

## Common Acute Lymphoblastic Leukemia Antigen Expressed on Leukemia and Melanoma Cell Lines Has Neutral Endopeptidase Activity

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### Abstract

We have previously reported that the amino acid sequence of the common acute lymphoblastic leukemia antigen (CALLA, CD10) translated from a normal human kidney cDNA clone is identical to that of neutral endopeptidase (NEP, EC 3.4.24.11). In this study, we show that by flow cytometry, a monoclonal antibody (135A3) produced against rabbit NEP reacted selectively with leukemia and melanoma cell lines expressing CALLA on their surface. A glycoprotein of apparent  $M_r$  100,000 was immunoprecipitated from surface labeled NALM-1 leukemia or Mel-1477 melanoma cells with monoclonal antibodies to NEP (135A3) or CALLA (44C10). mRNAs hybridizing to a NEP-specific probe were present in CALLA<sup>+</sup> leukemia and melanoma cell lines, but absent from CALLA<sup>-</sup> lines. NEP enzymatic activity was detected on intact cells from CALLA<sup>+</sup> lines, but not CALLA<sup>-</sup> lines. The activity was blocked by two selective inhibitors of NEP, thiorphan and phosphoramidon. CALLA antigen purified from the NALM-6 leukemic cell line by affinity to 44C10-IgG Sepharose retained a peptidase activity that was completely blocked by thiorphan and phosphoramidon. Thus the CALLA antigen present at the surface of leukemia and melanoma cell lines is an enzymatically active neutral endopeptidase.

### Introduction

The common acute lymphoblastic leukemia antigen (CALLA; CD10)<sup>1</sup> is a membrane glycoprotein of  $M_r$  95,000–100,000 (1–6) that was first identified on lymphoblasts of patients with the common (pre-B) type of acute lymphoblastic leukemia (ALL) (1, 2, 7, 8). While CALLA is not expressed on normal T

and B lymphocytes and on monocytes, it is found on lymphoid precursors in fetal liver and bone marrow, and on normal granulocytes (9–11). It is also present on several nonhemopoietic tissues: in kidney, on the brush border of proximal tubules and on glomerular epithelium (1, 3, 12, 13); in adult breast, on myoepithelial cells (12); and in fetal small intestine, on the microvillar epithelium (12).

We have recently isolated a cDNA clone coding for CALLA from a human kidney library (14). The cDNA sequence can be translated into a protein of 749 amino acids, whose sequence is identical to that reported for CALLA from the NALM-6 leukemic cell line (15), and to that of the recently cloned human neutral endopeptidase (NEP, EC 3.4.24.11) (14, 16). This enzyme, initially purified from rabbit renal brush border (17, 18), is a membrane-bound zinc-containing metalloproteinase with a specificity related to that of thermolysin (17–21). The presence of NEP has been detected by its enzymatic activity and/or with antibodies in several normal tissues (including brain) in rat, rabbit, pig, and man (16–30). NEP has its active site exposed to the extracellular environment, and is capable of hydrolyzing a number of peptides. NEP activity can be distinguished from that of other peptidases by its selective inhibition by the synthetic peptides thiorphan and phosphoramidon (17–19, 23, 27–29). Known natural substrates of NEP include angiotensins, enkephalins, atrial natriuretic peptide, substance P, chemotactic peptide, and several other biologically active peptides (17–19, 28, 29, 31).

NEP activity has never been reported in malignant cells. On the other hand, CALLA is found on lymphoblastic leukemias, on certain lymphomas, and on melanomas and glioma cell lines (1, 5, 7, 8, 32). We show in the current study that CALLA expressed on malignant cells expresses antigenic determinants of neutral endopeptidase and is enzymatically active.

### Methods

**Cell lines.** The pre-B ALL cell lines NALM-1 (5) and NALM-6 (33), the T-ALL cell lines Jurkat and HSB-2 (34), the Burkitt's lymphoma line Daudi (5), and the melanoma lines Mel-1477, LSD22 (35), and A375 (34) were grown in RPMI 1640 medium supplemented with 10% FCS.

**Monoclonal antibodies.** MA b 44C10 (IgG2b,  $\kappa$ ), 44G4 (IgG1,  $\kappa$ ), and 44E3 (IgG1,  $\kappa$ ) were produced by immunization of Balb/c mice with the pre-B ALL cell line HOON; 44C10 was shown to react with CALLA antigen (36), while the two others were used as negative controls. MA b 135A3 (IgG2b) was derived by immunization of Balb/c mice with tubular cells from rabbit renal cortex as described for pre-

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1. *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; CALLA, common acute lymphoblastic leukemia antigen; DAGNPG, dansyl-D-Ala-Gly-Phe(pNO<sub>2</sub>)-Gly; NEP, neutral endopeptidase.

