IMMUNOCHEMICAL IDENTIFICATION OF THE CELL SURFACE BOUND LEUCINE AMINOPEPTIDASE, THE TARGET ENZYME FOR THE IMMUNOSTIMULANT BESTATIN

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The microbial product bestatin is known to inhibit soluble microsomal- and cytosolic leucine aminopeptidase (Leu-APm and Leu-APc) as well as aminopeptidase B (AP-B). To clarify which of these enzymes is the target for bestatin on the cell surface, indirect immunofluorescence studies with antisera raised against purified Leu-APm and AP-B were performed. These antibodies (anti-Leu-APm and anti-AP-B) were found to react with intracellularly localized Leu-APm and AP-B of ethanol-treated L5178y cells. Using non-treated L5178y cells fluorescence was detected only on the cell surface after incubation with anti-Leu-APm. To confirm the supposition that only Leu-APm is present on the cell surface, the AP from the cell membrane was solubilized and analyzed electrophoretically. Based on relative migration data it could be shown, that the cell surface is charged with Leu-APm and not with detectable amounts of Leu-APc or AP-B. Moreover, it could be demonstrated that the solubilized Leu-APm binds to [^{3}H]bestatin.

Based on the initial work of SCHNEBLI and BURGER[1], it has now been experimentally shown that cell surface-bound proteases are involved in cell to cell adhesion[2] during embryonic morphogenesis. Moreover, it has been suggested that the breakdown of tissue integrity and the dynamic development of breast tissue may be mediated by proteases[3]. Analytical studies of UMEZAWA and coworker[4] revealed, that activities of aminopeptidases are located on the cell surface of mammalian cells. In order to elucidate their biological function UMEZAWA rationally screened for specific inhibitors of microbial origin of these enzymes[5,6]. Among a series of inhibitors isolated and physico-chemically described, one microbial metabolite was identified which has already gained therapeutic importance namely, bestatin[7,8]. This dipeptide, [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, was found to enhance immune responses and to cause antitumor effects *in vivo*[9,10,11].

Studies on an enzymic level revealed bestatin to be a strong competitive inhibitor of cytosolic leucine aminopeptidase (Leu-APc; EC 3.4.11.1), microsomal leucine aminopeptidase (Leu-APm; EC 3.4.11.2) and of aminopeptidase B or arginyl aminopeptidase (AP-B; EC 3.4.11.6)[9,5,12]. These published kinetic experiments were performed with the substrates L-leucine-β-naphthylamide (Leu-NA) and L-arginine-β-naphthylamide (Arg-NA). It was shown that Leu-APc hydrolyzed Leu-NA and AP-B hydrolyzed only Arg-NA, while Leu-APm degraded both Leu-NA and Arg-NA[4,12]. Hence, additional characteristics of the aminopeptidase(s) had to be used in order to identify which cell surface-bound enzyme interacts with bestatin. In a preceding contribution[12] we described, that the solubilized, cell surface-bound aminopeptidase electrophoretically co-migrates with Leu-APm. Moreover it was

shown[12], that the cell surface-bound aminopeptidase is inhibited by the microbial product amastatin, an inhibitor of Leu-AP but not of AP-B[13]. From these data we concluded that Leu-APm is the enzyme which interacts with bestatin on the surface of L5178y cells. In the present contribution we apply immunochemical methods to demonstrate, that Leu-APm is the target enzyme for bestatin on the cell surface.

Materials and Methods

Materials

The following materials were obtained: L-leucine-β-naphthylamide·HCl, L-arginine-β-naphthylamide·HCl and leucine aminopeptidase (microsomal, from swine kidney; 25 units/mg) from Sigma, St. Louis, Mo.; leucine aminopeptidase (cytosolic, from swine kidney; 100 units/mg) from Boehringer, Mannheim, Germany. Unlabeled bestatin was prepared by Nippon Kayaku Co. Ltd. using the method described by SUDA *et al.*[14] Radioactively labeled bestatin was prepared as described previously[15]; the specific activity of [^{3}H]bestatin was 55 Ci/mmole.

L5178y mouse lymphoma cells, a Thy-1, 1-bearing, Fc receptor positive T-lymphoma, were grown in suspension as described[12,16].

Aminopeptidase B

Aminopeptidase B was isolated from mouse liver and purified by the method of HOPSU *et al.*[17]. After differential $(NH_4)_2SO_4$-fractionation, Sephadex G-200 gel filtration and DEAE-cellulose chromatography, the AP-B enzyme preparation was free from Leu-AP and pure according to the described criteria[17]. The specific activity of AP-B was 3.9 units/mg protein using Arg-NA as substrate.

