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SUBCELLULAR LOCALISATION OF LEUCINE AMINOPEPTIDASE IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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Human polymorphonuclear leukocytes were homogenised in isotonic sucrose and subjected to analytical subcellular fractionation by sucrose density gradient centrifugation. The gradient fractions were assayed for leucine aminopeptidase and for principal organelle marker enzymes. Leucine aminopeptidase, when assayed with both L-leucine-7-amido-4-methyl-coumarin and leucyl-2-naphthylamide as substrate, showed a unimodal distribution with an equilibrium density of 1.18 $g \cdot cm^{-3}$. This distribution was quite distinct from that exhibited by marker enzymes for all the recognised subcellular organelles: there was no leucine aminopeptidase associated with the plasma membrane. Fractionation experiments with neutrophils treated with isotonic sucrose containing a low concentration of digitonin, and studies with the non-permeant ectoenzyme inhibitor, diazotised sulphanilic acid, confirmed that leucine aminopeptidase had a purely intracellular localisation. Fractionation experiments with neutrophils homogenised in sucrose medium containing digitonin, showed leucine aminopeptidase associated with a membrane fraction. It is suggested that leucine aminopeptidase is located to the membrane of a previously unrecognised population of cytoplasmic granules of the human neutrophil.

Introduction

5′-Nucleotidase was first used as a plasma membrane marker in the isolation of this subcellular organelle from rat liver [1]. Since then it has been localised to the plasma membrane of a variety of cell types and is considered to be the most specific enzymic plasma membrane marker available [2]. However, in human polymorphonuclear leukocytes the activity of this enzyme is quite low, the radioassay is time-consuming and it is necessary to include an additional substrate such as 2-glycerophosphate in the reaction mixture, to avoid measuring non-specific acid and alkaline phosphatases. An alternative plasma membrane marker, with a more convenient method of assay, would clearly be of interest in the further study of these cells.

Recently Nagaoka and Yamashita [3] claimed that leucine aminopeptidase is located solely to the external aspect of the plasma membrane in neutrophils from man, guinea pig and rabbit, and proposed it as a marker enzyme for this organelle. However, previous studies on human granulocytes have shown there to be three isoenzymes of leucine aminopeptidase in these cells [4]. One isoenzyme was found in the cytosolic fraction, a second was membrane-bound and extractable with detergent, and the third isoenzyme was found in a membrane fraction enriched with alkaline phosphatase.

Normal human polymorphonuclear leukocytes are a rich source of alkaline phosphatase which

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has recently been localised to a previously undescribed organelle, the phosphasome [5]. Since the function of this organelle is, as yet, unclear it was of interest to establish whether leucine aminopeptidase was also localised to this organelle. It was this controversy over the localisation of leucine aminopeptidase, its possible association with alkaline phosphatase, and the desirability of an alternative plasma membrane marker to 5′-nucleotidase, which prompted the present investigation.

The present study determines the subcellular localisation of leucine aminopeptidase in human polymorphonuclear leukocytes with analytical subcellular fractionation techniques. The distribution is compared to 5′-nucleotidase, the traditional plasma membrane marker, and marker enzymes for the other subcellular organelles. It was shown to be quite distinct from all those studied. It is suggested that human neutrophil leucine aminopeptidase is localised to a hitherto unrecognised intracellular organelle.

Materials and Methods

Materials. L-Leucine-7-amido-4-methylcoumarin was purchased from Uniscience (Cambridge) Ltd. and leucyl-2-naphthylamide-HCl was obtained from Sigma (London) Ltd. All other reagents were of Analar grade.

