COMMON ACUTE LYMPHOCYTIC LEUKEMIA ANTIGEN IS IDENTICAL TO NEUTRAL ENDOPEPTIDASE

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Common acute lymphocytic leukemia antigen (CALLA[1]; CD10) is an important cell surface marker in the diagnosis of human acute lymphocytic leukemia (ALL) (references 1–8). It is present on leukemic cells of pre-B phenotype, which represent 85% of cases of ALL, and is absent from normal PBMC. However, CALLA is not restricted to leukemic cells and is found on a variety of normal tissues (9–14). It is particularly abundant in kidney, where it is present on the brush border of proximal tubules and on glomerular epithelium (2, 9, 10). CALLA is a glycoprotein of M_r 9.4 × 10^4–10^5 (4–11, 14–19) for which no functional activity has yet been described. We report on the cloning of a cDNA coding for CALLA, and show that the amino acid sequence deduced from the cDNA sequence is identical to that of human membrane-associated neutral endopeptidase (NEP; EC 3.4.24.11), also known as enkephalinase. NEP cleaves peptides at the amino side of hydrophobic residues (21, 22) and inactivates several peptide hormones including glucagon, enkephalins, substance P, neurotensin, oxytocin, bradykinin, and the chemotactic peptide fMLF (20–24).

Materials and Methods

Purification and Partial Sequencing of CALLA. The production and purification of the 44C10 IgG2b mAb have been described previously (8). CALLA was purified from human kidney cortex by Triton X-100 solubilization, and sequential affinity chromatography with nonimmune mouse IgG-Sepharose and monoclonal 44C10 IgG-Sepharose. The antigen was eluted with 0.05 M diethylamine, pH 11.2, 0.1% Triton X-100, and was neutralized immediately; recovery of antigen was estimated by inhibition of a cellular RIA (8, 11). The antigenic preparation was fractionated by SDS-PAGE, and the M_r 9.4 × 10^4 protein band was eluted electrophoretically. CNBr fragments were generated, fractionated by reversed-phase HPLC (25), and analyzed in a gas-phase sequencer.

Isolation and Sequence Analysis of a CALLA cDNA Clone. Mixed oligonucleotide probes corresponding to the sequences of peptides 1 and 2 (Fig. 1) were used to screen an oligo-dT-primed human kidney cDNA library inserted in the λ gt10 vector; the library was the kind

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[1] *Abbreviations used in this paper:* ALL, acute lymphocytic leukemia; CALLA, common acute lymphocytic leukemia antigen; NEP, neutral endopeptidase.

gift of Dr. Graeme Bell, Chiron Corp., Emeryville, CA. One clone was selected that hybridized to both probes; two Eco RI fragments (0.7 and 1.6 kb) of this primary cDNA clone were subcloned into the Bluescript vector (Stratagene, San Diego, CA), and sequenced on both strands using the dideoxy chain terminator method. The sequences obtained were compared with all sequences in the GenBank database (version 56, March 1988), using the algorithm of Lipman and Pearson (26). The optimal alignment with rat NEP found by the computer program did not contain any gaps, and was used as the basis for the protein sequence comparison shown in Fig. 1.

Northern Blot Analysis. Total RNA was prepared from cultured cells by acid guanidinium thiocyanate-phenol-choroform extraction (27). 20-μg aliquots were separated on a 1.2% agarose gel under denaturing conditions and transferred to a nylon membrane (Hybond-N; Amersham Corp., Arlington Heights, IL). Hybridization with the 1.6-kb cDNA fragment labeled by random priming was performed in 50% formamide, 5× SSC, at 42°C, and the filter was washed in 0.1× SSC, 0.5% SDS at 55°C.

