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AMINOPEPTIDASE ACTIVITIES ON THE SURFACE OF MAMMALIAN CELLS

TAKAAKI AOYAGI, HIROYUKI SUDA, MACHIKO NAGAI, KEIJI OGAWA, JIRO SUZUKI, TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry, Kamiosaki 3-Chome, 14—23, Shinagawa-ku, Tokyo, 141 (Japan)

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Summary

Activities of hydrolytic enzymes on the surface of monkey kidney, canine kidney, L. FM3A and various tumor cells were determined and compared with those in the cell homogenate. Although aminopeptidase (EC 3.4.11.-) activities were always detected on the surface membrane in mammalian cells, trypsin, chymotrypsin and elastase activities were not detected while slight glycosidase activity was detected in a suspension of cultured cells. The activities of alanine-, leucine-, methionine- and phenylalanine-aminopeptidases were rather high but aminopeptidase A, proline-, valine-, glycyl proline dipeptidyl- and glycyl propyl leucine-tripeptidyl-aminopeptidases showed relatively low activities. Aminopeptidase activity was also demonstrated in the isolated membrane fractions. The specific activities of enzymes in these membrane fractions were not significantly greater than in cell homogenate so it was concluded that these enzyme activities were rather loosely bound to the cell membrane. Further evidence for the localization of the aminopeptidase activities on the cell surface was obtained by using glass-bead-bound substrate and detecting the release of the terminal residues. When bestatin, a specific inhibitor against aminopeptidase B and leucine aminopeptidase, was included in the assay system for the enzyme activities on the cell surface, the enzymes were commonly inhibited in all types of cells.

Introduction

The importance of the surface membrane in various functions of mammalian cells is widely recognised. It is known that the components of the cell sur-

Abbreviations used in this paper: TosArgOMe, *p*-toluenesulfonyl-L-arginine methyl ester; BzArgOEt, N^{α}-benzoyl-L-arginine ethyl ester; $AcAla_3OMe$, acetyl-L-alanyl-L-alanyl-L-alanine methyl ester. LE medium: Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate; L cell: established from connective tissues of C3H mouse; FM3A cell: established from mammary tumor of mouse strain C3H-He.

face play a key role in the processes of inflammation, immunity, oncogenesis, metastasis and virus infection. Mild treatment of the cells with proteolytic or glycosidic enzymes somehow modifies these components and thus often causes an alteration in cell function [1—3]. In previous papers, we reported the effect of sialidase treatment on the sialo compounds located on the cell surface and also the interaction of virus particles with components of the cell surface [4—6].

Recently, a plasminogen activator has been reported to exist on the surface of malignant cells [7—9] and proteolytic activity was also detected on the human erythrocyte membrane [10]. It is well known that contact inhibition in confluent monolayer culture is disconnected by mild treatment of the culture with trypsin [11]. However, no report has ever mentioned either the presence of exopeptidase activity on the cell surface or the effect of the enzyme on cell function, but these exopeptidases might also serve as modulators or triggers of reactions which take place on the cell surface.

In the present investigation, we determined the activities of exopeptidases, endopeptidases and glycosidases on the cell surface of intact monkey kidney, canine kidney, L. and FM3A cells as well as the corresponding activities in the cell homogenate, representing the total activity. Furthermore, we determined exopeptidase activities in the membrane fractions prepared from the above-mentioned cells and also from several additional tumor cells. The localization of hydrolytic enzymes on the cell surface was also demonstrated by using glass-bead-linked substrate in place of soluble substrate.

The results presented strongly support the view that aminopeptidases (EC 3.4.11.-) of mammalian cells are located on the cell surface, reacting with external substrates.

