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Analysis of Neutral Endopeptidase Activity in Lymphocytes

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Analysis of Neutral Endopeptidase Activity in Lymphocytes

By

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THESIS

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I. Enzymatic, Cellular and Functional Properties of Neutral Endopeptidase

A. NEP Regulates Enkephalins in the Central Nervous System

Cell membrane-bound ectopeptidases that cleave peptide mediators can modify cellular responses to these factors by decreasing the effective extracellular concentration of the primary mediator and generating peptide fragments with lower potency or even antagonistic activity. For example, neutral endopeptidase 24.11 (EC 3.4.24.11), a cell membrane-bound, Zn-metalloendopeptidase, down-regulates the effects of endogenous opioids in mammalian brains by hydrolyzing leucine- and methionine-enkephalin (Schwartz, Malfroy, et al., 1981; Erdos and Skidgel, 1989), rendering them incapable of binding to their cell membrane receptors. The importance of neutral endopeptidase (NEP) in the regulation of the enkephalins is suggested by the similarity in distribution of NEP in the brain with that of the two opioids (Gorenstein and Snyder, 1980; Llorens, Malfroy, et al., 1982; Back and Gorenstein, 1985; Waksman, Hamel, et al., 1986; Matsas, Kenny, et al., 1986; Back and Gorenstein, 1989) and the mu and delta opioid receptors (Malfroy, Swerts, et al., 1979; Back and Gorenstein, 1985; Waksman, Hamel, et al., 1986; Matsas, Kenny, et al., 1986). Moreover, the importance of NEP in regulating endogenous opioids is suggested by the naloxone-reversible analgesic

effects produced by selective NEP inhibitors (Roques, Fournie-Zaluski, et al., 1980; Roques, Lucas-Soroca, et al., 1983).

B. NEP Has Broad Specificity and Is Widely Distributed

Due to its effects on the enkephalins, NEP has been given the trivial name enkephalinase. However, this name is misleading, as numerous substrates have been reported for NEP. A partial list includes insulin B chain, bradykinin, angiotensins I and II, oxytocin, neurotensin, neurokinin A and B, atrial natriuretic factor (Erdos and Skidgel, 1989), cholecystokinin (Matsas, Turner, et al., 1984), vasoactive intestinal polypeptide (Goetzl, Sreedharan, et al., 1989), N-formyl-Met-Leu-Phe (Painter, Dukes, et al., 1988), and substance P (Matsas, Fulcher, et al., 1983). The common feature of these seemingly diverse peptide substrates is their low (less than 5000 daltons) molecular weight (Bond and Butler, 1987; Erdos and Skidgel, 1989) and their susceptibility to cleavage at the amino side of hydrophobic amino acids (Erdos and Skidgel, 1989). For example, Met-enkephalin, a pentapeptide (Tyr-Gly-Gly-Phe-Met), is cleaved at its Gly_3-Phe_4 bond (Gorenstein and Snyder, 1980).

In addition to having a wide range of substrates, NEP is widely distributed throughout the central nervous system (CNS) and in non-neural tissues. NEP has been identified by one or more assays in areas of the kidney and small intestine containing cells with brush borders (Ronco, Pollard, et al., 1988; Erdos and Skidgel, 1989), skin and

lung fibroblasts (Lorkowski, Zijderhand-Bleekemolen, et al., 1987; LeBien and McCormack, 1989), thyroid, seminal vesicle, prostate (Ronco, Pollard, et al., 1988), neutrophils (Painter, Dukes, et al., 1988), and some subsets of lymphocytes (Letarte, Vera, et al., 1988; Shipp, Vijayaraghavan, et al., 1989). The broad substrate specificity and presence of NEP in different organs and cell types suggests that NEP also has regulatory functions outside of the CNS.

Indeed, in neutrophils, NEP modulates chemotactic responses to N-formyl-Met-Leu-Phe (fMLP), the so-called chemotactic peptide. The mechanism of this effect of NEP, however, is complex and may involve cleavage of receptor-bound fMLP. On the cell membranes of neutrophils, NEP hydrolyzes fMLP at the Met-Leu bond (Painter, Dukes, et al., 1988), which removes the peptide's chemotactic properties (LeBien, McCormack, et al., 1989), suggesting that the presence of the peptidase would decrease the chemotactic response of neutrophils to fMLP. Yet when NEP is inhibited by anti-NEP monoclonal antibody (McCormack, Nelson, et al., 1986) or the selective NEP inhibitor phosphoramidon (Painter, Dukes, et al., 1988), chemotaxis is diminished rather than potentiated. Possibly these findings are due to a functional (but unestablished) interaction between NEP and the fMLP receptor (LeBien and McCormack, 1989); chemotaxis may be mediated by the cleavage of fMLP after it is bound to its receptor.

Like neutrophils, certain lymphocytes express NEP on their cell membranes. Indeed, NEP is an important cell surface marker in the classification of acute lymphoblastic leukemia (ALL) and is called common acute lymphoblastic leukemia antigen (CALLA). Immunologic studies, using antibodies to CALLA and antigens corresponding to different stages of B- and T-lymphocyte development, demonstrate that NEP is present on pre-B and early pre-B ALL lymphocytes and usually absent on T-ALL lymphocytes (Foon and Todd, 1986; Champlin and Gale, 1989). In normal lymphocytes, NEP shows a similar pattern of expression; it is present on early lymphoid progenitors, which are uncommitted to B or T lymphocyte differentiation, and early pre-B cells but is absent in mature T cells (Shipp, Richardson, et al., 1988; Shipp, Vijayaraghavan, et al., 1989). Due to this pattern of expression, NEP is used as a cell surface marker for lymphocyte development; its cluster designation is CD10 (Foon and Todd, 1986; Champlin and Gale, 1989).

Although NEP is a well characterized surface marker of lymphocytes, its function is unknown. But in view of its known substrates and functions, it likely modulates the actions of small regulatory peptides selectively.

C. Lymphocyte Recognition and Effects of Neuropeptides

Neuropeptides comprise one group of small, regulatory peptides that may be modulated by NEP of lymphocytes. Many of these peptides are known NEP substrates (Erdos and Skidgel, 1989), and many have regulatory effects on

lymphocytes (Sreedharan, Peterson, et al., 1989). For example, somatostatin (SOM) inhibits proliferation of murine splenic lymphocytes (Pawlikowski, Stepien, et al., 1985) and phytohemagglutinin stimulated T lymphocytes (Payan, Hess, et al., 1984). The demonstration of high affinity (kd 3-5 pM) SOM receptors on B and T lymphocytes by competitive-binding experiments (Sreedharan, Kodama, et al., 1989) further suggests the regulatory role of the neuropeptide.

Another neuropeptide that regulates lymphocytes is vasoactive intestinal polypeptide (VIP). For example, VIP inhibits mitogen-induced proliferation of murine-tissue derived mixed lymphocytes (Ottaway and Greenberg, 1984; Ottaway, 1987). In addition, VIP reduces the synthesis of IgA and increases that of IgM by lymphocytes taken from Peyer's patches (Stanisz, Befus, et al., 1986). As with SOM, the presence of high affinity VIP receptors on B and T lymphocytes corroborates the observed effects of VIP on lymphocytes (Ottaway and Greenberg, 1984; Wood and O'Dorisio, 1985; Calvo, Molinero, et al., 1986; Finch, Sreedharan, et al., 1989).

