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CD10/neutral endopeptidase 24.11 hydrolyzes bombesin-like peptides and regulates the growth of small cell carcinomas of the lung

(metalloendopeptidase/autocrine growth factors/cell surface enzyme/common acute lymphoblastic leukemia antigen)

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ABSTRACT **Bombesin-like peptides are essential autocrine growth factors for many small cell carcinomas (SCCas) of the lung. Herein, we demonstrate that these malignant pulmonary neuroendocrine cells express low levels of the cell surface metalloendopeptidase CD10/neutral endopeptidase 24.11 (CD10/NEP, common acute lymphoblastic leukemia antigen) and that this enzyme hydrolyzes bombesin-like peptides. The growth of bombesin-like peptide-dependent SCCas is inhibited by CD10/NEP and potentiated by CD10/NEP inhibition. The results provide evidence that CD10/NEP is involved in the regulation of tumor cell proliferation. Since SCCa of the lung occurs almost exclusively in cigarette smokers and cigarette smoke inactivates CD10/NEP, decreased cell surface CD10/NEP enzymatic activity may be causally related to the development of SCCa of the lung.**

Molecular cloning and expression studies have shown that CD10 (common acute lymphoblastic leukemia antigen) is the zinc metalloprotease neutral endopeptidase 24.11 (NEP, "enkephalinase," membrane metalloendopeptidase) (1–3). This cell membrane-associated enzyme cleaves peptide bonds on the amino side of hydrophobic amino acids and hydrolyzes a number of naturally occurring peptides including the endogenous opioid pentapeptides Met- and Leu-enkephalin, substance P, neurotensin, oxytocin, bradykinin, angiotensins 1 and 2, atrial natriuretic factor, endothelin, and the chemotactic peptide fMet-Leu-Phe (2). CD10/NEP, which is expressed on normal lymphoid progenitors, mature polymorphonuclear leukocytes, and a variety of nonhematopoietic cell types (4–6), functions in multiple organ systems to down-regulate induced responses to peptide hormones. For example, Met-enkephalin triggers inflammatory responses by inducing morphological changes, directed migration, and aggregation of CD10/NEP$^+$ neutrophils; inhibition of CD10/NEP enzymatic activity reduces the amount of Met-enkephalin required for neutrophil activation by several orders of magnitude (7, 8). CD10/NEP, which is expressed at high levels in the lung (9), similarly regulates responses to substance P, the primary mediator of neurogenic inflammation of the respiratory tract (10). Stimulation of afferent nerves in the bronchial mucosa triggers substance P release; resulting substance P-mediated responses include increased vascular permeability, neutrophil migration, bronchospasm, and cough (10). Inhibition of CD10/NEP dramatically increases the binding of substance P to bronchial membranes and the resulting physiological effects (11–14).

The bombesin-like peptides (BLPs) are another family of peptides that regulate pulmonary epithelial, mesenchymal, and neuroendocrine cell responses (15, 16). BLPs include the amphibian peptide bombesin and the mammalian counterparts, gastrin-releasing peptide [GRP-(1–27)] and neuromedin C [GRP-(18–27)]; these peptides share a 7-amino acid C terminus that is essential for receptor binding and biological activity (Table 1) (15, 16). BLPs are potent mitogens for normal bronchial epithelial (17) and neuroendocrine cells and fibroblasts (18, 19). These peptides also stimulate the growth of many small cell carcinomas (SCCas) of the lung (20), which are thought to result from neoplastic transformation of neuroendocrine cells in the bronchial epithelial cell layer (21). In these SCCas of the lung, an autocrine loop exists whereby tumor cells secrete BLP, express BLP receptors, and respond to BLP stimulation with increased proliferation (20). Antibodies to BLP completely inhibit SCCa colony formation and tumorigenesis in nude mice, indicating that the bombesin autocrine loop is essential for the growth of these cells (20). Clinical studies suggest that BLP may also regulate SCCa growth in patients. The levels of circulating BLP detected with a sensitive radioimmunoassay correlate with tumor burden in patients with SCCa of the lung and the levels of BLP detected in cerebrospinal fluid accurately predict leptomeningeal involvement with SCCa (22, 23).