Materials and Methods

Cells. Primary monkey kidney cells were prepared by trypsinization and were grown in LE medium according to the method that was described previously [4]. A continuous line of canine kidney cells were grown in Eagle F11 medium as described by Tobita et al. [12]. A continuous line of L, L-1210, FM3A, and SV40-C3H cells were grown in minimal essential medium as described previously [4]. SV40-C3H and FM3A cells were kindly given by Dr. K. Oda, Department of Tumor Virus Research, and Dr. T. Ando, Department of Virology, Institute of Medical Science, Tokyo University. The Ehrlich ascites tumor cells were grown for 7 days in 5-week-old male dd/Y mice. The Yoshida ascites were grown for 4 days in 8-week-old female Donryu rats. The tumor cells were separated by centrifugation of the ascitic fluid, which had been diluted 10-fold with Hank's solution in order to diminish the tendency towards cell agglutination and the cells were washed three times with the same solution.

Buffers. Buffers employed were as follows: Hank's solution, pH 7.2 without phenol red; phosphate buffered saline, 0.02 M and pH 7.2; Krebs-Ringer buffer, pH 7.2.

Substrates for enzyme assay: Sources of materials: *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride (TosArgOMe), *N*-acetyl-L-tyrosine ethyl ester (AcTyrOEt) and L-leucine β-naphthylamide hydrochloride from Tokyo Kasei

Kogyo Co., Japan; N^{α}-benzoyl-L-arginine ethyl ester hydrochloride (BzArg-OEt), phenolphthalein-glucuronic acid and N^{α}-acetyl-L-lysine methyl ester hydrochloride from Sigma Co., U.S.A.; *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-α-D-mannopyranoside from Calbiochem., U.S.A.; *p*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide from BDH Chemicals Ltd., England; L-glutamine β-naphthylamide hydrochloride, L-arginine β-naphthylamide hydrochloride, L-lysine β-naphthylamide hydrochloride, L-methionine β-naphthylamide and L-proline β-naphthylamide hydrobromide from Mann Research, U.S.A.; L-valine β-naphthylamide hydrochloride, glycine β-naphthylamide hydrochloride, L-leucyl-glycyl-glycine from Protein Research Foundation, Japan. Glycyl-L-proline β-naphthylamide and acetyl-L-alanyl-L-alanyl-L-alanine methyl ester ($AcAla_3OMe$) were kindly synthesized by Dr. R. Nishizawa, Nihon Kayaku Co. Ltd., Tokyo.

Preparation of L-leucyl-glycyl-glycyl-alkylamine/CPG-550: The amino group of L-leucyl-glycyl-glycine (L-Leu-Gly-Gly) was masked with *t*-butyloxycarbonyl group by *t*-butyloxycarbonyl azide. 140 mg of BOC-Leu-Gly-Gly was dissolved in 20 ml of tetrahydrofuran and 80 mg of dicyclohexylcarbodiimide was added at 0° C. After 30 min, 2 g of Alkylamine/CPG-550 (Pierce Chem. Co., U.S.A.) was added and the mixture was stirred gently on a rotary shaker for 16 h. The glass beads were washed with 500 ml of methanol on a glass filter and dried under reduced pressure. 3 M hydrochloric acid/methanol (1 : 1) was added to the glass beads and the mixture was kept standing for 1 h at 37° C. After successive washing with acetic acid, water and methanol, L-Leu-Gly-Gly · glass beads was dried under reduced pressure. Ninhydrin reaction showed that 0.05 μmol of L-Leu-Gly-Gly was attached per mg of the glass beads.

Determination of enzyme activities. In monkey kidney, canine kidney, SV40-C3H and L-1210 cells, enzyme activities were assayed on the monolayer cultures grown in glass vials for scintillation counting (diameter 2.0 cm, $2.5 \cdot 10^5$ cells). Without removing the cells from the vials, the growth medium was replaced with 1 ml of Hank's solution (pH 7.2) containing respective substrates corresponding to the species of enzymes. In L, FM3A cells and tumor cells, enzyme activities were assayed in a test tube (1.5 × 10 cm, $2.5 \cdot 10^5$ cells) with 1 ml of Hank's medium containing respective substrates. The vials and test tubes were incubated for 1 h at 37° C. The supernatant was withdrawn and centrifuged (3000 rev./min, 10 min) for further measurements. Cell homogenates were prepared in the same buffer with Dounce homogenizer (200 strokes). Microscopic observation indicated that the cells were completely destroyed under these conditions.