Isolation and homogenisation of polymorphonuclear leukocytes. Polymorphonuclear leukocytes were isolated from peripheral blood by dextran sedimentation and Ficoll-Hypaque centrifugation as previously described [6]. The cell preparations were examined microscopically and found to be at least 98% neutrophils. The cells were pelleted in 4 ml 0.2 mol/l sucrose containing 1 mmol/l Na_2EDTA. pH 7.2 and 5000 units heparin/litre, and disrupted with a Dounce homogeniser (Kontes Glass Co., Vineland, NJ, U.S.A.) by 30 strokes of a tight fitting (Type B) pestle. The homogenate was then centrifuged at $800 \times g$ for 10 min in a $4 \times$ 100 ml swing out rotor (Coolspin, MSE Scientific Instruments, Crawley, Sussex). The post-nuclear supernatant was removed, stored on ice and subjected to analytical subcellular fractionation and enzyme analysis as described previously [6].

Analytical subcellular fractionation. Approx. 5 ml of the post-nuclear supernatant were layered onto a 28 ml sucrose-density gradient, extending linearly with respect to volume, from a density of 1.05 $g \cdot cm^{-3}$ to one of 1.28 $g \cdot cm^{-3}$ and resting on a 6 ml cushion of density 1.32 $g \cdot cm^{-3}$ in a Beaufay automatic zonal rotor. All gradient solutions contained 1 mmol/l Na_2EDTA, pH 7.2 and 5000 units heparin/litre. The rotor was run at 35 000 rev./min for 35 min and, after slowing to 8000 rev/min, some 15 fractions were collected, weighed and their density determined. The integrated angular velocity during the centrifugation procedure was $3.3 \cdot 10^{10}$ radians^2/s.

Organelle marker enzymes. The gradient fractions were assayed for marker enzymes of the principal subcellular organelles. The enzymes assayed, with the organelle shown between parentheses, were: 5′-nucleotidase, EC 3.1.3.5 (plasma membrane); alkaline phosphatase, EC 3.1.3.1 (phosphasome granules); myeloperoxidase, EC 1.11.1.7 (azurophil, or primary, granule); *N*-acetyl-β-glucosaminidase, EC 3.2.1.30 (tertiary granules), particulate malate dehydrogenase, EC 1.1.1.37 (mitochondria) and lactate dehydrogenase, EC 1.1.1.27 (cytosol). Conditions for these assays have been described previously [5,7]. Unsaturated vitamin B-12 binding capacity (specific, or secondary, granule) was determined by the charcoal radioassay [8].

Assay for leucine aminopeptidase. Leucine aminopeptidase (EC 3.4.11.1) was assayed with two different substrates. In some assays an adaptation of the fluorimetric assays for γ-glutamyltransferase [9] was used, with L-leucine-7-amido-4-methyl-coumarin (L-leucine-AMC) as substrate. In the alternative assay the method of Peters et al. [10] was used, with leucyl-2-naphthylamide as substrate. The assay conditions were the same in each case. In brief, 0.2 ml 10 mmol/l L-leucine-AMC or leucyl-2-naphthylamide was diluted with 9.8 ml 0.1 mol/l Tris-HCl buffer, pH 8.0 containing 0.1% (w/v) Triton X-100. Enzyme activity was assayed by adding 0.1 ml suitably diluted neutrophil homogenate or gradient fraction to 0.25 ml substrate solution and incubating at 37°C for up to 60 min. The reaction was stopped by the addition of 2 ml ice-cold 0.05 mol/l glycine buffer, pH 10.4. The fluorescence

was measured with a Perkin-Elmer 204 fluorescence spectrophotometer with either 370 nm for excitation and 440 nm for emission wavelengths (L-leucine-AMC) or 340 nm for excitation and 410 nm for emission wavelengths (leucyl-2-naphthylamide).