Increased BLP production may be an early consequence of cigarette smoking (24). Asymptomatic cigarette smokers have increased levels of BLP in their bronchioalveolar lavage fluid and intense specific staining of neuroendocrine cells with anti-BLP reagents (24). Similarly, hamsters exposed to cigarette smoke develop increased levels of pulmonary BLP and neuroendocrine cell hyperplasia (25). That cigarette smokers have bombesin-related abnormalities of neuroendocrine cells is of particular interest because virtually all (>98%) SCCa patients have a history of heavy cigarette smoking (26). Recent studies indicate that cigarette smoke inactivates bronchial epithelial cell surface CD10/NEP and that the damage to the enzyme is mediated by free radicals (27). The fact that CD10/NEP regulates pulmonary responses to other peptide hormones and that the enzyme is decreased in cigarette smokers (27) who have increased levels of BLP prompted us to determine whether BLPs are CD10/NEP substrates and whether CD10/NEP regulates the growth of SCCa of the lung.

MATERIALS AND METHODS

Hydrolysis of BLP and K_i Determination. Bombesin, GRP, GRP-(14–27), and [$^{13}\psi^{14}$,CH_2NH]bombesin (bombesin in

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Abbreviations: SCCa, small cell carcinoma; NEP, neutral endopeptidase 24.11; BLP, bombesin-like peptide; GRP, gastrin-releasing peptide; mAb, monoclonal antibody.

Table 1. CD10/NEP hydrolyzes BLPs

BLP	Cleavage sites	K_i, μM	K_{cat}, min^{-1}
Bombesin	p-Ė(1) Q R L G N Q Ẇ(8) ↑ A V G H ↑ L Ṁ(14)-amide	117 ± 12	70
GRP	...Ṁ(14) Y P R G N H Ẇ(21) ↑ A V G H ↑ L Ṁ(27)-amide	34 ± 3	2
GRP-(14–27)	Ṁ(14) Y P R G N H Ẇ(21) ↑ A V G H ↑ L Ṁ(27)-amide	18 ± 3	35

Bombesin, GRP-(1–27), and GRP-(14–27) were hydrolyzed by CD10/NEP at the indicated alanine and leucine residues (↑) within the conserved 7-amino acid C terminus required for biologic activity (boldface letters). The K_i values of bombesin, GRP, and GRP-(14–27) were determined by using the BLP as alternate substrate inhibitors of a reference peptide (dansyl-D-Ala-Gly-[NO_2]-Phe-Gly). Estimates for V_{max} based on the HPLC analyses and utilized to calculate K_{cat} values were 777.4, 22.8, and 388.6 nmol per min per mg of enzyme for bombesin, GRP, and GRP-(14–27), respectively.

which the peptide bond between residues 13 and 14 has been replaced with CH_2NH) were obtained from Peninsula Laboratories and CD10/NEP was purified as described (28). The sites of cleavage were determined by incubation of 400 pmol of peptide with 1 pmol of CD10/NEP in 100 μl of 20 mM Mops (pH 6.5) at 37°C for 60 min. Reaction products were subsequently analyzed on a Hewlett–Packard HPLC equipped with a 4.6 × 250 mm Brownlee RP300 column eluted at 1 ml/min with a 3%/min gradient of 0.1% aqueous trifluoroacetic acid and 0.1% trifluoroacetic acid in acetonitrile by using a diode array UV detector and a fluorescence detector. Appropriate peaks were collected for subsequent amino acid analysis using phenyl isothiocyanate as described (29). K_i values were obtained from Dixon plots using dansyl-D-Ala-Gly-[NO_2]-Phe-Gly as substrate at a fixed concentration of 25 μM and an inhibitor range of 0–100 μM for GRP and GRP-(14–27) and 0–400 μM for bombesin. Data were fit to a weighted least squares program of Cleland (30). K_i values were calculated from the following relationship: $K_i = K_i(obs) + K_i(1 + [S]/K_m)$, where $K_i(obs)$ is the experimentally observed value and $[S]$ is the substrate concentration (25 μM). K_m was independently determined to be 87 μM.