Isolation of plasma membrane. Plasma membranes were isolated from the liver of adult male rats, L and Ehrlich cells. Isolation was carried out according to the method of Ray [13] with a modification as follows: 2 g of liver or $3 \cdot 10^9$ L cells and $6 \cdot 10^9$ Ehrlich cells were homogenized in 20 ml of medium A (0.25 M sucrose, 5 mM Tris · HCl (pH 7.4), 1 mM $MgCl_2$) in a Dounce homogenizer with 200 strokes. The homogenate was diluted with medium A equivalent to 100 times of the wet weight of fresh liver, L or Ehrlich cells and centrifuged at 5000 rev./min for 10 min. The pellet was suspended in the same buffer and homogenized with 30 strokes. The suspension was diluted 50 times with the same buffer and centrifuged again for 10 min. The pellet was taken up in a

small volume of the same buffer and then mixed with 70% ice-cold sucrose solution (w/w) so that the final concentration of sucrose was 48% (w/w). The sucrose suspension was layered using 8 ml of 45% sucrose, then 10 ml of 41% sucrose and finally 5 ml of 37% sucrose successively. The tubes were then spun for 2 h at 25 000 rev./min in a Spinco SW-25 centrifuge. The plasma membrane layer appeared at the interface between 37% and 41% sucrose in the form of a thin compact sheet. The membrane layer was taken out with a Pasteur pipette, washed free of sucrose and then suspended in an appropriate amount of buffer.

Enzyme preparations. Aminopeptidases (EC 3.4.11.-) and glycosidases (EC 3.2.1.-) were partially purified from rat or bovine liver according to the procedure described by Hopsu [14] and Chytil [15]. Leucine aminopeptidase (EC 3.4.11.1) and elastase (EC 3.4.4.7) were purchased from Boehringer Mannheim, Germany. Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and β-glucuronidase (EC 3.2.1.31) were purchased from Sigma Chemical Co., and Nutritional Biochem. Co., U.S.A. These enzymes were used in the experiment for Table I.

Method of determination of exopeptidase reactions. For aminopeptidase assay, the reaction mixture included 0.25 ml of 2.0 mM β-naphthylamide derivatives. After the reaction, the mixture was processed as described previously [16] and the absorbance at 525 nm of the final mixture was determined.

Method of determination of endopeptidase reactions. For endopeptidase assay, the reaction mixture included 0.1 ml of 75 mM ester derivatives. After the reaction, the mixture was processed as described previously [17] and the absorbance at 525 nm of the final mixture was determined.

Method of determination of glycosidase reactions. For glycosidase assay, the reaction mixture included 0.05 ml of 50 mM *p*-nitrophenyl or phenolphthalein derivatives. After the reaction, the mixture was processed as described previously [5] and the absorbance at 550 nm of the final supernatant was determined.

Protein determination. The method of Lowry et al. [18] was used with bovine serum albumin as standard.

Results

Selection of media suitable for the determination of enzyme activities

In determining the enzyme activities located on the cell surface of monolayer cells or suspended cells, we have to choose a medium that does no damage the cell surface when added with the substrate. We determined the rate of hydrolysis of various substrates by intact L cells suspended in phosphate-buffered saline, Krebs-Ringer and Hank's solution with and without the addition of 2.5% albumin [19] and compared the recovery of enzyme activities in different media. When the enzymes which can not be detected on the cell surface were to be determined, commercial preparations of the corresponding enzyme were employed. As shown in Table I, the highest recovery was always obtained in phosphate-buffered saline and the recovery in Hank's solution ranked next. However, the determination of the fluidity of the cell membrane by the method of Inbar and Sachs [20,21] indicated that Hank's solution is preferred to phosphate-buffered saline from the standpoint of membrane stability. Accordingly, when assaying the enzyme activities on the cell membrane, we used Hank's solution in order to avoid damage to the cell membrane. When assaying

TABLE I

RECOVERY OF ENZYME ACTIVITIES IN DIFFERENT MEDIA WITH PRESENCE AND ABSENCE OF ALBUMIN

Each figure represents the average of triplicate experiments.