Results

Fig. 1 shows the averaged density gradient distributions for leucine aminopeptidase and some of the principal organelle marker enzymes for human polymorphonuclear leukocytes. Leucine aminopeptidase when assayed with L-leucine-AMC or leucyl-2-naphthylamide as substrate, has a unimodal distribution with a modal density of 1.18 $g \cdot cm^{-3}$. This is quite distinct from 5′-nucleotidase, the traditional plasma membrane enzyme which has a modal density of 1.12 $g \cdot cm^{-3}$. The distribution profile and modal density of leucine aminopeptidase is also quite distinct from alkaline phosphatase (phosphasome granules), vitamin B-12 binding protein (specific granules), myeloperoxidase (azurophil granules) and *N*-acetyl-β-glucosaminidase (tertiary granules). However the modal density of leucine aminopeptidase is quite similar to that of particulate malate dehydrogenase (mitochondria), although the distribution profiles within the sucrose density gradient are dissimilar. In further experiments analytical subcellular fractionation was performed on neutrophils homogenised in isotonic sucrose containing 0.15 mg/ml digitonin. Digitonin exerts a dual action [11]. It is a selective membrane perturbant which binds to cholesterol in plasma membrane, selectively increasing the density of this organelle, and also acts as a detergent releasing lysosomal enzymes into the cytosolic fraction. The results of these experiments are shown in Fig. 2. Treatment with digitonin was shown to have a major effect on the gradient distribution of certain organelles of the neutrophil. Leucine aminopeptidase, as assayed with both L-leucine-AMC and leucyl-2-naphthylamide, and 5′-nucleotidase show denser, but still distinct distributions. Vitamin B-12 binding protein and *N*-acetyl-β-glucosaminidase showed a large increase in soluble and concomitant decrease in particulate component following digitonin treatment. In contrast, there was no change in the distribution profile or the model density of malate dehydrogenase. This is conclusive evidence that leucine aminopeptidase is not located to the mitochondria of the human polymorphonuclear leukocytes.

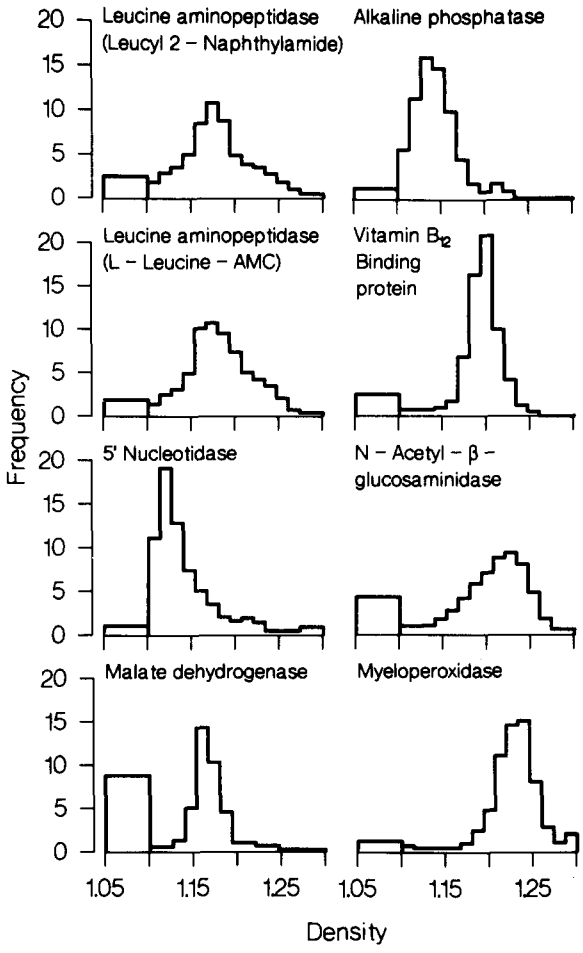

Fig. 1. Isopycnic centrifugation of post-nuclear supernatant prepared from neutrophil leukocyte homogenates. Results show mean distributions from four experiments. Frequency is defined as the fraction of total recovered activity present in the gradient fraction divided by the density span covered. The activity present over the density span 1.05–1.10 represents enzyme remaining in the sample layer. Recovered activities range from 87 to 105%.