Immunohistochemical Analysis of CD10/NEP. Immunohistochemical staining of serial sections of fetal lung was performed as described (31) using a rabbit anti-bombesin antiserum (31), an unreactive control monoclonal antibody (mAb, MOPC 21; Sigma), and the anti-CD10 mAb J5 (4). The specificity of J5 for CD10/NEP was confirmed by preincubating the mAb (2 ml of a 1:500 dilution of malignant ascites fluid) with purified CD10/NEP (100 μg) prior to immunostaining certain slides.

CD10/NEP Enzymatic Activity. Whole-cell suspensions of the SCCa cell lines NCI-H345, H209, H146, H82, and H69; the $CD10^+$ acute lymphoblastic leukemia cell lines Nalm6 and Laz 221; and the CD10 T-cell leukemia cell line J77 were evaluated for cell surface CD10/NEP enzymatic activity by using a fluorometric assay (2). SCCa cell lines were obtained from A. Gazdar (National Cancer Institute, Bethesda, MD).

Analysis of SCCa Proliferation. SCCa colony-forming assays were performed as described (20) using 5×10^4 cells per 30-mm well in serum-free Hites medium containing no additives, 10% (vol/vol) fetal calf serum, 10 or 50 μM phosphoramidon, 50 nM bombesin, 50 nM [$^{13}\psi^{14}$,CH_2NH]bombesin, or 50 nM [$^{13}\psi^{14}$,CH_2NH]bombesin and 50 nM bombesin or 50 μM phosphoramidon. In NCI H146 and NCI H345 colony-forming assays, 0.1% bovine serum albumin was added to the top agar to enhance cloning efficiency as described (20). To enumerate soft agar colonies, agar samples were dehydrated, methanol-fixed, and stained with Gill's hematoxylin solution.

SCCa cell lines were also cultured for 48 hr in serum-free Hites medium (20) with no addition or 200 μM GRP-(14–27) and the indicated amounts of purified soluble CD10/NEP that was enzymatically active or boiled to destroy enzymatic activity. [^{3}H]Thymidine incorporation was analyzed thereafter.

For coculture experiments, murine L cells were transfected with pZipneo (32) or pZipneo CD10/NEP, selected for G418 resistance, and analyzed for CD10/NEP enzymatic activity. Irradiated L cells were plated at 20,000 cells per well in 96-well plates and NCI H345 cells (10,000 cells per well) were added to the L-cell monolayer 24 hr later in serum-free Hites medium supplemented with [^{3}H]thymidine. Thymidine incorporation was determined 16 hr thereafter.

RESULTS AND DISCUSSION

BLP, bombesin, GRP, and GRP-(14–27) were each incubated with purified CD10/NEP enzyme (28) under conditions determined to be optimal for hydrolysis of known CD10/NEP substrates. HPLC analysis of the reaction products indicated that bombesin, GRP, and GRP-(14–27) were each efficiently hydrolyzed by CD10/NEP. The amino acid composition of the respective peptide fragments from each reaction mixture identified as CD10/NEP cleavage sites the Trp-Ala and His-Leu peptide bonds in the conserved 7-amino acid C terminus region required for BLP biologic activity (Table 1). Although each BLP was efficiently hydrolyzed by CD10/NEP, GRP-(14–27) was inactivated by 10 times less enzyme than GRP or bombesin (data not shown). These results are of interest because GRP-(14–27) is as potent a mitogen as GRP-(1–27) at 10 times lower molar concentrations (15) and a shorter GRP fragment [GRP-(18–27), neuromedin C (33–35)] mediates pulmonary BLP effects. The K_i values of GRP-(14–27), GRP-(1–27), and bombesin were determined by using these peptides as inhibitors of CD10/NEP hydrolysis of a reference peptide (dansyl-D-Ala-Gly-[NO_2]-Phe-Gly) (30) (Table 1). The observed 18 ± 3 to 117 ± 2 μM K_i values (Table 1) are comparable to the 20–80 μM K_i values of other peptides such as Met-enkephalin and Leu-enkephalin that are efficiently hydrolyzed by CD10/NEP. Estimates for K_{cat} for each BLP based on the HPLC analyses are also included in Table 1.