Enzymes	Substrates	Buffers					
		Phosphate-buffered saline		Krebs-Ringer		Hank's solution	
		—	2.5% albumin	—	2.5% albumin	—	2.5% albumin
Aminopeptidase A	Glu-β-naphthylamide	100	62.6	76.2	75.0	90.6	76.1
Aminopeptidase B	Arg-β-naphthylamide	100	130.6	42.9	83.7	66.0	105.7
Aminopeptidase B	Lys-β-naphthylamide	100	108.0	71.2	112.0	82.8	90.5
Glycine-aminopeptidase	Gly-β-naphthylamide	100	53.6	37.6	47.6	80.5	65.4
Alanine-aminopeptidase	Ala-β-naphthylamide	100	87.9	69.0	72.1	85.4	87.7
Serine-aminopeptidase	Ser-β-naphtylamide	100	84.8	70.0	60.2	79.2	46.5
Proline-aminopeptidase	Pro-β-naphthylamide	100	63.0	72.1	63.0	109.2	92.6
Valine-aminopeptidase	Val-β-naphthylamide	100	69.0	69.0	54.0	80.9	62.6
Leucine-aminopeptidase	Leu-β-naphthylamide	100	94.7	58.5	83.0	88.1	91.4
Methionine-aminopeptidase	Met-β-naphthylamide	100	113.0	73.9	87.0	91.7	105.7
Phenylalanine-aminopeptidase	Phe-β-naphthylamide	100	81.0	36.5	36.0	89.0	59.6
Gly-Pro-aminopeptidase	Gly-Pro-β-naphthylamide	100	67.5	108.3	95.8	103.7	91.3
Gly-Pro-Leu-aminopeptidase	Gly-Pro-Leu-β-naphthyl-amide	100	126.3	78.9	68.4	85.3	78.0
α-D-Glucosidase	*p*-nitrophenyl-α-Glc	100	160.0	91.9	91.9	88.7	93.3
β-D-Galactosidase	*p*-nitrophenyl-β-Gal	100	153.3	83.0	92.8	100.0	97.3
α-D-Mannosidase	*p*-nitrophenyl-α-Man	100	267.7	135.5	135.5	96.3	107.0
N-Ac-β-D-glucosaminidase	*p*-nitrophenyl-GlcNH$_2$	100	659.3	38.5	93.4	78.5	134.9
β-Glucuronidase	phenolphthalein-glucuronic acid	100	12.5	29.2	20.8	37.4	20.8
Trypsin	TosArgOMe	100	35.9	134.4	103.1	95.6	62.1
Trypsin	BzArgOEt	100	82.1	103.6	125.0	93.2	76.5
Chymotrypsin	AcTyrOEt	100	71.2	130.3	121.2	94.1	70.4
Elastase	AcAla$_3$OMe	100	102.7	135.1	140.5	87.7	75.0

