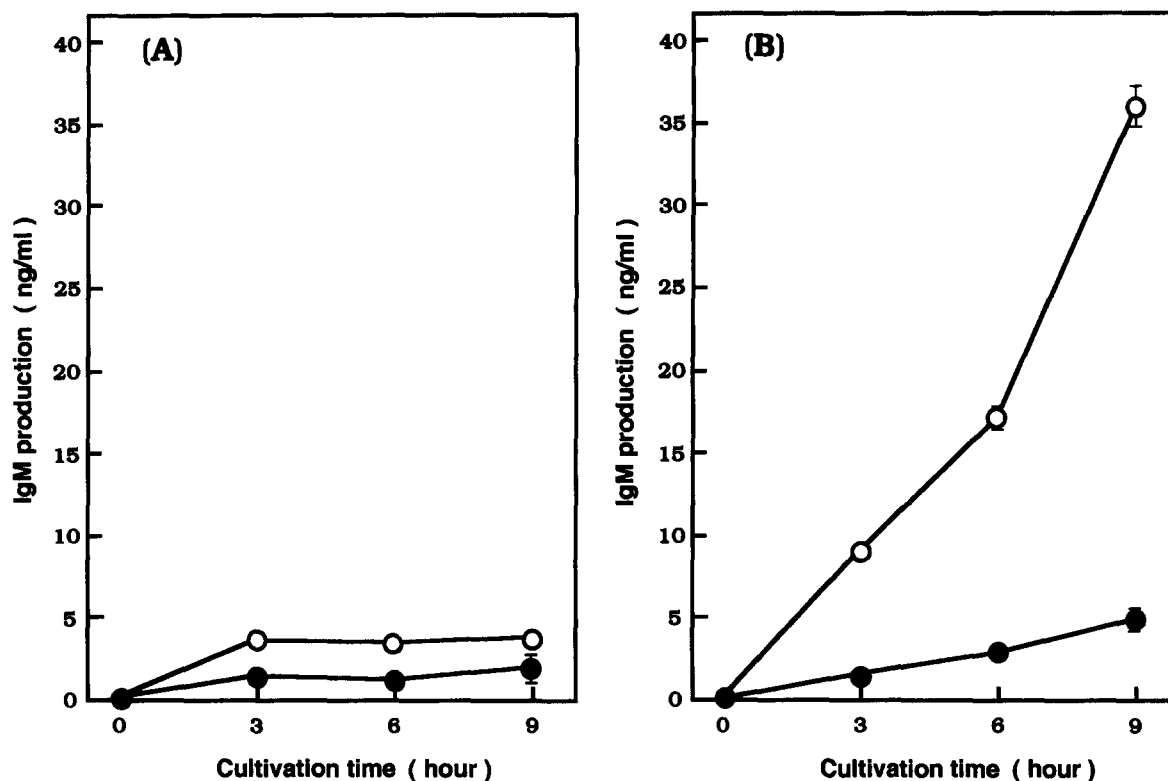


Fig. 4. IPSF effect of GAPDH on translation-suppressed HB4C5 cells. HB4C5 cells were cultured in ITES-ERDF medium containing 10 μ g/ml of cycloheximide to inhibit their translation activity during pre-incubation for 5 h. (A) After pre-incubation, the IPSF activity of GAPDH was assayed with 10 μ g/ml of cycloheximide to continue to inhibit translation activity. (B) After pre-incubation with cycloheximide the IPSF assay performed without cycloheximide to reactivate translation activity. Each symbol shows IgM accumulation in the medium containing GAPDH (○) or not (●). Results shown in (A) and (B) represent the mean \pm S.D. of three independent measurements.

A human–human hybridoma cell line, HB4C5 cells started to increase IgM production as soon as GAPDH was added to the medium, and the accelerated production was maintained for 3 days until the conditions of the hybridomas became worse due to a deficiency of nutrition and accumulation of cytotoxic metabolites. GAPDH stimulated the immunoglobulin production without any growth stimulating effects, so that the enzyme facilitated secretion or immunoglobulin production of the hybridomas. However, only a small amount of immunoglobulin was detectable inside HB4C5 cells in comparison with their productivity. This indicates that most of the immunoglobulin molecules are constitutively secreted by these hybridoma cells. This circumstantial evidence implies that the process of immunoglobulin secretion is not affected by GAPDH, and the enzyme enhances immunoglobulin productivity of each hybridoma cell and acts on the protein synthesis of the cell. This hypothesis is also supported by the results shown in Fig. 2 that indicates the durable stimulation of the immunoglobulin productivity.

