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Jurkat T cells express a functional neutral endopeptidase activity (CALLA) involved in T cell activation

Bernard Mari, Frederic Checler[1], Gilles Ponzio, Jean-François Peyron, Serge Manie, Dariush Farahifar, Bernard Rossi and Patrick Auberger[2]

INSERM U210, Faculté de Médecine, Avenue de Vallombrose, 06107 Nice cedex 02 and [1]IPMC, Institut de Pharmacologie Moléculaire et Cellulaire, 660 route des Lucioles, Sophia Antipolis, 06250 Valbonne, France
[2]Corresponding author

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We have characterized a T lymphocyte endopeptidase activity that hydrolyses succinyl-alanine-alanine-phenylalanine-paranitroanilide (Suc-Ala-Ala-Phe-pNa). Hydrolysis of this substrate by intact Jurkat T cells was markedly enhanced when exogenous aminopeptidase N was added to the incubation medium. It thus appears that the release of paranitroaniline from Suc-Ala-Ala-Phe-pNA results from the combination of two distinct enzymatic activities: (i) an endopeptidase activity that cleaves the substrate at the alanyl bond and (ii) an aminopeptidase activity that ultimately cleaves the phenylalanyl bond. This cleavage was further confirmed by HPLC analysis. Specific endopeptidase 24.11 inhibitors were shown to inhibit the endopeptidase activity. These features are reminiscent of the characteristics of neutral endopeptidase (NEP, also known as endopeptidase 24.11, CALLA or CD10). Anti-CD10 monoclonal antibodies (mAbs) recognized the CD10$^+$ B cell line Raji, but not Jurkat cells as assessed by FACS analysis. This is probably due to a lack of sensitivity of this method, the level of NEP activity in Jurkat T cells being 3-5% of that measured in B cell lines. Anti-CD10 mAbs immunoprecipitated endopeptidase 24.11 activities in both Jurkat T cells and Raji B cells, demonstrating that T lymphocytes express a CALLA-related endopeptidase. We also demonstrate that T and B cell endopeptidases have the same molecular weight, that T cells express less functional CALLA mRNA than B cells and that there are at least two shorter transcripts (1.8 and 0.8 kb) in both T and B cells. Finally, we report that specific endopeptidase inhibitors and an anti-CD10 mAb, which failed to affect the increase in intracellular calcium induced by T cell activation, significantly decreased IL-2 production in activated Jurkat T cells. Our findings demonstrate that human T cells express a functional CALLA involved in the regulation of IL-2 production.

Key words: endopeptidase 24.11/IL-2 production/T cell activation/T lymphocytes

Introduction

During the past decade, increasing evidence has accumulated to implicate serine proteases or esterases as key regulators of cell activation, proliferation and communication in the immune system. Involvement of serine proteases has been suggested in processes as diverse as cell killing by cytotoxic T lymphocytes (Ding-E Young *et al.*, 1986; Lobe *et al.*, 1986; Masson *et al.*, 1986; Pasternak *et al.*, 1986; Kramer and Simon, 1987), B and T cell activation (Schreiner and Unanue, 1976; Kishimoto *et al.*, 1979; Boyd-Bartlett and Troll, 1983; Ku *et al.*, 1983; Schön *et al.*, 1987) and T cell proliferation (Gutowski *et al.*, 1984; Wong *et al.*, 1987). Many of these studies were performed with specific protease inhibitors which have often been shown to inhibit several immune responses (Chang and Eisen, 1980; Utsunomiya and Nakanishi, 1986; Auberger *et al.*, 1989a,b). Naturally occurring protease inhibitors have also been found to affect the immune response (Ikuta *et al.*, 1982; Heumann and Visher, 1988; Petersen *et al.*, 1989).

Despite these well documented effects, lymphocyte-derived proteases remain poorly characterized. Recently, interest in this field has revived following several reports dealing with the characterization of cell surface peptidases on B and T lymphocytes and on macrophages. Of these enzymatic activities, dipeptidyl peptidase IV (Schön *et al.*, 1987; Morimoto *et al.*, 1989; Gorvel *et al.*, 1990; Hegen *et al.*, 1990) and a surface-associated neutral aminopeptidase (Amoscato *et al.*, 1989) have been proposed to be involved in regulating the proliferation of T lymphocytes. In addition, molecular cloning of the CD10 molecule has led to the unexpected discovery that it was identical to neutral endopeptidase (NEP, or endopeptidase 24.11) (Letarte *et al.*, 1988; Shipp *et al.*, 1989).

We have shown previously that chymotryptic-type protease inhibitors can block the production of IL-2 by activated Jurkat T cells, possibly through interaction with a membrane-associated protease (Auberger *et al.*, 1989a,b). The present study further characterizes this associated-membrane protease and defines in more detail its importance in regulating IL-2 production in T cells. The activity of this ectoprotease is reminiscent of CALLA (common acute lymphoblastic leukaemia antigen, or endopeptidase 24.11) and is correlated with the production of IL-2 by activated Jurkat T cells.

Results

Characterization of a CALLA-like activity at the surface of Jurkat T cells

Intact Jurkat T cells contain an ectoprotease activity that hydrolyses the metalloproteinase and chymotryptic-type substrate succinyl-alanine-alanine-phenylalanine-paranitro-

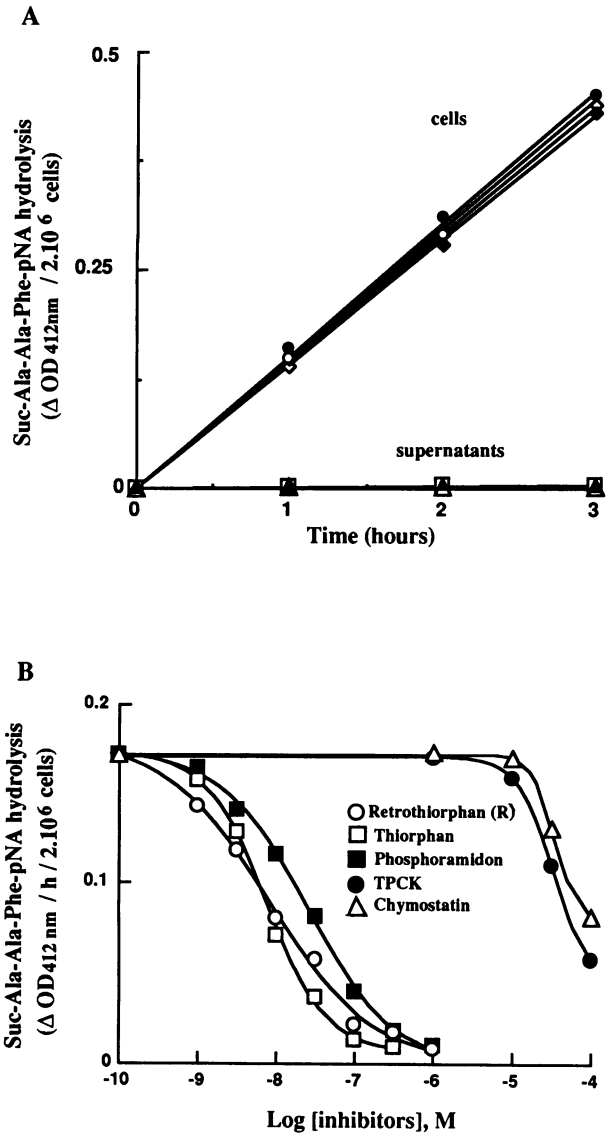


Fig. 1. Characterization of a surface-associated T cell protease. (**A**) A surface associated protease from Jurkat T cells hydrolyses Suc-Ala-Ala-Phe-pNA. Jurkat T cells (10^7/ml) were incubated in phosphate buffered saline (PBS) for 0, 1, 2 or 3 h at 37°C. At each time, cells were collected by centrifugation and resuspended in 200 μl of fresh PBS. Suc-Ala-Ala-Phe-pNA hydrolysis was determined at different times on each set of cells by the release of paranitroaniline 0 (○), 1 (●), 2 (◇) and 3 h (◆) and in the corresponding supernatants 0 (△), 1 (▲), 2 (□) and 3 h (■). (**B**) Effect of various protease inhibitors on the ectoprotease activity. T lymphocytes (10^7/ml) were preincubated for 10 min at 37°C in the presence of various concentrations of either retrothiorphan, thiorphan, phosphoramidon, TPCK or chymostatin. Suc-Ala-Ala-Phe-pNA (0.5 mM) was then added and the ectoprotease activity determined at different times as described before. Data points are means of triplicates.

anilide (Suc-Ala-Ala-Phe-pNA). In the assay, hydrolysis occurred linearly up to the first 3 h of incubation (Figure 1A). Hydrolysis cannot be accounted for by the secretion of a protease during the time of incubation nor by the non-specific release of internal proteolytic activities from lysed cells since cell supernatants collected at different times after the onset of the incubation failed to induce liberation of paranitroaniline.