D. Lymphocytes Express Neutral Endopeptidase-Like Activity

One function of NEP in lymphocytes may be to degrade VIP, thereby modulating its ability to bind to its receptors and exert cellular effects. Indeed, in studies of VIP receptors involving human myeloma cells of the U266 line and human leukemic T lymphocytes of the Jurkat line, VIP, a 28

amino acid peptide, was partially degraded during incubations with the intact cells (Finch, Sreedharan, et al., 1989). Resolution of the VIP cleavage fragments by reverse-phase high- performance liquid chromatography (HPLC) and subsequent microsequencing showed that VIP was cleaved preferentially at three sites with time- and temperature-dependence. The three principal fragments were VIP_{4-28} (A), VIP_{23-28} (B), and VIP_{15-28} (C) (Goetzl, Kodama, et al., 1989). Fragment C was generated by a trypsin-like activity, fragments A and B by a neutral endopeptidase-like activity, as suggested by the following: (1) fragments A and B were generated by cleavages on the amino side of hydrophobic amino acids (Ala, fragment A and Leu, fragment B), (2) the selective NEP inhibitors DL-thiorphan (Roques, Fournie-Zaluski, et al., 1980) and phosphoramidon (Alstein, Bachar, et al., 1983) inhibited the generation of fragment A and to a lesser extent B (but had no effect on C), whereas, inhibitors of trypsin, chymotrypsin and cysteine proteases had no such effect, and (3) cell membrane enriched preparations increased the generation of fragments A and B relative to C, consistent with the cellular localization of NEP.

Furthermore, fragments A and B are identical or similar to the principal fragments generated when VIP was incubated with human recombinant NEP (fig. 1) (Goetzl, Sreedharan, et al., 1989). Fragment A, the principal cleavage fragment in the lymphocyte studies, is identical to the principal cleavage fragment in the recombinant NEP study. Fragment B,

Fig. 1 (adapted from fig. 4 of Goetzl, Kodama, et al., 1989 and fig. 2 of Goetzl, Sreedharan, et al., 1989). Sites of cleavage of VIP_{1-28} by U266 and Jurkat lymphocytes and human recombinant NEP. The arrows above the sequence (pointing down) indicate the sites of cleavage by lymphocytes, the arrows below (pointing up), the sites of cleavage by human recombinant NEP. The length of the vertical arrows indicates the relative activity at the site.

however, is slightly different from its corresponding NEP fragment, VIP_{22-28}, which was cleaved on the amino side of Tyr_{22}.

II. Experimental Methods

A. Immunochemical Detection of NEP in Lymphocytes

1. Introduction

The presence of NEP-like activity in the U266 myeloma and Jurkat T cell lines is a surprising finding, for plasma membrane-bound NEP, as stated above, is generally considered to be specific for early stages of B cell differentiation and absent in T cells (LeBien and McCormack, 1989; Shipp, Vijayaraghava, et al., 1989). To further elucidate the NEP-like activity, the presence of NEP in the U266 and Jurkat cell lines, as well as the NEP-positive, pre-B ALL cell line Nalm 6 (LeBien, Kersey, et al., 1982; Greaves, Hariri, et al., 1983; Shipp, Vijayaraghavan, et al., 1989) and the NEP-negative myeloid cell line HL-60 (LeBien, Kersey, et al., 1982; Greaves, Hariri, et al., 1983), was assessed by flow cytometric analysis of cell populations and direct microscopic visualization of individual cells by immunofluorescence, both with (total) and without (surface only) cellular permeabilization, for comparison with results of enzymatic and genetic measurements. For the flow cytometric and microscopic immunofluorescence studies, a rabbit, polyclonal anti-NEP and two mouse monoclonal IgG anti-NEP antibodies were used to identify NEP on the cell

surface and in the cytoplasm. Both FACS and immunofluorescence detected NEP in the cytoplasm but not on the cell surfaces of U266 and Jurkat cells, whereas in the positive-NEP line Nalm 6, NEP was present both on the cell surface and in the cytoplasm, and was completely absent from the negative-NEP HL-60 line.

2. Materials and Methods

Cell Culture--Nalm 6 (Dr. M.S. O'Dorisio, Ohio State University), U266 (American Type Culture Collection), Jurkat (Dr. A. Weiss, University of California at San Francisco) and HL-60 (American Type Culture Collection) cells were grown in RPMI 1640 medium with 5-10% (v/v) filter-sterilized fetal bovine serum, 25 mM N-2-hydroxy-ethyl-piperazine-N'2-ethane sulphonic acid (HEPES; pH 7.4), penicillin (100 units/ml), and streptomycin (100 ug/ml; Cell Culture Facility, UCSF) and maintained at 37°C, 5% CO_2:95% air in 175-cm^2 flasks (Falcon Labware, Oxnard, CA). The cultures were subdivided every 48-72 hours to attain a cell density of 5 x 10^5/ml and used for studies when the density reached 1-2 x 10^6/ml. Before use, the cells were washed twice with Dulbecco's phosphate-buffered saline (PBS; Cell Culture Facility, UCSF).

Antibodies--Mouse monoclonal antibodies to NEP, J5 (Ritz, Pesando, et al., 1980) and 44C10 (Quackenbush and Letarte, 1985), were obtained from Dr. J. Ritz (Harvard University) and Dr. M. Letarte (Hospital for Sick Children, Toronto, Canada), respectively. Rabbit polyclonal

antibodies were obtained by immunizing New Zealand white rabbits with human recombinant NEP (Genentech) and extracting their sera after booster injections. Rhodamine-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antibodies were obtained from Cappel, West Chester, PA. Normal rabbit serum (NRS) was obtained from New Zealand white rabbits prior to immunization with NEP.

Solutions--A concentrated phosphate-buffered saline solution was prepared consisting of 8 mM NaH_2PO_4, 30 mM Na_2HPO_4, 600 mM NaCl, and 35 mM MgCl; it was diluted 1:4 (v/v) before use (PBS). A 4% paraformaldehyde solution was prepared by first dissolving, with gentle heat and stirring, 4 g of paraformaldehyde in 85 ml of a 0.1 M phosphate solution (1 M PO_4) consisting of 0.5 M NaH_2PO_4 and 0.5 M Na_2HPO_4 (pH 7.3). Then 5 ml of 0.001 M $CaCl_2$ were added, the solution cooled to room temperature, and filtered through Whatman No. 1 filter paper (BioRad, Emeryville, CA), before the addition of 10 ml of 0.1 M PO_4. The 4% paraformaldehyde solution was stable for 1-2 weeks at 4^oC and was diluted 1:2 (v/v) with 0.1 M PO_4, for use as a 2% solution, just before use. A 0.1% Triton X100 solution was prepared by diluting 100 ul of Triton X100 in 25 ml of the concentrated stock solution of PBS and 75 ml of distilled H_2O and stored at 4^oC. A 50 mM NH_4Cl solution was prepared by dissolving 1.34 g of NH_4Cl in 125 ml of the concentrated PBS solution and then diluting to 500 ml with distilled H_2O. A 0.25% bovine serum albumin (BSA) solution was prepared by dissolving

0.25g of BSA in 25 ml of the concentrated PBS and 75 ml of distilled H_2O and stored at 4^oC. All chemicals were purchased from Sigma, St. Louis, MO.

Flow Cytometry--For cell surface analysis, aliquots of 2×10^6 cells were suspended in 200 ul of Dulbecco's PBS and incubated with either J5 or rabbit polyclonal antibody at a final concentration of 1/250 and 1/2500, respectively, for 1 hour at 4^oC. After incubation with the primary antibody, the cells were washed 3 times with PBS, then suspended in 200 ul of PBS and incubated for 30 minutes at 4^oC with either rhodamine-conjugated goat anti-mouse IgG (for J5) or goat anti-rabbit IgG (for rabbit polyclonal antibody) at a final concentration of 1/100. Afterwards, the volume was brought up to 800 ul with PBS.