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vious MAb (25). IgG fractions were purified using standard procedures (23, 36). For competitive antibody binding studies (37), 44C10 IgG was radiolabeled with Na<sup>125</sup>I using Iodogen (Pierce Chemical Co., Rockford, IL).

**Cell surface-labeling and immunoprecipitation.** Cells from the NALM-1 or Mel-1477 lines ( $1 \times 10^7$ ) were labeled with Na<sup>125</sup>I using the lactoperoxidase/glucose oxidase-catalyzed reaction, and surface antigens were immunoprecipitated from a 1% Genapol X-080 (Calbiochem, Lucerne, Switzerland) extract as described earlier (38).

**Purification of CALLA antigen from NALM-6 cells.** NALM-6 cells ( $3 \times 10^9$ ) were harvested, washed three times with PBS and solubilized at  $2 \times 10^7$  cells/ml in 0.05 M Tris, pH 7.0, 0.15 M NaCl, 0.5% Triton X-100, 1 mM PMSF for 2 h at 4°C. The soluble extract (100,000 g) was incubated with 5 ml of control nonimmune murine IgG-Sepharose beads (2 mg IgG/ml) for 16 h, and with 7 ml of 44C10 IgG-Sepharose beads for 24 h at 4°C. The beads were washed, and the antigen was eluted with 0.05 M diethylamine, pH 11.2, 0.3% deoxycholate. The antigen preparation and the beads were immediately neutralized with 0.5 M Tris, pH 6.8. The 44C10 antigen preparation was concentrated by ultrafiltration and frozen in aliquots at -70°C.

**RNA preparation and Northern blot analysis.** Total RNA was isolated from freshly harvested cells by the acid guanidinium thiocyanate-phenolchloroform extraction procedure (39). Northern blots were probed with a 1.6 kb Eco RI fragment of kidney-derived CALLA cDNA, which we know codes for NEP (14).

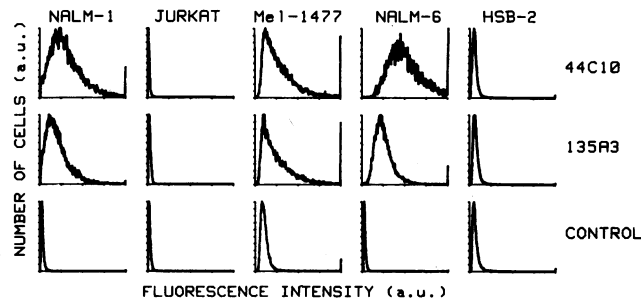
**Fluorimetric assay of NEP activity.** Assays were performed essentially as described (40), using the synthetic substrate dansyl-D-Ala-Gly-Phe(pNO<sub>2</sub>)-Gly (DAGNPG; Sigma Chemical Co., St Louis, MO). Cells were washed three times in PBS, once in Pipes buffer (50 mM Pipes, pH 6.4, 150 mM NaCl), and resuspended in Pipes buffer at  $4 \times 10^7$  ml<sup>-1</sup>. For each assay,  $2 \times 10^6$  cells were incubated in a 0.1-ml volume with 50 μM substrate in Pipes buffer at 37°C. The reaction was stopped by adding 0.4 ml of ice-cold Pipes buffer containing 5 mM EDTA, the cells were pelleted for 5 min at 10,000 g, and the fluorescence of the supernatant was measured in a (model LS-5; Perkin-Elmer Co., Norwalk, CT) luminescence spectrometer, using an excitation slit width of 5 nm at a wavelength of 342 nm and an emission slit of 10 nm at 562 nm.

To measure the activity of the purified CALLA preparation, the buffer was supplemented with 0.1% Genapol X-080 and with 50 μM Zn acetate as indicated. At increasing reaction times (0–120 min), 0.1-ml aliquots were quenched in ice-cold Pipes buffer containing 5 mM EDTA, centrifuged to remove the fine precipitate formed in the reaction, and processed as above. A doubling in the initial fluorescence intensity corresponds to the hydrolysis of 20% of the substrate (40).

## Results

**Reactivity of a monoclonal antibody to NEP with CALLA-positive tumor cells.** In a recent paper, we demonstrated that the sequence of a CALLA cDNA clone is identical to that coding for human neutral endopeptidase (14). The sequences of human, rabbit and rat NEP are 95% identical (14, 16, 20, 22). Therefore, we investigated the reactivity of CALLA<sup>+</sup> cells with MAb 135A3 (26), an antibody directed against rabbit NEP, which cross-reacts with human NEP.