In order to determine the specificity of the ectoprotease, we tested the capacity of various metallo- and chymotryptic-

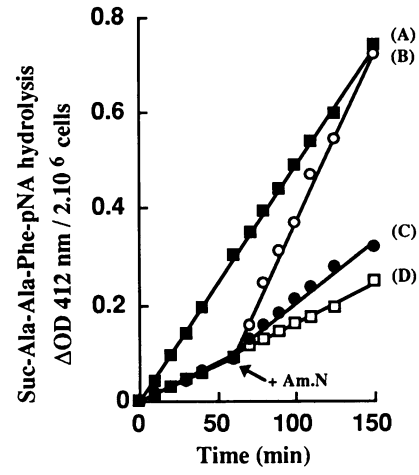


Fig. 2. Characterization of the enzymatic activities responsible for the release of paranitroaniline from Suc-Ala-Ala-Phe-pNA. Jurkat T cells (10^7/ml) were incubated at 37°C with 1 mM Suc-Ala-Ala-Phe-pNA as substrate in the presence (curve A) or the absence (curve D) of exogenously added aminopeptidase N (10 μg/ml). A second set of cells were first incubated for 1 h in the absence of aminopeptidase N, then purified enzyme was added (arrow) in the presence (curve C) or the absence (curve B) of 10 μM bestatin. Enzymatic activity was determined as previously described in Materials and methods. Data points are means of triplicates.

type protease inhibitors to block the ectoprotease activity towards Suc-Ala-Ala-Phe-pNA. Figure 1B shows that phosphoramidon, thiorphan, retrothiorphan, TPCK (*N*-α-*p*-tosyl-L-phenylalanine-chloromethylketone) and chymostatin inhibit the ectoprotease activity dose-dependently. As previously described for membrane preparations of Jurkat T cells (Auberger *et al.*, 1989b), the chymotryptic-type inhibitors TPCK and chymostatin partially inhibited the hydrolysis of Suc-Ala-Ala-Phe-pNA with a half-maximal inhibition (IC_{50}) of 50 μM. Thiorphan, retrothiorphan and phosphoramidon, three potent inhibitors of NEP, abolished enzymatic activity at 100 nM, with IC_{50}s at 7, 9 and 30 nM respectively (see also Figure 9C). Furthermore, addition of 1 μM Sepharose-coupled phosphoramidon also blocked the activity (not shown), supporting the hypothesis that this enzyme is located externally.

The blockade of the ectoprotease activity associated with the T cell membrane by well characterized NEP inhibitors suggested that hydrolysis of Suc-Ala-Ala-Phe-pNA was the result of the combined action of an endopeptidase and an aminopeptidase, the former hydrolysing the substrate on the NH_2 side of phenylalanine, allowing the aminopeptidase to liberate paranitroaniline from Phe-pNA. In order to test this hypothesis, we incubated intact Jurkat T cells in the presence or absence of purified aminopeptidase N. As shown in Figure 2, addition of aminopeptidase N to Jurkat cells caused a 3-fold increase in the initial velocity of hydrolysis of Suc-Ala-Ala-Phe-pNA, suggesting that hydrolysis of Phe-pNA represents the limiting step for pNA release. Addition of aminopeptidase N at 60 min increased the initial velocity of hydrolysis of Suc-Ala-Ala-Phe-pNA, an effect strongly inhibited by the aminopeptidase inhibitor bestatin. These results and those of Figure 1B clearly demonstrate that human T lymphocytes express an NEP-like activity at their surface.

We analysed the effect of various inhibitors on surface endopeptidase and aminopeptidase activities respectively with

Table I. Effect of various protease inhibitors on the activities of an aminopeptidase and an NEP on the surface of human T cells

Inhibitor	Hydrolysis activity		
	Leu-pNA (%)	Suc-Ala-Ala-Phe-pNA (%)	
		−Am N	+Am N
None	100 (±6)	100 (±5)	100 (±6)
Phosphoramidon (0.001 mM)	95 (±7)	5 (±1)	3 (±1)
Thiorphan (0.001 mM)	98 (±4)	4 (±1)	3 (±1)
Orthophenantroline (0.1 mM)	61 (±1)	5 (±1)	3 (±1)
Chymostatin (0.1 mM)	71 (±5)	50 (±3)	57 (±4)
TPCK (0.1 mM)	63 (±6)	64 (±4)	82 (±5)
NEM (2 mM)	58 (±3)	17 (±2)	98 (±5)
Amastatin (0.05 mM)	58 (±5)	60 (±4)	nd
Bestatin (0.05 mM)	52 (±4)	60 (±4)	nd

Jurkat T cells (10^7/ml) were preincubated for 10 min at 37°C in the presence or absence of various protease inhibitors in a final volume of 200 μl PBS. The appropriate substrates were then added at a final concentration of 1 mM. After 1 h at 37°C, enzymatic activities were determined at 412 nM as described in Materials and methods. Results are expressed as percent of control. Data are means ± SD of three determinations. nd, not determined.

Suc-Ala-Ala-Phe-pNA and H-Leu-pNA. To determine endopeptidase activity, purified exogenous aminopeptidase N was added or not at the onset of incubation. As shown in Table I, in the absence of aminopeptidase N, the most potent inhibitors of the endopeptidase activity were, in order of efficiency, thiorphan, phosphoramidon, *o*-phenanthroline, *N*-ethylmaleimide (NEM) and chymostatin. Aminopeptidase activity was inhibited by NEM, *o*-phenanthroline, aminopeptidase inhibitors (bestatin and amastatin), TPCK and chymostatin but not by thiorphan and phophoramidon. In the presence of aminopeptidase N, the same inhibition profile was obtained, except for NEM and TPCK whose inhibitory effects were highly reduced. This confirms that hydrolysis of Suc-Ala-Ala-Phe-pNA was not performed by a chymotrypsin-like enzyme. The observation that the effect of chymostatin is reduced only weakly in the presence of aminopeptidase N can be explained by our finding that chymotrypsin can inhibit purified aminopeptidase N (not shown). Thus, the possibility arises that inhibition of Suc-Ala-Ala-Phe-pNA hydrolysis by chymotryptic-type inhibitors (like TPCK and chymostatin), observed essentially in the absence of exogenous aminopeptidase N, results from the blockade of the endogenous neutral aminopeptidase. This is confirmed by the fact that Jurkat cells poorly hydrolyse the well characterized chymotrypsin-like substrates Suc-Ala-Ala-Pro-Phe-pNA and Suc-Phe-pNA (not shown).

The expression of endopeptidase 24.11 activity is not limited to the leukaemic T cell line Jurkat. Indeed, we have shown that normal highly purified T lymphocytes hydrolyse Suc-Ala-Ala-Phe-pNA (Table II), an effect inhibited by both thiorphan and retrothiorphan in the concentration range 1−100 nM (not shown).

Table II. Peripheral T lymphocytes express endopeptidase 24.11 activity

Cells	Endopeptidase 24.11 activity (nmoles/min·mg)
Jurkat T Cells	0.69 ± 0.06
Normal T cells	0.20 ± 0.05

Jurkat T cells or purified T lymphocytes (2×10^7/ml) were incubated with 1 mM Suc-Ala-Ala-Phe-pNA and 10 μg/ml aminopeptidase N in a final volume of 200 μl PBS, in the presence or absence of 1 μM retrothiorphan. Values represent for each set of cells the retrothiorphan-inhibitable endopeptidase 24.11 activity. Data obtained with Jurkat cells are the mean of three different experiments performed in triplicate. Values reported for peripheral T lymphocytes are the mean of three experiments performed in triplicate with blood samples obtained from three healthy donors.

HPLC analysis of the cleavage of Suc-Ala-Ala-Phe-pNA by intact Jurkat T cells

In order to define better the sites of cleavage of the substrate, the kinetics of Suc-Ala-Ala-Phe-pNA hydrolysis by intact Jurkat cells or by purified NEP were analysed by HPLC. The upper part of Figure 3 shows the kinetics of hydrolysis of this substrate by intact Jurkat T cells. The peak present at the beginning of the incubation, with a retention time of 44 min (panel A, peak 1), corresponds to intact substrate. The first degradation peak, with a retention time of 34 min (peak 2 corresponding to Phe-pNA), was observed after 1 h of incubation (panel B). This peak is identical to the one obtained in the presence of purified NEP (panel G). Interestingly, this peak was not observed when T cells (panel E) or purified NEP (panel H) were incubated in the presence of 1 μM phosphoramidon. A third peak with a retention time of 21 min (peak 3) was also visible in control cells after 3 h of incubation (panel C). It corresponds to a product of the degradation of peak 2 by a surface aminopeptidase. Indeed, addition of purified aminopeptidase N resulted in the replacement of peak 2 by peak 3, with either Jurkat T cells (panel F) or purified NEP (panel I) as the source of endopeptidase. Furthermore, amastatin, a specific inhibitor of aminopeptidases N and A, was found to protect peak 2 from degradation (not shown).