Although the results in Fig. 1 show it is unlikely that leucine aminopeptidase is located to the plasma membrane, as reflected by 5′-nucleotidase, further studies were carried out to confirm this. In these experiments analytical subcellular fractionation experiments were performed on neutrophils

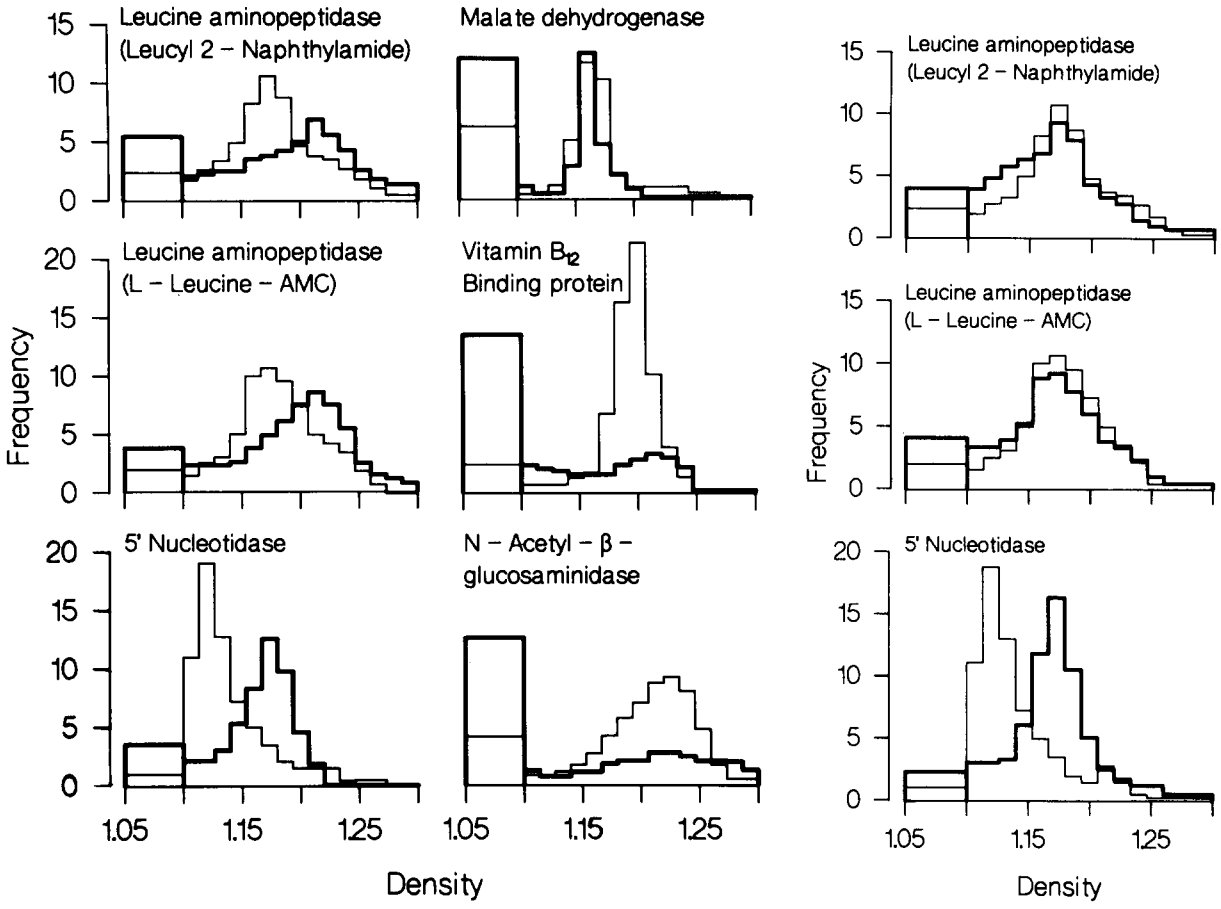

Fig. 2. Isopycnic centrifugation of post-nuclear supernatant prepared from control (———) and digitonin-treated (▬▬) neutrophil homogenates. Leukocytes were homogenised in 0.2 mol/l sucrose medium containing either no (control) or 0.15 mg/ml digitonin and fractionated. Averaged control data taken from Fig. 1. Digitonin data show the mean distributions from three experiments. Recovered activities for the digitonin experiments range from 80 to 98%. All other details as for Fig. 1.

