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Flow Cytometric Determination of Aminopeptidase Activities in Viable Cells Using Fluorogenic Rhodamine 110 Substrates

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Aminopeptidases (AP) are ubiquitously occuring, nonspecific exopeptidases involved in protein degradation. They cleave the N-terminal amino acid of peptides and occur in practically all mammalian cells and tissues. Physiological and pathological processes such as metastasis of tumors and inflammation have been thought to involve changes in AP activities. Determination of AP (EC 3.4.11.X) activity in viable cells by flow cytometry was the subject of this study because of its general biological and clinical interest. Different bis-substituted rhodamine 110 (R110) peptide derivatives were synthesised and used as AP- and exopeptidase (EC 3.4.13.X–EC 3.4.14.X) substrates for flow cytometric measurements. Intracellular AP activities in viable lympho-, mono-, granulo-, and thrombocytes were detected by fluorescence increase from R110 following intracellular substrate cleavage. Eukaryotic-AP do not cleave D-aminoacids and hence NH_2(D-Leu)$_2$R110 substrate served as negative control. Specific substrate cleavage by AP is shown by complete inhibition of fluorescence generation following preincubation of cells with leucine-chloromethylketone inhibitor. R110 AP- and exopeptidase substrates are suitable indicators for coupled endopeptidase reactions due to their rapid cleavage and largely pH independent generation of intracellular fluorescence. © 1995 Wiley-Liss, Inc.

Key terms: Rhodamine 110, protease substrate, exopeptidase, coupled endopeptidase reaction, flow cytometry

Lysosomes contain various proteolytic enzymes necessary for protein degradation in mammalian cells. These proteolytic enzymes comprise endopeptidases and exopeptidases. Exopeptidase act on peptide bonds adjacent to free amino or carboxyl groups. Aminopeptidases (E.C.3.4.11.X) catalyse the specific hydrolysis of single amino acids from the N-terminus of proteins or peptide substrates. They exhibit a broad specificity and a considerable number of them are Zn-metalloenzymes (17).

AP catalysis occurs on cell surfaces, in the cytoplasm, and within various cellular compartments and different categories of these enzymes exist in various mammalian tissues or cells (4). AP participate in fundamental biochemical and physiological processes and have been identified in eukaryotes and prokaryotes (4, 15). They are regularly found in the serum and urine (2, 5, 8, 13) and elevated amounts are observed in pathological conditions such as adenocarcinomas of the colon and pancreas, intravascular hemolysis, and tissue damage, as well as physiological states such as pregnancy and the postpartum, postoperative, and recovery periods, or in secreta from duodenum and small intestine (4, 6, 7, 12). Histochemical studies demonstrate the presence of AP in the perineoplastic stroma of tumors and inflammatory tissues (4, 5, 7). The intense AP activity in fibroblasts, macrophages, granulocytes, and mononuclear phagocytic cells is an indicator of proliferating tissue and wound cellularity (7).

AP activities can be determined by several different cell destructive biochemical assays (3, 6). Limitations of result interpretation originate from the changes of protease and inhibitor compartmentalization. Accurate determination of AP activity in human malignancies may be fundamental to the understanding of their invasive or metastatic behaviour and for the evaluation of strategies for treatment and management.

Our earlier work was concerned with the synthesis of R110 substrates for the flow cytometric determination of specific cysteine proteinase and serine proteinase activities in viable cells (9,14). The goal of the present work, however, was to determine AP activities that belong to the metalloproteinase class of enzymes. Instead of 7-amino-4-methyl coumarin, 4-methoxy-2-napthylamine or 7-amino-4-trifluoro-methyl coumarin, rhodamine substrates were found more advantageous because they are