CALLA is not expressed at the surface of the T cell line Jurkat as measured by FACS analysis

CALLA, also known as CD10, NEP or endopeptidase 24.11, is present on the surface of the B cell line Raji as determined by flow cytometry using the specific mAbs IOT5, IOT5a, Dako CALLA, K14 and K50 (Figure 4). In contrast, the leukaemic T cell line Jurkat was found to be negative for this marker, in agreement with a previous report (Letarte

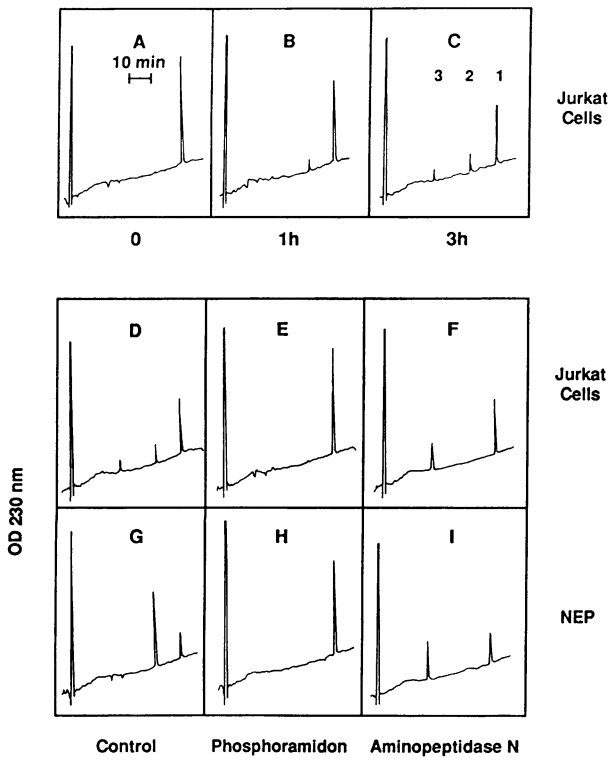


Fig. 3. HPLC analysis of the cleavage of Suc-Ala-Ala-Phe-pNA. T lymphocytes (10^7/ml) or purified NEP were incubated at 37°C with 1 mM Suc-Ala-Ala-Phe-pNA in a final volume of 100 μl PBS. The upper part of the figure shows the kinetics of hydrolysis of the substrate after 0 (panel **A**), 1 h (panel **B**) and 3 h (panel **C**). The lower part of the figure corresponds to a 3 h incubation of the cells (panels **D**, **E** and **F**) or purified NEP (panels **G**, **H** and **I**) with the substrate in the presence of 1 μM phosphoramidon (panels E and H) or 10 μg/ml of aminopeptidase N (panels F and I). For each condition, 5 μl of supernatant were acidified and analysed by HPLC as described in Materials and methods.

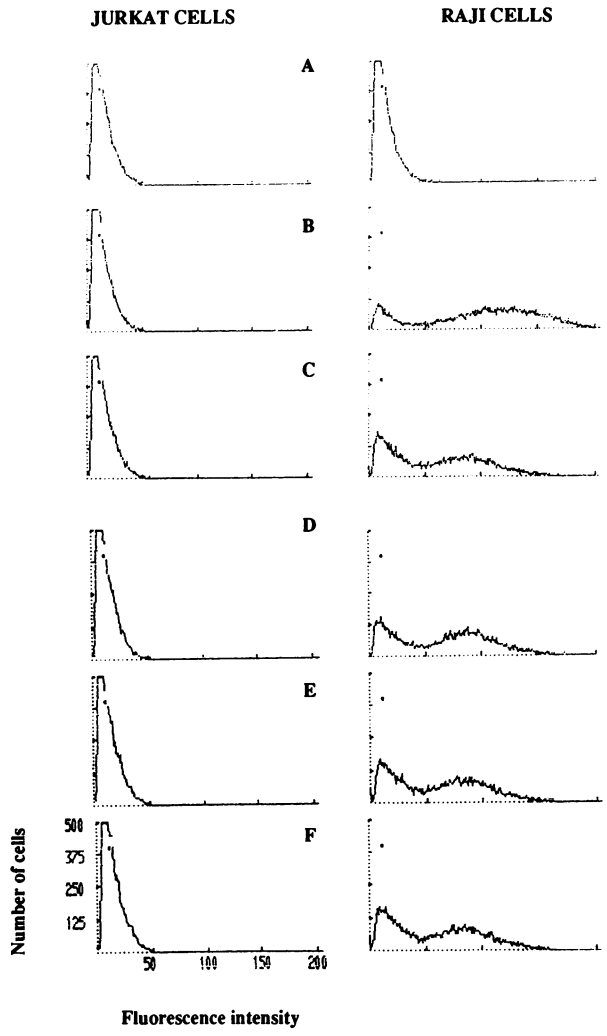


Fig. 4. Lack of expression of CALLA on the surface of T lymphocytes. Raji B cells or Jurkat T cells (10^6/ml) were incubated at 4°C with an anti-CD26 mAb as control (**A**) or with various anti-CALLA mAbs (panel **B**, IOT5; panel **C**, IOT5A; panel **D**, Dako CALLA; panel **E**, K14; and panel **F**, K50) and RAM-FITC, as described in Materials and methods. Analysis was performed using an ATC 3000 cytofluorimeter (ODAM).

et al, 1988) stating that this cell type does not express CALLA. The Ta1 antibody (anti-CD26 mAb) used as a control failed to label either of these cell types.

Anti-CD10 mAbs immunoprecipitated CALLA-like activities in both T and B cell lines

In order to determine whether the lack of staining of Jurkat cells observed by flow cytofluorimetry was due to a limitation of the sensitivity of this method, endopeptidase 24.11 activities present in membrane preparations from either Jurkat or Raji cells were immunoprecipitated in the presence of a panel of mAbs and protein A−Sepharose. Sepharose beads were then washed extensively and the endopeptidase activities of each protein A pellet and in the corresponding supernatant were determined by their ability to hydrolyse Suc-Ala-Ala-Phe-pNA in the presence of purified aminopeptidase N. As shown in Figure 5A, irrelevant mAbs X35 (anti-CD3), IOL1 (anti-CD45) and Ta1 (anti-CD26) were unable to recognize specific endopeptidase activity in T (black columns) or B cell (hatched columns) membrane preparations. Conversely, all the anti-CD10 mAbs precipitated such activity in membrane preparations of T or B lymphocytes. As expected, the corresponding supernatants were devoid of NEP enzymatic activity (not shown).

An SDS−PAGE analysis of IOT5 immunoprecipitates from detergent extracts of ^{125}I-surface-labelled Jurkat T cells and Raji B cells is presented in Figure 5B. Comparison of immunoprecipitates shows that T and B cell endopeptidases have similar molecular weights (100 kDa). Since 10 times more T cells were used than B cells, it appears that the amount of endopeptidase 24.11 at the surface of Jurkat cells is 3−5% that of Raji B cells. This is in good agreement with the level of endopeptidase activity measured by immunoprecipitation in both cell types.

Jurkat T cells express functional CALLA mRNA

Amplification of human T cell and B cell mRNAs was performed by reverse transcriptase PCR (RT-PCR) using various oligonucleotide primers corresponding to the translated region of CALLA (Figure 6). RT-PCR amplification of Raji B cell mRNAs using various pairs of primers revealed different bands of the expected sizes, i.e. 2.3 kb (primers 1 and 4), 1.7 kb (1 and 3) or 0.5 kb (2 and 4) (Figure 7A). As a positive control, Figure 7B shows that all these bands hybridized to the 2.3 kb probe corresponding to the total sequence of CALLA. Moreover, the 0.5 kb B

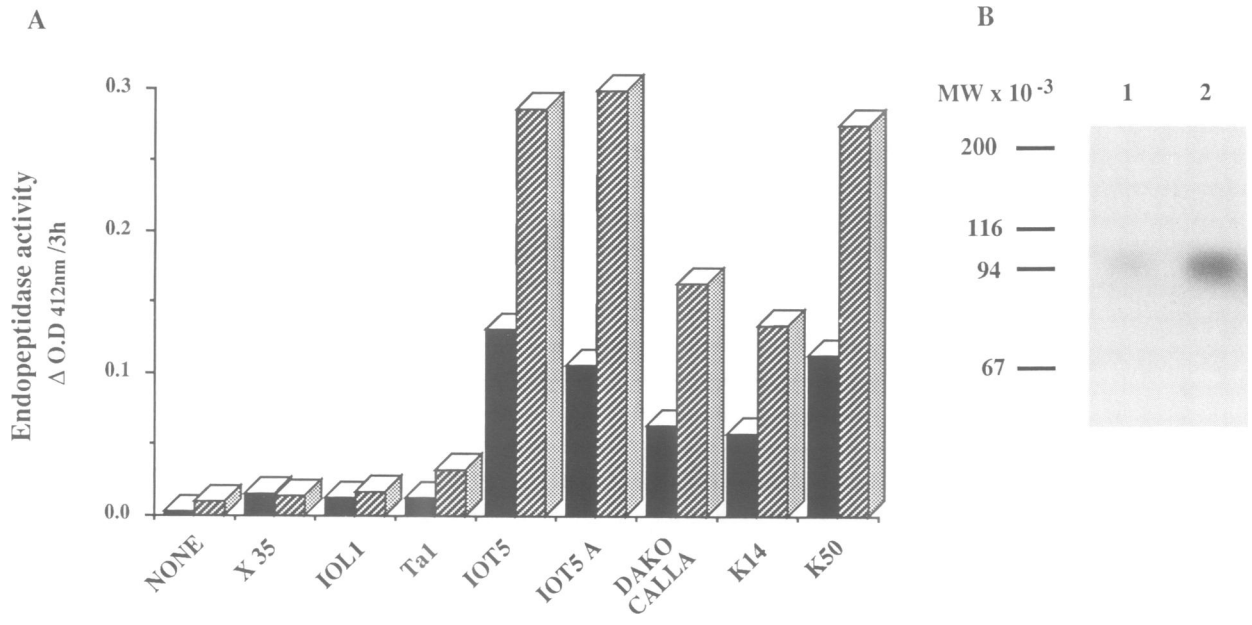
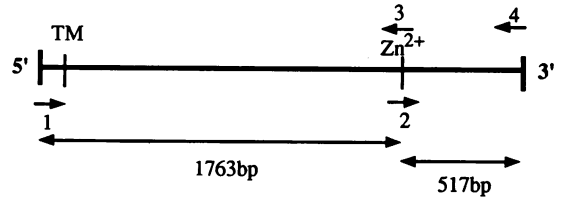


Fig. 5. Immunoprecipitation of CALLA in membrane preparations of Jurkat T cells and Raji B cells. (**A**) Solubilized membrane proteins from either Jurkat T cells (2 mg/ml, black columns) or Raji B cells (100 μg/ml, hatched columns) used to achieve approximately the same activity were immunoprecipitated with a panel of mAbs for 2 h at 37°C. Rabbit anti-mouse polyclonal antibody was then added in the presence of protein A–Sepharose. Beads were extensively washed and the endopeptidase activity of each protein A pellet was determined as described before. The figure represents a typical experiment chosen from three experiments. (**B**) SDS–PAGE analysis of ^{125}I-labelled membrane proteins after immunoprecipitation with a specific anti-CALLA antibody. 10^8 Jurkat T cells and 10^7 Raji B cells were surface-iodinated using the glucosoxidase lactoperoxidase technique. After solubilization in 1% Triton X-100, cell extracts were immunoprecipitated and analysed by SDS–PAGE as described in Materials and methods. Lanes 1 and 2 correspond to T and B cells enzyme respectively.

cell probe hybridized to the 2.3 kb PCR product, but not to the 1.7 kb fragment. Finally, we confirmed that the 1.7 kb probe hybridized to both the 1.7 and the 2.3 kb PCR product but not to the 0.5 kb probe, as shown in Figure 6.