For detection of total (surface membrane and cytoplasmic) NEP, cells (2×10^6) were suspended in 200 ul of 2% paraformaldehyde and incubated for 30 minutes at 4^oC, then rinsed twice with 50 mM NH_4Cl and twice with PBS. Afterwards, the cells were incubated in 200 ul of 0.1% Triton X100 for 5 minutes at -20^oC, washed twice with PBS, suspended in 200 ul of PBS, and incubated for 1 hour at 4^o with either J5 or rabbit polyclonal antibody at a final concentration of 1/500 and 1/5000, respectively. The cells were subsequently washed 3 times with PBS and incubated for 30 minutes at 4^o with either rhodamine-conjugated goat anti-mouse or anti-rabbit IgG at a final concentration of 1/100. Then the volume was brought up to 800 ul with PBS.

To control for non-specific binding of the secondary

antibody, the primary antibody was replaced with a corresponding volume of PBS for J5 and NRS for rabbit polyclonal antibody in duplicate experiments in both the analysis of surface and total (surface and cytoplasmic) NEP. The presence of NEP was assessed by comparing the mean channel fluorescence values of the corresponding experimental and control samples, which were measured with a Becton-Dickinson FACS IV equipped with a 2-watt argon-laser (Spectra-Physics, Inc., Mountain View, CA) excited at 528 nm and operated at 250 milliwatts. For each sample, 0.8-1 x 10^4 cells were examined.

Immunofluorescence--Cells (1-2 x 10^6/sample) were washed twice with PBS, incubated for 30 minutes at room temperature in 150 ul of 2% paraformaldehyde, then washed twice with 50 mM NH_4Cl and twice with PBS. Cells that were intended for cell surface staining of NEP were then rinsed with 0.25% BSA, whereas cells that were intended for total cell staining were first permeabilized by incubation in 150 ul of 0.1% Triton X100 for 3 minutes at -20^oC and then washed twice with PBS, before being rinsed in 0.25% BSA. After rinsing, the cells were incubated for 30 minutes at room temperature in either J5, 44C10, or rabbit polyclonal antibody diluted 1:200 for the two mouse monoclonal antibodies and 1:1000 (v/v) for rabbit polyclonal antibody in 0.25% BSA. Then the cells were rinsed three times with PBS and incubated for 30 minutes at room temperature in either rhodamine-conjugated goat anti-mouse IgG (if the

primary antibody was J5 or 44C10) or anti-rabbit IgG diluted 1:100 (v/v) in PBS. The cells were subsequently washed three times in PBS, suspended in 20 ul of 90% glycerol/10% 1 M Tris (pH 7.4), mounted on glass slides and covered with coverslips, which were sealed with clear nail polish.

To control for non-specific staining of the secondary antibody, an equivalent volume of 0.25% BSA was substituted for J5 and 44C10 and an equivalent dilution of NRS in 0.25% BSA was substituted for rabbit polyclonal antibody in duplicated samples. Staining was assessed with a Zeiss Photomicroscope (IIIRS) equipped with a Zeiss oil-lens objective (160/0.17) and Plan NeoFluar filters (63-0.17).

B. Quantification of Lymphocyte NEP by a Degradation of Synthetic Substrates

1. Introduction

Although NEP was detected in the cytoplasm of the U266 and Jurkat cell lines by both FACS and immunofluorescence, it cannot be inferred that the peptidase is functionally active. Moreover, despite the highly suggestive evidence that NEP is indeed present in the cytoplasm, it is possible that the anti-NEP antibodies recognized an antigenically similar protein. To further clarify the results of the preceding study, the presence of NEP was assessed using two selective NEP substrates: dansyl-D-Ala-Gly-Phe($\underline{p}NO_2$)-Gly (DAGNPG) and [tyrosyl-3,5-^{3}H]-D-Ala$_2$-Leu-enkephalin (^{3}H-Enk).

The former substrate, due to the presence of a dansyl group, is a fluorogenic peptide. In the intact state, the native fluorescence of the dansyl group is quenched by the spatial proximity of Phe(pNO_2) group, but when the peptide is cleaved by NEP at the Gly-Phe(pNO_2) bond, the fluorescence is released, which can be measured by spectrofluorimetry and used as an index of NEP activity (Florentin, Sassi, et al., 1984). Another feature of the dansyl group is that, along with the D-Ala residue, it protects the peptide against aminopeptidase and dipeptidylaminopeptidase activity.

Like DAGNPG, the ^{3}H-Enk substrate has a D-Ala residue in position 2 that protects the peptide against amino-peptidase and dipeptidylaminopeptidase activity (Llorens, Malfroy, et al., 1982). It is selectively cleaved by NEP at the Gly-Phe bond, producing the radiolabeled fragment ^{3}HTyr-D-Ala-Gly which can be isolated by such techniques as polystyrene bead chromatography and reverse- phase HPLC.

These two substrates were used to detect NEP activity in the four cell lines investigated in the previous study: Nalm 6, U266, Jurkat and HL-60. NEP activity was detected in intact Nalm 6 cells, in disrupted Nalm 6, U266, and Jurkat cells in equivalent amounts and in HL-60 cells in a lesser amount.

2. Materials and Methods

Cell Culture--The cell lines were maintained as described above.

Fluorimetric Assay of NEP Activity--Assays were performed essentially as described (Florentin, Sassi, et al., 1984) using DAGNPG (Sigma, St. Louis, MO). A 0.01 M DAGNPG/Ethanol stock solution was diluted to a final concentration of 50 uM in 50 mM Tris-HCl (pH 7.4), aliquoted in 100 ul samples and preincubated at 37°C for 15 minutes. Then the cells (2.5 x 10^6/sample) were added to begin the reaction, and the samples were incubated for 30 minutes at 37°C with shaking. In duplicate samples, cells were first preincubated in 10 uM DL-thiorphan before being added to the DAGNPG solution. After incubation, the enzyme reaction was stopped by boiling at 100°C for 5 minutes and the samples diluted with 1.35 ml of 50 mM Tris-HCl (pH 7.4) Then the cells were pelleted by centrifugation at 5000 x g for 15 minutes and the supernatants measured for fluorescence with a spectrofluorimeter (model 650-40, Perkin-Elmer Corp., Norwalk, CT) set at the following wavelengths: excitation 342 nm, emission 562 nm (slit widths 10 nm).

Radiometric Assay of NEP Activity--For assessing surface NEP activity, intact cells (5 x 10^6/sample) were suspended in 100 uM 50 mM Tris-HCl (pH 7.4) with 25 nM [tyrosyl-3,5-^{3}H]-D-Ala-Leu-enkephalin (New England Nuclear, Boston, MA) and incubated for 30 minutes at 25°C. Then 2 volumes of -20°C absolute ethanol were added and the solution incubated for 5 minutes at 4°C before centrifugation at 8000 x g for 15 minutes to pellet the precipitated proteins. The supernatants were placed in silanized tubes and completely dried in vacuo. The dried

peptides were then dissolved in 100 ul of 0.07% trifluoroacetic acid (TFA; Pierce Chemical Co., Rockford, IL) in distilled water for analysis by reverse-phase HPLC.