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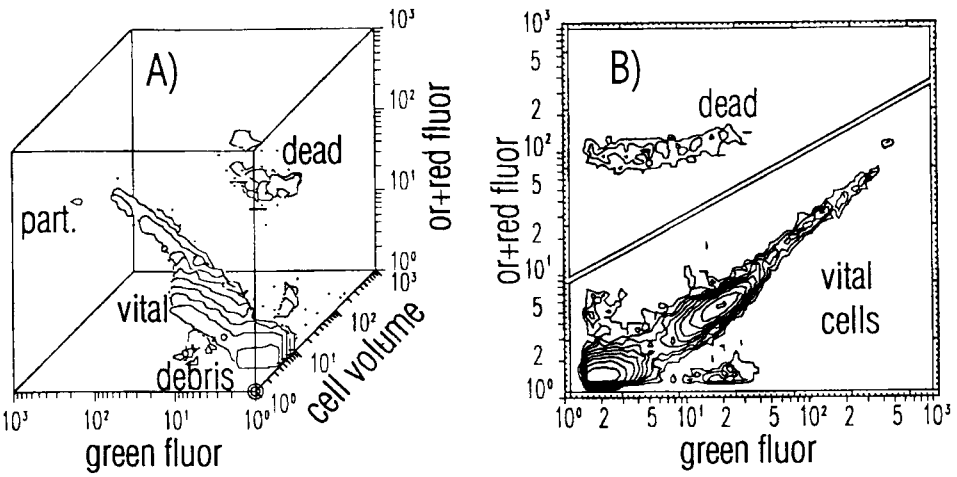

FIG. 1. R110/PI stained human blood cells. PI positive dead cells are well separated from vital cells (A). The vital cells can be quantitatively gated in the green/orange + red fluorescence projection histogram (B). A total of 16,129 cells were measured with a vitality of 97.4%. The maximum channel count was 592 (A) and 394 (B) cells. Contour lines were plotted at 10% (A) and at 10% intervals (B) of the maximum logarithmic channel counts.

nonfluorescent and penetrate readily into living cells where positively charged fluorescent R110 is released that favours cellular dye enrichment (14). In addition, the high quantum yield (>95%) upon excitation at 488 nm, e.g., by argon ion laser light and the practical pH independence of fluorescence emission within the range pH 3–9 make R110 substrates very suitable for flow cytometric assays.

MATERIALS AND METHODS

Chemicals

Amino acid derivatives were purchased from Bachem Biochemica GmbH (Heidelberg, Germany). Rhodamine 110 was from Exciton Chemical Co. (Dayton, OH). All other reagents and anhydrous solvents were from Aldrich Chemie GmbH (Steinheim, Germany).

Substrates

$(NH_2\text{-D-Leu})_2$R110, $(NH_2\text{-Leu})_2$R110, $(NH_2\text{-Phe})_2$R110, $(NH_2\text{-Gly})_2$R110, $(NH_2\text{-Pro})_2$R110, $(NH_2\text{-Ala})_2$R110, $(NH_2\text{-Val})_2$R110, $(NH_2\text{-Ala-Ala})_2$R110, $(NH_2\text{-Gly-Pro})_2$R110, $(NH_2\text{-Pro-Gly})_2$R110, $(NH_2\text{-Pro-Phe})_2$R110, $(NH_2\text{-Pro-Val})_2$R110, $(Z\text{-Arg-Phe})_2$R110, $(Z\text{-Arg-Arg-Leu})_2$R110, and $(Z\text{-Phe-Arg-Leu})_2$R110 [Z = carbo-benzoxy] were synthesised in analogy to Leytus et al. (10, 11) using the modification by Klingel et al. (9). All amino acids were of L-configuration if not otherwise noted. The purity of the substrates was checked by thin layer chromatography (10), reversed-phase HPLC, and ^{1}H-NMR. It was always >95%. The HPLC analysis was performed using a two-pump LKB Bromma system (Pharmacia, Freiburg, Germany) with UV detector (254 nm) and a Shimadzu fluorescence detector RF 535 (Latek GmbH, Eppelheim, Germany) with excitation monochromator at 480 nm and emission monochromator at 525 nm. The HPLC column (4 × 250 mm) was filled with Nucleosil 100 C18 (M. Grom, Herrenberg, Germany). The different amino acid containing substrates were analyzed in an acetonitrile (MeCN)/0.2 M triethylammonium acetate buffer pH 7.0 (gradient: 0–3 min 65% MeCN, 3–18 min to 80% MeCN).

Cells

Blood leukocytes and thrombocytes were isolated from 2–3 ml heparinized fresh venous blood following careful overlay onto a cushion of 5 ml Ficoll-Hypaque separation medium (Sigma Chemical, St. Louis, MO) in a 10 ml polycarbonate tube and sedimentation (1 × g) for 30 min at room temperature. Erythrocytes deplete themselves by forced aggregation and sedimentation at the Ficoll/blood interface leaving the thrombo- and leukocytes behind. The upper two-thirds of the supernatent containing thrombo-, lympho-, mono-, and granulocytes were carefully removed to avoid any contact with the separating medium. This is important because the separating medium significantly stimulates mono- or granulocytes and thereby potentially influences subsequent enzyme activity measurements.