Results and Discussion

CALLA was purified from kidney extracts by immunoaffinity chromatography using mAb 44C10, produced by immunisation with the CALLA-positive leukemic cell line HOON (8), and reactive with the same epitope as other widely used anti-CALLA mAb (2–8). The M_r of the antigens immunoprecipitated with mAb 44C10 were estimated at 9.4×10^4 (kidney) and 10^5 (HOON); after Endo F digestion, both polypeptides had an apparent M_r of 8×10^4. The CALLA glycoprotein was purified on average 200–300-fold relative to the crude kidney extract. From a whole kidney cortex, 6 mg of protein representing 40–50% of the CALLA antigenic activity were recovered after immunoaffinity chromatography. The protein, purified an additional 12-fold by electrophoretic elution from polyacrylamide gels, migrated as a single band; M_r, 9.4×10^4. The sequences of CNBr peptides 1 and 2 (Fig. 1) were determined for two independent preparations, with identical results.

A cDNA clone was isolated from a human kidney library using a mixed oligonucleotide probe corresponding to the sequence of peptide 2 (MNPEKK). This clone (~3.4 kb) also hybridized with a mixed oligonucleotide probe derived from a partial sequence of peptide 1 (MVIGHE). We determined the nucleotide sequence of two Eco RI fragments (0.7 and 1.6 kb) of this cDNA, and compared these sequences with all entries in the GenBank nucleotide sequence database. Both fragments showed a high degree of similarity (86% and 91%, respectively) with the sequence of a cDNA coding for the rat NEP (28). The 0.7-kb fragment corresponds to the 5′ untranslated and NH_2-terminal coding regions, while the 1.6-kb fragment covers two thirds of the coding region.

Examination of more recent entries into the data banks revealed that both rabbit (29) and human (20) NEP had also been cloned and sequenced. Compared with the human NEP cDNA, the CALLA cDNA sequence was missing a 60-bp Eco RI fragment between the two sequenced regions, and 39 bases of coding sequence before the termination codon. Translation of the CALLA cDNA into an amino acid sequence confirmed that the protein is identical to human NEP and 95% identical to rat or rabbit NEP (20, 21). Peptide 2, not found in the sequenced portion of the CALLA cDNA, is identical to residues 741–746 of human and rabbit NEP.

The 5′ end of the CALLA cDNA clone extends 143 nucleotides further than the human NEP cDNA clone and contains a stop codon located 6 bp 5′ of the first AUG codon, as seen for rat and rabbit NEP (28, 29). The main structural features of NEP

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          1                                                                                    80
NEP Rt    GrSESQMDIT DINaPKPKKK QRWTPLEISL SVLVLLLTiI AVTMIALYAT YDDGICKSSD CIKSAARLIQ NMDAsaEPCT
NEP Rb    GrSESQMDIT DINtPKPKKK QRWTPLEISL SVLVLLLTvI AVTMIALYAT YDDGICKSSD CIKSAARLIQ NMDAtaEPCT
CALLA     GkSESQMDIT DINtPKPKKK QRWTPLEISL SVLVLLLTiI AVTMIALYAT YDDGICKSSD CIKSAARLIQ NMDAttEPCT
NEP Hu    .kSESQMDIT DINtPKPKKK QRWTPLEISL SVLVLLLTiI AVTMIALYAT YDDGICKSSD CIKSAARLIQ NMDAttEPCT
                         |--ST---|     |-----Transmembrane-----|

          81                                                                                  160
NEP Rt    DFFKYACGGW LKRNVIPETS SRYsNFDILR DELEViLKDV LQEPKTEDIV AVQKAKtLYR SCiNEsAIDS RGGqPLLtLL
NEP Rb    DFFKYACGGW LKRNVIPETS SRYsNFDILR DELEViLKDV LQEPKTEDIV AVQKAKtLYR SCvNEtAIDS RGGqPLLkLL
CALLA     DFFKYACGGW LKRNVIPETS SRYgNFDILR DELEVvLKDV LQEPKTEDIV AVQKAKaLYR SCiNEsAIDS RGGePLLkLL
NEP Hu    DFFKYACGGW LKRNVIPETS SRYgNFDILR DELEVvLKDV LQEPKTEDIV AVQKAKaLYR SCiNEsAIDS RGGePLLkLL
                                                                               ***