For assessing cytoplasmic NEP activity, 5 x 10^6 cells were solubilized in 5 ul of Tris-buffered saline containing 1% octylglucoside as described previously (Devault, Nault, et al., 1988) using vortexing and repeated straining through 18-gauge syringe needles. The resulting extract was added to 95 ul of 50 mM Tris-HCl (pH 7.4) containing 25 nM of ^{3}H-Enk, and the solution was incubated for 30 minutes at 25^{o}C.

In both surface and cytoplasmic analysis, duplicate samples were run that contained 10 uM DL-thiorphan. NEP activity was assessed by reverse-phase HPLC using a Beckman Instruments system with dual 100 A metered pumps and a 4.6 x 250 mm column of 300 A pore octadecylsilane (Alltech Associates, Inc., Deerfield, IL) that was developed at 0.7 ml/minute for 10 minutes with 0.07% TFA in water (v:v) and 40 minutes with a linear gradient to 0.046% TFA in 72% acetonitrile: 28% water (v:v). Eluate was collected in successive 700-ul fractions, which were analyzed for radioactivity in a gamma-well counter (United Technologies, Packard Division, Downers Grove, IL).

C. Analysis of Genetic Message for NEP by Polymerase Chain Reaction

1. Introduction

The preceding studies provided immunologic and

functional evidence that NEP is present in one or more intracellular compartments of U266 and Jurkat cells, with limited surface expression. However, the results still do not eliminate alternative hypotheses. For example, possibly the findings were due to the presence of an other peptidase that has a similar structure and action to that of NEP. (Indeed, recently a novel class II Zn-metalloprotease, BP-1/6C3 antigen, which has a zinc binding size homologous to that of NEP, was cloned from murine pre-B lymphocytes (Wu, Lahti, et al., 1990)). Moreover, assuming that NEP is present in the cytoplasms of U266 and Jurkat cells, the previous studies do not provide much information about the structure of the cytoplasmic NEP. To address these problems, the cell lines were examined for the genetic message of NEP.

This study was made possible due to recent insights into the genetic and protein structure of NEP. The enzyme was first cloned by two groups, Malfroy, Shofield, et al. (1987) and Devault, Lazure, et al. (1987). The former group purified NEP from rat kidney membranes and determined its partial amino acid sequence, which they used to deduce oligonucleotide probes for selection of the entire cDNA message from cDNA libraries of rat kidney and brain. The latter group applied a similar strategy to clone the NEP gene from rabbit kidney. Human NEP was later cloned by screening a human placenta cDNA library with a partial rat cDNA clone (Malfroy, Kuang, et al., 1988).

From the nucleotide sequence of the gene, NEP was

determined to be an approximately 95 kiloDalton, 750 amino acid protein with a short N-terminal cytoplasmic domain (about 25 amino acids), one cell membrane-spanning domain (about 25 amino acids), as determined by hydrophobicity analysis, and a large carboxy-terminal, extracellular domain (about 700 amino acids). The primary structure of NEP was highly conserved among the three species from which it was cloned; human and rat NEP were 94% conserved, and rabbit NEP was 93% conserved with both human and rat NEP (Malfroy, Kuang, et al., 1988). Other features of the protein include six potential N-linked glycosylation sites in human (Malfroy, Kuang, et al., 1988) and rat NEP (Malfroy, Schofield, et al., 1987) and 12 cysteine residues in all three species, which probably form internal disulfide bridges (Erdos and Skidgel, 1989).

The cloning of NEP has also led to other important insights into its structure. For instance, it established NEP as a class 2 integral membrane protein (Shipp, Richardson, et al., 1988; Lemay, Waksman, et al., 1989). Such proteins, unlike those of class 1 membrane proteins, do not have a cleavable signal peptide in addition to their hydrophobic membrane-spanning sequence. Rather, their membrane-spanning region, which is located near the amino-terminus, also functions as a non-cleaved signal peptide (Wickner and Lodish, 1985; Lemay, Waksman, et al., 1989).

The cloning of NEP has also provided insights into its active site. This area was explored by oligonucleotide-

directed mutagenesis using expression vectors in which the complete coding sequence of NEP was placed downstream from the simian virus 40 (SV 40) early promoter for expression of NEP in COS-1 monkey kidney cells (Devault, Nault, et al., 1988; Devault, Sales, et al., 1988). Through this technique, Glu_{584} was shown to be essential for catalytic activity (Devault, Nault, et al., 1988), whereas, His_{583} and His_{587} were shown to be important Zn-coordinating residues (Devault, Sales, et al., 1988). Interestingly, these features of the active site correspond to features of the active site of thermolysin, a bacterial Zn-metalloendopeptidase that has a similar substrate specificity to NEP (Devault, Lazure, et al., 1987); Glu_{584} in NEP is thought to correspond to Glu_{143} in thermolysin (Devault, Nault, et al., 1988; Erdos and Skidgel, 1989), and His_{583} and HIS_{587} to His_{142} and His_{146} in thermolysin (Devault, Sales, et al., 1988; Erdos and Skidgel, 1989).

Another finding, in part attributable to the cloning of NEP, is that common acute lymphoblastic leukemia antigen (CALLA) was identical to NEP. CALLA was cloned from lymphocytes by screening Nalm 6 cDNA libraries with oligonucleotide probes deduced from the partial structure of NEP, which has been immuno-affinity purified from Nalm 6 cells with the monoclonal antibody J5 (Shipp, Richardson, et al., 1988). In addition, CALLA was also cloned from human kidney following immuno-affinity purification with the monoclonal antibody 44C10 (Letarte, Vera, et al., 1988). By deducing the amino acid sequence of the protein from the

gene and comparing it to known protein sequences stored in computer databases, CALLA was determined to be identical to NEP. Later CALLA was shown to exhibit NEP activity by demonstrating that CALLA+ lymphocytes and purified CALLA (Jongeneel, Quackenbush, et al., 1989), as well as CALLA- myeloma cells transfected with CALLA cDNA (Shipp, Vijayaraghavan, et al., 1989), acted on specific NEP substrates and were suppressed by NEP inhibitors.

Although NEP is functionally active in certain lymphocytes, it has never been described as an intracellular enzyme; it is usually studied on the surfaces of intact cells or after isolation from cell membranes. Furthermore, in the cloning studies, the deduced primary structure of NEP has always included a short transmembrane region near the N terminus, indicating that the enzyme is an integral membrane protein. However, the cytosolic and transmembrane portions are not essential for activity. When a cDNA encoding the sequence of a cleavable signal peptide from a secreted protein was fused in-frame with the complete extracellular domain of NEP downstream from an SV-40 derived expression vector, a functionally active NEP was secreted by COS-1 cells transfected with the vector (Lemay, Waksman, et al., 1989).

Possibly, then, the functionally-active, cytoplasmic NEP detected in U266 and Jurkat cells was derived from alternatively spliced messenger RNA that lacked the transmembrane coding sequence. Another possibility, of

course, is that the detected NEP is simply due to other proteins that share NEP epitopes or activity. These hypotheses were tested by analyzing the total RNA of U266 and Jurkat cells for the presence of NEP message using the extremely sensitive polymerase chain reaction (PCR).

PCR is so sensitive that a single DNA segment can be selectively amplified over a million fold. This is accomplished by using two oligonucleotide primers that hybridize to opposite strands on the 3' boundaries of the DNA segment (such that DNA synthesis occurs between the primers) and a thermocycler that raises and lowers the temperature through multiple cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers by a thermostable DNA polymerase. Each cycle doubles the amount of the specific DNA segment present in the previous cycle because the primers can anneal to the extension products, themselves. Thus, PCR can amplify the segment of interest 2^n times, where n equals the number of cycles (Saiki, 1990).