Alternatively, the tubes were centrifuged at 200 × g for 10 min to deplete erythrocytes and granulocytes in order to obtain a lympho-, mono-, and thrombocyte suspension. The cells were washed twice in 15 ml HBS buffer by centrifugation (10 min, 200 × g) prior to staining and resuspended in HBS buffer to a concentration between 1 and 10×10^6 cells/ml.

Cell Staining for Aminopeptidase Assays

A 250 µl cell suspension at a concentration of $1–10 \times 10^6$ cell/ml was incubated with 5 µl of a dye cocktail containing between 0.02 and 0.2 mM bis-substituted R110 substrates together with 3 mM propidium iodide (PI, Sigma) in dimethylformamide (DMF) for 10–30 min at 20°C or 37°C.

Table 1
Relative AP Activities Against Various Substrates in Human Blood Cells

Substrates	Cellular fluorescence (arbitrary fluorescence units)		
	Thrombocytes	Lymphocytes	Granulocytes
$(NH_2\text{-D-Leu})_2$R110 (control)	0	0	0
$(NH_2\text{-L-Leu})_2$R110	5	90	200
$(NH_2\text{-Phe})_2$R110	5	200	900
$(NH_2\text{-Val})_2$R110	5	55	250
$(NH_2\text{-Gly})_2$R110	0	20	25
$(NH_2\text{-Ala})_2$R110	0	20	25
$(NH_2\text{-Pro})_2$R110	0	0	0
$(NH_2\text{-Gly-Pro})_2$R110	5	20	40
$(NH_2\text{-Pro-Gly})_2$R110	0	0	0
$(NH_2\text{-Pro-Phe})_2$R110	0	9	9
$(NH_2\text{-Pro-Val})_2$R110	0	9	9
$(NH_2\text{-Ala-Ala})_2$R110	4	35	65

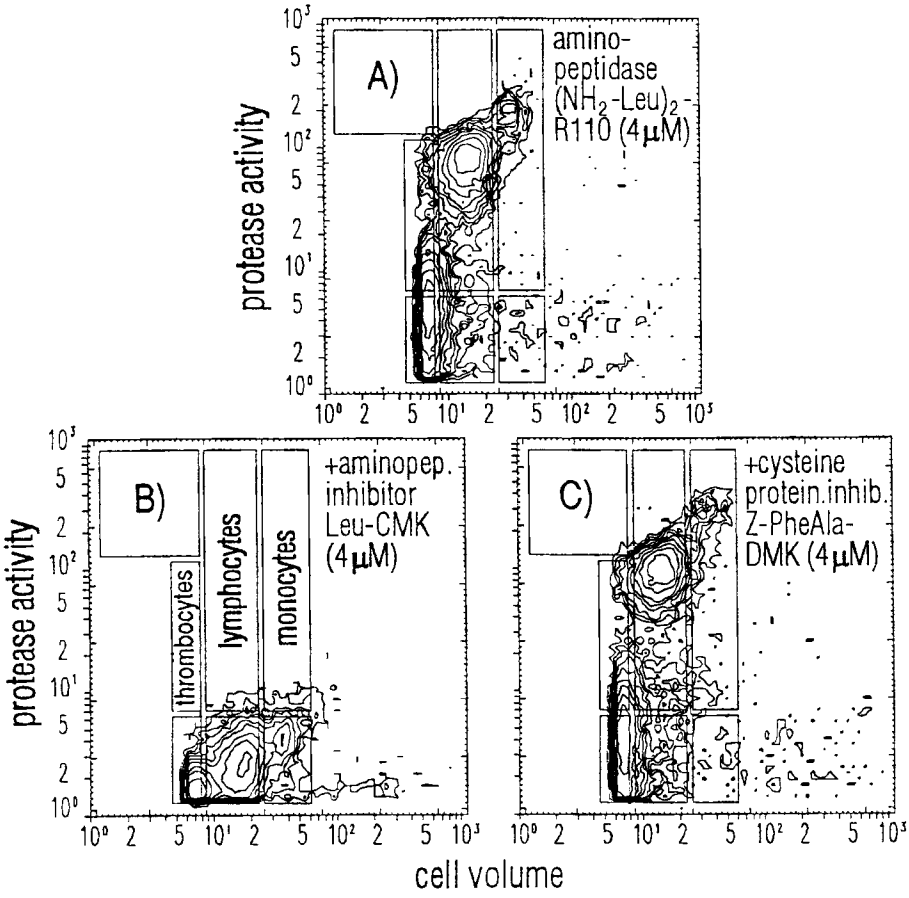

FIG. 2. $(NH_2\text{-Leu})_2$R110 cleavage by vital human blood cells (40 mW laser power). Display as in Figure 1B.