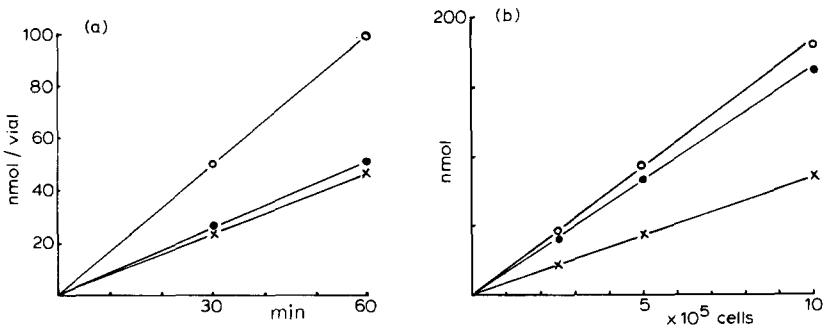

Fig. 1. (a) Time course of aminopeptidase activities on intact canine kidney cells. The hydrolysis of alanine-β-naphthylamide, lysine-β-naphthylamide and leucine-β-naphthylamide were determined on the surface of intact canine kidney cells. Enzyme activities are expressed as nmol of naphthylamine per vial bottle in the Hank's solution. Each point was obtained from the average of triplicate samples. ○———○, alanine aminopeptidase; ●———●, aminopeptidase B; X———X, leucine aminopeptidase. (b) Proportionality between surface enzyme activities and number of L cells. The hydrolysis of alanine-β-naphthylamide, arginine-β-naphthylamide and leucine-β-naphthylamide were determined on the surface of intact L cells. Enzyme activities are expressed in nmol of naphthylamine per h and per number of cells in the Hank's solution given abscissa. Each point was obtained from the average of triplicate samples. ○———○, alanine aminopeptidase; ●———●, aminopeptidase B; X———X, leucine aminopeptidase.

enzyme activities in the membrane fractions, phosphate-buffered saline was used.

The kinetics of the enzyme activities

Fig. 1 (a) illustrates the time course of the enzyme reaction which occurs on surface of canine kidney cells. The catalytic activities of alanine-, lysine- and leucine-aminopeptidases were constant for up to 60 min. As shown in Fig. 1 (b), aminopeptidase activities located on the surface of L cells were also proportional up to at least 10^6 cells in the assay medium. As the control experiment, cells were incubated for 60 min without substrate and the supernatant was checked for any release of these enzymes. Incubation of the cells without substrate did not cause any release of the enzymes.

Comparison of the enzyme activities located on the cell surface with those present in the cell homogenate

The enzyme activities on the cell surface were compared with those in the cell homogenates in monkey kidney and canine kidney cells grown in the form of monolayer and in L and FM3A cells grown in the form of suspension culture. Although aminopeptidase activities were always detected on the surface in any kind of cells, activities of trypsin, chymotrypsin and elastase, which are all endopeptidases, were not detected on the cell surface. While glycosidase activities were not detected in monolayer cells, they were detected in low concentration in the suspension cultured cells (Table II). Among the aminopeptidase activities detected on the cell surface, alanine-, leucine-, methionine- and phenylalanine-aminopeptidase showed rather high activities and aminopeptidase A, proline-, valine-, glycylproline dipeptidyl- and glycylprolylleucine tripetidyl-aminopeptidase showed relatively low activities. On the whole, the enzyme activities on the cell surface were higher in the suspension-cultured cells than in

TABLE II

COMPARISON OF ENZYME ACTIVITIES LOCATED ON THE CELL SURFACE WITH THOSE PRESENT IN THE CELL HOMOGENATE

The homogenates were prepared in Dounce homogenizer with 200 strokes. The enzyme activities were expressed as nmol/min per mg protein. The ratio of the activity on the cell surface to that in the homogenate was calculated for each species of the enzymes. The enzyme activity is expressed as given in Table I. Each figure represents the average of triplicate experiments.

