The aminopeptidase activity in the human T-cell lymphoma line (Jurkat) is not at the cell surface and is not aminopeptidase N (CD-13)

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Although lymphocytes are CD-13-negative and therefore should not express the ectoenzyme aminopeptidase N (AP-N), there have been a number of reports suggesting the presence of a cell-surface aminopeptidase with many similarities to AP-N. We have determined aminopeptidase activity with 4-methyl-7-coumarylamide (NMec) derivatives of alanine, leucine, lysine and arginine in Jurkat cells (a human T-cell lymphoma line) and in HL60 cells (a CD-13-positive myeloid leukaemia line) and compared the activities with those of purified pig AP-N and human renal microvillar membranes. Jurkat cell aminopeptidase activity doubled on disrupting the cells and the sensitivity to amastatin increased. When the cells were fractionated only 4% of the activity was recovered in the membrane fraction, compared with 87% recovery for alkaline phosphatase. The profile of activities for intact Jurkat cells was Leu > Ala > Lys > Arg, changing in the cytosolic fraction to Lys ≥ Arg > Leu = Ala; the profiles for intact HL60 cells and AP-N were identical, namely Ala > Leu > Arg > Lys. The K_m values for the hydrolysis of Ala-NMec and Leu-NMec by Jurkat cells were 65 μM and 11 μM, in each case some 6-fold lower than those for AP-N. The pH–activity curves for the hydrolysis of Ala-NMec by Jurkat cells and human renal microvillar membranes were displaced by almost 1 pH unit and the activity was not sensitive to the anionic composition of the buffers. However, a 3-fold activation of the cytosolic activity by 0.1 M NaCl was observed with Arg-NMec as substrate. With Ala-NMec as substrate, the sensitivity of the aminopeptidase activity to inhibitors increased markedly after disrupting the cells, but still differed from that observed with purified pig AP-N; the concentrations giving 50% inhibition were as follows (values for AP-N in parentheses): amastatin. 28 nM (150 nM); bestatin, 12 μM (43 μM), probestin, 100 nM (< 10 nM), puromycin, 30 μM (> 1 mM). Anion exchange chromatography on Mono Q revealed two activities: that of peak I preferentially hydrolysed Arg-NMec, was activated by NaCl and was insensitive to amastatin; while that of peak II was strongly inhibited by amastatin and had a broad specificity. Jurkat cells hydrolysed [Leu^5]enkephalin, the activity increasing 4-fold on cell disruption, of which 89% was recovered in the cytosolic fraction and less than 3% in the membrane pellet, contrasting with HL60 cells for which most of the activity was recovered in the 100000 *g* pellet. We conclude that there is no evidence for the presence of AP-N in Jurkat cells and that the cytosolic activity comprises two aminopeptidases, one resembling aminopeptidase B. The other, which accounted for 90% of the activity with Ala-NMec, was sensitive to amastatin, but not to metal chelators and was not activated by Cl^-. The differences in properties between the activities of intact and disrupted cells are explained by a permeability barrier to entry of substrates and inhibitors.

INTRODUCTION

In the last few years the applications of cDNA cloning and monoclonal antibodies have revealed several cluster differentiation (CD) antigens to be identical to cell-surface peptidases previously recognized on other cell types. Thus CD-10 (CALLA; common acute lymphoblastic leukaemia antigen) is endopeptidase-24.11 (Letarte et al., 1988; Jongeneel et al., 1989; Shipp et al., 1989), CD-26 is dipeptidyl peptidase IV (Ulmer et al., 1990), CD-13 is aminopeptidase N (AP-N) (Look et al., 1989b) and the murine BP-1/6C3 antigen is aminopeptidase A (Wu et al., 1990, 1991). CD-13, or AP-N is widely distributed on many cell types, including epithelia of kidney and intestine, fibroblasts, endothelial cells (Kenny et al., 1987) and, among leucocytes, on cells of the monocytic and granulocytic series (Griffin et al., 1981; Bowes and Kenny, 1987; Look et al., 1989a).

Although human lymphocytes are CD-13 negative (Ashmun and Look, 1990; Look et al., 1989b), there are several reports of surface aminopeptidase activity on human lymphocytes. Amoscato et al. (1988, 1989) have described the stepwise degradation of thymopentin (Arg-Lys-Asp-Val-Tyr) by human lymphocytes, the activity being inhibited by bestatin, amastatin and chelating agents. Alanine 4-nitroanilide was also hydrolysed by intact lymphocytes, the aminopeptidase activity apparently being associated with the cell surface and sensitive to bestatin. Mitogenic agents caused an increase in this activity over a period of 24–72 h. Another report (Ansorge et al., 1991) has claimed that AP-N is indeed present on human lymphocytes (both T- and non-T-cells) with an activity of about 6 nmol of Ala 4-nitroanilide hydrolysed/min per 10^7 cells. However, this activity differed in its sensitivity to the inhibitors probestin and actinonin compared with purified AP-N (Ansorge et al., 1991). An earlier study (Kohno and Kanno, 1985) concluded that the activity hydrolysing leucine *p*-nitroanilide had a microsomal distribution in homogenates of peripheral blood lymphocytes, while the cytosolic activity, hydrolysing leucine amide, was similar in properties to leucine aminopeptidase. A recent immunological study on T-cells from human synovial fluid reported positive staining for CD-13 in some clinical conditions (Riemann et al., 1993).

The activity that hydrolyses alanine and leucine nitroanilides has also been studied in several neoplastic cell lines of lymphoid and myeloid origin. In human myeloid leukaemia lines, including HL60 cells, aminopeptidase activity was abundant and correlated with CD-13 expression, while the activity in some CD-13-negative

Abbreviations used: Ala-NMec, Arg-NMec, Leu-NMec and Lys-NMec are the 4-methyl-7-coumarylamides of alanine, arginine, leucine and lysine respectively; AP-N, aminopeptidase-N, (EC 3.4.11.2); EC-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)butane.

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human lymphoid leukaemia lines (e.g. NALM 1) was at background levels (< 1 nmol/min per 10^7 cells), as was the case for circulating lymphocytes (Ashmun and Look, 1990). Neutral aminopeptidase activity has been demonstrated on several human tumour cell lines, including Jurkat cells, a line derived from a human T-cell lymphoma. The activity on intact Jurkat cells was inhibited by amastatin and bestatin (K_i values of 50 μM and 2 μM respectively). Other lymphoid cell lines, e.g. Raji cells, exhibited both low- and a high-affinity binding of amastatin (the former similar to that of Jurkat cells, while the latter had a K_i of 1 μM), implying the presence of two aminopeptidase activities (Amoscato et al., 1990).