The effect of GAPDH on transcription-suppressed hybridomas was examined to investigate how this enzyme affected the immunoglobulin synthesis of the hybridoma. GAPDH stimulated immunoglobulin productivity of HB4C5 cells in spite of the inhibition of the transcription activity. This result indicates that GAPDH does not accelerate transcription of RNA molecules. The immediate IPSF effect of GAPDH on Act-D treated HB4C5 cells suggests that the enzyme does not stabilize the mRNA for immunoglobulin molecule to enhance the produc-

tivity. This is also supported by the fact that the immunoglobulin mRNA level in HB4C5 cells was not increased by the treatment with GAPDH [4]. All these results suggest that the immunoglobulin production stimulating activity of GAPDH is not derived from acceleration of the transcription process. Further investigation revealed that GAPDH was not able to stimulate immunoglobulin productivity of translation-suppressed hybridomas. This fact suggests that this enzyme facilitates translation activity of the hybridoma to enhance immunoglobulin production. In conjunction with the stimulation of the translation activity, GAPDH was added to the rabbit reticulocyte cell-free translation system to examine the effect of the enzyme on this system. The translation of immunoglobulin peptide was enhanced 1.5-fold by the addition of this enzyme [9]. However, translation stimulating effect of GAPDH on the cell-free translation system was much lower than that on intact cells. Some reasons could be suggested for the lower IPSF activity of the enzyme in cell-free translation. First, it comes from essential features of the cell-free translation system, is that the cell-free translation system is inferior to in vivo translation and exhibits only limited translation activity. Second, the cell-free translation systems contain endogenous GAPDH [10], so that the further enhancing effect was not observed by elevated concentrations of this enzyme. Finally, the most possible reason, that GAPDH may be a precursor of the IPSF active substance, and some modification, such as enzymatic digestion, is necessary for GAPDH to act as IPSF. This hypothesis could also explain why the endogenous GAPDH does not enhance

immunoglobulin productivity of the hybridoma. We suppose that GAPDH would have to be extracellularly added and modified to stimulate immunoglobulin productivity of the hybridoma.

GAPDH has been studied by many workers for its associating property with cytoplasmic membrane proteins and cellular organelles, such as human erythroid cell membrane [11–14], plasma membrane of the intact human red blood cell [15], microtubules of a human colon tumor cell line [16], the rod outer segment of retinal rod photoreceptor cell [17], and calsequestrin of skeletal muscle [18]. The enzyme has a specific affinity for highly acidic regions of certain proteins and the association of the enzyme with these organelles is inhibited by physiological ionic strength [13,18]. We, however, investigate the existence of receptors for GAPDH on the surface of the hybridoma, and the existence of a specific receptor was not verified (data not shown). From this result, it is supposed that GAPDH associates with the surface of the hybridoma in the same way as with the cytoplasmic membranes or cellular organelles.

Besides these functions which are mentioned previously, GAPDH has other properties, too. The enzyme associates with single-stranded DNA [19], RNA [20] and mono- and polyribosomes [21]. Moreover, this enzyme is one of the three major RNA-binding proteins of rabbit reticulocytes [10]. We suppose that these features of this enzyme are concerned with the IPSF activity of the enzyme, because some DNA-binding proteins, such as histone H1, H2A, and H2B, have IPSF activities [22]. In addition, lactate dehydrogenase which associates with single-stranded DNA [23] also has IPSF activity (Sugahara et al., in press). From these results, it is assumed that the enzyme associates with the surface of HB4C5 cells in an electrostatic manner. Following this, the enzyme is internalized by pinocytosis, and modified by degradative enzymes in lysosomes to serve as IPSF. The modified GAPDH stimulates the translation activity of hybridoma.