Because NEP has been cloned, it was possible to design oligonucleotide primers to specific regions of the gene (fig. 2). Based on the previous studies, it seemed likely that the active site was present in U266 and Jurkat cells, whereas the transmembrane-signal region near the NH_2-terminus was absent. Consequently, the cell lines were studied with two sets of primers directed to the active site region in the latter half of the NEP gene. In addition to

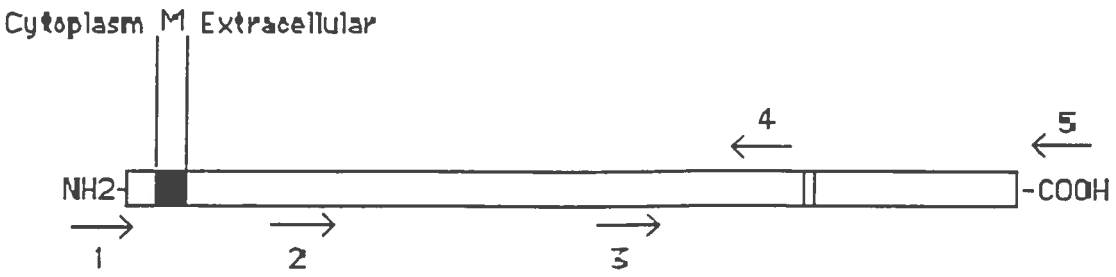

Fig. 2 (adapted from fig. 1 of Erdos and Skidgel 1989). Schematic primary structure of human NEP based on sequence information provided by Malfroy, Kuang, et al. (1988). The dark box represents the transmembrane portion of the protein, the small, open box near primer 4, the region containing the active site Glu_{577} and the two histidines involved in the binding of the zinc cofactor, His_{576} and His_{580}. The arrows indicate the relative positions and orientations of the oligonucleotide PCR primers.

the U266 and Jurkat lines, AF10, a U266 subclone, and the NEP-positive Nalm 6 and NEP-negative HL-60 lines were studied. PCR detected NEP only in Nalm 6, Jurkat and AF10.

2. Materials and Methods

Cell Lines--AF10 (Dr. D. Adelman, University of California at San Francisco) and other cell lines were maintained as described above.

Isolation of RNA--The cells (0.5-1 x 10^8) were washed three times with cold PBS (4°C) then lysed in 7.5 ml of a 4 M guanidinium thiocyanate (GU-SCN) solution (4 M GU-SCN, 0.5% (w/v) sodium N-laurylsarcosine, 25 mM EDTA, 0.13% (v/v) anti-foam A, and 0.1 M B-mercaptoethanol (pH 7.0)) by vortexing and repeated straining through 22-gauge needles. The resulting mixture was layered over a cesium chloride gradient consisting of 3 ml of a 5.7 M CsCl solution (5.7 M CsCl, 25 mM sodium acetate and 10 mM (EDTA)) in the bottom layer and 0.7 ml of a 2.4 M CsCl solution (2.4 M CsCl, 25 mM sodium acetate and 10 mM EDTA) in the top layer. Then the gradients were centrifuged at 30,000 rpm at 18°C for 24 hours in an SW 41Ti rotor with a Beckman ultracentrifuge. Afterwards, the supernatant was aspirated, and the RNA pellet was dissolved in 200 ul of TES (10 mM Tris (pH 7.5), 5 mM EDTA, and 1% sodium dodecyl sulfate) plus 10 ul of phenol. The RNA solution was then transferred to 1.5 ml Eppendorf tubes, vortexed and heated at 95°C for 5 minutes. Then 200 ul of phenol was added to the solution and the solution was vortexed for 5 minutes and subsequently

centrifuged at 15,000 rpm for 15 minutes. The upper aqueous phase was removed and placed into a clean 1.5 ml Eppendorf tube and extracted with one volume of chloroform, following the phenol extraction procedure described above. The aqueous layer was placed in a clean 1.5 ml Eppendorf tube and 3 M sodium acetate (pH 5.5) was added to a final concentration of on 0.2 M followed by 2.5 volumes of absolute ethanol. The solution was vortexed and the RNA precipitated at -20°C overnight. Afterwards, the RNA was pelleted by centrifugation at 15,000 rpm for 30 minutes, dried in vacuo and reconstituted in DEPC-treated, autoclaved distilled water. The quantity of RNA was determined spectrophotometrically, and the material was stored in 10 ug aliquots at -80°C.

cDNA Synthesis--Synthesis of cDNA was done following the procedure outlined in the Promega (Madison, WI) Riboclone manual. Ten ug of total RNA was dissolved in 3.5 ul of RNAase-free water, and 2 ul of oligo-dT mix was added. This mixture was heated at 70°C for 5 minutes and allowed to cool to room temperature. Then the following components were added: 2.5 ul of 10 x first strand buffer (500 mM Tris-HCl (pH 8.3), 750 mM KCl, 100 mM $MgCl_2$, 5mM spermidine), 2.5 ul 100 mM DTT, 2.5 of ul of 10 mM dNTP mix, 0.8 ul (25u) of RNasin, 2.5 ul of 4.0 mM sodium pyrophosphate and 10 ul of AMV reverse transcriptase solution consisting of a 1/10 dilution of 28,000 units of AMV reverse transcriptase in 10 ul of enzyme buffer. The solution was mixed by gentle tapping and incubated for 1

hour at 42°C. Afterwards, the reaction was cooled on ice, and 20 ul of 0.3 M NaOH, 0.03 M EDTA was added. This solution was incubated at 60°C for 1 hour to hydrolyze the RNA. Then 3.4 ul of 3 M sodium acetate (pH 5.2) was added to neutralize the solution followed by 2 volumes of -20°C absolute ethanol to precipitate the cDNA (Grady and Campbell, 1989). After drying, the cDNA was reconstituted in 250 ul of sterile, distilled water and used in 10 ul aliquots in PCR studies.

Polymerase Chain Reaction--The following components were assembled in a 500 ul Eppendorf tube: 10 ul of 10x reaction buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM MgCl, and 0.1% (w/v) gelatin), 16 ul dNTP mix (1.25 mM each dNTP), 1 ul each of primers 1 and 2 (100 pmol/ul), 10 ul of template cDNA, 61 ul of sterile, distilled water, and 0.5 ul of Taq DNA polymerase (2.5 units). This mixture was overlayed with mineral oil before use to prevent evaporation. All chemicals were obtained from Perkin-Elmer Cetus Emeryville, CA, with the exception of template cDNA (see above).

A thermocycler (Perkin-Elmer Cetus) was used to raise and lower the temperature to programmed settings for denaturation, annealing and polymerization. To amplify the 800 base-pair segments between primers 3-5 and 4-6 (see figure), 35-40 cycles of the following program were used: 94°C, 1.5 min (denaturation); 55°C, 2 min (annealing) and 72°C, 5 min (polymerization). To amplify the complete 2.5

kilobase gene, an identical program was used with the exception of the polymerization step which was extended to 10 min at 72°C, as longer periods are required for amplification of larger segments (Grady and Campbell, 1989).

After the PCR had finished, 100 ul of chloroform were added to extract the DNA from the mixture. The DNA solution was then mixed with 40 ul of 10 M NH_4OAC and precipitated in 2 volumes of ethanol. After this, the DNA was pelleted by centrifugation, dried in vacuo and reconstituted in 10 ul of distilled water. This DNA solution was then mixed with 2 ul of 0.25% bromphenol blue, 30% glycerol and subjected to gel electrophoresis, using 1% agarose gels, which were developed at 80 V for about one hour. DNA bands were stained with ethidium bromide and illuminated by ultraviolet light. Phi X-Hae III and lambda-Hind III DNA ladders (Gibco-BRL, Grand Island, NY) were electrophoresed in the gels concurrently to allow size determination of the DNA samples.

