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RELEASE OF A PLASMA MEMBRANE-BOUND TRIAMINOPEPTIDASE ACTIVITY FROM MAMMALIAN CELLS BY THERMOLYSIN

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SUMMARY: Aminopeptidase and other enzyme activities on cellular surface were determined in the presence and absence of endopeptidases. Gly-Pro-Leu-AP activity was specifically released into the medium by thermolysin treatment, while the other activities retained on the cellular surface were markedly decreased. A similar phenomenon was also observed in rat liver membrane, mouse FM3A, spleen lymphocyte and other cells. Structural rearrangement of some protein components in the cell plasma membrane was suggested.

Introduction

Previously we reported that several kinds of enzymes such as aminopeptidase, alkaline phosphatase and esterase were located on the surface of mammalian cells. Furthermore, our experiments with specific inhibitors against these enzymes demonstrated that these enzymes played an important role in various functions of mammalian cells (1-4). For example, bestatin (5,6) a specific inhibitor of aminopeptidase B and leucine aminopeptidase enhanced delayed-

Abbreviations:

AP-A, aminopeptidase A; AP-B, aminopeptidase B; Ala-AP, alanine aminopeptidase; Leu-AP, leucine aminopeptidase; Met-AP, methionine aminopeptidase; Phe-AP, phenylalanine aminopeptidase; Tyr-AP, thyrosine aminopeptidase; Gly-Pro-AP, glycyl-proline aminopeptidase; Gly-Pro-Leu-AP, glycyl-prolyl-leucine aminopeptidase; β-NA, β-naphthylamide

C3H2K: established from embrionic kidney of mouse strain C3H.

3Y1: Fisher rat embryo.

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type hypersensitivity (3) and blastogenesis of small lymphocytes by concanavalin A (4).

It is well known that mild treatment of cellular surface with trypsin induces cell proliferation or enhances agglutinability of lectins (7). These phenomena are considered to be mediated by conformational changes in the cellular surface. It is an essential step in elucidation of cellular physiology to understand the relationship between various conformational changes in the plasma membrane and the corresponding functional changes of cells. In the present experiments we studied how the activities of aminopeptidase and other enzymes on the plasma membrane of mammalian cells behaved after treatment with endopeptidases.

Materials and Methods

Cells: Mice were killed by cervical dislocation, and spleen cell suspension was prepared and cleared of debris by the procedures of Mishell and Dutton (8). Spleen lymphocytes were obtained through removal of adherent cells in plastic petri dishes (9). A continuous line of FM3A was grown in minimal essential medium as described previously (10). Plasma membrane was isolated from the liver of adult male rat according to the method previously described (1). WI-38 cells were kindly supplied by Dr. Y. Nagai of Tokyo Metropolitan Institute of Gerontology. C3H2K, SV40-C3H2K, 3Y-1 and SV40-3Y1 cells were kindly supplied by Dr. K. Oda at Institute of Medical Science, University of Tokyo.

Buffer: Buffers employed were as follows; Hank's medium, pH 7.2 without phenol red; phosphate buffered saline, 0.02 M and pH 7.2.

Commercial sources of enzymes and substrates: Trypsin from Worthington Biochem. Co., U.S.A.; papain, α-chymotrypsin and elastase from Sigma Co., U.S.A.; thermolysin from Daiwa Kasai K. K., Japan; L-α-glutamic acid β-naphthylamide hydrochloride (L-α-Glu-NA·HCl), L-Arg-NA·HCl and L-Met-NA from Mann Research, U.S.A.; L-Ala-NA, L-Leu-NA, L-Phe-NA, L-Tyr-NA, Gly-L-Pro-NA and Gly-L-Pro-L-Leu-NA from Bachem Feinchemikalien AG, Schweiz; p-nitrophenyl phosphate from BDH Chemicals Ltd., England; p-nitrophenyl acetate from Tokyo Kasei Co., Japan.

Determination of enzyme activities: Lymphocyte cells, FM3A cells or rat liver membrane fraction were dispensed into test tubes (1.5 x 10 cm) with 1 ml Hank's medium containing respective substrates. Monolayer cell culture was carried out in petri dishes with diameter of 3 cm. Culture medium was replaced with 1 ml Hank's medium contain respective substrates. The test tubes and petri dishes were incubated for 1 hr at 37°C. The supernatant was withdrawn and centrifuged (3000 rev./min, 10 min) for determination of absorbance at 525 nm for the assay of aminopeptidase, at 420 nm for the assay of alkaline phosphatase and at 400 nm for the assay of esterase.