Cell Membranes and Solubilization

The cell membranes were isolated from mouse liver according to the "Tris method"[18]. The membranes were solubilized in Triton X-100 as described[12].

In Situ Determination of AP Activity by Gel Electrophoresis

Enzyme preparations were subjected to polyacrylamide gel electrophoresis in the presence of Triton X-100[19,12]. After separation, the gels were either stained with Coomassie brilliant blue to identify protein bands or assayed for distribution of enzyme activity[12], using 300 μM Leu-NA as a substrate.

In Situ Binding Studies

For the determination of the binding capacity of both the isolated Leu-APs and the membrane bound AP, the enzymes were first separated by polyacrylamide gel electrophoresis in the presence of Triton X-100[19,12]. Subsequently the gels were thoroughly washed in PBS and then incubated (30 minutes, 37°C) in a 100 mM Tris-maleate buffer (pH 7.2), supplemented with 20 μM [^{3}H]bestatin (50 mCi/mmole). After incubation, the non-bound radioactivity was washed off and the distribution of the radioactivity was determined after slicing the gels[20].

Preparation of Antibodies

Antisera to Leu-APm and AP-B were produced in female New Zealand rabbits (weighing about 3.5 kg) by 3 to 6 two-weekly subcutaneous injections with 0.5 ml of complete Freund's adjuvant, containing 50 μg of enzyme protein. These antibodies will be referred to as anti-Leu-APm or anti-AP-B. The immunoglobulin fraction was isolated from antisera by precipitation with 37% saturated ammonium sulfate.

Immunological Procedures

Microdiffusion was carried out on glass microscope slides coated with 1% agarose in PBS[21]. The precipitin lines were stained with Coomassie brilliant blue or with Leu-NA and Fast Garnet GBC[15].

For immunofluorescence studies, suspensions of L5178y cells (5×10^6 cells/ml) were assayed in PBS and were either fixed (5 minutes, 4°C) in 95% ethanol or let remain untreated and processed for indirect immunofluorescence. Antisera (1: 64 dilution) were added to the cultures for 15 minutes at 20°C in a moist chamber. After washing for 10 minutes in PBS, the cells were incubated in the presence of fluore-

scein-conjugated goat anti-rabbit IgG (15 minutes) followed by two washes in PBS (5 minutes each). The processed cells were mounted with buffered glycerol (pH 7.2) and the cells were observed for fluorescence using narrow-band filters selective for fluoresceine. Normal rabbit serum controls were included.

Results

Affinities of Microsomal Leucine Aminopeptidase to Bestatin

The commercially available Leu-APm preparation was analyzed gel chromatographically under non-denaturing conditions and determined to consist only of one protein species which coincided with the enzyme activity (Fig. 1 A, a and b). Addition of 15 μM bestatin during the incubation of the gels in the presence of 300 μM Leu-NA, reduced the intensity of the staining of Leu-APm band by more than 90% (Fig. 1 B, a and b).

In a previous contribution we have shown[12], that the solubilized cell membrane bound AP co-migrates in polyacrylamide gels with Leu-APm but not with Leu-APc. This enzyme activity could also be inhibited by bestatin. These findings already implied that the solubilized membrane bound AP is the target enzyme for bestatin. To support this assumption in a direct way, *in situ* binding studies in polyacrylamide gels were detected after incubation in the presence of Leu-NA and Fast Garnet GBC[15]. The relative mobilities of the APs were as follows; Leu-APm: 0.25; Leu-APc: 0.35 and membrane bound AP: 0.25 (Fig. 2 A, a~c). After incubation of the separated gels in the presence of [^{3}H]bestatin

Fig. 1. *In situ* determination of purified Leu-APm by gel electrophoresis.

A: Separation of 10 μg of Leu-APm; (a) gel assayed for distribution of enzyme activity and (b) gel stained with Coomassie brilliant blue.

B: Separation of 1 μg of Leu-APm followed by staining for enzyme activity in the absence (a) or presence of 15 μM of bestatin (b).

F=front.