Depending upon the type of cells used, the incubation temperature and substrate concentration varied. Blood cells required 4 μM final substrate concentration, whereas 0.4 μM was sufficient for guinea pig kidney cells. The plateau phase of substrate cleavage is usually attained after 15–30 min at 37°C.

Cells were preincubated for 15 min at 20°C or 37°C with 5 μl of a 0.5 mM solution of AP inhibitor leucine-chloromethylketone (CMK, Bachem) or cysteine proteinase inhibitor Z-Phe-Ala-diazomethylketone (DMK, Bachem) to study the specificity of the intracellular R110-AP substrates.

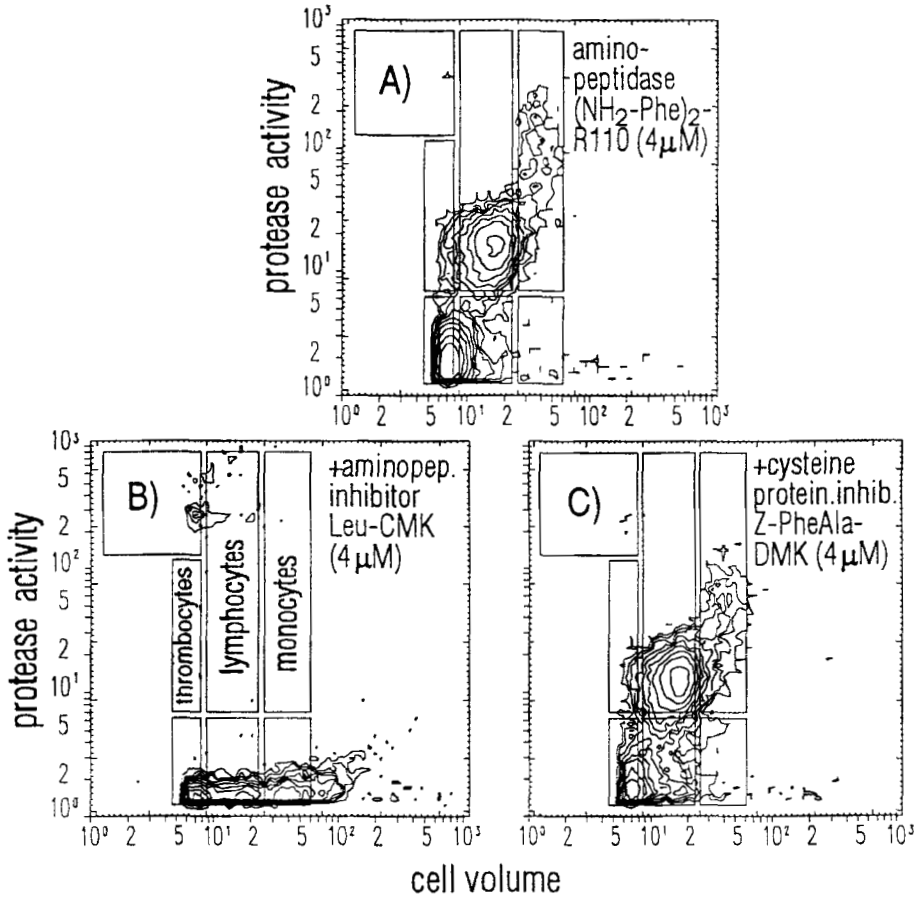

FIG. 3. $(NH_2\text{-Phe})_2$R110 cleavage by vital human blood cells (4 mW laser power). Display as in Figure 1B.