Fig. 3. Isopycnic centrifugation of post-nuclear supernatant prepared from control (———) and digitonin-treated (———) neutrophils. Prior to homogenisation, the leukocytes were resuspended in 23 ml of 0.34 mol/l sucrose containing either no (control) or 0.04 mg/ml digitonin, centrifuged at $120 \times g$ and washed in digitonin-free 0.2 mol/l sucrose. The cells were then homogenised and fractionated. Averaged control data were taken from Fig. 1. Digitonin data show the mean distributions from three experiments. Recovered activities for the digitonin experiments range from 83 to 95%. All other details as for Fig. 1.

which had been resuspended in isotonic sucrose containing 0.04 mg/ml digitonin and then washed in digitonin-free sucrose medium. Intracellular organelles, which are inaccessible to the digitonin, would be unaffected by this treatment. The results of these experiments are shown in Fig. 3. 5'-Nucleotidase, the plasma membrane marker, was markedly affected within the sucrose density gradient and the modal density increased from 1.12 $g \cdot cm^{-3}$ to 1.18 $g \cdot cm^{-3}$. In contrast, the distribution of leucine aminopeptidase was unaffected by the digitonin treatment.

Further evidence in support of an intracellular localisation of leucine aminopeptidase is shown in Fig. 4. Diazotised sulphanilic acid is a poorly permeant reagent which has been shown to selectively inactivate ectoenzymes [12]. When intact cells were incubated with varying concentrations of diazotised sulphanilic acid, 5'-nucleotidase, an enzyme known to be located on the external surface

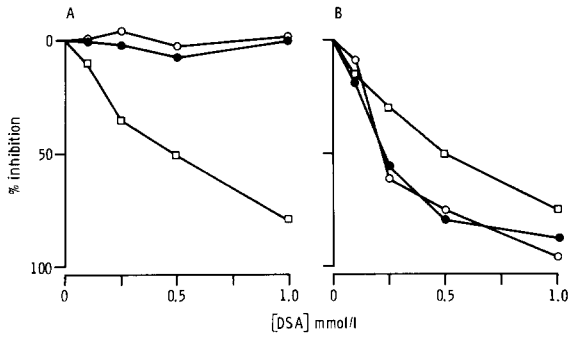

Fig. 4. (A) Intact polymorphonuclear leukocytes, suspended in phosphate-buffered saline, were incubated for 15 min at 37°C with various concentrations of diazotised sulphanilic acid (DSA). The cells were then washed twice with ice-cold phosphate-buffered saline, sonicated for 30 s and assayed for leucine aminopeptidase (●), 5′-nucleotidase (□) and lactate dehydrogenase (○). (B) Polymorphonuclear leukocytes were suspended in phosphate-buffered saline, sonicated for 30 s and then incubated for 15 min at 37°C with various concentrations of diazotised sulphanilic acid. The treated sonicates were then assayed for leucine aminopeptidase (●), 5′-nucleotidase (□) and lactate dehydrogenase (○). In both studies enzyme activities are expressed as percentages of those measured for untreated cells.

of the plasma membrane [13] shows a progressive and almost complete inhibition by this reagent, whilst leucine aminopeptidase and lactate dehydrogenase, the cytosolic marker, were both unaffected. When sonicated cells were incubated with diazotised sulphanilic acid under identical conditions, all three enzyme activities were inhibited by diazotised sulphanilic acid to a similar extent. These results confirm that leucine aminopeptidase has an intracellular localisation in human polymorphonuclear leukocytes.

Discussion

The present study has shown that human polymorphonuclear leukocytes have significant levels of leucine aminopeptidase activity which can be reliably measured by fluorimetric assays with either L-leucine-AMC or leucyl-2-naphthylamide as substrate. It has been shown during this study that the distribution of the activity in the sucrose density gradients were identical whether leucyl-2-naphthylamide or L-leucine-AMC was used as substrate. Since neither L-leucine-AMC nor its product are carcinogenic it is suggested that this is a more desirable substrate for this assay. It also has the added advantage of being approx. 10-fold more sensitive than leucyl-2-naphthylamide [9].