RT-PCR amplification of Jurkat T cells mRNAs revealed two fragments of 0.5 and 1.7 kb which were indistinguishable from those observed in B cells (Figure 7A, lanes a and c). By contrast, the 2.3 kb product was faintly visible (lane b). As expected, the 0.5 kb T cell probe hybridized not only to itself but also to the 0.5 kb Raji PCR product (Figure 7B, panel 1, lanes a and d). There was no detectable hybridization of the T cell probe to the 1.7 kb fragment, while longer exposure of the autoradiogram revealed the 2.3 kb full length Jurkat PCR product (not shown). Finally, we confirmed that the 2.3 kb B cell probe revealed the 0.5 and 1.7 kb Jurkat PCR products and to a lesser extent the 2.3 kb one (panel 4, lanes a, c and b).

In order to determine whether Jurkat T cells express a functional CALLA mRNA, we probed a Northern blot of RNA extracted from Raji B cells and Jurkat T cells with the four different probes (i.e. the 0.5, 1.7 and 2.3 kb Raji PCR products and the 0.5 kb Jurkat PCR product). Depending on the probes used, RNA species of 6.6, 3.8, 1.8 and 0.8 kb were visualized. The two larger species corresponding to those described for CALLA mRNAs (Letarte *et al.*, 1988), were consistently found in B cell RNA preparations using the 0.5, 1.7 and 2.3 kb Raji PCR probes (Figure 8B, panels 2, 3 and 4). However, in contrast to the statement of Letarte *et al.*, we found that T cells do express low but significant amounts of the two larger 6.6 and 3.8 kb forms. These transcripts were observed with the three B cell probes, but were barely detectable with the 0.5 kb T cell probe (Figure 8B, panel 1). Again, longer exposure



PRIMER	SEQUENCE	POSITION
1 (sense)	GTGATGGGCAAGTCAGAAAGTCAG	-3 - 22
2 (sense)	GGTCATCGGACATGAAATCACACA	1741 - 1765
3 (reverse)	TGTGTGATTTCATGTCCGATGACC	1741 - 1765
4 (reverse)	AGATCACCAAACCCGGCACTTCTT	2234 - 2258

Fig. 6. Oligonucleotides used for RT-PCR amplification of T cell and B cell endopeptidase 24.11. The sequences of primers 1–4 are given and their orientations are representated by the arrows. Nucleotides were numbered according to Malfroy *et al.* (1987). TM, transmembrane domain; Zn^{2+}, Zn^{2+} binding site.

of the autoradiogram allowed detection of these two transcripts (not shown).

Two other lower transcripts (1.8 and 0.8 kb) were detected in both T and B cell mRNA preparations. These mRNAs hybridized to various extents to the different probes used, and more particularly to the 2.3 kb B cell probe and the 0.5 kb T cell probe. The fact that the 0.8 and 1.8 kb mRNAs hybridized to the 0.5 kb B cell probe faintly as compared with the T cell probe may appear puzzling. This apparent discrepancy is likely to reflect some differences in the sequences of these two probes as we used very stringent

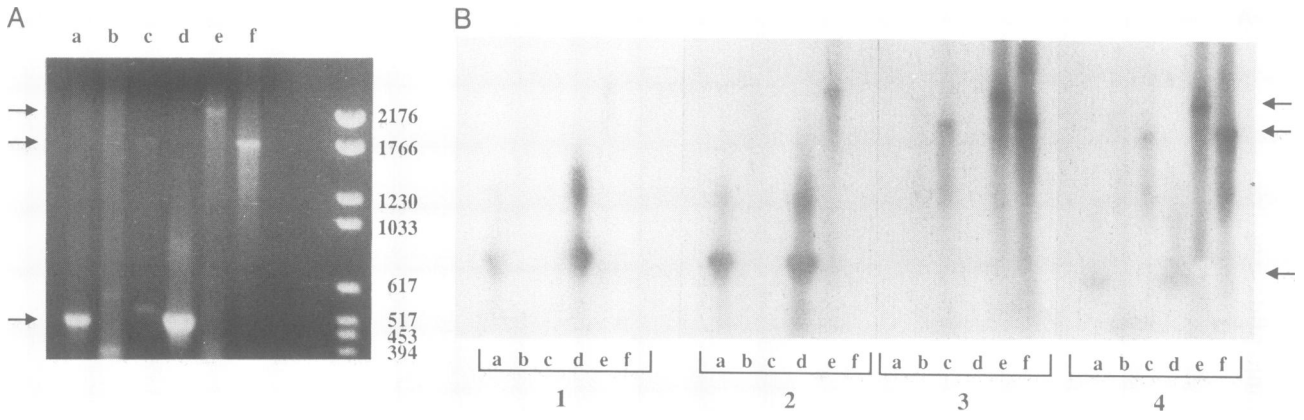


Fig. 7. Amplification of cDNA by PCR. (**A**) Amplifications were performed using T cell or B cell poly(A)$^+$ RNA between different pairs of primers as shown in Figure 6. 10 μl of the reaction was subjected to electrophoresis through a 1% agarose gel and DNAs were visualized by ethidium bromide. The positions of markers of known sizes in base pairs are given on the right of the figure. Lanes a–c correspond to Jurkat DNAs and lanes d–f to Raji DNAs. Amplification was performed between primers 2 and 4 (517 bp fragment), lanes a and d, primers 1 and 4 (2261 bp fragment), lanes b and e and primers 1 and 3, lanes c and f (1763 bp fragment). (**B**) Southern blot analysis of PCR products. The different radioactive probes were amplified, purified and labelled with [^{32}P]dCTP as described in Materials and methods. Panel 1, 517 bp T cell probe; panel 2, 517 bp B cell probe; panel 3, 1763 bp B cell probe; and panel 4, 2261 bp B cell probe. Lanes a–f are the same as in Figure 7A.

hybridization conditions. Finally, we were unable to detect hybridization of the 1.7 kb probe to the shortest mRNA species (Figure 8B, panel 3). The 1.7 kb probe corresponds to 75% of the total CALLA open reading frame but does not contain the catalytic active site of the enzyme which is part of the 500 bp fragment. This observation suggests that this mRNA encodes the C-terminal part of the enzyme.

Implication of the endopeptidase activity in the regulation of IL-2 production

Together with our previous report indicating that chymotryptic-type protease inhibitors block Jurkat activation (Auberger *et al.*, 1989b), the characterization in this study of a surface CALLA activity raises the question of its physiological significance. We have proposed that these inhibitors affect T cell responses through their interaction with a chymotryptic-type protease. However, the slight inhibition of Suc-Ala-Ala-Phe-pNA hydrolysis by TPCK, measured in the absence of exogenous aminopeptidase N, is due to a blockade of the endogenous aminopeptidase that liberates paranitroaniline rather than to a direct effect on endopeptidase 24.11.

In order to determine whether the activity of endopeptidase 24.11 is involved in T cell activation, we tested the ability of various concentrations of different endopeptidase 24.11 inhibitors to block IL-2 production in Jurkat T cells activated in the presence of phytohaemagglutinin (PHA) or a coated anti-CD3 mAb (X35). All the endopeptidase inhibitors tested diminished the production of IL-2 dose-dependently (Figure 9A). The half-maximal inhibitions of IL-2 production by retrothiorphan, thiorphan, phosphoramidon, N-CBX Me-Phe-Leu (*N*-carboxymethyl-phenylalanine-leucine) and Suc-Ala-Ala-Phe-pNA are indicated in Figure 9C. These concentrations are compatible with the half-maximal effect of each inhibitor on endopeptidase 24.11 activity. The inhibition is restricted to endopeptidase 24.11 since bestatin, amastatin and arphamenine B, which are potent blockers of aminopeptidase N, A and B respectively, failed to affect IL-2 production in the same conditions (not shown). Moreover, another specific metalloprotease