Flow Cytometry

Cell volume was measured in a hydrodynamically focused electrical sizing orifice of 80 μm diameter in a PASIII flow cytometer (Partec, Münster, Germany). Cellular fluorescence was excited by a 488 nm argon laser (4–40 mW) and measured simultaneously with cell volume. The R110 fluorescence was collected in the green channel (512–542 nm) and the propidium iodide fluorescence in the orange + red channel (572–750 nm) of the flow cytometer following logarithmic amplification of all signals by three decade logarithmic amplifiers. The amplified signals were digitized simultaneously in parallel by 4,096-step analog digital converters and stored as FCS1.0 list mode files. The list mode files were transferred to a personal computer with MS-DOS operating system and evaluated graphically and quantitatively by the CLASSIF1 (16) program system (Partec).

Monosized yellow/green (YG) fluorescent particles (Polysciences, Warrington, PA) of 4.2 μm diameter were used for the long-term standardization of the flow cytometer.

RESULTS

The bis-substituted peptide derivatives of R110 were cleaved rapidly by intracellular AP resulting in substantial R110 fluorescence release after cell incubation at a substrate concentration of 4 μM for 10 min at 22°C.

Propidium iodide stained dead cells were readily distinguished from vital cells (Fig. 1A). The dead cells were quantitatively eliminated during further analysis by gating on the vital cells (Figs. 1B, 2,3,5).

AP-activities were observed in lympho-, mono-, granulo-, and thrombocytes of human blood. Arbitrary fluorescence units for the AP-activity are listed in Table 1 as compared to $(NH_2\text{-D-Leu})_2$R110. $(NH_2\text{-D-Leu})_2$R110 was taken as control since eukaryotic-APs do not cleave D-aminoacids.

Granulocytes have approximately twice the volume of lymphocytes. This volume difference is paralleled by an increase of protease activity for $(NH_2\text{-Leu})_2$R110, $(NH_2\text{-Ala-Ala})_2$R110, and $(NH_2\text{-Gly-Pro})_2$R110 (Table 1). A substantially higher protease activity concentration for $(NH_2\text{-Phe})_2$R110 and $(NH_2\text{-Val})_2$-R110 (Table 1) is, however, observed in granulocytes as compared to lymphocytes.

Monocyte (Figs. 2,3) protease activities can be determined following granulocyte depletion by centrifugation over Ficoll instead of 1 × g sedimentation. Their protease activity is overlapping with the lower end of the granulocyte cell cloud but otherwise follows the same charac-