III. Results

Immunochemical Detection of NEP--NEP-like activity has been demonstrated in the U266 myeloma line and the Jurkat mature T cell line which lack surface expression of NEP antigen (Goetzl, Kodama, et al., 1989). To determine whether this activity was due to NEP, NEP antigen in these cells was evaluated by flow cytometry and immunofluorescence with and without prior permeabilization to expose intracellular proteins. Both of these techniques detected

NEP in the intracellular compartments of U266 and Jurkat cells, as well as the positive control cells, Nalm 6; however, surface NEP was detected only on Nalm 6 cells.

In the flow cytometry studies, intact or permeabilized cells were incubated with either rabbit polyclonal anti-NEP antibody or J5, a mouse monoclonal antibody to NEP, followed by staining with a rhodamine-labeled anti-rabbit or anti-mouse IgG antibody. Background fluorescence was determined by substituting the anti-NEP antibody with either NRS or PBS in control samples and comparing intensity of fluorescence with that of the corresponding experimental sample (fig. 3a, b). This technique detected NEP in the intracellular compartments but not the surfaces of U266 and Jurkat cells (Table 1).

Direct microscopic visualization of NEP staining by immunofluorescence confirmed the findings (Table 2). In these experiments, intact or permeabilized cells were probed for NEP with 44C10, a mouse monoclonal anti-NEP antibody, as well as the two anti-NEP antibodies used in flow cytometry, and stained with rhodamine-conjugated secondary antibody before microscopic visualization. All three antibodies detected intracellular but not surface NEP of U266 and Jurkat cells. In permeabilized Nalm 6, U266 and Jurkat cells, fluorescent staining was observed throughout the cell, often collected in dense patches. In contrast, permeabilized in HL-60 cells were not stained. Only Nalm 6 cells were stained in intact cell preparations, as

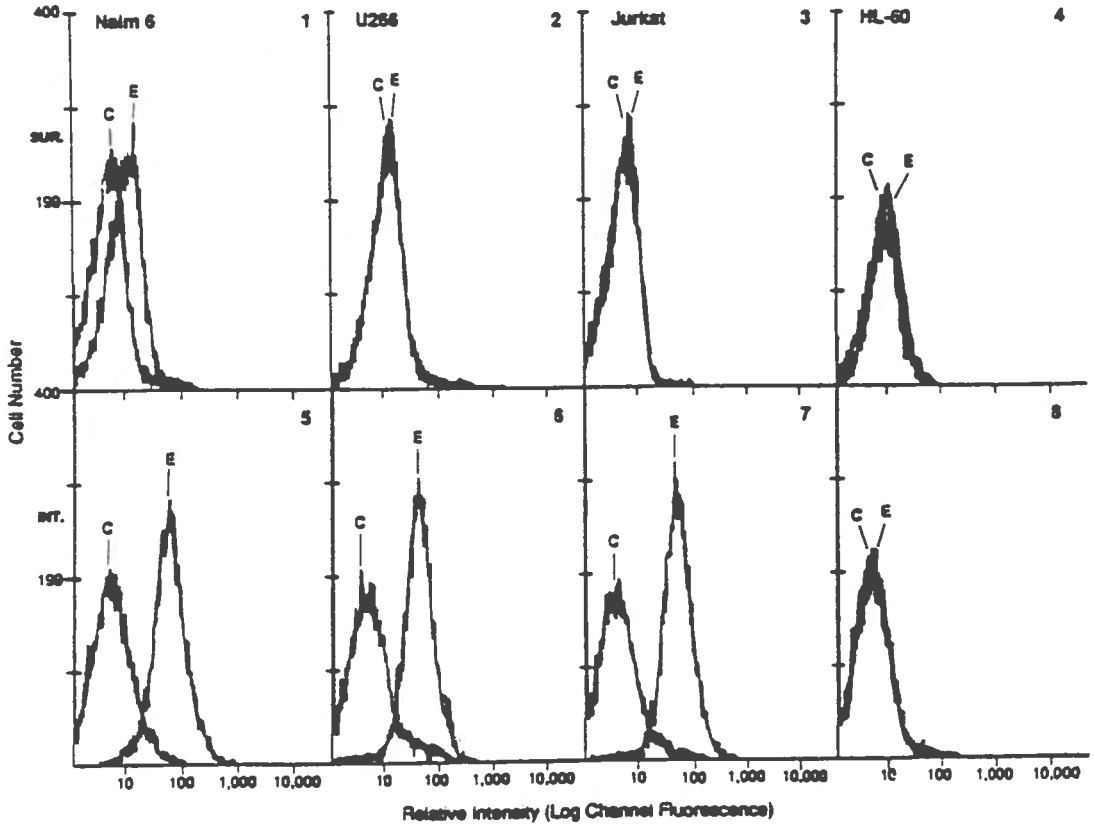

Fig. 3a. Flow cytometric analysis of surface and intracellular NEP using rabbit polyclonal anti-NEP antibody. Experimental curves (E) were generated by first incubating the cells with rabbit anti-NEP polyclonal antibody, then with rhodamine-labeled goat anti-rabbit IgG. Control curves (C) were generated by replacing the rabbit polyclonal anti-NEP antibody with normal rabbit serum. These conditions were applied to intact cells to assess surface NEP and Triton X100 permeabilized cells to assess intracellular NEP.