There is one finding that GAPDH mRNA is a major interleukin 2-induced transcript in the helper-T lymphocyte, and the translation of the GAPDH mRNA was enhanced [24]. This finding makes us consider the possibility that the enzyme is produced by IL-2 activated helper-T lymphocytes, and its enhances immunoglobulin productivity of plasma cells in vivo. Because the enzyme can also strongly enhance immunoglobulin productivity of peripheral blood lymphocytes (manuscript in preparation). However, this hypothesis contains the inconsistency that the enzyme is an intercellular protein, hence that the enzyme is not usually secreted outside of the cell. Uncovering

the mechanisms of the actions of GAPDH will contribute not only to the effective enhancement of cellular productivities of immunoglobulin of hybridomas, but also to the interpretation of the regulation of protein synthesis in animal cells.

References

- [1] Toyoda, K., Sugahara, T., Inoue, K., Yamada, K., Shirahata, S. and Murakami, H. (1990) *Cytotechnology* 3, 189–197.
- [2] Yamada, K., Akiyoshi, K., Murakami, H., Sugahara, T., Ikeda, I., Toyoda, K. and Omura, H. (1989) *In Vitro Cell. Dev. Biol.* 25, 243–247.
- [3] Sugahara, T., Shirahata, S., Yamada, K. and Murakami, H. (1991) *Cytotechnology* 5, 255–263.
- [4] Sugahara, T., Shirahata, S., Akiyoshi, K., Isobe, T., Okuyama, T. and Murakami, H. (1991) *Cytotechnology* 6, 115–120.
- [5] Sugahara, T., Nakajima, H., Shirahata, S. and Murakami, H. (1992) *Cytotechnology* 10, 137–146.
- [6] Murakami, H., Hashizume, S., Ohashi, H., Shinohara, K., Yasumoto, K., Nomoto, K. and Omura, H. (1985) *In Vitro Cell. Dev. Biol.* 21, 593–596.
- [7] Murakami, H., Masui, H., Sato, G.H., Sueoka, N., Chow, T.P. and Kono-Sueoka, T. (1982) *Proc. Natl. Acad. Sci. USA* 79, 1158–1162.
- [8] Sinden, F.V. (1955) *Methods Enzymol.* 1, 401–406.
- [9] Murakami, H., Sugahara, T. and Nakajima, H. (1993) *Cell Biology and Biotechnology*, Springer-Verlag, New York, pp. 35–62.
- [10] Ryazanov, A.G. (1985) *FEBS Lett.* 192, 131–134.
- [11] Shin, B.C. and Carraway, K.L. (1973) *J. Biol. Chem.* 248, 1436–1444.
- [12] Kant, J.A. and Steck, T.L. (1973) *J. Biol. Chem.* 248, 8457–8464.
- [13] Kliman, H.J. and Steck, T.L. (1980) *J. Biol. Chem.* 255, 6314–6321.
- [14] Allen, R.W., Trach, K.A. and Hoch, J.A. (1987) *J. Biol. Chem.* 262, 649–653.
- [15] Rogalski, A.A., Steck, T.L. and Waseem, A. (1989) *J. Biol. Chem.* 264, 6438–6446.
- [16] Launay, J.F., Jellali, A. and Vantier, M.T. (1989) *Biochim. Biophys. Acta* 996, 103–109.
- [17] Hsu, S.-C. and Molday, R.S. (1990) *J. Biol. Chem.* 265, 13308–13313.
- [18] Caswell, A.H. and Corbett, A.M. (1984) *J. Biol. Chem.* 260, 6892–6898.
- [19] Perucho, M., Salas, J. and Salas, M.L. (1977) *Eur. J. Biochem.* 81, 557–562.
- [20] Perucho, M., Salas, J. and Salas, M.L. (1980) *Biochim. Biophys. Acta* 606, 181–195.
- [21] Ryazanov, A.G., Ashmarina, L.I. and Mironetz, V.I. (1988) *Eur. J. Biochem.* 171, 301–305.
- [22] Sugahara, T., Sasaki, T. and Murakami, H. (1994) *Biosci. Biotechn. Biochem.* 58, 2212–2214.
- [23] Grosse, F., Nasheuer, H.-P., Sholtissek, S. and Schomburg, U. (1986) *Eur. J. Biochem.* 160, 456–467.
- [24] Sabath, D.E., Broome, H.E. and Prystowsky, M.B. (1990) *Gene* 91, 185–191.