Enzymes	Monkey kidney			Canine kidney			L			FM 3A		
	Homogenate	Surface	Ratio	Homogenate	Surface	Ratio	Homogenate	Surface	Ratio	Homogenate	Surface	Ratio
Aminopeptidase A	6.0	0.7	11.6	2.9	0.3	10.2	1.8	0.3	16.6	1.7	0.3	17.6
Aminopeptidase B	7.9	0.2	2.5	8.0	0.3	3.8	8.7	2.1	24.0	10.3	2.7	26.2
Aminopeptidase B	8.3	1.8	21.7	19.2	4.0	20.8	5.6	2.2	39.3	8.9	3.3	37.1
Glycine-aminopeptidase	5.9	0.7	11.9	11.6	3.0	25.8	4.1	1.6	39.0	6.5	2.5	38.4
Alanine-aminopeptidase	19.6	4.5	23.0	23.2	4.0	17.2	16.2	6.5	40.1	19.9	9.3	46.7
Serine-aminopeptidase	2.2	0.4	18.2	4.8	1.1	22.9	1.9	0.6	32.2	5.0	1.7	34.0
Proline-aminopeptidase	3.3	0.7	21.2	2.4	0.5	20.8	1.2	0.4	32.8	6.3	1.3	20.1
Valine-aminopeptidase	3.8	0.8	21.0	5.8	1.3	22.4	0.7	0.2	28.6	0.8	0.2	25.0
Leucine-aminopeptidase	19.4	3.6	18.6	16.4	3.8	23.2	12.8	5.9	46.0	15.6	6.4	41.0
Methionine-aminopeptidase	26.6	6.2	23.3	41.8	6.6	15.8	19.4	7.4	38.1	3.0	1.4	46.6
Phenylalanine-aminopeptidase	25.6	5.7	22.2	23.8	5.1	21.4	9.0	4.1	45.6	13.2	6.5	49.2
Gly-Pro-aminopeptidase	3.6	0.9	25.0	14.0	3.6	25.7	1.1	0.3	27.2	0.8	0.2	25.0
Gly-Pro-Leu-aminopeptidase	3.8	1.0	26.3	8.2	2.3	28.0	2.4	0.9	37.5	1.8	0.7	38.9
α-D-Glucosidase	0.4	0	0	0.6	0	0	2.4	0.3	12.5	1.0	0.2	20.0
β-D-Galactosidase	0.6	0	0	0.4	0	0	0.4	0.1	25.0	0	0	0
α-D-Mannosidase	0.4	0	0	0.8	0	0	2.8	0.6	21.4	0.7	0.2	28.5
N-Ac-β-D-Glucosaminidase	0.8	0	0	1.6	0	0	2.8	0.4	14.2	1.2	0.2	16.6
β-Glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0
Trypsin	24.6	0	0	10.4	0	0	3.6	0	0	6.2	0	0
Trypsin	18.1	0	0	11.5	0	0	4.6	0	0	3.4	0	0
Chymotrypsin	36.5	0	0	40.8	0	0	46.2	0	0	20.0	0	0
Elastase	4.8	0	0	1.5	0	0	4.0	0	0	41.6	0	0

TABLE III

ENZYME ACTIVITIES DETECTED IN THE MEMBRANE FRACTIONS WITH THOSE IN THE CELL HOMOGENATE

Results are expressed as nmol/min per mg protein. Preparation of the cell homogenate and the membrane fraction proceed as described in the text. The enzyme activity is expressed as given in Table I. Each figure represents the average of triplicate experiments.

Aminopeptidases	Rat liver			L			Ehrlich		
	Homogenate	Membrane	Ratio	Homogenate	Membrane	Ratio	Homogenate	Membrane	Ratio
A	3.0	3.3	1.1	0.9	1.4	1.6	1.1	4.2	3.8
B (Arg)	9.1	2.7	0.3	3.6	3.3	0.9	14.0	18.3	1.3
B (Lys)	6.7	3.9	0.6	5.9	14.1	2.4	—	—	—
Glycine	1.3	2.7	2.1	2.3	2.6	1.1	4.1	8.3	2.0
Alanine	6.4	7.8	1.2	6.7	12.8	1.9	19.1	41.5	2.2
Serine	0.4	0.6	1.5	2.2	2.6	1.2	3.5	4.2	1.2
Proline	2.0	2.2	1.1	1.0	1.3	1.3	7.0	4.6	0.7
Valine	3.8	2.2	0.6	0.6	0.7	1.2	1.4	4.9	3.5
Leucine	4.7	7.5	1.6	8.3	26.8	3.2	13.2	22.5	1.7
Methionine	14.9	7.4	0.5	10.4	7.0	0.7	15.3	18.4	1.2
Phenylalanine	7.2	9.2	1.3	4.6	15.3	3.3	15.3	24.5	1.6
Gly-Pro	7.9	22.3	2.8	0.8	3.8	5.0	1.4	3.4	2.4
Gly-Pro-Leu	2.0	1.6	0.8	1.0	0.7	0.7	2.0	1.8	0.9

the monolayer cells, probably because the suspension-cultured cells have a larger surface area. When any type of cell was incubated only with medium, no enzyme activity was detected in the supernatant and therefore it was concluded that the cells do not release enzymes into the medium during incubation. Furthermore, after such cells were incubated with the substrate, electron microscopy of the cells revealed that they remained intact. The viability of the cells was not decreased by incubation and nigrosin staining also showed the intactness of the cells after incubation. In certain species of aminopeptidase, the enzyme activities on the cell surface were rather high, compared with those in the homogenate. In order to get further confirmation of the localization of enzymes on the cell surface, enzyme activities were determined on membrane fractions prepared from several different kinds of cells.