Thus there is a paradox in that Jurkat cells, which, in common with cells of the lymphocytic lines, are CD-13-negative, seem to express a surface aminopeptidase with some similarities to AP-N. In this paper we have compared the aminopeptidase activity of Jurkat cells with that of the CD-13-positive cell line, HL60, as well as with a highly purified preparation of AP-N, and report a number of crucial differences.

EXPERIMENTAL

Materials

Cells

Jurkat cells were a gift from D. P. Nathan (ICI Pharmaceuticals, Alderley Park, Macclesfield, U.K.). HL60 cells were donated by Dr. C. Bunce (Department of Immunology, Birmingham University, U.K.). Foetal calf serum was obtained from Applied Protein Products (Leys Road, Brockmoor, Brierley Hill DY5 3UP, U.K.). Medium and additives were obtained from Gibco.

Substrates

The 4-methyl-7-coumarylamides Ala-NMec, Arg-NMec, Lys-NMec and Leu-NMec were from Bachem Feinchemikalien AG (CH4416 Bubendorf, Switzerland). [Leu]enkephalin was from Sigma Chemical Co.

Inhibitors

Amastatin, bestatin, puromycin, E-64, bacitracin, *N*-ethylmaleimide, di-isopropylfluorophosphate and arphamenine-B were obtained from Sigma Chemical Co., and probestin was a gift from Dr. Takaaki Aoyagi (Institute of Microbial Chemistry, Tokyo 141, Japan). Captopril was a gift from the Squibb Institute for Medical Research (Princeton, NJ, U.S.A.), and enalaprilat was a gift from Dr. A. A. Patchett (Merck, Sharpe & Dohme Laboratories, Rahway, NJ, U.S.A.). Stock solutions (10 mM) of these inhibitors were made up in water. Captopril and phosphoramidon were added to assays with [Leu]enkephalin as substrate (Stephenson and Kenny, 1987).

Monoclonal antibodies to CD13

MCS2 was kindly provided by Dr. A. T. Look (Department of Haematology, St Jude Children's Research Hospital, Memphis, TN 38101, U.S.A.); 22A5 was a gift from Dr. M. Horton (ICRF Laboratories, Lincoln's Inn Fields, London WC2A 3PX, U.K.); WM15 was bought from Sera Lab (Crawley Down, W. Sussex, U.K.).

Methods

Cell culture

Both cell lines were grown in RPMI 1640 supplemented with 10% foetal calf serum, pyruvate (1 mM), glutamine (5 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml) to densities of approx. 10^6 cells/ml.

Subcellular fractionation of kidney and cells

Human kidney microvillar membranes (human P4) were prepared as previously described for rabbit kidneys (Booth and Kenny, 1974). Purified pig AP-N was prepared by immunoaffinity chromatography with the monoclonal antibody GK8C1 (Gee and Kenny, 1985).

Cultured cells were fractionated as follows. Cells were washed in PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4), centrifuged (100 *g* for 10 min), and resuspended at 1×10^7 cells/ml in PBS containing 2 mM $CaCl_2$. The resulting cell suspension was disrupted by loading into a pre-chilled PARR Cell Disruption Bomb (Scientific and Medical Products, Manchester, U.K.) pressurized to 800 lb/in^2 (5360 kPa) and kept on ice for 10 min before releasing the pressure. The resulting homogenate was centrifuged (1000 *g* for 10 min) to produce supernatant 1 (SN1) which was further centrifuged (Beckman ultracentrifuge; 100000 *g* at 4 °C for 90 min) to yield the membrane fraction (P2) and the second supernatant (SN2). The P2 fraction was resuspended in PBS in a volume equivalent to 1×10^7 cells/ml and used for assays. P2 was sometimes washed with 10 ml of 500 mM NaCl, 10 mM sodium phosphate, pH 7.4, and recentrifuged and resuspended as above. For detergent extraction, the cell suspension (10^7 cells/ml) was incubated with 2% Triton X-100 for 30 min at room temperature and the supernatant was used for assay after a brief centrifugation to remove insoluble material.

Chromatography of fraction SN2 by f.p.l.c.

Several runs were performed, but losses of activity were high during preliminary dialysis, to remove buffer salts, and the cell disruption was then done in 0.25 M sucrose. Two such runs were carried out using 8–10 ml of SN2 (activities in the range 3.9–5.9 nmol/min per ml, with Ala-NMec as substrate) loaded on to a Mono Q column (Pharmacia) equilibrated with 10 mM Tris/HCl, pH 7.5, at room temperature and developed with a gradient of NaCl (0–0.5 M; volume 60 ml); 1 ml fractions were collected for assay. In the second of these runs, chromatography was completed on the same day as the cell fractionation and the fractions were collected on ice; the recovery of activity in the fractions under these conditions was 74%.

Protein estimation

Protein was estimated using the bicinchoninic acid protein assay (Sigma Chemical Co.; procedure no. TPRO-562) in 96-well microplates using BSA as standard.

Immunoblotting

This was performed as previously described (Barnes et al., 1989).

Enzyme assays

Alkaline phosphatase was measured by the hydrolysis of 4-nitrophenyl phosphate (Bessey et al., 1946). Cells or fractions (volume 100 μl) were incubated with substrate buffered at pH 10 (1 ml) for 30 min at 37 °C. The reaction was stopped by the addition of 5 ml of 0.04 M NaOH and activity was determined by measuring the A_{400}.

Aminopeptidase activity was normally assayed fluorimetrically using the NMec substrates, as described by Fulcher and Kenny

Table 1 Aminopeptidase activity in intact Jurkat and HL60 cells, and effects of cell disruption and Triton X-100 treatment

See the Experimental section for details. Activities are expressed as nmol/min per 10^7 cells and are means ± S.E.M. (numbers of experiments are shown in parentheses; where none is shown, $n = 4$). The activity in the presence of 100 μM amastatin is also shown, with the percentage inhibition within square brackets.

	Aminopeptidase activity (nmol/min per 10^7 cells)			
	Jurkat cells		HL60 cells	
Cells	Total	+ Amastatin	Total	+ Amastatin
Intact cells	3.27 ± 0.23 (24)	2.21 ± 0.48 (16) [32%]	7.71 ± 0.07	3.53 ± 0.37 [54%]
Disrupted cells	7.32 ± 0.90 (11)	1.54 ± 0.17 (9) [79%]	–	–
Triton X-100-treated cells	6.25 ± 1.50 (4)	2.02 ± 0.27 [68%]	17.70 ± 2.50	0.80 ± 0.11 [95%]

(1983). In brief, 1.9 ml of 0.2 mM substrate in 0.1 M Tris/HCl, pH 7.0, was brought to 37 °C in a thermostatted fluorimeter cuvette. The reaction was started by the addition of 0.1 ml of cells or extracts and the increase in fluorescence was followed for 3–5 min (excitation 370 nm; emission 442 nm). At the cell densities used, no interference with the fluorescence assay was noted. For inhibitor studies, samples of cells or extracts were preincubated with inhibitors at the stated concentrations for 30 min at room temperature before addition to the substrate/buffer mixture.