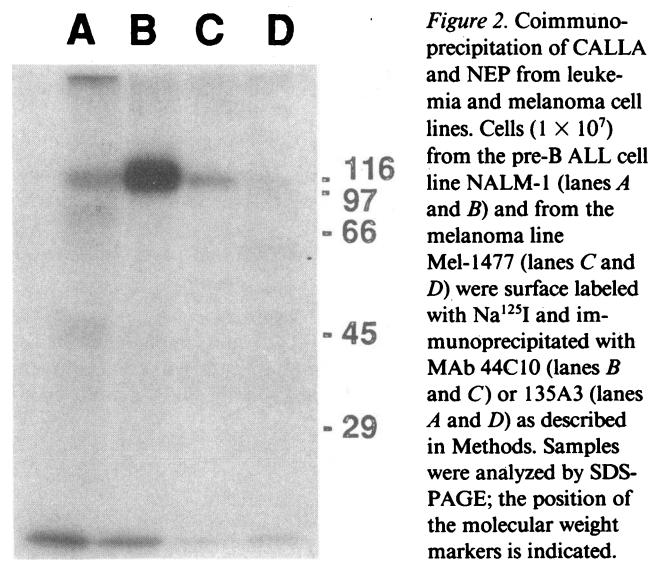
The fluorescence histograms shown in Fig. 1 demonstrate that MAb 135A3 reacted with two CALLA<sup>+</sup> pre-B ALL cell lines, NALM-1 and NALM-6, but not with two CALLA<sup>-</sup>T-ALL cell lines, Jurkat and HSB-2. The CALLA<sup>+</sup> melanoma cell line Mel-1477 was also stained by MAb 135A3, but to a lesser degree than the pre-B leukemic lines. The LSD22 and Daudi cell lines expressed levels of CALLA in the same range as the pre-B ALL cell lines, while the melanoma line A375 was CALLA<sup>-</sup> (data not shown).



**Figure 1.** Reactivity of anti-NEP antibody with CALLA<sup>+</sup> cells assessed by flow cytometry. Cells ( $5-10 \times 10^5$ ) from the NALM-1, NALM-6, Jurkat, HSB-2, and Mel-1477 lines were incubated with saturating levels of MAb 44C10, 135A3 or control IgG and with FITC-conjugated goat anti-mouse IgG. After three washings in PBS, the cells were analyzed by flow microfluorometry with a FACS II (Becton Dickinson, Mountain View, CA).

**Coimmunoprecipitation of NEP and CALLA from leukemia and melanoma cell lines.** Both 135A3 (anti-NEP) and 44C10 (anti-CALLA) MAb precipitated a major polypeptide band of apparent  $M_r$  100,000 from surface radiolabeled NALM-1 or Mel-1477 cell lines (Fig. 2). This is in agreement with previous data indicating that both CALLA and NEP are glycoproteins with  $M_r$  90,000–100,000 (1, 2, 4–6, 10–12, 17–19, 24–26, 30, 32, 36). The amount of antigen reactive with 44C10 MAb was higher in the NALM-1 line than in the Mel-1477 line, consistent with the data shown in Fig. 1. The amount of antigen precipitated by the anti-NEP antibody was less than that observed with anti-CALLA, and contaminating bands were also seen (Fig. 2). This difference is consistent with the lower intensity of staining, even at saturating concentrations, observed with 135A3 MAb compared to 44C10 MAb (Fig. 1), and is probably due to differences in the avidity of the two MAb towards related epitopes, as indicated by competitive antibody binding studies (data not shown).

**Detection of NEP mRNA in lymphoid and melanoma cell lines expressing CALLA on their surface.** We probed Northern blots of RNA prepared from various tumor cell lines with a



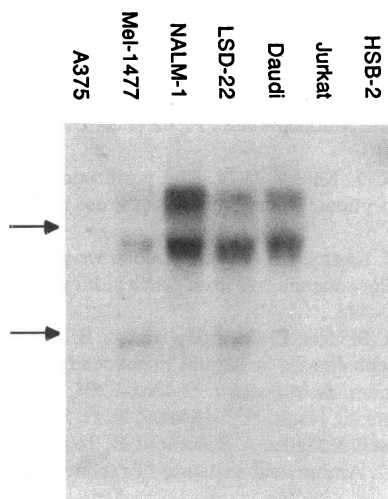
**Figure 2.** Coimmunoprecipitation of CALLA and NEP from leukemia and melanoma cell lines. Cells ( $1 \times 10^7$ ) from the pre-B ALL cell line NALM-1 (lanes A and B) and from the melanoma line Mel-1477 (lanes C and D) were surface labeled with Na<sup>125</sup>I and immunoprecipitated with MAb 44C10 (lanes B and C) or 135A3 (lanes A and D) as described in Methods. Samples were analyzed by SDS-PAGE; the position of the molecular weight markers is indicated.