          161                                                                                 240
NEP Rt    PDiYGWPVAs qNWEQtYGtS WtAEKsIAQL NSkYGKKVLI NFFVGTDDKN StqHiIHfDQ PRLGLPSRDY YECTGIYKEA
NEP Rb    PDvYGWPVAt qNWEQtYGtS WsAEKsIAQL NSnYGKKVLI NFFVGTDDKN SmnHiIHiDQ PRLGLPSRDY YECTGIYKEA
CALLA     PDiYGWPVAt eNWEQkYGaS WtAEKaIAQL .......... .........N SvnHvIHiDQ PRLGLPSRDY YECTGIYKEA
NEP Hu    PDiYGWPVAt eNWEQkYGaS WtAEKaIAQL NSkYGKKVLI NLFVGTDDKN SvnHvIHiDQ PRLGLPSRDY YECTGIYKEA

          241                                                                                 320
NEP Rt    CTAYVDFMIs VArLIRQEqr LPIDENQlsl EMNKVMELEK EIANATtKpE DRNDPMLLYN KMtLAklQNN FSLEINGKPF
NEP Rb    CTAYVDFMIa VAkLIRQEeg LPIDENQisv EMNKVMELEK EIANATtKsE DRNDPMLLYN KMtLAqiQNN FSLEINGKPF
CALLA     CTAYVDFMIs VArLIRQEer LPIDENQlal EMNKVMELEK EIANATaKpE DRNDPMLLYN KMtLAqiQNN FSLEINGKPF
NEP Hu    CTAYVDFMIs VArLIRQEer LPIDENQlal EMNKVMELEK EIANATaKpE DRNDPMLLYN KMpLAqiQNN FSLEINGKPF
                                                         ***                         * **

          321                                                                                 400
NEP Rt    SWsNFTNEIM STVNInIqNE EeVVVYAPEY LtKLKPILTK YspRDLQNLm SWRFIMDLVS SLSRnYKeSR NAFRKALYGT
NEP Rb    SWsNFTNEIM STVNInIpNE EdVVVYAPEY LiKLKPILTK YfpRDFQNLf SWRFIMDLVS SLSRtYKdSR NAFRKALYGT
CALLA     SWlNFTNEIM STVNIsItNE EdVVVYAPEY LtKLKPILTK YsaRDLQNLm SWRFIMDLVS SLSRtYKeSR NAFRKALYGT
NEP Hu    SWlNFTNEIM STVNIsItNE EdVVVYAPEY LtKLKPILTK YsaRDLQNLm SWRFIMDLVS SLSRtYKeSR NAFRKALYGT
             ***        ***

          401                                                                                 480
NEP Rt    TSEtATWRRC ANYVNGNMEN AVGRLYVEAA FAGESKHVVE DLIAQIREVF IQTLDDLTWM DAETKKkAEE KALAIKERIG
NEP Rb    TSEsATWRRC ANYVNGNMEN AVGRLYVEAA FAGESKHVVE DLIAQIREVF IQTLDDLTWM DAETKKkAEE KALAIKERIG
CALLA     TSEtATWRRC ANYVNGNMEN AVGRLYVEAA FAGESKHVVE DLIAQIREVF IQTLDDLTWM DAETKKrAEE KALAIKERIG
NEP Hu    TSEtATWRRC ANYVNGNMEN AVGRLYVEAA FAGESKHVVE DLIAQIREVF IQTLDDLTWM DAETKKrAEE KALAIKERIG

          481                                                                                 560
NEP Rt    YPDDIiSNeN KLNNEYLELN YKEeEYFENI IQNLKFSQSK QLKKLREKVD KDEWIsGAAv VNAFYSSGRN QIVFPAGILQ
NEP Rb    YPDDIvSNdN KLNNEYLELN YKEdEYFENI IQNLKFSQSK QLKKLREKVD KDEWItGAAi VNAFYSSGRN QIVFPAGILQ
CALLA     YPDDIvSNdN KLNNEYLELN YKEdEYFENI IQNLKFSQSK QLKKLREKVD KDEWIsGAAv VNAFYSSGRN QIVFPAGILQ
NEP Hu    YPDDIvSNdN KLNNEYLELN YKEdEYFENI IQNLKFSQSK QLKKLREKVD KDEWIsGAAv VNAFYSSGRN QIVFPAGILQ