In another aminopeptidase assay, the release of tyrosine from [Leu^5]enkephalin was measured by h.p.l.c., as previously described by Matsas et al. (1985). Briefly, the incubation volume was 100 μl, and the substrate concentration was 0.5 mM in 0.1 M Tris/HCl, pH 7, containing 1 μM phoshoramidon and 1 μM captopril. The incubation time was 15 min at 37 °C and the reaction was terminated by heating to 100 °C. After centrifuging, the sample was loaded on to a μBondapak column and eluted with a 4.5–30% (v/v) acetonitrile gradient, under which conditions tyrosine had a retention time of 5 min. The quantity of cells or extract was adjusted to give suitable rates (< 40% hydrolysis), as follows. For HL60 cells: 2×10^5 cells; P2, 2–4 μg of protein; Triton X-100-solubilized cells, 1×10^6 cells; for Jurkat cells: 2×10^6 cells; P2, 20–40 μg of protein; homogenate, SN1 and SN2, 80 μg of protein; Triton X-100-solubilized cells, 1×10^7 cells. Amastatin sensitivity was determined by preincubation in the assay buffer mixture for 30 min at room temperature, the reaction being started by the addition of the substrate (10 μl to 90 μl of the buffer mixture containing cells or extracts).

pH–activity curves

The buffers used were 100 mM Na_2HPO_4/H_3PO_4 for the range pH 6–8 and 100 mM Mes/NaOH for the range pH 5–6.

Kinetic experiments on Ala-NMec and Leu-NMec

When pig AP-N was studied, the substrate concentrations ranged from 25 μM to 1 mM. For intact Jurkat cells the same range was used for Ala-NMec, and a range of 2–20 μM was used for Leu-NMec. K_m values were computed from Lineweaver–Burk plots.

RESULTS

Hydrolysis of Ala-NMec by Jurkat cells

Aminopeptidase activity was consistently detected when intact Jurkat cells were incubated with Ala-NMec as substrate (Table 1). Cells were repeatedly checked at the end of an assay period for viability by the exclusion of Trypan Blue; > 90% were seen to be viable by this criterion. Only a minor part of this activity (30%) was inhibited by 100 μM amastatin but, when the cells were disrupted or solubilized by detergent, the activity increased 2-fold and the amastatin sensitivity rose to about 70%. The increase in fluorescence was linear after addition of the cells, and no initial lag was noted. When cells were preincubated in PBS and then removed by centrifugation, no aminopeptidase activity was detected in the medium.

A simple scheme for subcellular fractionation after disruption in a nitrogen bomb was developed (Table 2). After removal of a low-speed pellet, the supernatant (SN1) was centrifuged at 100000 *g* for 90 min to yield a membrane pellet (P2) and a second supernatant (SN2). Alkaline phosphatase was assayed as a plasma membrane marker; it was enriched 3.6-fold in P2 with a recovery of 87%. This contrasted with the aminopeptidase activity, for which the P2 fraction had only 10% of the specific activity of the homogenate, with a recovery of 4%, while the supernatant fractions SN1 and SN2 were very slightly enriched (1.1–1.2-fold) and contained most of the homogenate activity (93% and 70% respectively). These results are consistent with aminopeptidase activity being predominantly localized in the cytosol. Sensitivity to amastatin was unchanged in SN1 and SN2 compared with the homogenate. In two experiments HL60 cells were fractionated in the same manner; 45% of the aminopeptidase activity was recovered in SN2 and 35% in P2.

Comparison of aminopeptidase activity in Jurkat cells with that in HL60 cells and with purified pig AP-N

HL60 cells are CD-13-positive and hence express AP-N on their surface. They showed a somewhat higher activity in hydrolysing Ala-NMec than Jurkat cells and about half of this was amastatin-sensitive (Table 1). When HL60 cells were treated with Triton X-100 the activity increased more than 2-fold and all of it was now inhibited by amastatin. In these respects Jurkat cells were not dissimilar: activity was doubled by detergent treatment and amastatin-sensitivity was also increased.

The substrate profiles for the hydrolysis of four NMec derivatives of amino acids were explored for Jurkat and HL60 cells and compared with that of pure pig AP-N (Table 3). The relative rates of hydrolysis of the NMec derivatives by pure AP-N were in the ranking order Ala > Leu > Arg > Lys, and an identical pattern was observed for intact HL60 cells. In contrast, intact Jurkat cells showed a quite different order: Leu > Ala > Lys > Arg, which changed to Lys ≥ Arg ≫ Leu = Ala for the SN2 fraction.

The pH–activity curve for the SN2 fraction from Jurkat cells is shown in Figure 1, together with the curve obtained with a

Table 2 Subcellular fractionation of Jurkat cells

See the Experimental section for details. The aminopeptidase activity was determined with Ala-NMec as substrate, and alkaline phosphatase activity with *p*-nitrophenyl phosphate as substrate. H, homogenate; SN1, supernatant fraction after centrifuging H at 1000 *g* for 10 min; SN2, fraction after centrifuging SN1 at 100000 *g* for 90 min; S.A., specific activity (nmol/min per mg of protein); E, enrichment factor (relative to SA of H); R, recovery of homogenate activity in each fraction; Am (%), proportion of aminopeptidase activity sensitive to inhibition by 100 μM amastatin. The values are means of four experiments (±S.E.M.), except for one result (shown *) which was a single determination.

	Alkaline phosphatase			Aminopeptidase			
Fraction	SA	E	R (%)	SA	E	R (%)	Am (%)
H	8.7±2.1	1.00	100	5.75±0.87	1.0	100	77±7
SN1	6.1±1.3	0.76	68±5	6.10±0.65	1.1	93±4	73±8
SN2	3.1±0.8	0.36	23±2	6.63±1.40	1.2	70±9	74±6
P2	30.9±6.8	3.60	87±11	0.60±0.20	0.1	4.1±1	90*

Table 3 Hydrolysis of four amino acid NMec substrates by Jurkat and HL60 cells and by pure pig AP-N

See the Experimental section for details. The assays (in duplicate) were performed on samples of 10^6 cells (or the SN2 fraction prepared from 10^6 cells) or with 0.4 μg of pig AP-N (an amount selected to give a rate comparable with those of the cells with Ala-NMec; range 5–15 nmol/min). The results are means of three experiments±S.E.M. and are expressed (as percentages) relative to the rate of hydrolysis of Ala-NMec.