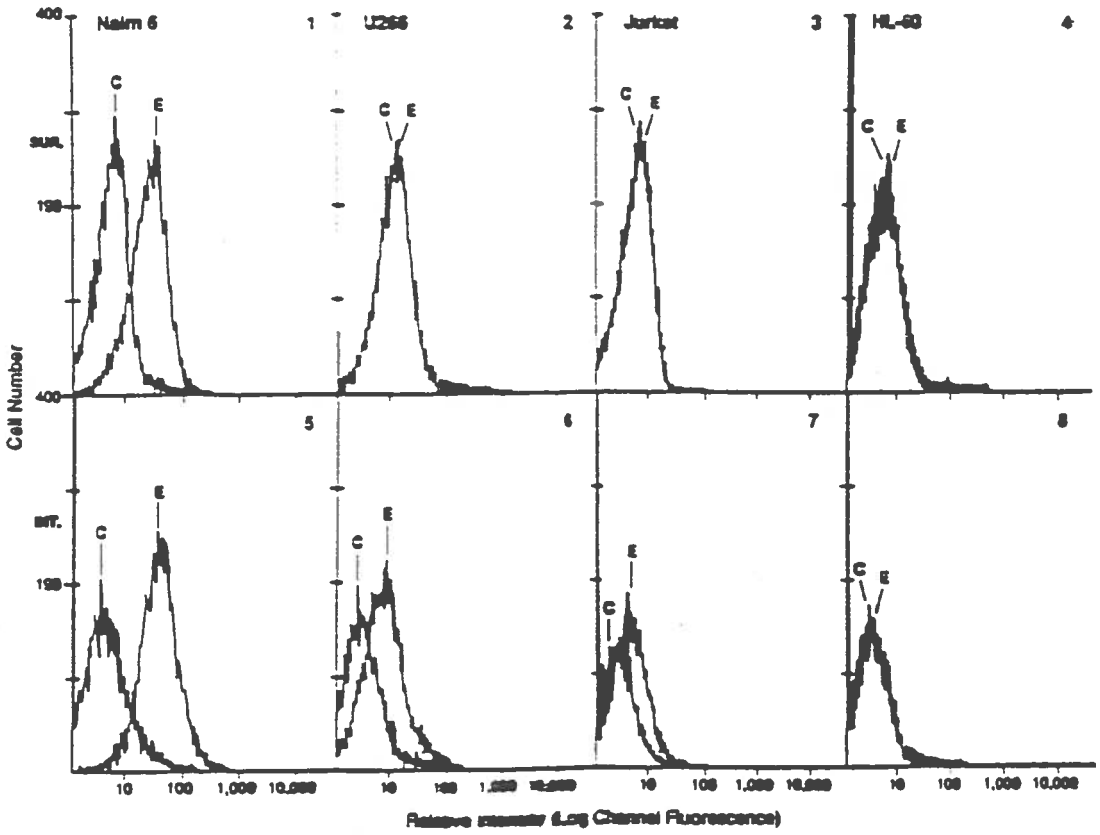

Fig. 3b. Flow cytometric analysis of surface and intracellular NEP using the mouse monoclonal anti-NEP antibody J5. Experimental curves (E) were generated by first incubating the cells with J5, then with rhodamine-labeled goat anti-mouse IgG. Control curves (C) were generated by replacing J5 with PBS. These conditions were applied to intact cells to assess surface NEP and Triton X100 permeabilized cells to assess intracellular NEP.

Table 1. Detection of Surface and Intracellular NEP by Flow Cytometry

Cell line	Surface		Intracellular	
	Rabbit polyclonal anti-NEP	J5	Rabbit polyclonal anti-NEP	J5
Nalm 6	4.5	6.2	9.6	6.6
U266	0.6	1.0	9.3	4.8
Jurkat	0.4	1.8	6.0	2.3
HL-60	1.1	0.3	0.0	-0.4

Data are measured as log channel fluorescence values, which were obtained by subtracting the mean log channel fluorescence generated by the control sample, in which the primary antibody was substituted with either normal rabbit serum (for rabbit polyclonal anti-NEP) or PBS (for J5), from that of its corresponding experimental sample. Each value represents the mean of 2 or 3 experiments.

Table 2. Detection of Surface and Intracellular NEP by Immunofluorescence

Cell line	Surface			Intracellular		
	Rabbit polyclonal anti-NEP	J5	44C10	Rabbit polyclonal anti-NEP	J5	44C10
Nalm 6	+	+	+	+	+	+
U266	0	0	0	+	+	+
Jurkat	0	0	0	+	+	+
HL-60	0	0	0	0	0	0

Positive NEP staining is designated as '+', negative staining as '0'. Three or four experiments were performed per value.

Table 3. Radiometric Assessment of Surface and Intracellular NEP Activity Using the Selective NEP Substrate [tyrosyl-3,5-^{3}H]-D-Ala-Leu-Enkephalin

	Surface		Intracellular	
Cell lines	DL-thiorphan 10 uM	fmol/hr/10^{6}cell	DL-thiorphan 10 uM	fmol/hr/10^{6}cells
Nalm 6	-	60	-	132
	+	37	+	83
U266	-	12	-	199
	+	5	+	52
Jurkat	-	22	-	207
	+	12	+	47
HL-60	-	13	-	55
	+	8	+	1

Surface values were generated by incubating the ^{3}H-Enk with intact cells and resolving the characteristic cleavage fragment by reverse-phase HPLC. Intracellular values were obtained by incubating the substrate with cell lysates. Each value represents the mean of 2 experiments.

visualized by fluorescent staining around the cell periphery.

Cleavage of Synthetic NEP Substrates by Lymphocytes-- After having immunochemically detected intracellular NEP in U266 and Jurkat cells, the cells were examined for surface and intracellular NEP activity by the ability to cleave two selective, synthetic NEP substrates: dansyl-D-Ala-Gly-Phe(pNO_2)-Gly(DAGNPG) and [tyrosyl-3,5-^{3}H]-D-Ala_2-Leu-enkephalin (^{3}H-Enk).

When intact cells were incubated with DAGNPG, only Nalm 6 cells were able to cleave the substrate, as assessed by spectrofluorimetry. However, when ^{3}H-Enk was used as the substrate, NEP activity was detected in intact cells of all cell lines, as assessed by reverse-phase HPLC resolution of the NEP cleavage product from the intact substrate (Table 3). The highest levels were seen in Nalm 6 cells with approximately 3-5 fold lower levels in the other cells. This activity was inhibited by 10 uM DL-thiorphan.

To assess total cell (intracellular and membrane) NEP activity, ^{3}H-Enk was incubated with total cell lysates, prepared in octylglucoside. Total cell NEP activity was markedly greater than surface activity in all cell lines, particularly in U266 and Jurkat (Table 3), suggesting that the intracellular compartment has active NEP. Lysing the cells increased the activity of U266 and Jurkat cells 16 and 9-fold, respectively, to higher levels than that of Nalm 6 cells. This activity was inhibited by 10 uM DL-thiorphan. Thus, U266 and Jurkat cells express intracellular NEP-like

activity.

Detection of NEP Genetic Message by PCR--The above results suggest that NEP is present intracellularly in U266 and Jurkat cells. To further confirm the presence of NEP and to investigate the molecular features of the protein, PCR was used to detect the NEP genetic message in U266 and Jurkat cells, as well as the U266 subclone AF10. Because NEP has been cloned, it was possible to design oligonucleotide primers to specific regions of the gene (fig. 2). Different combinations of primers were employed in PCR reactions to determine whether the message was intact in cDNA prepared from total RNA of the cell lines. After PCR, the samples were run on agarose gels and stained with ethidium bromide to allow visualization of the DNA upon illumination with ultraviolet light.

When primers 1 and 5 were used to amplify the complete coding region of NEP, only Nalm 6 cDNA produced an observable band. However, when the primers were directed downstream from the transmembrane region (2 and 4; 3 and 5), AF10 and Jurkat cDNA, as well as Nalm 6, produced observable bands (fig. 4). Thus, Jurkat and AF10 express at least a partial message for NEP.

IV. Discussion

These results indicate that U266 and Jurkat cells express intracellular, active NEP. For example, both FACS and indirect immunofluorescence detected NEP in the

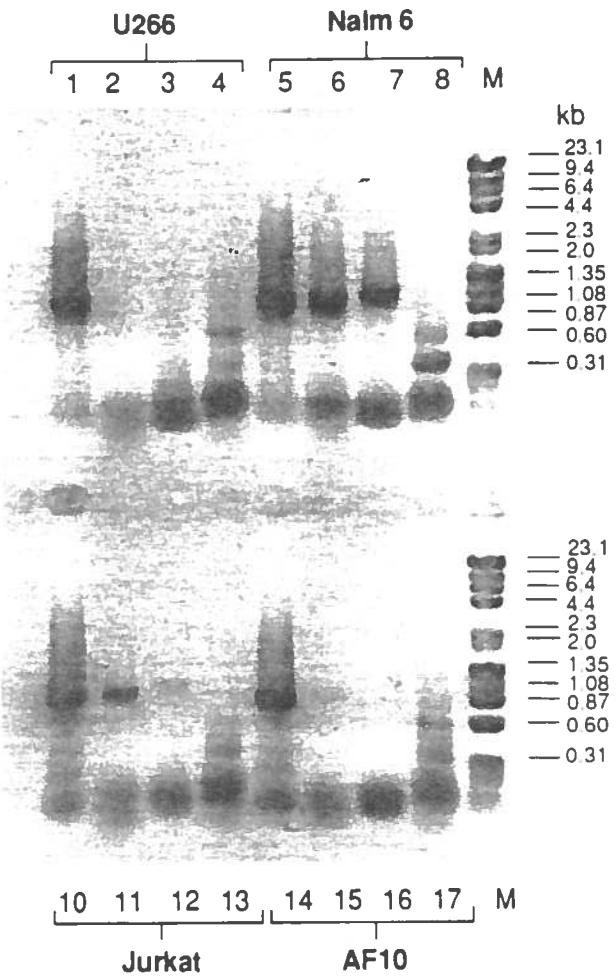

Fig. 4. Detection of NEP Genetic Message by Polymerase Chain Reaction. All lanes in this 1% agarose gel contain samples of PCR reactions that were run under identical conditions (see materials and methods), with the exception of cDNA source and oligonucleotide primers 3 and 5; lanes 3, 7, 12 and 16, NEP primers 2 and 4. As a control for cDNA integrity, actin primers were used in samples in lanes 1, 5, 10 and 14.