          561                                                                                 640
NEP Rt    PPFFSArQSN SLNYGGIGMV IGHEITHGFD DNGRNFNKDG DLVDWWTQQS AnNFKdQSQC MVYQYGNFtW DLAGGQHLNG
NEP Rb    PPFFSAqQSN SLNYGGIGMV IGHEITHGFD DNGRNFNKDG DLVDWWTQQS AnNFKeQSQC MVYQYGNFsW DLAGGQHLNG
CALLA     PPFFSAqQSN SLNYGGIGMV IGHEITHGFD DNGRNFNKDG DLVDWWTQQS AsNFKeQSQC MVYQYGNFsW DLAGGQHLNG
NEP Hu    PPFFSAqQSN SLNYGGIGMV IGHEITHGFD DNGRNFNKDG DLVDWWTQQS AsNFKeQSQC MVYQYGNFsW DLAGGQHLNG
                             |---------1-----------|                              ***
                                  |--Zn-|

          641                                                                                 720
NEP Rt    INTLGENIAD NGGiGQAYRA YQNYvKKNGE EKLLPGlDLN HKQLFFLNFA QVWCGTYRPE YAVNSIKTDV HSPGNFRIIG
NEP Rb    INTLGENIAD NGGiGQAYRA YQNYvKKNGE EKLLPGiDLN HKQLFFLNFA QVWCGTYRPE YAVNSIKTDV HSPGNFRIIG
CALLA     INTLGENIAD NGGlGQAYRA YQNYiKKNGE EKLLPGlDLN HKQLFFLNFA QVWCGTYRPE YAVNSIKTDV HSPGNFRIIG
NEP Hu    INTLGENIAD NGGlGQAYRA YQNYiKKNGE EKLLPGlDLN HKQLFFLNFA QVWCGTYRPE YAVNSIKTDV HSPGNFRIIG