	Hydrolysis (%)			
Substrate	Pig AP-N	HL60 cells (intact)	Jurkat cells (intact)	Jurkat cells (fraction SN2)
Ala-NMec	100	100	100	100
Leu-NMec	76±4	64±11	223±64	105±29
Arg-NMec	26±3	26±3	50±6	331±78
Lys-NMec	16±3	20±2	75±11	376±81

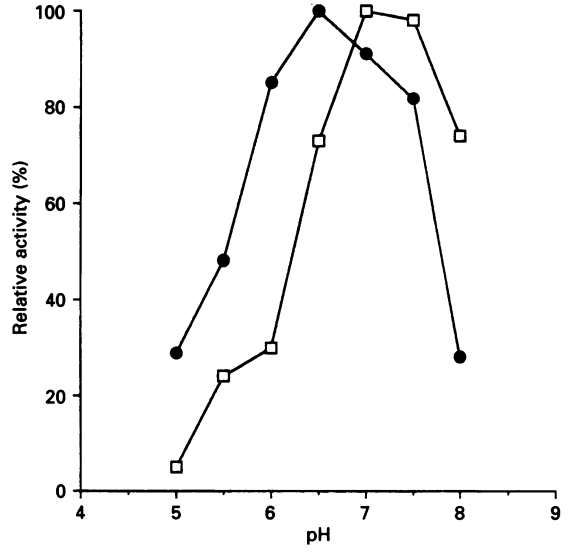

Figure 1 pH–activity curves for the hydrolysis of Ala-NMec by the cytosol fraction of Jurkat cells and by a microvillar membrane fraction prepared from human kidney cortex

See the Experimental section for details. The activities have been normalized to the peak activity of each preparation (= 100%). Buffers were Mes/NaOH for pH 5–6 and sodium phosphate for pH 6–8 (the two buffers gave same activity at pH 6). ●, Jurkat cell SN2 fraction; □, human kidney microvillar membranes.

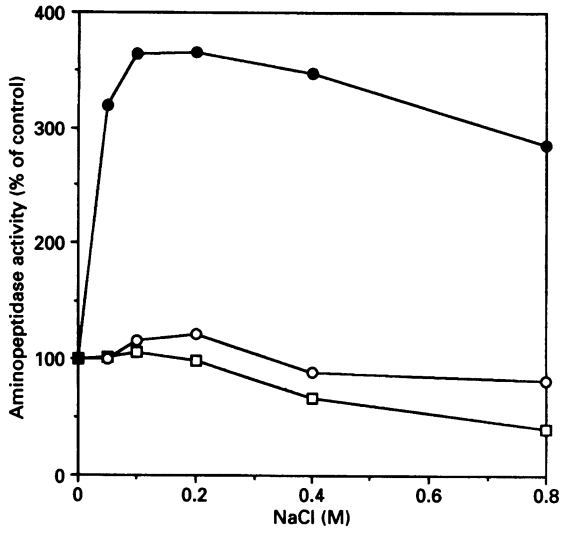

Figure 2 Effect of NaCl on the aminopeptidase activity of Jurkat cell SN2 and pig AP-N

Jurkat cells were disrupted in 0.25 M sucrose and the SN2 fraction was added to 50 mM sodium phosphate buffer, pH 6.5, containing NaCl over the range 0–0.8 M. After 15 min at room temperature, the assay was started by the addition of 0.08 ml of 5 mM substrate, 0.92 ml of buffer containing NaCl, and 1 ml of the treated SN2. The control activities (no NaCl) (nmol/min per ml) were: AP-N, 6.6 (Ala-NMec); SN2, 4.94 (Ala-NMec) and 6.31 (Arg-NMec). □, AP-N (Ala-NMec); ○, SN2 (Ala-NMec); ●, SN2 (Arg-NMec).

preparation of human kidney microvillar membranes, selected as the best alternative to purified human AP-N, which was not available. The amount of membranes in the assays was adjusted to give an activity comparable with that of the cells (7 nmol/min with Ala-NMec as substrate). The buffer ions did not appear to affect either activity; thus Mes and phosphate buffers and Tris/HCl and phosphate buffers gave similar activities at pH 6 and pH 7 respectively, and when 0.15 M NaCl was added to the 0.1 M Tris/HCl buffer the activity increased by no more than 25%. The pH maxima for the two activities differed by almost 1 pH unit.

The effect of chloride ions on activity was studied with purified pig AP-N and the SN2 fraction from Jurkat cells (Figure 2). Over the range of NaCl concentration of 0–0.8 M no activation occurred for AP-N or SN2 from Jurkat cells with Ala-NMec as substrate, but with Arg-NMec a 3-fold activation was observed for Jurkat cells which was maximal at 0.1 M NaCl.