In other cellular systems in which CD10/NEP has been shown to have a physiological role, the cells that respond to a CD10/NEP peptide substrate express both the peptide receptor and the cell surface enzyme. Since BLPs are CD10/NEP substrates and pulmonary neuroendocrine cells produce and respond to BLP, we evaluated these cells for CD10/NEP expression using an anti-CD10 mAb, J5. As shown in Fig. 1 *a* and *e*, CD10/NEP is expressed by epithelial cells in both small and large airways in fetal lung. The specificity of J5 immunoreactivity for CD10/NEP was determined by examining the staining pattern of J5 mAb preincubated with purified CD10/NEP prior to immunostaining (Fig. 1*b*) and by comparing staining of the same tissue with an irrelevant control mAb (Fig. 1*c*). Pulmonary neuroendocrine cells were identified in thin serial sections from fetal lung by location, morphology, and expression of BLP (Fig. 1*d*). Neuroendocrine cells that were present in serial sections of the same fetal lung (Fig. 1 *d* and *e*) expressed CD10/NEP as did additional cells within the bronchial epithelial cell layer

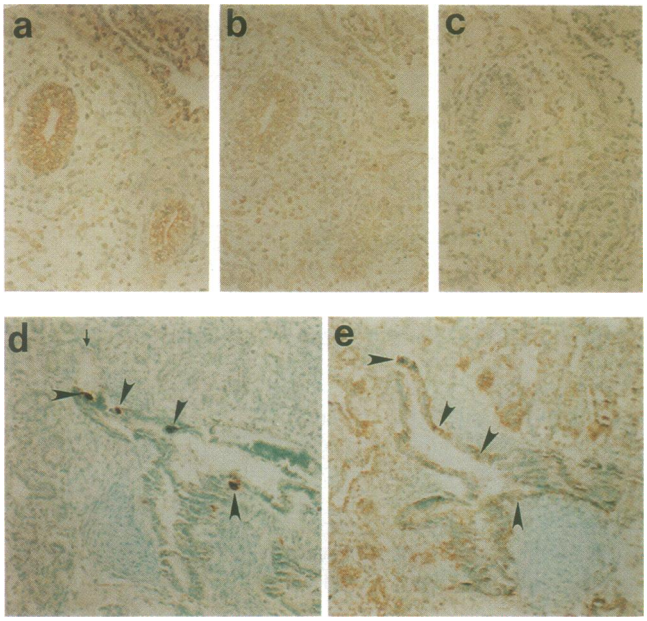

FIG. 1. Immunohistochemical analysis of CD10/NEP in fetal lung. Fetal lung at 12 weeks of gestation (*a–c*) and at 18 weeks of gestation (*d* and *e*) was stained with the anti-CD10 mAb J5 (*a* and *e*), J5 preincubated with CD10/NEP (*b*), the unreactive control mAb MOPC 21 (*c*), or the polyclonal rabbit anti-bombesin antiserum (*d*). Neuroendocrine cells within the bronchial epithelial cell layer are indicated with large arrowheads (*d*). CD10/NEP immunoreactivity in corresponding cells from a serial section is also indicated with large arrowheads (*e*). Small arrow in *d* denotes a region of epithelial compression in this paraffin section.

(Fig. 1 *a* and *e* and ref. 9). Similar results were obtained in adult lung (data not shown).

The relationship of BLP secretion, CD10/NEP expression, and growth of malignant neuroendocrine cells was further analyzed using a panel of SCCa cell lines [NCI H345, H209, H146, H82, and H69 (20)], reported to have variable levels of BLP secretion and BLP receptor binding (20, 36, 37). Previous studies indicated that NCI H209 secretes high, H345 and H69 secrete intermediate, H146 secretes low, and H82 secretes undetectable levels of BLP (20) and that NCI H345 expresses higher numbers of BLP receptors than the other SCCa cell lines (37). In previous studies, SCCa cell lines including NCI H345, H209, H146, and H69 responded to exogenous and autocrine BLP stimulation with increased thymidine incorporation and colony formation in soft agar and increased tumor growth in a nude mouse model (20, 38, 39). We analyzed GRP transcripts in these SCCa cell lines by performing reverse PCR using oligonucleotide probes derived from the human GRP cDNA sequence {5′ sense [base pairs (bp) 125–150] and 3′ antisense (bp 492–497)} (33). Reverse PCR products from four of the five SCCa cell lines contained the appropriately sized 372-bp GRP cDNA fragment (data not shown). The only cell line that did not have detectable GRP transcripts (NCI H82) was one that had been unresponsive to BLP in earlier functional assays (20).