          721                          749
NEP Rt    tLQNSaEFad AFhCrKNSYM NPErKCRVW
NEP Rb    sLQNSvEFse AFqCpKNSYM NPEkKCRVW
CALLA     tLQNSaEFse AFhCrK.... .........
NEP Hu    tLQNSaEFse AFhCrKNSYM NPEkKCRVW
                              |--2--|
```

FIGURE 1. Protein sequences of rat (*Rt*), rabbit (*Rb*), and human (*Hu*) NEP, and of the CALLA antigen. Small letters indicate positions where variations in sequence are observed between species. (· · ·) Positions in the CALLA sequence still missing from the cDNA sequence. The following features are indicated below the sequences: the stop transfer (*ST*) and transmembrane fragments that give the protein its polarity (cytoplasmic NH_2 terminus), the potential *N*-linked glycosylation sites found in the human sequence (***), and the consensus zinc-binding sequence (*Zn*). The sequences of the two CALLA peptides (1 and 2) used to design the mixed oligonucleotide probes are also marked. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00811.

are conserved between species: the transmembrane domain, the stop transfer sequence, the positions of the cysteine residues, the potential glycosylation sites (five out of six), and the pentapeptide consensus sequence (H-E-[I, L, M]-x-H) of zinc-binding metalloproteases (30, and Bouvier, J., A. Bairoch, and C. V. Jongeneel, submitted for publication; see Fig. 1).

We probed Northern blots of RNA extracted from two leukemic cell lines, HOON and NALM-6, and from human and mouse fibroblasts, with the 1.6-kb CALLA cDNA fragment (Fig. 2). The amounts of CALLA mRNA correlated with the amounts of CALLA detected at the cell surface by flow cytometry. The probe hybridized to two RNA species of 3.8 and 6.6 kb. These sizes correspond to those described for the rat NEP mRNAs (28). In RNA extracted from NALM-6 cells, we could detect one additional minor species of 5.0 kb. The same three mRNA species were observed in human kidney, at a higher abundance than in NALM-6; however, they were absent from RNA extracted from the T leukemia line Jurkat (data not shown). No CALLA mRNA could be detected in mouse fibroblasts (Fig. 2).

CALLA is a very useful diagnostic marker of common (pre-B) ALL, being absent from normal lymphocytes and monocytes; it is however present on some stem cells in fetal liver, bone marrow and thymus (12, 13), on some lymphomas (2, 3) and on melanoma and glioma cell lines (17, 18). It is, thus, neither leukemia specific nor confined to a single tissue but is expressed on normal and neoplastic cells of diverse origins. The presence of NEP on malignant cells or tissue has not been documented. Both CALLA and NEP have been independently observed on epithelial cells of kidney and gut (9–11, 21–23, 31), on neutrophils (14–16, 19, 24), and on cultured fibroblasts (15, 32). Within the kidney, where they are particularly abundant, both CALLA and NEP are found on the brush border of proximal tubules and on glomerular epithelium (9–11, 21–23, 31). CALLA antigens from leukemic cells (4–8, 16), kidney (9, 11), melanoma (17), glioma (18), granulocytes (14, 15, 19), or fibroblasts (15), and NEP from kidney (21–23, 28–29), brain (28), or fibroblasts

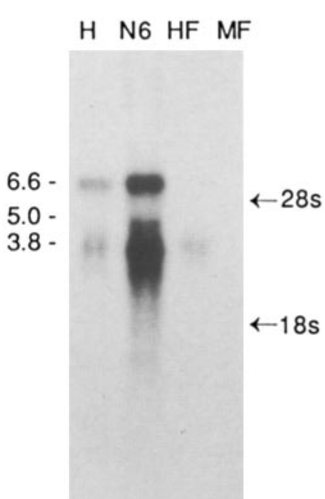

FIGURE 2. Northern blot of human leukemic cells and fibroblasts probed with CALLA cDNA. RNA samples (20 µg) prepared from the HOON (*H*) and NALM-6 (*N*) pre-B ALL cell lines and from human (*HF*) or mouse (*MF*) fibroblasts were fractionated on a denaturing agarose gel. After transfer to a nylon membrane, the RNAs were probed with ^{32}P-labeled 1.6-kb CALLA cDNA fragment.

(32), are all glycoproteins of $M_r \sim 9.4 \times 10^4$–10^5. The variation in apparent M_r has been attributed to heterogeneity of glycosylation.

NEP has been studied extensively in brain as an inactivator of enkephalins; inhibitors of the enzyme have analgesic properties (28). Although there are no reports of the presence of CALLA in brain, it has been described on several glioma cell lines (18).

Kidney and neutrophil NEP can cleave the chemotactic peptide, fMLF, and polyclonal antibody to human kidney NEP can inhibit the hydrolysis of the tripeptide by a neutrophil membrane fraction enriched in NEP (24). It is of interest that mAb to CALLA can inhibit 20% of fMLF-induced neutrophil chemotaxis (19). Since cleavage of chemotactic peptides is required for neutrophil degranulation and chemotaxis, CALLA (NEP) might be important in neutrophil function (19, 24).

Our observation that the structure of CALLA corresponds to that of an enzyme able to hydrolyze a variety of polypeptide hormones opens new avenues for testing the function of this molecule in leukemic cells and its potential role in the process of malignancy. In this context, it is of interest that transin, a zinc metalloprotease belonging to the same superfamily as NEP, can be induced by oncogenes and is expressed more abundantly in malignant than in benign tumors (33).

Summary

We purified CALLA from human kidney and isolated a cDNA clone reactive with two oligonucleotide probes corresponding to two distinct peptides. The amino acid sequence translated from the CALLA cDNA revealed 100% identity with that of human neutral endopeptidase (NEP, enkephalinase). The distributions of CALLA antigen and NEP in normal tissues are similar.

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Note added in proof: The sequence of the CALLA cDNA has been completed and is identical to that shown in Fig. 1 for human NEP. While this manuscript was in press, the sequence of a CALLA cDNA isolated from the NALM-6 leukemic cell line has been reported (34) and is identical to our sequence.

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