cytoplasms but not on the cell membranes of U266 and Jurkat cells. The validity of this finding is supported by the relative absence of fluorescence in all samples where the primary antibody was replaced with either PBS or NRS, suggesting that detection of NEP (which is assessed as intensity of fluorescence) is not due to non-specific binding or trapping of the rhodamine-conjugated secondary antibody in the permeabilized cells. Furthermore, the ability of the techniques to discriminate between the positive-control cells and the negative-control cells (NEP was detected in both the cell surface and cytoplasm of Nalm 6 cells but was completely absent in HL-60) suggests that detection of cytoplasmic NEP in U266 and Jurkat cells was not due to trapping of the secondary antibody or nonspecific binding of the primary antibody to common cellular antigens.

In addition, the consistency in the results produced by the three independently generated antibodies suggests that the detection of NEP was not due to nonspecific binding of the primary antibody. In particular, the finding that two monclonal antibodies, presumably recognizing different epitopes of NEP, produce identical staining patterns is highly suggestive that NEP is present in the cytoplasms of U266 and Jurkat cells.

Furthermore, the manifold difference in the cleavage of ^{3}H-Enk by cell lysates of U266 and Jurkat cells over that of intact cells suggests that NEP is present in the intracellular compartment and that it is active. The low levels of NEP-like activity observed in incubations of the

substrate with intact cells may be due to the presence of lysed cells in the mixture. Or possibly they may have been due to expression of very low amounts of surface NEP, undetectable by fluorescent techniques. Indeed, membrane preparations obtained from cells classified as NEP negative by fluorescent techniques, such as macrophages and mature B chronic lymphoblastic leukemia cells, were able to cleave the ^{3}H-Enk substrate (Beaumont, Brouet, et al., 1989). Sensitivity of the assay may also account for the low levels of NEP activity seen in the HL-60 cells.

Another possibility is that a different enzyme with similar substrate specificity is present, which is able to cleave the substrate. This may account for the relative inability of the DL-thiorphan to completely inhibit the cleavage of the substrate, as indicated by the higher levels of activity of the DL-thiorphan treated intact Nalm 6 preparations compared to that of the HL-60, for example.

However, the PCR findings indicate that NEP is indeed present in Jurkat and the U266 subclone AF10. The fragments which were generated by PCR were appropriately sized, indicating that they represented specifically amplified portions of NEP. Furthermore, the relatively high temperature used for primer annealing (55^{o}C) suggests that the primers specifically bound to NEP message, producing the observed fragments.

With the exception of the U266 line, the PCR findings agree with the other findings described in this thesis.

Interestingly, AF10, a subclone of U266, showed traces of NEP fragment, whereas U266 did not show any evidence of NEP by PCR. FACS and indirect immunofluorescence detected cytoplasmic NEP in both AF10 (data not shown) and U266 cells. Perhaps the discrepancy between the PCR and immunologic studies can be explained by the instability of the NEP transcript relative to the protein. For example, NEP may be transcribed only during certain phases of the cell cycle. Indeed, NEP can be induced in certain NEP-negative cells, such as the chronic myeloid leukemia line K562, by altering the normal cycle of the cell with agents like sodium butyrate (Fraser and Berridge, 1987).

It is possible that the NEP detected by the studies discussed in this thesis represent alternatively spliced NEP message. Indeed, the immunologic studies, the radiometric enzymatic assay and PCR suggest that protein is not intact, that it lacks the transmembrane-signal domain. This is suggested by the absence of immunologically detectable NEP on the surfaces of the U266, AF10 and Jurkat lines, despite the presence of cytoplasmic NEP. The PCR findings also support this finding, as the intact NEP fragment (indeed any fragment that used a primer directed upstream from the transmembrane region) could not be amplified in the Jurkat or AF10 lines, unlike the fragments near the active site. As mentioned above, the transmembrane region is not essential for activity (Lemay, Waksman, et al., 1989). Thus, it is possible that the intracellular NEP activity observed in U266 and Jurkat cells is due to the absence of a

transmembrane-signal domain on an otherwise functional protein.

Without having investigated other mature B and T cell lines, it cannot be determined whether the intracellular NEP detected by the above studies represent normal, physiologically active enzymes or aberrant cleavage products due to relaxed genetic control in the malignant cells. Most of the earlier studies of NEP expression used flow cytometry and immunofluorescence solely to investigate surface expression (LeBien, Kersey, et al., 1982; Greaves, Hariri, et al., 1983; Jongeneel, Quackenbush, et al., 1989). The few studies that could have detected cytoplasmic NEP in mature T cells but did not used Northern Blot analysis of total RNA (Devault, Nault et al., 1988; Shipp, Richardson, et al., 1988); possibly the negative results were due to the inherent instability of RNA and the lower abundance of NEP transcripts in the mature T cells studied, which included the Jurkat line, relative to that of the positive lines. Alternatively, the cytoplasmic NEP detected in this study may be specific to the particular U266 and Jurkat lines employed, which may have become phenotypically different than their parent lines.

To clarify some of the questions raised by the studies, future studies should attempt to clarify the molecular aspects of the intracellular NEP. One approach would be to use PCR to generate oligonucleotide probes to NEP in Jurkat cells which could be used to screen Jurkat cDNA libraries for the intact coding region. The coding region could then

be sequenced and compared with the previously described sequences for NEP. Alternatively, the anti-NEP antibodies that were used in the immunodetection studies described earlier in this thesis could be used to purify the intracellular NEP to obtain partial primary sequence of the protein, which could be compared to previously reported sequences. If significantly different, oligonucleotide probes could be deduced from the sequence and used to screen U266 and Jurkat cDNA libraries. More indirectly, PCR could be used to clone the entire coding region of NEP with the exception of the transmembrane-signal region, which could be spliced into a eucaryotic expression vector and transfected into COS-1 cells. These transfected COS-1 cells could then be analyzed for intracellular NEP by immunodetection and the ability to cleave synthetic NEP substrates.

Until further studies such as the ones mentioned above are performed, the physiologic relevance of the findings discussed in this thesis will remain unclear. At present, however, it seems reasonable to consider that the findings of this study may have some implications in the diagnoses of cancer, apart from any physiologic role of intracellular NEP. Indeed, NEP was used as an important diagnostic marker for ALL years before it was identified as a functional enzyme (an enzyme still without a known physiologic role). Perhaps future studies employing the Triton X100 permeabilizing step to other cancerous cells will discover a similar diagnostic use for cytoplasmic NEP--or other cell markers.

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