Comparison of the enzyme activities in isolated membrane fractions with those in cell homogenates

Aminopeptidase activities were determined in the membrane fractions prepared from rat liver, L and Ehrlich cells. As shown in Table III, the specific activities in the membrane fraction were higher than those in the cell homogenate for most species of aminopeptidase and this indicates the localization of the enzyme activities in the membrane fractions. However, for certain kinds of aminopeptidase, the specific activities of the membrane fraction do not exceed those in the homogenates. The inactivation of the enzymes during preparation of the membrane fractions and the release of the activities from the membranes may be responsible for this. It seems likely that aminopeptidase activity on the cell membrane is rather loosely bound to the membrane.

Further proof for the localization of enzyme activity on the cell surface

In order to give a further proof that the enzymatic activities so far described are actually located on the cell surface, the enzymatic activities of intact cells were determined by using L-Leu-Gly-Gly or L-Leu-Gly-Gly · glass beads as the substrate. Purified preparation of leucine aminopeptidase from rat liver hydrolyzed both L-Leu-Gly-Gly and L-Leu-Gly-Gly · glass beads. The rate of hydrolys-

TABLE IV

RELEASE OF LEUCINE FROM THE SUBSTRATE AND THE GLASS BEAD-BOUND SUBSTRATE

The cells were grown in Falcon dishes (35 × 10 mm) and washed with phosphate-buffered saline, 2 ml of phosphate-buffered saline containing L-Leu-Gly-Gly (10 mg) or L-Leu-Gly-Gly · glass beads (100 mg) were added and the dishes were incubated for 1 h at 37°C. The supernatant was centrifuged for 10 min at 3000 rev./min and concentrated by evaporation in vacuo. The residue was dissolved in a small amount of water and submitted to thin-layer chromatography.

Substrates	Leucine released (μmol/10^6 cells)				
	SV40-C3H	Ehrlich	L-1210	Monkey kidney	Canine kidney
L-Leu-Gly-Gly	1.33	1.38	1.52	1.30	1.21
L-Leu-Gly-Gly · glass beads	0.25	0.30	0.38	0.25	0.22
Glass beads	0	0	0	0	0

TABLE V

EFFECT OF BESTATIN AGAINST AMINOPEPTIDASE B AND LEUCINE AMINOPEPTIDASE IN VARIOUS CELLS

Inhibitory activity against aminopeptidase B and leucine aminopeptidase were assayed as described previously [16].

Enzymes	Final concn. of inhibitor (μg/ml)	Percent inhibition						
		Rat liver	Monkey kidney	Canine kidney	L	Ehrlich	L-1210	Yoshida
Aminopeptidase B	10	76.8	84.5	71.2	66.7	90.4	51.0	84.8
	2	61.0	71.0	47.6	47.1	73.6	44.8	63.1
	0.5	44.9	52.3	26.8	30.2	53.6	30.5	30.4
	0.1	24.4	34.0	11.5	14.6	26.8	18.7	10.2
Leucine aminopeptidase	50	48.2	56.0	60.9	43.6	40.7	26.5	65.4
	10	31.6	38.8	42.2	32.3	18.5	22.2	55.8
	2.5	23.8	24.8	28.3	23.7	6.5	17.6	37.5
	1.0	18.4	17.5	20.6	18.0	2.8	7.8	29.0

is was much higher for the substrate of the smaller molecule. Both substrates were incubated for 1 h at 37° C with SV40-C3H, Ehrlich, L-1210, monkey kidney and canine kidney cells, respectively and the supernatant after incubation was concentrated and submitted to silica gel thin-layer chromatography with *n*-butanol/acetic acid/water (4 : 1 : 1) as the solvent. As detected by Ninhydrin spraying, a spot corresponding to leucine with R_F value of 0.44 appeared only when the cells were incubated with substrates. The leucine spot was scanned with an Ozumor densitometor at 530 nm using the slit 2 × 8 mm. As shown in Table IV, the terminal leucine residue was clearly released from L-Leu-Gly-Gly · glass beads, although its amount was about one fifth of that released from the low molecular substrate, L-Leu-Gly-Gly. This result shows that at least leucine aminopeptidase is distributed on the cell surface. When the cells were incubated with only glass beads under the similar condition, the enzyme activities were not released into the supernatant. Furthermore, the intactness of the cells after incubation with glass beads was also ascertained by microscopic observation.