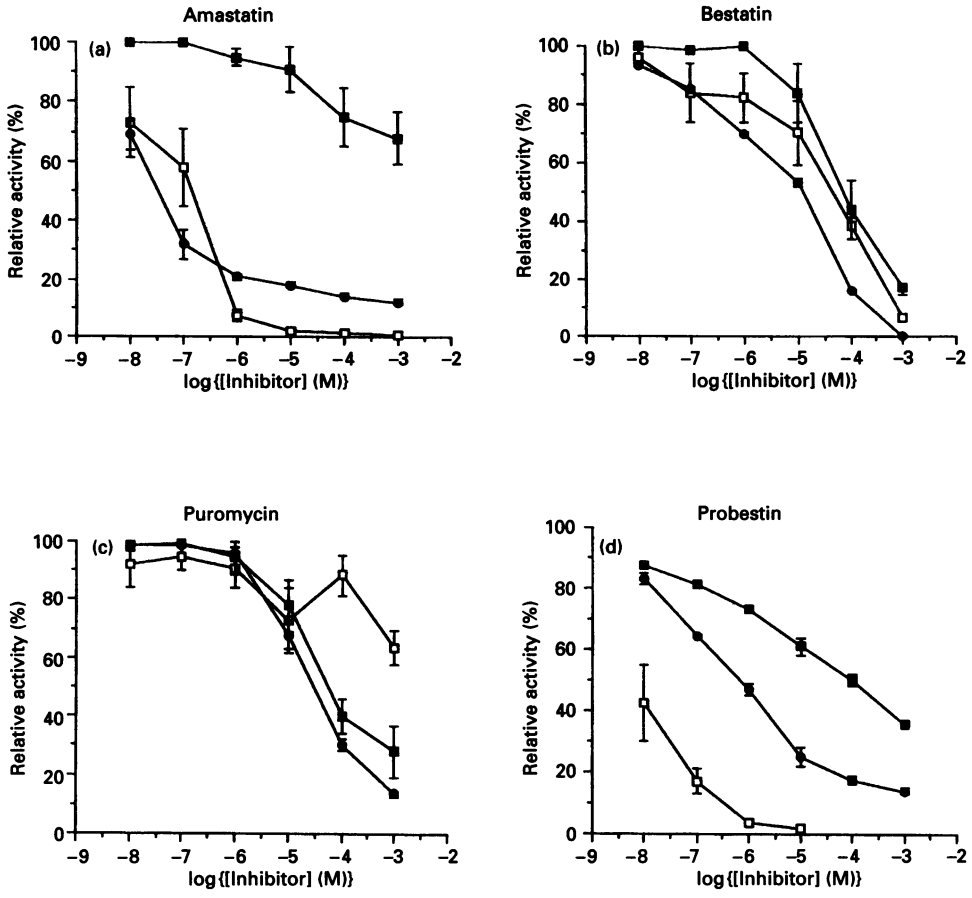

Figure 3 Effect of inhibitors on the hydrolysis of Ala-NMec by Jurkat cells, the cytosol fraction (SN2) from Jurkat cells and purified pig AP-N

See the Experimental section for details. The results are the means of two experiments and are percentages of the uninhibited activity. Bars indicate the range. ■, Intact Jurkat cells; ●, SN2 fraction of Jurkat cells; □, pig AP-N. Inhibitors: (**a**) amastatin, (**b**) bestatin, (**c**) puromycin, (**d**) probestin.

K_m values for the hydrolysis of Ala-NMec and Leu-NMec by Jurkat cells and by purified AP-N

For each substrate the K_m values were about 6.5 times higher for purified AP-N than those determined with intact Jurkat cells. For AP-N the K_m values (means ± S.E.M. with numbers of experiments in parentheses; in μM) were Ala-NMec, 418 ± 77 (4); Leu-NMec, 75 ± 9 (4), and for Jurkat cells they were Ala-NMec, 65 ± 6.5 (3) and Leu-NMec, 11.3 ± 2.4 (3). Given that there appears to be a barrier to the entry of these substrates into Jurkat cells, the K_m values are likely to be overestimated.

Effect of inhibitors on aminopeptidase activity in Jurkat cells and on pure AP-N

The effects of four inhibitors of aminopeptidases (amastatin, bestatin, probestin and puromycin) on the hydrolysis of Ala-NMec by intact Jurkat cells, fraction SN2 of Jurkat cells and purified pig AP-N were determined over a concentration range of 10 mM–1 mM (Figure 3, Table 4). Intact cells were very resistant to amastatin (IC_{50} > 1 mM), but after disruption the activity became susceptible in the SN2 fraction and the IC_{50} value (28 nM) was then lower than that for AP-N (150 nM); however, it was noted that some 10% of the SN2 activity was resistant to inhibition by 1 mM amastatin (Figure 3a). Bestatin did not discriminate so clearly, but the activity in the SN2 fraction was somewhat more sensitive than that of AP-N or that in intact cells (Figure 3b). AP-N was very sensitive to probestin (IC_{50} < 10 nM) compared with either intact cells or the SN2 fraction, for which the IC_{50} values were more than 10-fold higher (Figure 3d). AP-N was relatively resistant to puromycin (as was the aminopeptidase activity of human renal microvilli to 0.1 mM puromycin), while the SN2 fraction was slightly more sensitive than intact cells (Figure 3c).

A number of other potential inhibitors were studied in single experiments (after 30 min of preincubation) with the SN2 fraction

Table 4 IC_{50} values for aminopeptidase inhibitors of the hydrolysis of Ala-NMec by Jurkat cells, SN2 and AP-N

See the Experimental section for details. The values are the means of two experiments and are derived from the curves in Figure 2.

	IC_{50}		
Inhibitor	Jurkat cells (intact)	Jurkat cells (fraction SN2)	AP-N
Amastatin	> 1 mM	28 nM	150 nM
Bestatin	64 μM	12 μM	43 μM
Probestin	630 nM	100 nM	< 10 nM
Puromycin	55 μM	30 μM	> 1 mM

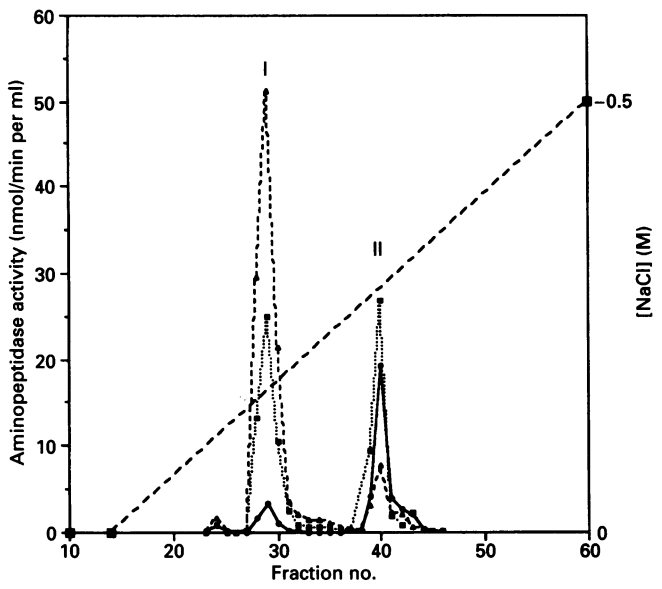

Figure 4 Chromatography of the SN2 fraction from Jurkat cells on a Mono Q column

See the Experimental section for details. The applied sample (9.5 ml in 0.25 M sucrose) had an activity of 5.9 nmol/min per ml with Ala-NMec and 17.2 nmol/min per ml with Arg-NMec as substrate. The fraction volume was 1 ml. Aminopeptidase activities: ———, Ala-Nmec; – – – – – –, Arg-Nmec; · · · · · · · · · ·, Lys-NMec. ■, [NaCl]. The protein elution pattern is omitted for clarity; the baseline level of fractions 23–48 was about 0.1 mg/ml with peaks at fractions 27–29 (0.18 mg/ml) and 37–40 (0.28 mg/ml); the latter peak did not coincide with the activity peak II.

and gave the following results (as percentage inhibition): 1 mM EDTA, 16%; 1 mM 1,10-phenanthroline, 0%; 1 mM E-64, 1%; 0.1 mM enalaprilat, 11%; 10 mM captopril, 30%; 0.1 mM di-isopropylfluorophosphate, 0%; 1 mM *N*-ethylmaleimide, 71%; 0.1 mM *N*-ethylmaleimide, 27%; 0.01 mM *N*-ethylmaleimide, 0%; 1 mM arphamenine B, 72%; 0.1 mM arphamenine, 26% (means of two experiments); 1 mM bacitracin, 80%; 0.1 mM bacitracin, 47%. Purified AP-N was not inhibited by 1 mM bacitracin. Thus the Jurkat cell aminopeptidase is relatively insensitive to chelating agents, angiotensin I-converting enzyme inhibitors, a serine peptidase reagent and E-64. It was inhibited by bacitracin and arphamenine B, as well as by the thiol reagent *N*-ethylmaleimide. It was slightly activated (+35%) by 1 mM dithiothreitol.