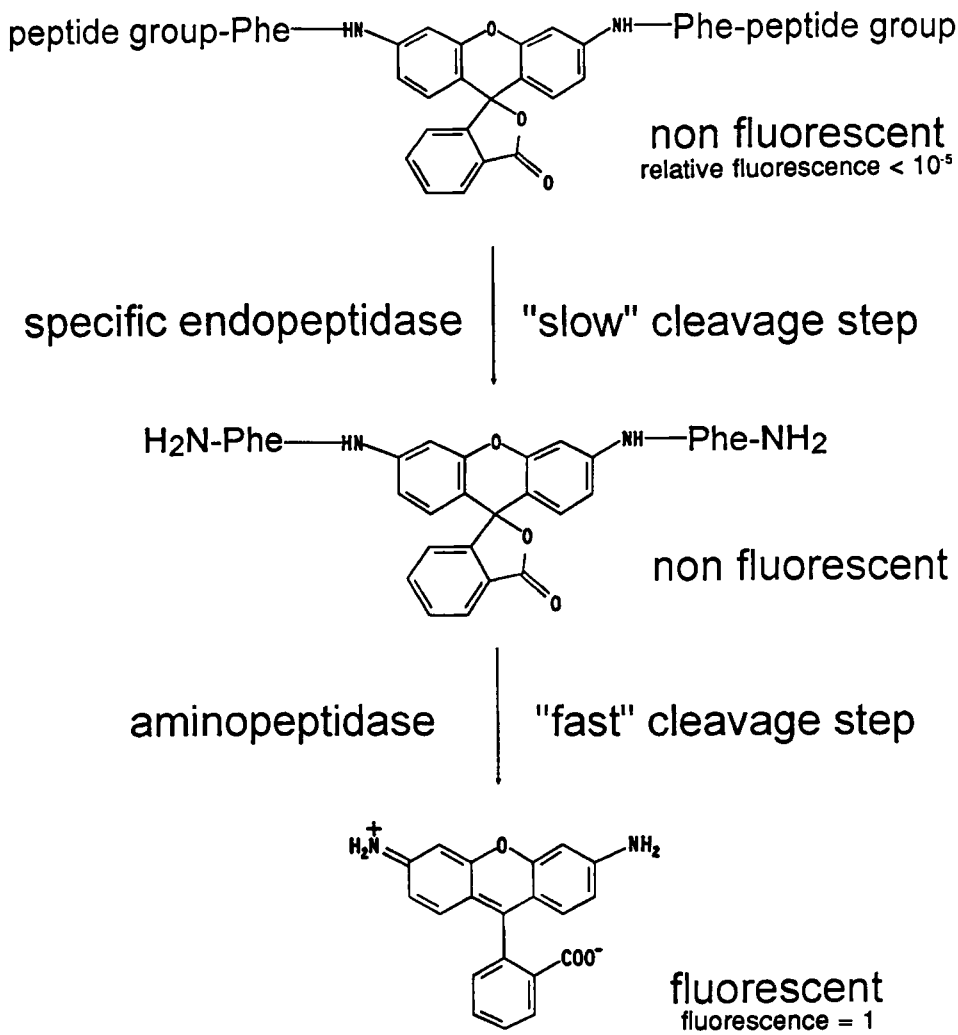

FIG. 4. Coupled enzyme cleavage reaction: Endopeptidases in a first reaction step cleave at the specific intrapeptide cleavage point without fluorescence generation. This exposes free N-terminal amino acids which in a second step are cleaved by ubiquitously occuring aminopeptidase. Aminopeptidase consecutively set free the remaining amino acids to finally liberate highly fluorescent R110 as indicator substance.

teristics with regard to $(NH_2\text{-Leu})_2$R110 (Fig. 2) and $(NH_2\text{-Phe})_2$R110 protease activity (Fig. 3). The specificity of the AP generated fluorescence was shown by the inhibition of fluorescence increase by preincubation of cells with the AP inhibitor CMK for intracellular and membrane bound AP but not by the cysteine protease inhibitor DMK (Figs. 2,3).

Since cellular AP activity is present at high levels in the various blood cell types, it was thought to use these activities in coupled enzyme reactions for the determination of endopeptidase activities in viable cells. The model substrates $(Z\text{-Arg-Phe})_2$R110, $(Z\text{-Arg-Arg-Leu})_2$R110, and $(Z\text{-Phe-Arg-Leu})_2$R110 were synthesized for this purpose. With their cathepsin B/H/L endopeptidase cleavage site at the Arg-Phe or Arg-Leu peptide bond, these substrates should be initially cleaved to nonfluorescent $(NH_2\text{-Phe})_2$R110 or $(NH_2\text{-Leu})_2$R110 (Fig. 4). Cellular AP should consecutively cleave off fluorescent R110. This indeed occurred (Fig. 5A) in monocytes that exhibit significant cathepsin B/H/L levels. Provided the coupled reaction is specific, it should be inhibitable by either cathepsin B/H/L inhibitor DMK or AP inhibitor CMK, which was the case (Fig. 5B, C).

DISCUSSION

Nonfluorescent aminoacids and peptide derivatives of the fluorogene rhodamine 110 permit the determination of various AP-activities in living cells (Figs. 2,3). The cleavage of the substrates is completely inhibited by specific inhibitors (Figs. 2,3). The defined degradation of the substrate to free R110 without intermediate steps (14) allows a rapid assay in viable cells.

The increase in fluorescence due to the cleavage of bis-substituted R110 derivatives, namely, $(NH_2\text{-Phe})_2$ R110, $(NH_2\text{-Val})_2$R110, $(NH_2\text{-Leu})_2$R110, $(NH_2\text{-Gly})_2$ R110, and $(NH_2\text{-Ala})_2$R110, indicates the presence of