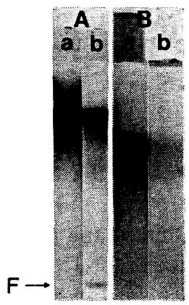
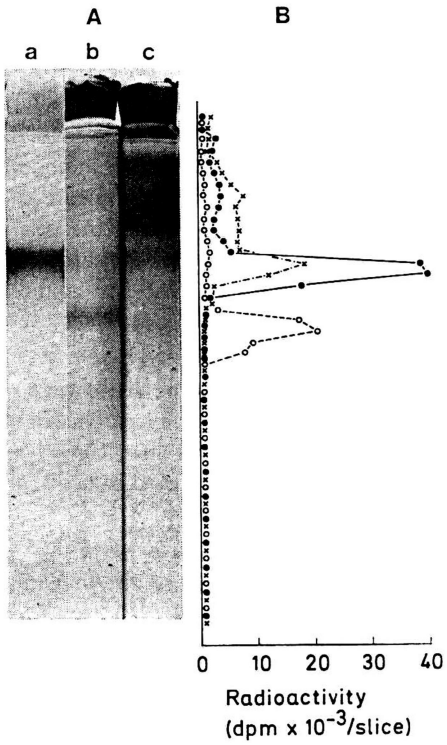


Fig. 2. *In situ* determination of bestatin binding to cell membrane bound AP.

A: Separation of purified Leu-APm (10 μg/gel) (a), of purified Leu-APc (40 μg/gel) (b) and of solubilized membrane fraction (supernatant obtained after Triton extraction of 500 μg isolated membranes) (c).

The gels were incubated with 300 μM Leu-NA to trace the enzyme activity. B: *In situ* binding of [^{3}H]bestatin to Leu-APm (●—●), to Leu-APc (○---○) and membrane bound AP (×-·-×). Enzyme preparation, at the same concentrations as used under (A), was separated by polyacrylamide gel electrophoresis and subsequently incubated with [^{3}H]bestatin as described under "Methods".



(Fig. 2 B), the radioactivity bands in the gels for the respective enzymes coincided with their enzyme activity bands. These findings demonstrate, that the solubilized, membrane associated AP has the same electrophoretic mobility as the Leu-APm and binds bestatin at a comparable affinity.

Antisera Against Aminopeptidases

Antisera were produced against purified Leu-APm and AP-B. Their specificities were tested by double diffusion analysis. Testing anti-Leu-APm (Fig. 3) it was found that a 1: 128 diluted antiserum gave only one band with Leu-APm in Ouchterlony tests (Fig. 3 A). Amounts of enzymes as low as 0.01 μg could be traced by staining the slides for enzyme activity with Leu-NA/Fast Garnet GBC (Fig. 3 B). The enzyme remains active after completion of the precipitin reaction (Fig. 3 C). Even under this condition (precipitation of the enzyme by the antibody) the enzyme could be inhibited by bestatin (Fig. 3 D). As shown in Fig. 3 B, anti-Leu-APm was found to be specific for Leu-APm; no precipitin reaction could be detected for Leu-APc and AP-B.

Anti-AP-B was determined to form a strong precipitin line with AP-B which had been visualized both by staining for protein (Fig. 4 A) and for enzyme activity (Fig. 4 B). The antiserum did not cross-react with Leu-APm or Leu-APc.

Localization of Leucine Aminopeptidase

The immunological determination of the localization of APs was performed with L5178y cells. Ethanol fixed cells were incubated with both anti-Leu-APm (Fig. 5 B) and anti-AP-B (Fig. 5 C) and developed for indirect immunofluorescence. In both series, 100% of the cells were shown to be intracellularly stained; no difference in the intensity between nucleus and cytoplasm could be detected. In most cases the antisera strongly reacted with a small particle of unknown nature (size: 0.9 ± 0.2 μm). In control experiments, using sera from untreated rabbits, no fluorescence could be detected (Fig. 5 A).

Using non-fixed L5178y cells only anti-Leu-APm reacted with the surface of these cells (Fig. 6 B), while anti-AP-B (Fig. 6 D) and control serum (Fig. 6 A) caused no detectable fluorescence. As in the double diffusion experiments, bestatin had no influence on the binding of anti-Leu-APm to the cell surface (Fig. 6 C). Of interest might be the fact that the FITC label of anti-Leu-APm-treated cells was not uniformly distributed on the cell surface but concentrated on $0.3 \sim 0.6$ μm areas. These findings strongly suggested that the Leu-APm is not only distributed in the cytoplasm and perhaps also in the nucleus, but it is also present on the surface of L5178y cells.