Results

Under our experimental conditions, activities of aminopeptidase, alkaline

phosphatase and esterase were determined in the presence and absence of endopeptidases. So we checked whether the preparations of endopeptidases used had any other contaminant activity to hydrolyze the substrates of these above mentioned enzymes. Although the results are not shown here, none of the substrates was hydrolyzed by endopeptidases used. Therefore, if hydrolysis of the substrates should happen under our experimental conditions, it can be concluded that the hydrolysis was due to enzymes originaged from cells or membrane fractions and not due to contaminant activity of endopeptidases.

First, rat liver plasma membrane fraction was chosen becuase it showed a high level of enzyme activities. Enzyme activities in the fraction were determined both in the presence and absence of endopeptidases. As shown in Table 1, trypsin, papain, chymotrypsin, elastase and thermolysin inactivated aminopeptidase activities in the plasma membrane fractions. However, there was a marked enhancement in the Gly-Pro-Leu-AP when the determination was carried out in the presence of thermolysin. It seems likely that Gly-Pro-Leu-AP activity on the plasma membrane fraction is in a cryptic state and that thermolysin in the assay system activates the activity of aminopeptidase in the membrane fractions. We reported that alkaline phosphatase and esterase activities were also detected on the surface of mammalian cells (2). As shown in Table 1, thermolysin treatment decreased the activity of phosphatase but it had little effect on the activity of esterase.

Aminopeptidase activities on the surface of mouse spleen lymphocyte cells were compared in the presence and absence of endopeptidases (Table 2). Activities of AP-A and AP-B were inactivated with any kind of endopeptidases. Activities of Leu-AP and Gly-Pro-AP were not affected by the treatment. There was a marked enhancement in the activity of Gly-Pro-Leu-AP when the lymphocytes were treated with thermolysin. The result coincides with that observed in the rat liver plasma membrane fraction treatment with thermolysin. When aminopeptidase activities on the surface of FM3A cells treated with endopeptidases were determined, it was shown that Gly-Pro-Leu-AP activity was significantly increased

Table I. Changes in aminopeptidase activities of rat liver membrane fraction after treatment with endopeptidases

Treated enzymes	μg	Change in enzyme activities (%)										
		AP-A	AP-B	Ala-AP	Leu-AP	Met-AP	Phe-AP	Tyr-AP	Gly-Pro-AP	Gly-Pro-Leu-AP	Phosphatase	Esterase
Trypsin	10	-38	+10	-48	-51	-14	-52	-55	-8	-20	-47	-2
Papain	10	-27	-20	-36	-10	-5	-40	-38	-7	-5	-5	+11
α-Chymotrypsin	10	-62	-43	-58	-55	-20	-46	-36	-10	-33	-53	+12
Elastase	10	-38	-38	-39	-37	-10	-55	-51	-4	-15	-50	+11
Thermolysin	10	-49	-43	-61	-53	-19	-64	-67	-3	+100	-32	-2

Aminopeptidase activities were determined according to the methods described in Methods. Each tube contained 100 μg protein of rat liver membrane fractions. Values obtained with the tubes including 10 μg of endopeptidase were devided by values obtained with control tubes and were expressed as precentage. The amount of hydrolyzed substrates in control tubes was approximately in the range of 25 to 40 nmols. Each figure represents the average of triplicate experiments.

by thermolysin treatment. Activities of other aminopeptidases in FM3A cells behaved almost in a similar way against endopeptidase treatment to those in mouse spleen cells. It is suggested that Gly-Pro-Leu-AP activity is released from the cell membrane when the lymphocytes and FM3A cells are treated with thermolysin. It seems unlikely that the cells could be damaged by the endopeptidases and that all the kinds of aminopeptidase activities could be released into the media unspecifically. The enhancement of the activity are strictly restricted to Gly-Pro-Leu-AP.

When the increment of hydrolyzed Gly-L-Pro-L-Leu-NA by the presence of thermolysin in three kinds of preparations were plotted against incubation time, there was a strict linearity between the amount of hydrolysis and incubation time (Fig. 1a). Furthermore, a proportionality between increased activities and number of cells treated with thermolysin was also demonstrated (Fig. 1b).