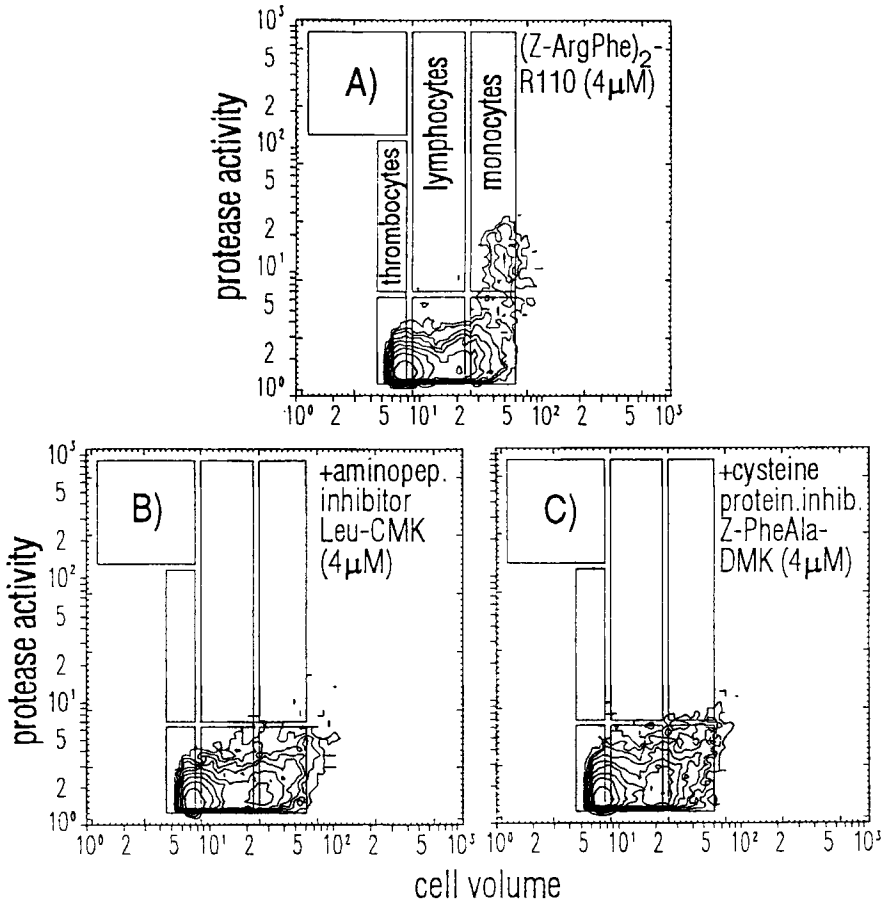

FIG. 5. Combined cleavage of $(Z\text{-Arg-Phe})_2$R110 by cysteine and aminopeptidase proteinases in blood cells and their inhibition by CMK and DMK (40 mW laser power). Display as in Figure 1B.

amino acid arylamidases in lympho-, mono-, granulo-, and thrombocytes. These enzyme activities are usually determined in serum by clinical chemistry methods to differentiate between liver and bone pathology (6). It is conceivable that the analysis of cellular proteases opens new possibilities for the diagnosis of inflammatory or other diseases.

Absence of fluorescence in the case of $(NH_2\text{-Pro})_2$R110 (Table 1) is in accordance with the fact that amino acid arylamidases preferentially split alanine and not proline (5). Because of the unique structure of proline peptides, they are frequently resistant to the action of many peptidases. However, a series of peptidases exists that can uniquely recognize the pyrrolidine ring of proline. The investigation of arylamidases-"clusters" in human serum provides a significant diagnostic feature of prolinaryl-amidases in rheumatic, tumor, and tissue damage as compared to carcinoembryonic antigen (CEA) and Tennessee polypeptide antigen and 3-dipeptidylarylamidase (1). Whereas blood cells do not contain appreciable levels of such enzymes (Table 1), it is conceivable that they are demonstrable in cells of other organs such as kidney or liver.

In addition, dipeptidase [prolyl dipeptidase (EC 3.4.13.8); prolidase (EC 3.4.13.9)] and dipeptidyl-peptidase [DPPII (EC3.4.14.2); DPPIV (EC 3.4.14.5)] exopeptidases were determined using dipeptidyl bis-substituted rhodamine 110 derivatives, namely, $(NH_2\text{-Pro-Gly})_2$ R110, $(NH_2\text{-Pro-Phe})_2$R110, $(NH_2\text{-Pro-Val})_2$R110, and $(NH_2\text{-Gly-Pro})_2$R110. Although $(NH_2\text{-Gly-Pro})_2$R110 proved to be an excellent substrate for prolidase and dipeptidyl peptidases II and IV activity determination in living cells, the other exopeptidase substrates were only insignificantly cleaved by prolyldipeptidases (Table 1).

Due to rapid cleavage, R110 AP-substrates are also suitable indicators for coupled endopeptidase reactions (Figs. 4,5). Coupled R110-substrate protease reactions allow principally the specific determination of other intracellular endopeptidases like the aspartate proteases: collagenase, cathepsin D, renin or retroviral proteinases (e.g., HIV-proteases). This seems an interesting area for further studies.

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