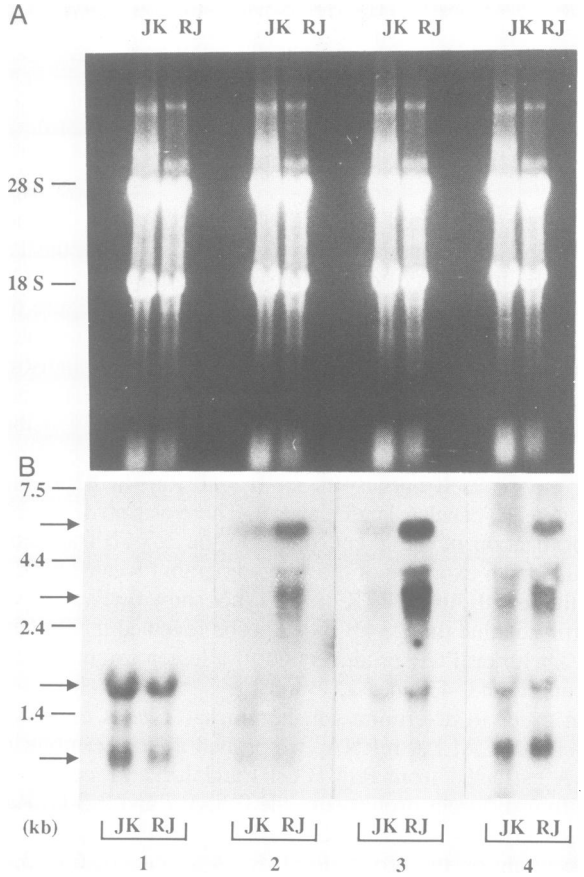
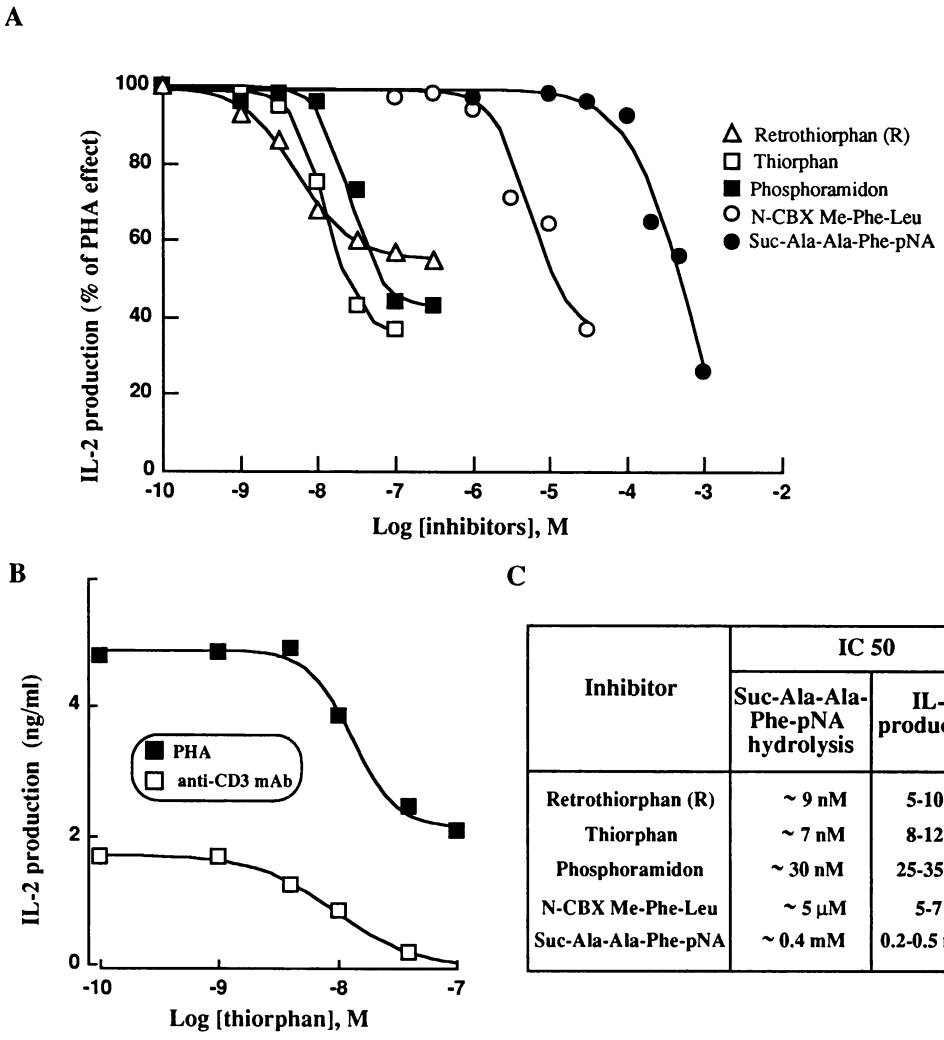


Fig. 8. Northern blot analysis of total RNAs from Jurkat T cells and Raji B cells. **A**. 20μg of total Jurkat or Raji RNA were subjected to electrophoresis through 1% agarose and visualized by ethidium bromide. **B**. RNAs were transferred to Hybond N$^+$ membrane and hybridized to specific ^{32}P-labelled probes. Panel 1, 517 bp Jurkat probe; panel 2, 517 bp Raji probe; panel 3, 1763 bp Raji probe; and panel 4, 2261 bp Raji probe. Arrows indicate the different RNA transcripts. The positions of markers of known sizes are given on the left of the figure.



Inhibitor	IC 50	
	Suc-Ala-Ala-Phe-pNA hydrolysis	IL-2 production
Retrothiorphan (R)	~ 9 nM	5-10 nM
Thiorphan	~ 7 nM	8-12 nM
Phosphoramidon	~ 30 nM	25-35 nM
N-CBX Me-Phe-Leu	~ 5 μM	5-7 μM
Suc-Ala-Ala-Phe-pNA	~ 0.4 mM	0.2-0.5 mM

Fig. 9. Effect of increasing concentrations of different NEP inhibitors on IL-2 production by activated Jurkat T cells. Jurkat T cells (10^6/ml) were incubated for 15 h with various concentrations of either retrothiorphan, thiorphan, phosphoramidon, N-CBX Me-Phe-Leu or Suc-Ala-Ala-Phe-pNA in the presence of PHA (20 μg/ml) (**A**) or with various concentrations of thiorphan in the presence of either PHA (20 μg/ml) or coated anti-CD3 mAb (X35, 5 μg/ml) (**B**). The IL-2 content of each supernatant was determined in triplicate using an EIA as described in Materials and methods. Data are from a single experiment performed in duplicate and are typical of four such experiments. Panel **C** indicates the half maximal inhibition concentration (IC_{50}) of each inhibitor on both endopeptidase activity and IL-2 production.

Table III. Effect of IOT5 on endopeptidase 24.11 activity and IL-2 production by PHA-activated Jurkat T cells.

	Endopeptidase 24.11 activity		IL-2 production	
Conditions	nmol/min·mg	% of control	ng/ml	% of control
Control	0.69 ± 0.06	100 ± 9	8.5 ± 1.1	100 ± 13
IgG1 (1 μg/ml)	0.67 ± 0.05	97 ± 7	9.9 ± 0.5	116 ± 5
IOT5 (1 μg/ml)	0.57 ± 0.03	83 ± 5	5.8 ± 0.4	68 ± 7
IgG1 (5 μg/ml)	0.74 ± 0.05	107 ± 7	10.2 ± 1.1	120 ± 11
IOT5 (5 μg/ml)	0.42 ± 0.03	61 ± 7	5.0 ± 0.5	59 ± 10
IgG1 (10 μg/ml)	0.73 ± 0.06	106 ± 8	nd	
IOT5 (10 μg/ml)	0.34 ± 0.03	49 ± 9	nd	

Jurkat cells (10^7/ml) were incubated for 2 h in the presence of various concentrations of either IOT5 or an irrelevant mAb of the same isotype. Cells were washed and resuspended at 10^7/ml in 200 μl of PBS in the presence of 1 mM Suc-Ala-Ala-Phe-pNA and 10 μg/ml aminopeptidase N. Endopeptidase 24.11 activity was determined on each set of cells, as described before. Values are the mean ± SD of three independent experiments performed in duplicate. Jurkat cells (10^6/ml) were preincubated for 2 h in the presence of various concentrations of an irrelevant IgG1 mAb or IOT5 mAb. Cells were then activated by 20 μg/ml PHA for 15 h at 37°C. The IL-2 content of each supernatant was determined in triplicate using an EIA as described in Materials and methods. Values are the mean ± SD of a single experiment performed in triplicate and are typical of three different experiments.

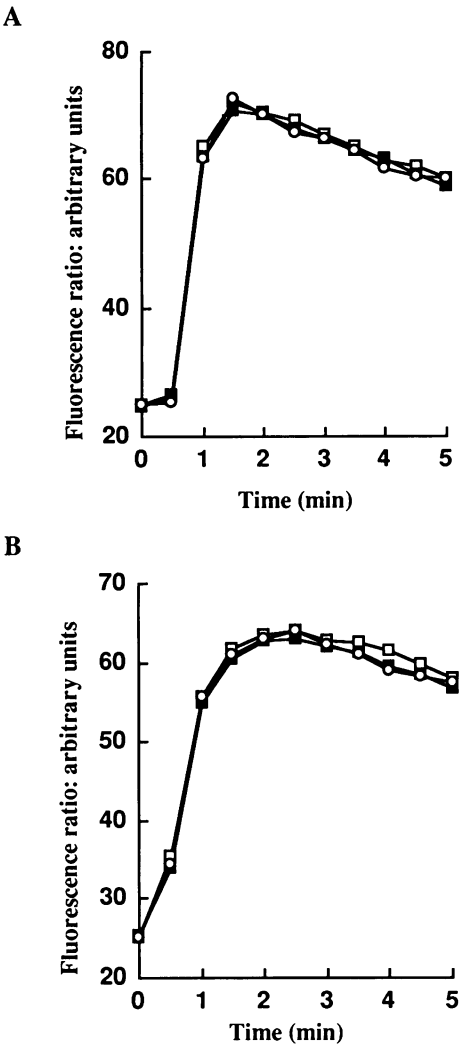


Fig. 10. Effect of thiorphan and phosphoramidon on the rise in intracellular Ca^{2+} concentration induced by X35 and PHA in Jurkat T cells. Cells were loaded with indo-1 and fluorescence was monitored as a function of time. Thiorphan (□) or phosphoramidon (■) at 1 μM concentration were added 2 min before the stimulus. **A**, X35 (1 μg/ml) (○). **B**, PHA (20 μg/ml) (○).

inhibitor, captopril, which blocks angiotensin converting enzyme (ACE), was found to be ineffective (not shown). It is noteworthy that, when coated X35 was used in place of PHA, the effect of thiorphan was more pronounced (Figure 9B). This may reflect the existence of a second transduction pathway involved in the secretion of IL-2 under PHA stimulation which is not controlled by the endopeptidase activity.