Partial purification of aminopeptidase activity from the SN2 fraction of Jurkat cells

The SN2 fraction was chromatographed on a Mono Q column. After dialysing the fraction, to lower the NaCl concentration before chromatography, the recovery of activity was poor, and hence the subcellular fractionation was repeated in 0.25 M sucrose in place of PBS. This increased the recovery of activity to 29%. The aminopeptidase activity hydrolysing Ala-NMec eluted at about 0.3 M NaCl. When further precautions were taken to avoid losses (chromatography within 4 h of cell fractionation and collection of fractions on ice), recovery was increased to 74% and a small peak eluting at 0.16 M NaCl was now observed. On assaying fractions with Lys- and Arg-NMec as substrates, two clear peaks were now defined, designated I and II (Figure 4). The activities of peaks I and II differed in several respects. The relative substrate profiles (Ala, Lys, Arg) were 100:765:1570 for I and 100:139:40 for II; inhibition by 100 μM amastatin was weak (4% for Arg-NMec and 22% for Ala-NMec) for the pooled fractions of peak I, but strong (76% for Arg-NMec and 96% for Ala-NMec) for those of peak II; activation by 200 mM NaCl was very marked (4–27-fold) for the pooled fractions of peak I, but absent (indeed, it was slightly inhibitory) for the pooled fractions of peak II.

Immunoblotting with monoclonal antibodies to CD-13

Three monoclonal antibodies (MCS2, 22A5 and WM15) were tested singly and in combination against Jurkat cells, HL60 cells (5×10^6 cells) and the Jurkat cell SN2 fraction after SDS/PAGE and PAGE in non-denaturing conditions, substituting 10% Triton X-100 for SDS. A stained band was seen with HL60 cells, but no positive results were obtained with Jurkat cells (results not shown).

Hydrolysis of [Leu^5]enkephalin by Jurkat and HL60 cells

The aminopeptidase activities of Jurkat and HL60 cells were studied by determining the release of free tyrosine from the pentapeptide substrate [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu; Table 5). The activity of intact HL60 cells was about 8-fold higher than that of Jurkat cells, and in both cases the activity was increased 3-fold by detergent treatment. Fractionation of Jurkat

Table 5 Subcellular distribution of aminopeptidase activity, with [Leu]enkephalin as substrate, in Jurkat and HL60 cells

See the Experimental section for details. The release of tyrosine was monitored by h.p.l.c. and activity is expressed as nmol/min per 10^7 cells; results are means ± S.E.M. with the numbers of experiments in parentheses. The values in square brackets are activities (%) relative to those in the suspension of disrupted Jurkat cells (homogenate), or after detergent treatment in the case of HL60 cells. Am (%) is the proportion of aminopeptidase activity sensitive to 100 μM amastatin.

	Jurkat cells			HL60 cells		
Fraction		Activity	Am (%)		Activity	Am (%)
Cells		4.9 ± 0.11 (7)	69		37.7 ± 9.6 (3)	95
Triton X-100-treated cells		15.7 ± 3.0 (4)		[100]	128.9 ± 25 (3)	98
Homogenate	[100]	20.4 ± 3.4 (3)	81			
SN1	[100]	20.4 ± 3.7 (3)	82			
SN2	[89]	18.2 ± 1.1 (3)	84			
P2	[14]	2.9 ± 0.73 (6)	86	[71]	92.1 ± 8.9 (3)	98
P2, after NaCl wash	[5]	1.0 (2)		[33]	42.5 (1)	

cells after disruption in a nitrogen bomb showed that most of the activity was associated with the supernatant fractions and only 14% was recovered in the P2 fraction; this was reduced to 5% after a single wash with 0.5 M NaCl. In contrast, the recovery of activity in P2 prepared from HL60 cells was high, namely 71% of that of a detergent-treated batch of cells, diminishing to 33% after the salt wash. Amastatin sensitivity of the Jurkat cells and fractions was relatively high (69–86%) compared with that observed when Ala-NMec was the substrate.

DISCUSSION

Properties of the aminopeptidase activity in Jurkat cells compared with that in HL60 cells and with AP-N

In these studies we have compared the activity in Jurkat cells with that from three other sources, namely CD-13-positive HL60 cells, pure pig AP-N and a preparation of human kidney microvillar membranes, which is rich in AP-N. Significant differences were observed in each comparison. First, the relative rates of hydrolysis of four fluorogenic substrates by Jurkat cells and Jurkat cell cytosol (fraction SN2) were unlike those for HL60 cells, which in turn exhibited a profile identical to that for pure AP-N. Secondly, the K_m values with Jurkat cells were 6-fold less for Ala-NMec and Leu-NMec compared with those with AP-N. Thirdly, the pH optima for the SN2 fraction of Jurkat cells and human kidney microvilli differed by almost 1 unit. Fourthly, the inhibition patterns for amastatin, bestatin, probestin and puromycin for Jurkat cells and fraction SN2 were unlike those for AP-N. By none of these criteria can the aminopeptidase of Jurkat cells be identified with AP-N, a conclusion consistent with these cells being CD-13-negative. All attempts to demonstrate CD-13 in Jurkat cells by immunoblotting using a mixture of monoclonal antibodies to CD-13 were unsuccessful.