NEP cDNA fragment. Cells from the NALM-1, LSD22 and Daudi lines, which are all CALLA<sup>+</sup>, contained major mRNA species of 6 and 3.8 kb (Fig. 3), in agreement with values reported previously for NALM-6 and other cell types (14, 15). The Mel-1477 line, which expressed lower levels of CALLA on its surface (Figs. 1 and 2), also contained less NEP-specific mRNA (Fig. 3). The RNAs prepared from the CALLA<sup>-</sup> lines Jurkat, HSB-2 and A375 did not react with the NEP cDNA probe. In the two CALLA<sup>+</sup> melanoma lines, a minor RNA species of 1.8 kb was more abundant than in the other cell types.

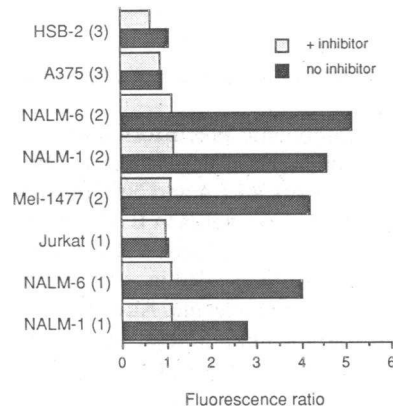
**Demonstration of specific NEP activity in suspensions of CALLA-positive cells and in purified CALLA antigen preparations.** We tested lymphoid and melanoma tumor cell suspensions for their ability to hydrolyze the fluorogenic peptide, DAGNPG, a selective substrate for NEP (40). Fig. 4 illustrates that all cell lines expressing CALLA were able to hydrolyze this substrate. The activity could be blocked by more than 90% by thiorphan or phosphoramidon, two specific inhibitors of NEP. The CALLA<sup>-</sup> lines (Jurkat, A375) had no detectable NEP activity. Thus NEP activity correlates with expression of CALLA at the cell surface.

CALLA antigen purified ~ 200-fold from NALM-6 cells by MAb immunoadsorption was tested for NEP activity. No hydrolysis of the substrate could be detected in the initial experiments. Since 0.05 M diethylamine at pH 11.2 had been used for elution of the antigen from the immunoadsorbent, we suspected that demetalization of the enzyme might have occurred. Addition of 50  $\mu$ M Zn<sup>2+</sup> did indeed restore enzymatic activity to the preparation. This effect was seen at Zn<sup>2+</sup> concentrations between 10 and 500  $\mu$ M (data not shown).

The activity of the purified CALLA preparation (at ~ 4  $\mu$ g/ml protein, the  $V_i$  was 250 nM min<sup>-1</sup>) was inhibited more than 20-fold by phosphoramidon and thiorphan at concentrations as low as 1  $\mu$ M. The specific activity of the preparation (0.06  $\mu$ mol  $\cdot$  min<sup>-1</sup>  $\cdot$  mg<sup>-1</sup>) was ~ 10-fold less than that reported for purified NEP (40). Since we know that CALLA purified by this rapid affinity procedure can be enriched another 10-fold by preparative gel electrophoresis, we can conclude that the specific activity of CALLA extracted from a leukemic cell line is of the same order of magnitude as that of NEP extracted from normal kidney.



**Figure 3.** Expression of NEP mRNA in leukemic and melanoma cells. Total cell RNA (10  $\mu$ g per lane) was separated in a 1.4% denaturing agarose gel, transferred to a nylon membrane, and probed with a NEP-specific DNA fragment. The arrows indicate the positions of the 18 S and 28 S ribosomal RNA bands detected by ethidium bromide staining.



**Figure 4.** NEP activity in intact pre-B and melanoma cell lines. In three separate experiments (indicated in brackets), cells ( $2 \times 10^6$ ) were incubated with DAGNPG as described in Methods. Fluorescence ratio is sample/control; in the control, the reaction was stopped immediately after mixing cells and substrate. The inhibitor was 10  $\mu$ M thiorphan (Exp. 1) or 5  $\mu$ M phosphoramidon (Exps. 2 and 3). Incubation times were 80 min (Exp. 1) or 120 min (Exps. 2 and 3).