To confirm that NEP is involved in the regulation of IL-2 production, we tested the effect of an anti-CD10 mAb, IOT5, on Suc-Ala-Ala-Phe-pNA hydrolysis and IL-2 production. As depicted in Table III, IOT5 inhibits endopeptidase activity at the surface of Jurkat T cells dose-dependently and significantly blocks PHA-induced IL-2 production. The inhibition of IL-2 production approximately reflects the blockade of endopeptidase 24.11 activity. This result was confirmed with two other mAbs, K14 and K50 (not shown).

Finally, we tested the effect of thiorphan and phosphoramidon, the most potent inhibitors of endopeptidase 24.11, on the Ca^{2+} rise induced by different stimuli in Jurkat T cells. Using the fluorescent probe indo-1, the increase in Ca^{2+} concentration was measured every 30 s after addition of 100 nM phosphoramidon or thiorphan, 2 min prior to activation. Neither phosphoramidon nor thiorphan affected the increase in Ca^{2+} concentration induced by PHA or X35 (Figure 10). Furthermore, an anti-CD10 mAb, IOT5, failed to affect this short term response induced by T cell activation (not shown).

Discussion

Using Suc-Ala-Ala-Phe-pNA as a substrate and a panel of protease inhibitors, we have obtained evidence supporting the involvement of a membrane-bound endopeptidase in the production of IL-2 consecutive to T cell activation. We demonstrate here that human T lymphocytes carry at their surface proteolytic activities that cleave Suc-Ala-Ala-Phe-pNA first on the NH_2 side of Phe and then on the COOH side of Phe to liberate paranitraniline. The two enzymatic activities responsible for this cleavage display ectoenzyme behaviour based on the following criteria: (i) intact T cells hydrolyse an extracellular substrate, (ii) the activity cannot be accounted for by secretion or non-specific release of (a) proteolytic enzyme(s) from cells, and (iii) phosphoramidon coupled to Sepharose beads drastically inhibited the hydrolysis Suc-Ala-Ala-Phe-pNA by intact Jurkat T cells (not shown). Addition of purified aminopeptidase N to Jurkat T cells strongly increased the rate of Suc-Ala-Ala-Phe-pNA hydrolysis, an effect markedly inhibited by the amino-peptidase inhibitor bestatin. In the absence of exogenous aminopeptidase N, hydrolysis of Suc-Ala-Ala-Phe-pNA can be abolished by the protease inhibitors, retrothiorphan, thiorphan, phosphoramidon and *o*-phenanthroline, and to a lesser extent by NEM, TPCK, chymostatin, amastatin and bestatin. The most striking effects were obtained with retrothiorphan, thiorphan and phosphoramidon, three potent and selective inhibitors of neutral metalloendopeptidase (Roques *et al.*, 1980, 1983; Roques, 1985). In the presence of aminopeptidase N, hydrolysis of Suc-Ala-Ala-Phe-pNA was insensitive to NEM and poorly inhibited by TPCK and chymostatin, indicating that cleavage of this substrate could not be attributed to a cellular chymotryptic-type protease. This assumption was confirmed by HPLC analysis showing that the initial site of cleavage of this substrate by the Jurkat enzyme is identical to that of purified NEP. Hydrolysis of H-Leu-pNA was inhibited by NEM, bestatin, amastatin, *o*-phenanthroline and by chymostatin and TPCK, but not by thiorphan and phosphoramidon. These results are consistent with the participation of both a NEP and an aminopeptidase in the release of paranitraniline by intact Jurkat T cells when Suc-Ala-Ala-Phe-pNA is used as substrate.

The characterization in this report of an NEP-like activity is somewhat puzzling. Indeed, NEP has been shown to be present only on lymphoid tissues (Bowes and Kenny, 1987), leukaemic cells of pre-B phenotype (Letarte *et al.*, 1988) and neutrophils (Connelly *et al.*, 1985; Painter *et al.*, 1988). In this study, we have been unable to detect CD10 expression on the surface of Jurkat T cells as measured by FACS analysis with anti-CALLA mAbs, even in optimal conditions of detection. Accordingly, it has been shown that CALLA$^-$ murine myeloma cells transfected with the CALLA open reading frame acquired significant NEP activity despite a poor cell surface expression as measured by FACS (Shipp *et al.*, 1989). However, using more sensitive methods, we

found that several anti-CD10 mAbs immunoprecipitated endopeptidase activities present in membrane preparations of both B and T cells, demonstrating that T cells express CALLA. It has been reported that Jurkat T cells do not express CALLA mRNA (Letarte *et al.*, 1988) although purified T lymphocytes have NEP activity (Beaumont *et al.*, 1989). However, we have confirmed in this study that highly purified peripheral T lymphocytes express detectable endopeptidase 24.11 activity. Thus, the possibility remains that T cells express a protein whose mRNA is not recognized by the 1.6 kb CALLA probe used by Letarte, but which shares with CALLA an endopeptidase 24.11-type of activity.

We obtained several other lines of evidence that Jurkat T cells do express CALLA. (i) T and B cell enzyme have similar molecular weights (100 kDa) as judged by SDS−PAGE analysis after immunoprecipitation of ^{125}I-surface-labelled membrane proteins by a specific anti-CD10 mAb (IOT5). (ii) RT-PCR indicates that T cells express a low but significant amount of a mRNA corresponding to the total open reading frame of CALLA. (iii) mRNAs species of 6.6 and 3.8 kb corresponding to those already described for the NALM-6 pre-B cell line (Letarte *et al.*, 1988) were consistently found in both B and T cells. Furthermore, the relative amounts of these transcripts in B and T cells are in good agreement with the respective CALLA activities of both cell lines.

Using several CALLA B cell probes we were able unambiguously to detect functional CALLA mRNA in Jurkat T cells (i.e. the well characterized 3.8 and 6.6 kb transcripts). However, in this study we observed that hybridization of these transcripts to the 0.5 kb B cell probe was stronger than that to the 0.5 kb T cell probe (Figure 8B, compare panels 1 and 2) This may reflect some slight differences in the C-terminal part of the two sequences.

Our results thus demonstrate for the first time that T cells possess CALLA mRNA which encodes a functional cell surface associated NEP.

The observation that both B and T cells express at least two other smaller mRNAs species is intriguing. The 1.8 kb form was always detected with the different CALLA PCR products, while the 0.8 kb transcript was never observed using the 1.7 kb PCR fragment. The fact that this transcript hybridized to the 0.5 and 2.3 kb PCR products but not to the 1.7 kb fragment suggests that it lacks two-thirds of the 5′-terminal sequence of CALLA or possesses a different 5′-terminal RNA sequence. A recent report described a novel potential metallopeptidase derived from the NEP gene by alternative splicing (Llorens-Cortes *et al.*, 1990). This mRNA encodes the cytosolic, the transmembrane and the C-terminal region of NEP but lacks the middle part of the sequence (nucleotides 175−1640) and appears to encode a putative membrane metallopeptidase of 255 amino acids with a specificity distinct from that of NEP. Such alternative splicing may account for the presence of the 0.8 kb mRNA in B and T cells. The function of the small mRNAs observed in both B and T cells remains to be elucidated. Experiments are currently in progress to characterize these different transcripts.

Finally, a major point of interest of our study is the demonstration that several potent and selective inhibitors of NEP (i.e. retrothiorphan, thiorphan, phosphoramidon and N-CBX Me-Phe-Leu), a general metallopeptidase inhibitor (*o*-phenanthroline; not shown) and a substrate (Suc-Ala-Ala-Phe-pNA) can block IL-2 production by Jurkat cells at concentrations that inhibit the surface endopeptidase activity. It has been described that thiorphan inhibits ACE (K_i = 140 nM; Roques *et al.*, 1983). The inhibition of IL2 production we observed in this study cannot be accounted for by the interaction of this inhibitor with ACE for the following reasons. (i) Retrothiorphan is a selective tool used to differentiate NEP and ACE activities (K_i = 6 nM and >10 μM respectively; Roques *et al.* 1983). (ii) Captopril, an inhibitor of ACE, fails to affect IL-2 production (not shown). Moreover, the fact that an anti-CD10 mAb, IOT5, can inhibit both enzymatic activity and IL-2 production rules out the possibility that the inhibitors used have a non-specific effect and demonstrates the link between endopeptidase 24.11 activity and the regulation of IL-2 production. Such an immunomodulatory effect has been already reported *in vivo* for N-CBX Me-Phe-Leu (Jankovic *et al.*, 1991). The inhibition is more pronounced when coated anti-CD3 was used in place of PHA or PHA + PMA (phorbol myristate acetate), suggesting that the endopeptidase may control T cell activation more specifically via the TCR/CD3 pathway. In contrast, a short-term response consecutive to T cell activation i.e. the increase in Ca^{2+} concentration induced by PHA or an anti-CD3 mAb was not affected. The level of action of endopeptidase 24.11 in the regulation of IL-2 production remains to be determined; nevertheless, the step affected by endopeptidase inhibitors appears to be independent of or posterior to the increase in Ca^{2+} concentration induced by T cell activation. Our results demonstrate that human T cells express at their surface functional endopeptidase 24.11 whose enzymatic activity is involved in the control of IL-2 production.