The SCCa lines were then evaluated for cell surface CD10/NEP enzymatic activity using a previously described sensitive fluorometric assay (Table 2) (2). All five SCCa lines had low but detectable levels of CD10/NEP enzymatic activity (123–906 nmol per hr per 10^6 cells) that were less than 3–30% of the levels of known CD10/NEP$^+$ controls (Laz 221 and Nalm-6) (Table 2). In contrast, CD10/NEP$^-$ cells (such as J77) had virtually no detectable CD10/NEP enzymatic activity (<17 nmol per hr per 10^6 cells). We also confirmed that the SCCa cell lines transcribed CD10/NEP by performing reverse PCR using oligonucleotide probes derived from the human CD10/NEP sequence [5′ sense (bp 1200–1224) and 3′ antisense (bp 1586–1611)] (1). Reverse PCR products from each of the five SCCa cell lines contained the appropriately sized 411-bp CD10/NEP cDNA fragment (data not shown). Four of the SCCa cell lines (NCI H209, H345, H146, and H69) that had detectable GRP transcripts, cell surface CD10/NEP enzymatic activity, and BLP responsiveness in *in vitro* assays were chosen for further analysis.

Table 2. CD10/NEP enzymatic activity in SCCa cell lines

Cell line	CD10/NEP enzymatic activity, nmol per hr per 10^6 cells
NCI H345	335
NCI H209	906
NCI H146	257
NCI H82	123
NCI H69	559
Nalm-6	>4000
Laz 221	3136
J77	17

Values represent the mean of triplicate samples of phosphoramidon-inhibitable CD10/NEP enzymatic activity.

The most sensitive assay for analyzing the effects of BLP on SCCa cell growth is colony formation in soft agar (20, 39). Colony formation by SCCa cells is markedly enhanced by the addition of exogenous BLP (refs. 20 and 39 and Fig. 2) and inhibited by the addition of anti-bombesin mAbs (20). As indicated in Fig. 2, SCCa colony formation was significantly increased when cells were plated with the CD10/NEP inhibitor phosphoramidon. In a representative experiment, NCI H209 cells plated without additives formed 34 colonies of 30–100 cells per colony and 1 colony of >100 cells, whereas NCI H209 cells plated with phosphoramidon formed 114 colonies of 30–100 cells per colony and 41 colonies of >100 cells per colony ($P = 0.002$ and $P < 0.001$, respectively) (Fig. 2). There was also increased colony formation when the other SCCa cell lines (NCI 345, H69, and H146) were plated in the presence of phosphoramidon. In a representative experiment, inhibition of NCI H146 CD10/NEP increased the number of colonies containing 20–30 cells and containing 30–100 cells by factors of 4.6 ($P = 0.005$) and 4.3 ($P = 0.003$), respectively (Fig. 2). Representative NCI H209 colonies formed in the presence or absence of phosphoramidon are shown in Fig. 3. SCCa cell lines were also cultured with one of two additional chemically related enzymatic inhibitors,

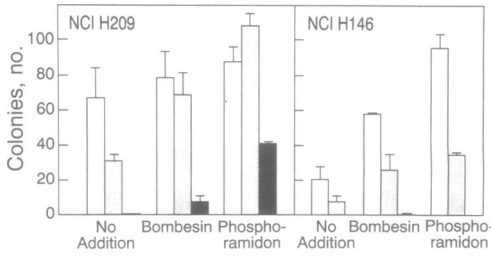

FIG. 2. SCCa colony formation in soft agar is potentiated by CD10/NEP inhibition. The numbers of SCCa colonies obtained when NCI H209 or NCI H146 cells were incubated without additives, with 50 nM bombesin, or with 10 μM phosphoramidon are shown. Colony sizes are as follows: 20–30 cells, open bars; 30–100 cells, stippled bars; >100 cells, solid bars. Values shown are the mean ± SD of duplicate samples in a representative experiment. The differences in NCI H209 and NCI H146 colony formation in the presence or absence of phosphoramidon were evaluated using a one-sided Student's *t* test. For NCI H209, no addition vs. phosphoramidon, 20- to 30-cell colonies, $P = 0.137$; 30- to 100-cell colonies, $P = 0.002$; >100-cell colonies, $P = <0.001$. For NCI H146, no addition vs. phosphoramidon, 20- to 30-cell colonies, $P = 0.005$; 30- to 100-cell colonies, $P = 0.003$.