Discussion

Since the discovery by UMEZAWA, that low molecular weight protease inhibitors[22] modulate the immune response[5], detailed investigations were performed to elucidate their mode of action on cellular, subcellular and biochemical level (surveys: References 13, 11, 23). Among the 50 inhibitors of various enzymes discovered[6], increasing interest was given to bestatin because of its potential use in human chemotherapy[8]. It is well established that bestatin, a potent inhibitor of isolated Leu-APm, Leu-APc and AP-B[5,12], binds to the cell surface[24,15]. Evidence is presented, indicating that Leu-AP and AP-B are also associated with the cell surface[4]. Detailed biochemical studies revealed that it is the Leu-APs which are the target enzymes for bestatin[24,12]. However, these enzymic studies were performed with Leu-NA, which is known not to be a special substrate for this group of enzymes[25]. Therefore, we applied in the present study immunological means (indirect immunofluorescence technique) to identify the cell surface bound AP. Antibodies, prepared against purified Leu-APm and AP-B, were determined to function mono-specifically with respect to the enzyme spectrum Leu-APm, Leu-APc and AP-B. In

Fig. 3. Agar gel diffusion experiments with antibodies against Leu-APm (anti-Leu-APm).

The center well contained anti-Leu-APm in a dilution of 1:128. The outer wells in A, C and D contained: (a), (d), (e) and (h) saline; (b) and (g) 6 μg of Leu-APm and (c) and (f) 1 μg of Leu-APm. A was stained with Coomassie brilliant blue, C and D for enzyme activity with 300 μM Leu-NA. The incubation assay in D contained in addition to 300 μM Leu-NA 15 μM of bestatin. B: Immunodiffusion of anti-Leu-APm against (a) 1 μg Leu-APm, (b) 0.1 μg Leu-APm, (c) 0.01 μg Leu-APm, (d) 10 μg Leu-APc, (e) 0.5 μg Leu-APc, (f) 10 μg AP-B, (g) 0.5 μg AP-B and (h) saline. The slide was stained to determine enzyme activity.

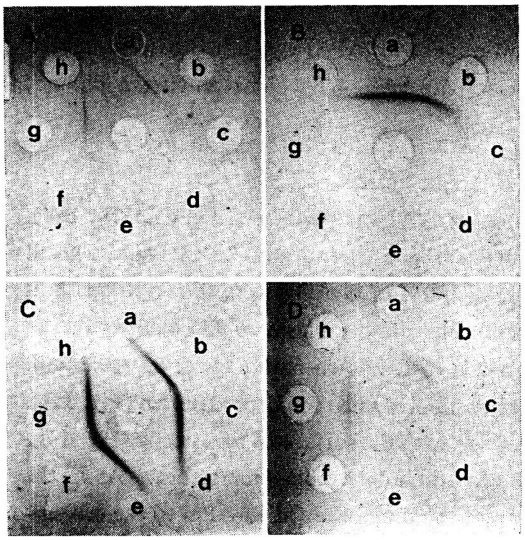


Fig. 4. Immunodiffusion studies with anti-AP-B (1:128 dilution; center well).

A and B contained in the outer wells the following components: (a) saline; (b) and (g) 10 μg of AP-B, (c) and (f) 1 μg of AP-B, (d) 10 μg of Leu-APm, (e) 1 μg of Leu-APm and (h) 3 μg of Leu-APc.

A was stained with Coomassie brilliant blue and B with Leu-Na/Fast Garnet GBC.

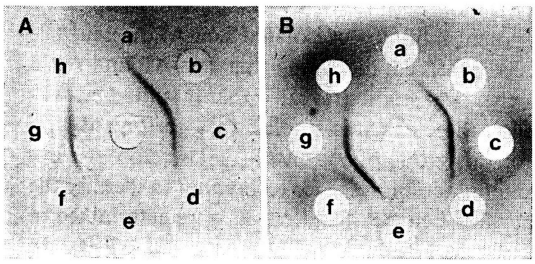


Fig. 5. Distribution of APs in ethanol-fixed L5178y cells.

After fixation, the cells were incubated with anti-Leu-APm (B), with anti-AP-B (C) or with control serum (A).

Further details are given under "Methods". Bar: 5 μm.