## Discussion

CALLA<sup>+</sup> cell lines reacted with a MAb produced against rabbit kidney and shown to be specific for neutral endopeptidase. The presence of NEP correlated with the presence of CALLA as determined by flow cytometry (Fig. 1). Coimmunoprecipitation of NEP and CALLA was observed for both a pre-B ALL cell line and a melanoma line (Fig. 2).

The CALLA/NEP antigens precipitated from the pre-B ALL cell line NALM-1 and from the melanoma cell line Mel-1477 had the same apparent  $M_r$  (100,000), which corresponds to that reported previously for CALLA precipitated from other pre-B or melanoma cell lines, or from granulocytes and fibroblasts (4-7, 11-13, 30). The identity of CALLA and NEP is further substantiated by the presence in CALLA<sup>+</sup> leukemia and melanoma cell lines of mRNA species reactive with a probe specific for human NEP (14, 16). These mRNA species were absent from the two CALLA<sup>-</sup> T-ALL cell lines and from the A375 CALLA<sup>-</sup> melanoma line (Fig. 3). The 6.0 and 3.8 kb mRNA species have been reported previously in rat brain and kidney (22). They are also present in CALLA<sup>+</sup> human lymphoblastic (14, 15) and non-lymphoblastic lines including fibroblasts (14, 15) and a colorectal carcinoma line (15), but absent from CALLA<sup>-</sup> lines (15). A third mRNA species (~1.8 kb) was more abundant in the melanoma lines than in the leukemic lines tested (Fig. 3). It is unclear whether this has any functional significance.

It is of particular interest that CALLA expressed on the surface of malignant cells has neutral endopeptidase activity. Intact cells hydrolyzed the selective substrate DAGNPG in a reaction that was entirely inhibited by specific inhibitors of NEP. Moreover, in the presence of zinc, CALLA purified by immunoadsorption had a specific activity similar to that of kidney-derived NEP.

NEP is capable of inactivating a wide range of bioactive peptides (17-19, 28, 29, 31). In the nervous system, it plays a central role in the metabolism of neuropeptides (31). It was recently demonstrated that purified NEP can inactivate the  $\beta$  form of IL-1, a pleiotropic inflammatory mediator (41). The presence of NEP on reticular cells of pig thymus and lymph

nodes (42, 43) as well as on human granulocytes (6, 29) suggests a role for the enzyme in the regulation of inflammatory responses.

The demonstration of an enzymatic activity for CALLA raises several fundamental questions: (a) does the presence of the enzyme at early stages of B cell differentiation imply a function in normal development? (b) does the enzyme play a role in malignancy? (c) does the presence of the enzyme on malignant cells influence the physiological state of cancer patients?

CALLA is present on hemopoietic precursors found in bone marrow and fetal liver, but not on mature B cells (9, 44). The demonstration of the identity of CALLA and NEP implies that this enzyme is associated with early stages in B cell ontogeny (9, 44). This might be linked to the production of a specific peptide whose expression is transiently needed in maturation, possibly by hydrolysis of a precursor molecule. Alternatively, the appearance of the enzyme on hemopoietic precursors might cause the inactivation of a regulatory peptide and facilitate a differentiation event.

NEP is a member of a large family of zinc-dependent endopeptidases, all of which share a common metal-binding pocket (44a). Other members of this family include the collagenases, whose increased expression is associated with a malignant phenotype (45, 46). CALLA is present on malignant cells of diverse origins: common ALL (1, 2, 7, 8), Burkitt's and other poorly differentiated lymphomas (8, 47), and tumors of neuroectodermal origin such as melanomas (5) and gliomas (32). It has been demonstrated that ALL cells express CALLA at higher levels than CALLA<sup>+</sup> B cell precursors isolated from normal bone marrow (44). Examination of brain sections from glioma patients (Schreyer, M., unpublished results) suggests that CALLA is found at a higher level in the tumor than in the surrounding normal tissue. Future studies should establish if an increased expression of CALLA/NEP is associated with other tumors, and if this expression has an effect on malignancy.

Many patients with common ALL have elevated amounts of CALLA in their serum (48, 49). A reduction of these levels is seen during remission (48). The presence of circulating CALLA, added to the large number of CALLA<sup>+</sup> lymphoblasts, would probably result in high levels of NEP activity in these patients. Since many regulatory peptides can be inactivated by NEP, this could lead to perturbations of their neuroendocrine balance. The potential inactivation of other substrates, such as IL-1, could also have systemic effects.

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