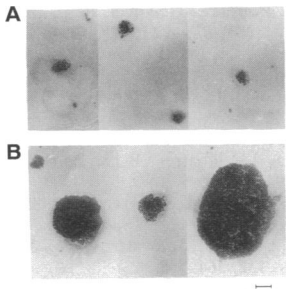

FIG. 3. SCCa (NCI H209) colonies in soft agar obtained in the absence (*A*) or presence (*B*) of phosphoramidon. Colonies were photographed using a Zeiss Axiophot with a ×20 objective. (Bar = 50 μM.)

thiorphan [*N*-(DL-2-benzyl-3-mercaptopropionyl)glycine (40)], which inhibits both CD10/NEP and peptidyl dipeptidase, or captopril [D-3-mercapto-2-methylpropanyl-L-proline (40)], which inhibits peptidyl dipeptidase but is inactive against CD10/NEP (40). Thiorphan increased SCCa proliferation whereas captopril was without effect (data not shown), strongly suggesting that the potentiation of SCCa cell growth was a specific consequence of the inhibition of cell surface CD10/NEP.

Indirect evidence that CD10/NEP functions primarily by modulating BLP levels was obtained by treating SCCa cells with phosphoramidon in the presence or absence of the bombesin receptor antagonist [$^{13}\psi^{14}$,CH_2NH]bombesin (41). As shown in Table 3, this bombesin receptor antagonist inhibits NCI H345 SCCa colony formation resulting from the addition of either phosphoramidon or bombesin.

Since BLPs are CD10/NEP substrates and SCCa cells secrete the autocrine growth factors and express low levels of the cell surface enzyme, we next evaluated whether exogenously added CD10/NEP inhibited SCCa cell growth. SCCa cell lines were cultured in the presence of specific concentrations of purified soluble CD10/NEP (28) that was enzymatically active or previously boiled to destroy enzymatic activity and [^{3}H]thymidine incorporation of cells cultured with active CD10/NEP was compared to that of cells cultured with inactive (boiled) CD10/NEP. The percent reduction in thymidine incorporation that resulted from the addition of active enzyme in a representative experiment is shown in Fig. 4*A*. As indicated, soluble active CD10/NEP enzyme inhibited thymidine incorporation of unstimulated or BLP-stimulated SCCa cells in a dose-dependent manner.

Since CD10/NEP normally functions as a membrane-bound cell surface endopeptidase, we next determined the effects of cell surface CD10/NEP enzyme on SCCa growth. It was not possible to directly transfect SCCa cells with CD10/NEP. For this reason, SCCa cells were cocultured with irradiated murine L cells transfected with CD10/NEP or vector alone and analyzed for [^{3}H]thymidine incorporation. As L cells are thymidine kinase-negative, the transfected adherent L cells themselves incorporated low levels of exogenous thymidine (Fig. 4*B*). L cells transfected with vector alone had virtually undetectable CD10/NEP enzymatic activity (<25 nmol per 10^6 cells per hr) whereas L cells transfected with CD10/NEP had ≈1360 nmol per hr per 10^6 cells of cell surface enzyme. In a representative experiment, NCI H345 cells cocultured with the CD10/NEP$^+$ L-cell transfectants incorporated ≈60% less thymidine than NCI H345 cells cocultured with vector-only L-cell transfectants (P = 0.018) (Fig. 4*B*).