Concerning the physiological role of the endopeptidase described here, one may assume that it contributes to the regulation of the concentration of lymphokines and/or to the processing of an activation molecule at the surface of or inside the T lymphocyte.

In conclusion, we have clearly and for the first time demonstrated that human T lymphocytes express functional CALLA mRNA and cell-surface activity. This enzyme is involved in Jurkat T cell activation and thus swells the limited numbers of peptidases (DAP IV and THAM) (Schön *et al.*, 1987; Dang *et al.*, 1990; Gorvel *et al.*, 1990; Hegen *et al.*, 1990; Vivier *et al.*, 1991) known to be capable of delivering an activation signal to T lymphocytes.

Materials and methods

Materials

TPCK, Suc-Ala-Ala-Phe-pNA and chymostatin were obtained from Serva. Phosphoramidon, thiorphan, bestatin, *o*-phenanthroline, N-CBX me-Phe-Leu, PHA and PMA were purchased from Sigma. Anti-CD3 mAb (X35), anti-CD10 mAbs (IOT5 and IOT5a) were from Immunotech (Marseille, France), anti-CD26 mAb (Ta1) from Coulter and Dako CALLA from Dako. AMV reverse transcriptase and *Taq* polymerase were purchased from Boehringer.

Cells

The human leukaemic T cell line Jurkat and the B lymphoma Raji were cultured in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate and 0.1 μM 2-mercaptoethanol as previously described (Mary *et al.*, 1987).

Peripheral lymphocyte preparation

Human peripheral mononuclear cells (PBMC) were isolated, under sterile conditions, from leukophoresis samples obtained from the 'Centre de Transfusion Sanguine A.Tzanck' (St Laurent du Var, France). Briefly, PBMC were diluted 1:1 with PBS and layered over lymphocyte separation

medium (d = 1.077, Eurobio, Les Ulis, France) in a 2:1 ratio and centrifuged at 400 g for 30 min at 20°C. The mononuclear cell interface was collected and the contaminating platelets and residual erythrocytes were removed by repeated centrifugations. Mononuclear cells were suspended in complete medium in the presence of 1% FCS and monocytes were removed by their adherence to 150 mm diameter tissue culture plate following a 1 h incubation at 37°C in a humidified 5% CO_2 atmosphere. B lymphocytes were removed by rosetting twice with 2-aminoethyl isothiouronium bromide treated sheep erythrocytes. Rosetted cells were collected and erythrocytes were lysed twice with H_2O. T lymphocytes were washed and resuspended in complete medium. The purity of the T cell preparation was assessed by flow cytometry both for CD2, CD3, CD4 and CD8 expression and for the lack of CD14 and CD20 expression and, finally, by the lack of PHA-induced [^{3}H]thymidine incorporation.

Determination of ectoprotease activity

Cells were extensively washed in PBS and resuspended at 10^7 cells/ml in the same buffer. Ectoprotease activity was determined using the metalloprotease and chymotryptic-type substrate Suc-Ala-Ala-Phe-pNA. Briefly, $1-2\times10^6$ cells were incubated in 200 μl of PBS containing 1 mM of substrate. At different times, proteolytic activity was measured at 412 nm in the presence or absence of exogenously added aminopeptidase N (10 μg/ml). In some experiments, various inhibitors of peptidase and protease activities were preincubated for 10 min at 37°C before determination of the ectoprotease activity.

Flow cytometry

Raji B cells or Jurkat T cells (10^6/ml) were washed twice with RPMI 5% FCS and 1 mM NaN_3, resuspended in 200 μl of the same buffer containing a 1:50 dilution of anti-CALLA monoclonal antibodies and incubated for 60 min at 4°C. After washing twice in PBS, fluoresceinated rabbit anti-mouse antibody was added for 60 min at 4°C. After four more washes in PBS, cells were analysed using an ATC 3000 cytofluorograph (ODAM, Wissembourg, France).

Purification of endopeptidase 24.11 from porcine renal brush border by K14 affinity chromatography

Membrane brush borders were prepared as described by Vannier *et al.* (1976). 20 mg of purified renal brush border were solubilized in 20 mM Tris−HCl, pH 7.5, O.5 M NaCl, 1% (w/v) Triton X-100 for 2 h at 4°C. The 30 000 g supernatant was applied to an immunoadsorbent column, made by coupling 10 mg of purified K14 mAb to 1 g of cyanogen-bromide activated Sepharose CL4B (Sigma, France) and extensively washed with 20 mM Tris−HCl, pH 7.5, 0.5 M NaCl, 0.05% Triton X-100. Endopeptidase 24.11 activity was eluted with 0.1 M $NaHCO_3$/0.5 M NaCl/0.05% Triton X-100, pH 10.4. The active fractions were immediately adjusted to pH 7.5, pooled and dialysed against 10 mM Tris−HCl (pH 7.5), 0.05% Triton X-100. Specific activity was estimated as 2400 nmol/min·mg using Suc-Ala-Ala-Phe-pNA as substrate. When subjected to SDS−PAGE, the enzyme sample revealed a single band of apparent molecular weight 94 kDa.

HPLC analysis

Cells (10^6/ml) or purified NEP were incubated at 37°C with 1 mM Suc-Ala-Ala-Phe-pNA in a final volume of 100 μl PBS, in the presence or absence of aminopeptidase N (10 μg/ml). Inhibition experiments were performed with phosphoramidon (1 μM). At various times, cells were centrifuged and 5 μl of the different supernatants were collected and acidified with 95 μl 0.1 N HCl. Each aliquot (50 μl) was subjected to HPLC under conditions previously described (Barelli *et al.*, 1988). Briefly, elutions were carried out at room temperature by means of a 40 min linear gradient of 0.1% trifluoroacetic acid (TFA), 0.05% triethanolamine (TEA)/0.1% TFA, 0.05% TEA in acetonitrile from 90:10 (v/v) to 60:40 (v/v) at a flow rate of 1 ml/min. Absorbance was monitored at 230 nm.

Immunoprecipitation of endopeptidase from Raji and Jurkat cells

Lymphocyte membranes were obtained following the procedure of Jett *et al.* (1977). 400 μg of solubilized T cell membrane proteins or 20 μg of B cell membrane proteins in PBS/1% Triton X-100 were used to achieve approximately the same final specific activities. Each set of solubilized proteins was incubated with 20 μg/ml of the following mAbs: X35 (anti-CD3), IOL1 (anti-CD45), Ta1 (anti-CD26) or IOT5, IOT5a, Dako CALLA, K14 and K50 (anti-CD10) for 2 h at 25°C in a final volume of 100 μl PBS/1% Triton X-100. Rabbit anti-mouse antibody (200 μg/ml) and protein A−Sepharose (100 μg/ml) were then added for 1 h at 4°C. Beads were extensively washed in PBS/1% Triton X-100. The endopeptidase activity of each protein A pellet and in the corresponding supernatant was measured as described above.

Cells were surface-iodinated by the glucosoxidase-coupled lactoperoxidase technique (Hubbard and Cohn, 1975). Labelled cells (10^8 Jurkat or 10^7 Raji) were washed three times in PBS, pH 7.4 and lysed in 1% Triton X-100 for 60 min at 4°C. Lysates were then centrifuged at 100 000 g and precleared for 30 min at 4°C on rabbit anti-mouse antibody coupled to protein A−Sepharose. Immunoprecipitation by a specific anti-CD10 antibody (100 μg/ml) was performed overnight at 4°C using IOT5. Protein A−Sepharose (100 μg/ml) was then added for the last 30 min. Sepharose beads were washed five times in PBS containing 0.5% Triton X-100 and twice more in PBS. Immunoprecipitates were lysed in sample buffer containing 3% SDS under non-reducing conditions, boiled and analysed by SDS−PAGE (7.5% acrylamide). The gel was dried and applied to 3M film at −80°C.

RT-PCR amplification and Southern blot analysis

Highly purified oligonucleotide primers were synthesized by Appligene. AMV reverse transcriptase (25 U) was used to synthesize (1 h, 42°C) a single stranded cDNA from 1 μg of Jurkat or Raji poly(A)$^+$ RNA in the presence of 10 μg/ml oligo(dT), 1 mM dNTP, 0.5 mM spermidine and 50 U RNase inhibitor in 40 μl of AMV reverse transcriptase buffer. The reaction was stopped by adding 150 μl H_20 and incubated for 10 min at 65°C. 5 μl of each cDNA solution were used to amplify several fragments of CALLA cDNA in the presence of 2.5 U *Taq* polymerase, 1 μM primers and 0.2 mM dNTP in 50 μl of *Taq* polymerase buffer.

PCR products were electrophoresed in 1% agarose gels and blotted onto nitrocellulose filters (Hybond N$^+$, Amersham, France). cDNA probes generated by PCR were purified and labelled by the random prime labelling system (Amersham, France). Prehybridization was performed at 65°C for 15 min and hybridization at 65°C for 16 h in rapid hybridization buffer (Amersham, France) with probes at 10^6 c.p.m./ml. Filters were washed twice in 1×SSC, 0.1% SDS for 10 min at 65°C and twice in 0.1×SSC, 0.1% SDS for 20 min at 65°C. Autoradiography was performed at −80°C using Amersham Hyperfilm.