Localization of Jurkat cell aminopeptidase activity

Lymphocytes are more difficult to disrupt than epithelial cells, requiring, in our experience, treatment in a nitrogen bomb (Howell et al., 1991). When Jurkat cells were so treated, the aminopeptidase activity increased 2-fold and became more sensitive to amastatin. While the marker for plasma membrane (alkaline phosphatase) was substantially enriched and mainly recovered in the high-speed membrane pellet (P2), aminopeptidase was predominantly recovered in the soluble fraction (SN2) and was impoverished in P2. Comparable results were obtained when a different aminopeptidase substrate, [Leu]-enkephalin, was used to assay the subcellular fractions; the very small proportion of the Jurkat cell activity in P2 contrasted with the results with HL60 cells, in which nearly all the activity was recovered in this fraction. The recovery of the activity of HL60 cells in P2, when assayed with Ala-NMec, was 35%, almost 10-fold greater than that in P2 from Jurkat cells.

Differences in properties of the aminopeptidase activity of Jurkat cells on disruption

Not only did the aminopeptidase activity double on cell disruption or detergent treatment, but striking differences in substrate preference and inhibitor responses were revealed. Thus intact cells hydrolysed Leu- and Ala-NMec faster than the Lys- and Arg- derivatives, while the cytosol fraction, SN2, hydrolysed the latter pair of substrates 3–4 times faster than Ala-NMec. For each of the four inhibitors amastatin, bestatin, probestin and puromycin, the sensitivity increased remarkably on disruption of the cells; in the case of amastatin the IC_{50} changed from 1 to 28 nM. This might be explained by the presence of aminopeptidases in two locations, one on the surface and the other intracellular, with the former being insensitive and the latter sensitive to the inhibitors and also having a different substrate profile. Our inability to demonstrate any significant membrane-bound aminopeptidase activity in Jurkat cells would contradict this explanation. We therefore propose that all of the aminopeptidase activity is intracellular and that the observed differences between intact and disrupted Jurkat cells depend on the plasma membrane permeability of substrates, products and inhibitors. If, as is likely, permeability is a function of hydrophobicity, the NMec substrates could be placed in the order Leu- > Ala- > Arg- = Lys-, essentially in agreement with their observed rates of hydrolysis. Only when the membrane barrier was abolished did the true specificity become apparent, with the basic substrates now preferred to the neutral ones. Similarly, we would argue that the inhibitors are largely excluded from access to the enzyme in intact cells and that the true sensitivity is revealed only after disruption. Thus the apparent activity of intact Jurkat cells is, in our view, attributable to cytosolic aminopeptidases, the activity of which requires penetration of substrates, including [Leu]-enkephalin, to the interior of the cell. In a very few cells (< 10%), this might be through a damaged membrane, but, more importantly, in > 90% of the viable cells, it must be by as yet unexplored mechanisms.

The identity of the Jurkat cell aminopeptidases

Chromatography of the SN2 fraction on a Mono Q column initially revealed only one activity when fractions were assayed with Ala-NMec. The second activity became apparent when the protocol was modified to minimize losses and the eluate was monitored with basic substrates. This aminopeptidase (peak I, Figure 4) was activated by NaCl, was relatively insensitive to amastatin and strongly favoured basic substrates. Peak II had a broader specificity, was not activated by NaCl and was strongly inhibited by amastatin. The studies on SN2 therefore represent the combined effects of these two activities, but almost all of the activity revealed by experiments with Ala-NMec can be attributed to that of peak II. This is because the recovered activity (nmol/min) of peak I was 6.1, compared with 64.2 for peak II, and peak I activity was apparently more labile than that of peak II, since it was not observed in several previous runs on Mono Q.

Other workers have noted the presence of multiple aminopeptidases in the cytosol of human leucocytes. Four were resolved by DEAE chromatography from the soluble fraction prepared from the buffy coat (Rautenberg and Tschesche, 1984), one of which was most active against basic substrates and was inhibited by bestatin, but it was not defined in detail and its cellular location was not studied. In another study on relatively pure preparations of human cells, 90% of the activity hydrolysing Arg-2-naphthylamide was in the cytosol of lymphocytes, was chloride-activated and exhibited a broad specificity, but was not defined by inhibitors (Grdisa and Vitale, 1991). Bauvois (1990) has reported the hydrolysis of Leu- and Ala-*p*-nitroanilides by intact murine thymocytes, with activities comparable with those we have observed with Jurkat cells with different substrates. The activity was insensitive to 5 mM EDTA, but more specific aminopeptidase inhibitors were not studied. The K_m values were 1.4 mM and 4 mM for the Leu- and Ala- substrates respectively, two orders of magnitude greater than those we report for the NMec substrates in Jurkat cells. Another activity hydrolysing arginine substrates was isolated from human erythrocytes, but

leucocytes contained 10 times more activity. It was activated by Co^{2+} and showed a preference for Lys- and Arg- substrates but also hydrolysed Leu- and Ala-2-naphthylamides at 48% and 12% respectively of the rate of the Lys- substrate. It was also sensitive to EDTA, being fully inhibited at 1 mM, and had IC_{50} values of 27 μM and 150 μM for amastatin and bestatin respectively (Vitale et al., 1981). A later report on what seems to be a similar enzyme gave further IC_{50} values: EDTA, 0.9 μM; amastatin, 90 nM; bestatin, 1 μM; puromycin, 2.5 μM. The K_m values were in the range 2.8–9.3 μM for Lys-, Arg- and Leu-2-naphthylamides, and $k_{cat.}$ values were very similar for the three substrates (Abramic and Vitale, 1992). The sensitivity to EDTA contrasts with the failure of chelating agents to inhibit the Jurkat cell activity, but the effect of the more specific inhibitors and the broad specificity may resemble the properties of our peak II.

Aminopeptidase B-like enzymes (EC 3.4.11.6), which are distributed in the cytosol of many tissues and cell types including leucocytes, are characteristically chloride-activated (2-fold by 0.2 M NaCl) and show a specificity that is strongly directed to Arg- and Lys- substrates, with Ala-2-naphthylamide being hydrolysed at < 1% of the rate for the basic substrates. Bestatin and arphamenine B are potent inhibitors (McDonald and Barrett, 1986; Söderling, 1982, 1983). Peak I activity from Jurkat cells thus has some properties in common with aminopeptidase B. Chloride ion activation is characteristic of another distinct enzyme, leukotriene A_4 hydrolase. This is a cytosolic enzyme, first isolated from neutrophils but widely distributed in many cells, including lymphocytes. It contains zinc and exhibits aminopeptidase activity, is able to hydrolyse a range of amino acid *p*-nitroanilides, is inhibited by bestatin and captopril and has a marked dependence on anions (20-fold stimulation by 100 mM NaCl and zero activity in phosphate buffer). It has high K_m values for the Ala- and Leu-*p*-nitroanilides ($\sim$ 0.5 mM), which are preferred to basic substrates, and is not inhibited by amastatin (Minami et al., 1990; Orning et al., 1990, 1991; Evans and Kargman, 1992; Wetterholm and Haeggström, 1992). It thus differs from the Jurkat cell activities.