As shown in Table 3, the increased portion of Gly-Pro-Leu-AP activity was released into the supernatant and the cells retained almost the same amount of the activity after the treatment. When the cells were incubated without the addition of thermolysin, there was no release of Gly-Pro-Leu-AP activity.

The effect of concentration of thermolysin on the increase in Gly-Pro-

Table 2. Changes in aminopeptidase activities on the surface of mouse spleen lymphocyte cells after treatment with endopeptidases

Treated enzymes	μg	AP-A	AP-B	Leu-AP	Gly-Pro-AP	Gly-Pro-Leu-AP
Trypsin	2	-86	-54	-10	-16	-5
Papain	2	-43	-10	-4	0	0
α-Chymotrypsin	2	-43	-39	-4	0	0
Elastase	2	-71	-46	0	0	0
Thermolysin	2	-71	-73	-4	-13	+49
	0.5	-57	-51	0	-7	+19

Activities of AP-A, AP-B, Leu-AP, Gly-Pro-AP and Gly-Pro-Leu-AP were determined using 2×10^7 cells, 1.5×10^7 cells, 2×10^6 cells, 2×10^7 cells and 5×10^6 cells, respectively. Incubation was carried out for 1 hr at 37°C. Changes in the enzyme activities were expressed as the percentage of the values obtained with the tubes including endopeptidases divided by the values in control tubes. Each figure represents the average of triplicate experiments.

Leu-AP activity in several kinds of cells was shown in Table 4. The treatment with thermolysin always resulted in the enhancement of the activity no matter which kinds of cells was employed.

Discussion

The present experiments demonstrates that the activity of Gly-Pro-Leu-AP is specifically released from the cells after thermolysin treatment. Any other kind of aminopeptidase is not released.

As shown in Table 3, the enzyme activity retained on the cellular surface is almost unchanged after the release of the enzyme activity into the medium. The fact leads to the conclusion that Gly-Pro-Leu-AP is coupled to a certain membrane component through thermolysin sensitive bondings. Furthermore, it is suggested that rearrangement of enzyme proteins in the cellular membrane is induced by the treatment in order to keep the enzyme activity unchanged. The possibility that a certain zymogen is activated by thermolysin seems neglible.

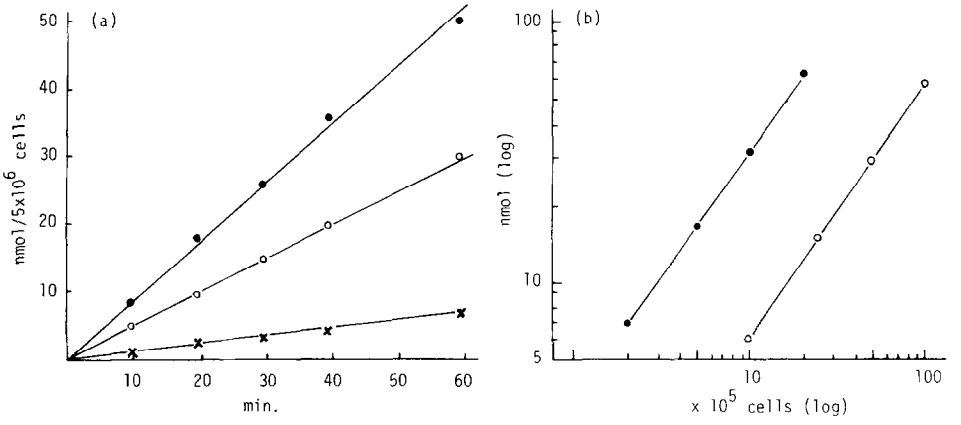

Figure 1a. Time course of increased Gly-Pro-Leu-AP activities on the cell surface and rat liver membrane treated with thermolysin. An increased hydrolysis of Gly-Pro-Leu-NA by the presence of 2 μg/ml of thermolysin were determined on the surface of intact mouse spleen lymphocyte cells, FM3A cells and rat liver membrane. Enzyme activities are expressed as nmol of naphthylamine per 5 x 10^6 lymphocyte cells, 2 x 10^6 FM3A cells and 100 μg protein of rat liver membrane. Each point was obtained from the average of triplicate samples. o ——— o lymphocytes; • ——— • FM3A; x ——— x rat liver membrane;