The effect of a specific inhibitor of aminopeptidase B and leucine aminopeptidase located on the cell surface

Bestatin is a specific inhibitor of aminopeptidase B and leucine aminopeptidase isolated from the culture filtrate of *Streptomyces olivoreticuli* by Umezawa et al. [16,22]. As shown in Table V, it strongly inhibited all the activties previously detected on the surface of various cultured cells, tumor cells and the rat liver homogenate. This phenomenon seems to be common to both enzymes in every cell type tested.

Discussion

The present experiments clearly indicate for the first time that at least some aminopeptidases are located on the cell surface of monkey kidney, canine kidney, L, FM3A and various tumor cells.

As shown in Table II, aminopeptidase activities were demonstrated on the surface of monkey kidney, canine kidney, L and FM3A cells while endopeptidases and glycosidases were not detected on the cell surface. However, low glycosidase activities were detected in suspended culture cells. When we prepared the membrane fractions from rat liver, L and Ehrlich cells, aminopeptidase activities were actually detected in the membrane fraction but no glycosidase nor endopeptidase activity could be detected in this fraction (Table III). When the cells were incubated with L-Leu-Gly-Gly · glass beads, the terminal residue of leucine was hydrolyzed (Table IV). This gives further proof of the localization of the enzyme on the cell surface. The amount of leucine residue released from L-Leu-Gly-Gly · glass beads was almost one fifth of that released from L-Leu-Gly-Gly. This is probably because the affinity of the enzyme for the glass bead-bound substrate was decreased by steric hindrance.

When substrates with low molecular weight were incubated with the cells in order to determine the enzyme activities located on the cell surface, we cannot eliminate the possibility that the enzyme activities located on the cell surface were over-estimated. The reason is that low-molecular-weight substrates can

penetrate into the cytoplasm and that the corresponding hydrolysis products are released back into the medium. This might explain our observation in certain species of aminopeptidase that the enzyme activities on the cell surface were rather high, compared with those in the homogenate (Table II). Alternatively, the presence of inhibitor in the homogenate could be assumed and we might have observed in the cell homogenate enzyme activities which are artificially low.

The viability of the cells examined remained constant and no cell damage was observed after the cells had been incubated with the substrate. Therefore, it is reasonable to assume that aminopeptidases and some glycosidases really are located on the cell surface and that the enzyme activities observed under our experimental conditions are not due to release from lysosomes of damaged or punctured cells.

ATPase is known as an example of the enzymes which are tightly bound to the membrane of most species of mammalian cells [23]. Glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase are also reported to be present on the membrane of Ehrlich tumor cells but the association between the enzymes and the membrane are suggested to be rather loose [19]. Aminopeptidases, which we detected for the first time on the surface of mammalian cells, can be considered to be bound loosely to the membrane as is the case of the latter.

Bestatin, a specific inhibitor of aminopeptidase B and leucine aminopeptidase, similarly inhibited enzymes on various cell surfaces (Table V). It is suggested that bestatin binds with these enzymes on the cell surface and causes some change in the cell function. As will be reported in another paper [24] we found that bestatin produces a strong enhancement of delayed-type hypersensitivity in mice to sheep red blood cells as determined by the footpad thickness. The compound also augmented the immune resistance to cancer and suppressed the growth of transplanted tumors. Since many kinds of hydrolytic enzyme are located on or near the cell surface, it is possible that these enzymes in the cell periphery might play an important role in the processes of inflammation, immunity, oncogenesis, metastasis or virus infection. How the surface aminopeptidases behave in the various cell functions in such cases is also an interesting question.

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