Northern blot analysis

Total RNA was prepared from Jurkat and Raji cells by acid guanidium thiocyanate−phenol−chloroform extraction (Chomczynski and Sacchi, 1987). 25 μg aliquots were separated on a 1% agarose gel under denaturing conditions and transferred to a nylon membrane (Hybond N$^+$, Amersham, France). The different probes were generated by PCR, purified and labelled by random priming. Hybridization was performed overnight at 65°C and the filters were washed at 65°C following the procedure of Church and Gilbert (1984).

IL-2 production and quantification

Jurkat cells (10^6/ml) were activated with 20 μg/ml PHA or 5 μg coated anti-CD3 mAb in the presence or absence of various concentrations of either thiorphan, phosphoramidon, N-CBX me-Phe-Leu, *o*-phenanthroline or Suc-Ala-Ala-Phe-pNA and incubated for 15 h at 37°C. Supernatants from stimulated cells were distributed in 96-well microtitre plates (3072 Falcon, Oxnard, CA) in 100 μl of fresh RPMI-FCS medium before determining the amount of IL-2 by an enzyme immunoassay as previously described (Ferrua *et al.*, 1987). Briefly, the lymphokine to be assayed was first extracted from supernatants of Jurkat cells using anti-rIL-2 rabbit IgG immobilized on to polystyrene microtitre plates and revealed by an anti-IL2-Fab' fragment conjugated to peroxidase. Peroxidase activity was assayed using 0.1 M phosphate citrate buffer (pH 5.5), hydrogen peroxide (0.02%) and *o*-phenylene diamine (3 mg/ml) (final pH 5.0). The standard used was rIL-2 (Biogen, Geneva, Switzerland). It was checked that protease inhibitors did not interfere with the EIA.

Cytosolic calcium measurement.

Cytoplasmic free calcium levels were determined using the fluorescent dye indo-1 (Grynkiewiz *et al.*, 1985) and an ATC 3000 cytofluorograph (ODAM, Wissembourg, France) as described elsewhere (Auberger *et al.*, 1989b). Briefly, cells were incubated for 1 h with 4 μM indo-1, in a buffer containing 140 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose and 0.1% BSA (pH 7.4) at a final concentration of 5×10^6 cells/ml. After a 5-fold dilution in the same medium, the mean violet:blue fluorescence ratio of 3000 cells was computed every 30 s after the addition of effectors. For inhibition studies, cells were preincubated for 2 min with the different inhibitors before the addition of effectors.

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References

Amoscato,A.A., Alexander,J.W. and Babcock,G.F. (1989) *J. Immunol.*, **142**, 1245–1252.

Auberger,P., Mary,D., Jambou,D., Aussel,C. and Fehlmann,M. (1989) *Cell. Signal.*, **1**, 289–294.

Auberger,P., Mary,D., Breittmayer,J.P., Aussel,C. and Fehlmann,M. (1989) *J. Immunol.*, **142**, 1253–1259.

Barelli,H., Girard,F., St Pierre,S., Kitabji,P., Vincent,J.P. and Checler,F. (1988) *Neurochem. Int.*, **12**, 351–359.

Beaumont,A., Brouet,J.C. and Roques,B.P. (1989) *Biochem. Biophys. Res. Commun.*, **160**, 1323–1328.

Bowes,M.A. and Kenny,A.J. (1987) *Immunology*, **60**, 247–253.

Boyd-Bartlett,Y. and Troll,W. (1983) *Biochem. Biophys. Res. Commun.*, **114**, 600–603.

Chang,T.W. and Eisen,H.N. (1980) *J. Immunol.*, **124**, 1028–1034.

Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.

Church,G. and Gilbert,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.

Connelly,J.C., Skidgel,R.A., Schulz,W.W., Johnson,A.R. and Erdös,E.G. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 8737–8741.

Dang,H.N., Torimoto,Y., Sugita,K., Daley,J.F., Schow,P., Prado,C., Schlossman,S.F. and Morimoto,C. (1990) *J. Immunol.*, **145**, 3963–3971.

Ding-E Young,J., Leong,L.G., Liu,C.C., Damiano,D.A., Wall,D.A. and Cohn,Z.A. (1986) *Cell*, **47**, 183–194.

Ferrua,B., Aussel,C. and Fehlmann,M. (1987) *J. Immunol. Methods*, **97**, 215–219.

Gorvel,J.P., Vivier,I., Naquet,P., Brekelmans,P., Rigal,A. and Pierres,M. (1990) *J. Immunol.*, **144**, 2899–2907.

Grynkiewiz,G., Poenie,M. and Tsien,R.Y. (1985) *J. Biol. Chem.*, **260**, 3440–3450.

Gutowski,J.K., Mukherji,B. and Cohen,S. (1984) *J. Immunol.*, **133**, 3068–3074.

Hegen,M., Niedobitk,C., Eberhard,K., Stein,H. and Fleisher,B. (1990) *J. Immunol.*, **144**, 2908–2914.

Heumann,D. and Vischer,T.L. (1988) *Eur. J. Immunol.*, **18**, 755–760.

Hubbard,A.L. and Cohn,Z.A. (1975) *J. Cell. Biol.*, **64**, 438–460.

Ikuta,T., Okubo,H., Kudo,J., Ishibashi,H. and Inoue,T. (1982) *Biochem. Biophys. Res. Commun.*, **104**, 1509–1516.

Jankovic,B.D., Veljic,J., Pesic,G. and Maric,D. (1991) *Int. J. Neurosci.*, **59**, 45–51.

Jett,M., Seed,T.M. and Jamieson,G.A. (1977) *J. Biol. Chem.*, **252**, 2134–2142.

Kishimoto,T., Kikutani,H., Nishigawa,Y., Sakagushi,N. and Yumamura,Y. (1979) *J. Immunol.*, **123**, 1504–1511.

Kramer,M.D. and Simon,M.M. (1987) *Immunol. Today*, **8**, 140–142.

Ku,G.S.B., Quigley,J.P. and Sultzer,B.M. (1983) *J. Immunol.*, **131**, 2494–2499.

Letarte,M., Vera,S., Tran,R., Aoblies,J.B.L., Onizuka,R.J., Quackenbuoh,E.J., Joongneel,C.V. and MacInnes,R.R. (1988) *J. Exp. Med.*, **168**, 1247–1253.

Lobe,C.G., Finlay,B.B., Pranchych,W., Paetkan,V.H. and Bleacklay,R.C. (1986) *Science*, **232**, 858–861.

Llorens-Cortes,C., Giros,B. and Schwartz,J.C. (1990) *J. Neurochem.*, **55**, 2146–2148.

Malfroy,B., Schofield,P.R., Kuang,W.J., Seeburg,P.H., Mason,A.J. and Henzel,W.J. (1987) *Biochem. Biophys. Res. Commun.*, **144**, 59–66.

Mary,D., Aussel,C. and Fehlmann,M. (1987) *J. Immunol.*, **139**, 1179–1184.

Masson,D., Nabholz,M., Estrade,C. and Tschopp,J. (1986) *EMBO J.*, **5**, 1595–1600.

Morimoto,C., Torimoto,Y., Levinson,G., Rudd,C.E., Schrieber,M., Dang,N.H., Letvin,N.L. and Schlossman,S.F. (1989) *J. Immunol.*, **143**, 3430–3439.

Painter,R.G., Dukes,R., Sullivan,J., Carter,R., Erdös,E.G. and Johnson,A.R. (1988) *J. Biol. Chem.*, **263**, 9456–9461.

Pasternak,M.S., Verret,C.R., Liu,M.A. and Eisen,H.N. (1986) *Nature*, **322**, 743–745.

Petersen,C.M., Ejlersen,E., Moestrup,S.K., Jensen,P.H., Sand,O. and Sottrup-Jensen,L. (1989) *J. Immunol.*, **142**, 629–635.

Roques,B.P. (1985) *J. Pharmacol.*, **16**, 5–11.

Roques,B.P., Fournié-Zaluski,M.C., Soroca,E., Lecomte,J.M., Malfroy,B., Llorens,C. and Schwartz,J.C. (1980) *Nature*, **288**, 286–288.

Roques,B.P., Lucas-Soroca,E., Chaillet,P., Costentin,J. and Fournié-Zaluski,M.C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3178–3182.

Schön,E.J., Sigbert,J., Kiessig,S.T., Demuth,H.R., Neubert,K., Barth,A., Von Baehr,R. and Ansorge,S. (1987) *Eur. J. Immunol.*, **17**, 1821–1826.

Schreiner,G.F. and Unanue,E.R. (1976) *Adv. Immunol.*, **24**, 37–165.

Shipp,M.A., Vijayaraghavan,J., Schmidt,E.V., Masteller,E.A., D'Adamio,D.A., Hers,L.B. and Reinhertz,E.L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 297–301.

Utsunomiya,N. and Nakanishi,M. (1986) *J. Biol. Chem.*, **261**, 16514–16517.

Vannier,C., Louvard,D., Maroux,S. and Desnuelle,P. (1976) *Biochim. Biophys. Acta*, **455**, 185–199.

Vivier,I., Marguet,D., Naquet,P., Bonicel,J., Black,D., Xiao-Yan-Li,C., Bernard,A.M., Gorvel,J.P. and Pierres,M. (1991) *J. Immunol.*, **147**, 447–454.

Wong,R.L., Gutowski,J.K., Katz,M., Goldfarb,R.H. and Cohen,S. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 241–245.

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