Analytical subcellular fractionation studies have shown that leucine aminopeptidase is located solely to a population of cytoplasmic granules with a modal density of 1.18 $g \cdot cm^{-3}$. These are quite distinct from the specific, azurophil, tertiary and phosphasome granules. Studies on neutrophils homogenised in isotonic sucrose containing digitonin show these granules are quite distinct from the mitochondria. These experiments also indicate that leucine aminopeptidase is associated with a cholesterol-rich membrane, but there was no activity associated with the plasma membrane of these cells. This intracellular localization is in agreement with the results of Folds et al. [14] who found leucine aminopeptidase in a low density population of granules associated with acid *p*-nitrophenyl phosphatase. An intracellular localisation was also reported by Lundgren et al. [4], although these workers found three isoenzymes of leucine aminopeptidase localised to the cytosol and to two granule fractions of different density, one of which was enriched with alkaline phosphatase, The present study, however, shows no association between leucine aminopeptidase and alkaline phosphatase, clear indication that leucine aminopeptidase is not located to the phosphasome granule.

The subcellular localisation of leucine aminopeptidase has also been investigated in polymorphonuclear leukocytes from both peritoneal exudates [15] and peripheral blood [16] of the rabbit. Using the technique of differential centrifugation Davies et al. [15] reported that leucine aminopeptidase was associated with an undefined intracellular membrane fraction, whilst our own studies [16], using analytical subcellular fractionation on sucrose density gradients, has shown the activity to be located solely to the tertiary granules. Indirect evidence of an intracellular localisation in polymorphonuclear leukocytes from mouse peritoneal exudates also comes from the work of Wachsmuth [17] who noted that intact cells exhibited no plasma membrane leucine aminopeptidase activity as detected by immunofluorescence techniques.

Our results are clearly in direct contrast to

those of Nagaoka and Yamashita [3]. In their study they incubated intact human, rabbit and guinea pig polymorphonuclear leukocytes with diazotised sulphanilic acid at 37°C for up to 15 min. The results they obtained varied in the three species studied. With rabbit neutrophils they reported that leucine aminopeptidase showed a progressive inhibition whilst 5′-nucleotidase remained unaffected by the inhibitor, but with guinea pig neutrophils both enzyme activities were inhibited. With human polymorphonuclear leukocytes leucine aminopeptidase activity was inhibited by diazotised sulphanilic acid, although they were unable to detect any 5′-nucleotidase in these cells. This is quite different from our results with the same reagent in which we found that, whilst leucine aminopeptidase was totally unaffected by diazotised sulphanilic acid, 5′-nucleotidase showed a progressive and almost complete inhibition, a result which we have repeatedly confirmed [12,18].

The failure of Nagaoka and Yamashita [3] to demonstrate 5′-nucleotidase activity in human polymorphonuclear leukocytes is also of interest, since in recent years not only has it been convincingly demonstrated that human neutrophils do possess 5′-nucleotidase [7] it has also been localised to the external surface of the plasma membrane in these cells [12,18]. In their study Nagaoka and Yamashita [3] measured 5′-nucleotidase using an unspecified modification of the method of Touster et al. [19], a colorimetric assay based on the measurement of phosphate released from adenosine 5′-monophosphate. In contrast, the assay for 5′-nucleotidase used in the present study is a highly specific radioassay with [^{3}H]adenosine 5′-monophosphate as substrate, and the incubation mixture, optimized for human neutrophil 5′-nucleotidase, also includes a large excess of 2-glycerophosphate, an alternative substrate for non-specific acid and alkaline phosphatases. Since 5′-nucleotidase is an enzyme with a particularly low K_m [19], and there is a much higher alkaline phosphatase activity in these cells, it is possible that Nagaoka and Yamashita in their studies [3,20,21] have been in part assaying alkaline phosphatase activity.

The nature of the leucine aminopeptidase-containing granule of human neutrophils is uncertain and further studies are clearly warranted on the nature of this organelle and its function and possible involvement in neutrophil pathology.

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