The lysosomal enzyme cathepsin H, for which the spleen is a rich source, exhibits both endopeptidase and aminopeptidase activities at neutral pH. The latter activity shows a broad specificity, hydrolysing naphthylamides in the order Arg- > Lys- > Ala- > Leu-, with a factor of only 2 between the first and last in this list. It is strongly inhibited by E-64, but insensitive to inhibition by 20 μM bestatin or 100 μM puromycin (McDonald and Barrett, 1986), properties which serve to distinguish it from the Jurkat cell activities.

In summary, the cytosol of Jurkat cells contains at least two aminopeptidases, one of which is similar to aminopeptidase B and accounts for some of the activity hydrolysing basic substrates. The other, revealed by the neutral substrates, typically Ala-NMec, can be distinguished from the cell surface enzyme AP-N, and differs in properties from other reported cytosolic aminopeptidases. An important lesson from this work is that aminopeptidase activity demonstrated by intact cells, even with a pentapeptide substrate, does not necessarily imply a cell surface activity.

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REFERENCES

Abramic, M. and Vitale, L. (1992) Biol. Chem. Hoppe-Seyler **373**, 375–380
Amoscato, A. A., Balasubramaniam, A., Alexander, J. W. and Babcock, G. F. (1988) Biochim. Biophys. Acta **955**, 164–174
Amoscato, A. A., Alexander, J. W. and Babcock, G. F. (1989) J. Immunol. **142**, 1245–1252
Amoscato, A. A., Sramskoski, R. M., Babcock, G. F. and Alexander, J. W. (1990) Biochim. Biophys. Acta **1041**, 317–319
Ansorge, S., Schön, E. and Kunz, D. (1991) Biomed. Biochim. Acta **50**, 799–807
Ashmun, R. A. and Look, A. T. (1990) Blood **75**, 462–469
Barnes, K., Ingram, J. and Kenny, A. J. (1989) Biochem. J. **264**, 335–346
Bauvois, B. (1990) Eur. J. Immunol. **20**, 459–468
Bessey, O. A., Lowry, O H. and Brock, M. J. (1946) J. Biol. Chem. **164**, 321–329
Booth, A. G. and Kenny, A. J. (1974) Biochem. J. **142**, 575–581
Bowes, M. A. and Kenny, A. J. (1987) Immunology **60**, 247–253
Evans, J. F. and Kargman, S. (1992) FEBS Lett. **297**, 139–142
Fulcher, I. S. and Kenny, A. J. (1983) Biochem. J. **211**, 743–753
Gee, N. S. and Kenny, A. J. (1985) Biochem. J. **230**, 753–764
Grdisa, M. and Vitale, L. (1991) Int. J. Biochem. **23**, 339–345
Griffin, J. D., Ritz, D. J., Nadler, L. M. and Schlossman, S. F. (1981) J. Clin. Invest. **68**, 932–941
Howell, S., Murray, H., Kenny, A. J. and Turner, A. J. (1991) Biochem. J. **278**, 417–421
Jongeneel, C. V., Quackenbush, E. J., Ronco, P., Verroust, P., Carrel, S. and Letarte, M. (1989) J. Clin. Invest. **83**, 713–717
Kenny, A. J., Stephenson, S. L. and Turner, A. J. (1987) in Mammalian Ectoenzymes (Kenny, A. J. and Turner, A. J., eds.), pp. 169–210, Elsevier, Amsterdam
Kohno, H. and Kanno, T. (1985) Biochem. J. **226**, 59–65
Letarte, M., Vera, S., Tran, R., Addis, J. B. L., Onizuka, R. J., Quackenbush, E. J., Jongeneel, C. V. and McInnes, R. R. (1988) J. Exp. Med. **168**, 1247–1253
Look, A. T., Ashmun, R. A., Shapiro, L. H., O'Connell, P. J., Gerkis, V., D'Apice, A. J., Sagawa, K. and Peiper, S. C. (1989a) in Leukocyte Typing IV (Knapp, W., Dorken, B., Gilks, W. R., Rieber, E. P., Schmidt, R. E., Stein, H. and Borne, A. E. G. K., eds.), pp. 784–787, Oxford University Press, Oxford
Look, A. T., Ashmun, R. A., Shapiro, L. H. and Peiper, S. C. (1989b) J. Clin. Invest. **83**, 1299–1307
Matsas, R., Stephenson, S. L., Hryszko, J., Kenny, A. J. and Turner, A. J. (1985) Biochem. J. **231**, 445–449
McDonald, J. K. and Barrett, A. J. (1986) Mammalian Proteases: A Glossary and Bibliography, vol. 2: Exopeptidases, pp. 48–55, Academic Press, London
Minami, M., Ohishi, N., Mutoh, H., Izumi, T., Bito, H., Wada, H., Seyama, Y., Toh, H. and Shimuzi, T. (1990) Biochem. Biophys. Res. Commun. **173**, 620–626
Orning, L, Krivi, G., Bild, G., Gierse, J., Aykent, S. and Fitzpatrick, F. A. (1990) J. Biol. Chem. **266**, 16507–16511
Orning, L., Krivi, G. and Fitzpatrick, F. A. (1991) J. Biol. Chem. **266**, 1375–1378
Rautenberg, W. and Tschesche, H. (1984) Hoppe-Seyler's Z. Physiol. Chem. **365**, 49–58
Riemann, D., Schwarchula, A., Hentschel, M. and Lagner, J. (1993) Immunobiology **187**, 24–35
Shipp, M. A., Vijayaraghavan, J., Schmidt, E. V., Masteller, E. L., D'Adamio, L., Hersh, L. B. and Reinherz, E. L. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 297–301
Söderling, E. (1982) Arch. Biochem. Biophys. **216**, 105–115
Söerling, E. (1983) Arch. Biochem. Biophys. **220**, 1–10
Stephenson, S. L. and Kenny, A. J. (1987) Biochem. J. **241**, 237–247
Ulmer, A. J., Mattern, T., Feller, A. C., Heyman, E. and Flad, H. D. (1990) Scand. J. Immunol. **31**, 429–435
Vitale, L., Zubanovic, M. and Abramic, M. (1981) Acta Biol. Med. Germ. **40**, 1489–1495
Wetterholm, A. and Haeggström, J. Z. (1992) Biochim. Biophys. Acta **1123**, 275–281
Wu, Q., Lahti, J. M., Air, G. M., Burrows, P. D. and Cooper, M. D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 993–997
Wu, Q., Cooper, M. D., Pierres, M. and Gorvel, J. P. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 676–680

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