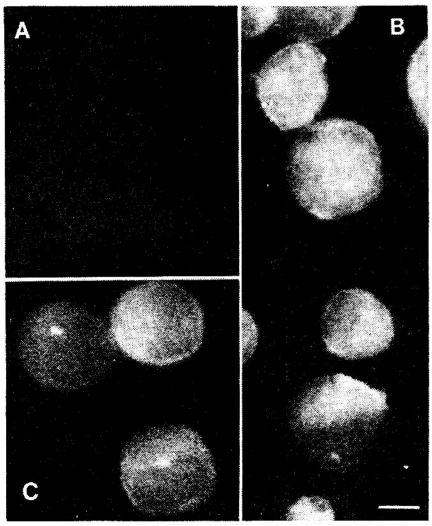


Fig. 6. Localization of APs in non-fixed L5178y cells.

The cells were incubated with anti-Leu-APm (B), with anti-Leu-APm in the presence of 20 μM bestatin (C), with anti-AP-B (D) or with control serum (A).

Bar: 5 μm.

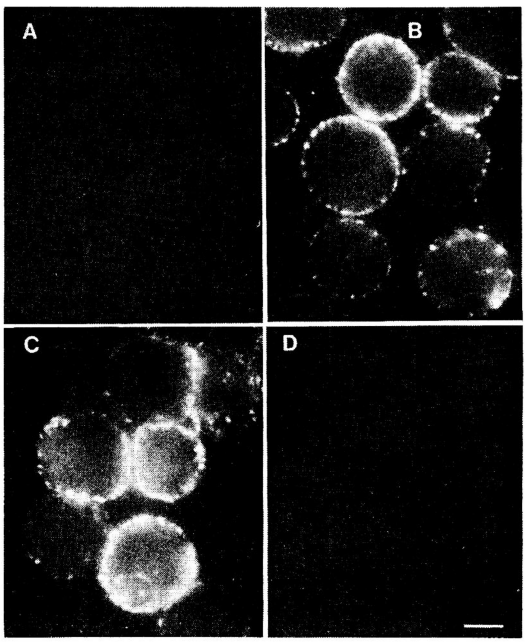


Table 1. Stoichiometric comparison between the number of bestatin binding sites and of Leu-APm molecules on the surface of a series of cell types.
Details are given in Discussion.

Cells	Binding of [^{3}H]bestatin (molecules/cell)	Aminopeptidase	
		Activity moles/cell × minute	Leu-APm molecules/cell
L5178y (cell culture)	13.4×10^5	1.15×10^{-15}	4.1×10^5
P 815	18.7×10^5	1.13×10^{-15}	4.0×10^5
T-Lymphocytes (spleen)	25.4×10^5	0.34×10^{-15}	1.2×10^5
B-Lymphocytes (spleen)	9.2×10^5	0.50×10^{-15}	1.8×10^5
Macrophages	145.9×10^5	11.41×10^{-15}	40.7×10^5

contrast to an earlier report[26], these antibodies caused no influence on enzymic activity of these enzymes. Using permeabilized L5178y cells fixed with ethanol, it was established that both Leu-APm and AP-B are almost homogeneously distributed intracellularly. Tracing enzyme activity in non-treated cells, fluorescence could be developed with anti-Leu-APm only; anti-AP-B failed to label the cells. Moreover it was demonstrated, that fluorescence after incubation with anti-Leu-APm is restricted to the cell surface. Therefore, it seems very probable that only Leu-APm is localized in detectable amounts on the cell surface.

In continuation of earlier biochemical approaches[12] it is now demonstrated, that radioactively labeled bestatin binds to the solubilized, cell surface bound AP. Electrophoresis studies revealed, that the relative mobilities of the enzyme activity and the bestatin-binding activity in the solubilized fraction are identical and correspond to that of Leu-APm. They differ from the Rf value of Leu-APc.

The conclusion, that the cell surface associated Leu-APm is the target enzyme for bestatin is additionally supported by a stoichiometric comparison (Table 1). From earlier contributions, both the bestatin-binding capacity[15] as well as the cell surface AP activity[12] of a series of cells are known. Taking the facts, that firstly the isolated Leu-APm is chromatographically pure (this report), secondly the molecular weight of this enzyme is 280,000[27] and thirdly the specific activity for Leu-APm is 5.8 μmoles/mg × minutes[12] into consideration, almost congruent values for the number of bestatin binding sites and of Leu-APm molecules are calculated. This intriguing correlation, together with the biochemical and immunochemical data strongly suggest, that the influence of bestatin on cell metabolism is initially caused by the binding to the cell surface associated Leu-APm.

Acknowledgments

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