Figure 1b. Proportionality between increased surface Gly-Pro-Leu-AP activities and number of cells treated with thermolysin. An increased hydrolysis of Gly-Pro-Leu-NA by the presence of 2 μg/ml of thermolysin were determined on the surface of intact lymphocyte and FM3A cells. Enzyme activities are expressed in nmol of naphthylamine per hr and per number of cells in the Hank's solution given abscissa. Each point was obtained from the average of triplicate samples. o ——— o lymphocytes; • ——— • FM3A

Table 3. Release of Gly-Pro-Leu-AP activity from cellular surface after thermolysin treatment

Lymphocyte	Thermolysin (μg)	First incubation	Substrate	Second incubation	nmol
2.5 x 10^6	–	———	Gly-Pro-Leu-NA	———→	10.2
2.5 x 10^6	–	Sup	Gly-Pro-Leu-NA	———→	0
		Cell	Gly-Pro-Leu-NA	———→	10.5
2.5 x 10^6	2	Sup	Gly-Pro-Leu-NA	———→	22.5
		Cell	Gly-Pro-Leu-NA	———→	9.7
2.5 x 10^6	2	———	Gly-Pro-Leu-NA	———→	33.0
—	2	———	Gly-Pro-Leu-NA	———→	0

Lymphocytes were incubated in Hank's solution for 10 min at 37°C (first incubation) with and without thermolysin at 2 μg/ml. Two cases were centrifuged for 3 min at 2000 rpm to obtain supernatant and cell fractions. The cell fractions were resuspended in Hank's solution. All cases were incubated again for 1 hr at 37°C (second inoculation) after the addition of the substrates. Each figure represents the average of triplicate experiments.

Table 4. Change in Gly-Pro-Leu-AP activities of various cell surface after treated with thermolysin

Thermolysin (μg)	released β-naphthylamide (nmol)							
	WI-38		C3H2K	SV40-C3H2K	3Y-1	SV40-3Y-1	Lymphocyte	FM3A
	31	42						
2	13.9	11.1	12.8	12.4	21.7	17.9	12.8	30.9
0.5	4.3	0.4	3.2	3.6	7.1	5.4	6.0	9.6
0.1	0.7	0	0	1.7	0.7	1.4	2.1	1.4
Cell No.	5×10^5	4.8×10^5	2.9×10^5	3.8×10^5	5.0×10^5	5.8×10^5	5×10^6	2×10^6

In mouse lymphocytes and FM3A cells the increase in Gly-Pro-Leu-AP activity was determined as described in the legend of Table 2. In other kinds of cells the increase was determined using the petri dishes (diameter 3 cm) in which the cells had been grown for the determination of Gly-Pro-Leu-AP activity. Each figure represents the average of triplicate experiments.

Thermolysin (EC 3.4.4. group) is a metalloendopeptidase obtained from Bacillus thermoproteolyticus and selectively hydrolyzes peptide bonds on the amino side of hydrophobic amino acids (11). Substrate specificity of the enzyme is completely different from other endoproteases. It is reasonable to assume that Gly-Pro-Leu-AP is arranged on the cellular membrane in a different way from other enzyme proteins. In all the cells tested in Table 4, the Gly-Pro-Leu-AP activity was enhanced by thermolysin treatment. The arrangement or structural configuration of some components in the cellular membrane should have some common features in these mammalian cells.

Previously we reported that the binding of influenza virus to cell surface increases the number of sialic acid residues which are accessible to the action of sialidase. We concluded that it is due to the rearrangement of sialo-compounds which are located on the cell membrane (10). Enhancement of the agglutinability of cells to phytohemagglutinin after trypsin treatment is also explained by the configulational change on the cellular membrane (7). The re-arrangement of some components of the cellular membrane does not seem an unusual phenomenon.

The tripeptide which was isolated from human serum promotes the growth

of hepatoma cells (12,13) and also enhances the growth and differentiation of carcical neurons. It is also reported to inhibit the growth of glial elements (14). Although the origin of the above tripeptide is not known and the substrate specificity of Gly-Pro-Leu-AP is not yet elucidated, it is possible that the triaminopeptidase has some significant role in the process of cancer and other malignant diseases.

Phosphoramidon (15-17), a specific inhibitor against metalloendopeptidase containing thermolysin which we isolated recently, should be an useful tool for examining the possibility.

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