Table 3. Bombesin receptor antagonist inhibits phosphoramidon effects on SCCa colony formation

Addition	Number of colonies	% change
None	16.5 ± 1.5	—
[$^{13}\psi^{14}$,CH_2NH]bombesin	20 ± 5	21
Bombesin	125.5 ± 2.5	660
Bombesin + [$^{13}\psi^{14}$,CH_2NH]bombesin	10.5 ± 0.5	36
Phosphoramidon	119.5 ± 9.5	624
Phosphoramidon + [$^{13}\psi^{14}$,CH_2NH]bombesin	21 ± 3	27

Number of colonies (>20 cells per colony) obtained when NCI H209 cells were incubated without addition, with [$^{13}\psi^{14}$,CH_2NH]bombesin at 50 nM, bombesin at 50 nM, bombesin + [$^{13}\psi^{14}$,CH_2NH]bombesin, phosphoramidon at 50 μM, or phosphoramidon + [$^{13}\psi^{14}$,CH_2NH]-bombesin are shown as are percentage changes in number of colonies formed.

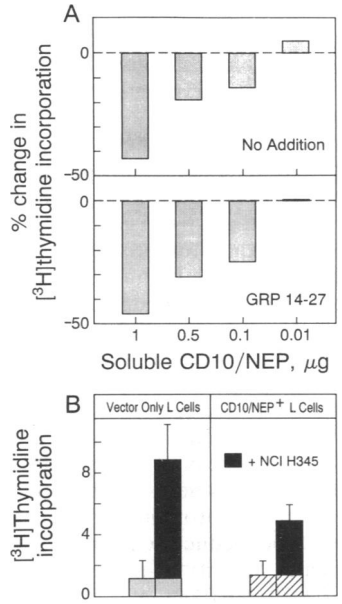

FIG. 4. CD10/NEP inhibits the thymidine incorporation of SCCa cell lines. (*A*) NCI H345-cell [^{3}H]thymidine incorporation after the addition of soluble CD10/NEP. Purified soluble CD10/NEP (28) that was enzymatically active or boiled to destroy enzymatic activity was added at the indicated concentrations to unstimulated or GRP-(14–27)-stimulated NCI H345 cells. Thymidine incorporation of NCI H345 cells cultured with active CD10/NEP was compared to that of NCI H345 cells cultured with inactive (boiled) CD10/NEP (one-sided Student's *t* test) and the percent reduction in thymidine incorporation resulting from the addition of active CD10/NEP [1 μg = 5239 nmol/hr (enzymatic activity)] is displayed. Actual values for [^{3}H]thymidine incorporation (×10^{-4}) of NCI H345 cultured with given concentrations (μg per well) of active or boiled enzyme (active/boiled) are as follows: For no additives, 1 μg (4.67 ± 1.09/8.13 ± 0.6, P = 0.004), 0.5 μg (5.69 ± 0.4/7.04 ± 0.5, P = 0.01), 0.1 μg (5.55 ± 0.15/6.47 ± 0.83, P not done), and 0.01 μg (7.3 ± 0.35/6.93 ± 0.62, P not done). For GRP-(14–27), 1 μg (5.51 ± 0.23/10.22 ± 0.26, P < 0.001), 0.5 μg (6.69 ± 0.49/9.74 ± 0.71, P = 0.001), 0.1 μg (7.15 ± 0.57/9.49 ± 0.13, P not done), and 0.01 μg (9.06 ± 1.15/9.01 ± 0.92, P not done). Values are the mean ± SD of triplicate samples in a representative experiment. (*B*) Coculture of NCI H345 cells with CD10/NEP$^+$ L cells. [^{3}H]Thymidine incorporation (cpm × 10^{-3}) of L-cell-vector-only transfectants (stippled bar), L-cell-vector-only transfectants plus NCI H345 cells (solid-above-stippled bar), L-cell CD10/NEP transfectants (hatched bar), and L-cell CD10/NEP transfectants plus NCI H345 cells (solid-above-hatched bar) are shown. Values are the mean ± SD of triplicate samples in a representative experiment. The differences in the [^{3}H]thymidine incorporation of NCI H345 cells cultured in the presence of L cells transfected with vector only vs. L cells transfected with CD10/NEP were determined using a one-sided Student's *t* test (P = 0.018).

In earlier studies, a phosphoramidon inhibitable membrane bound peptidase from gastric tissue with characteristics of NEP was reported to cleave a dipeptide from neuromedin C [GRP-(18–27)] after prolonged incubation (42, 43). We find that CD10/NEP rapidly inactivates each of the BLP family

members by cleaving the peptides at two sites within the 7-amino acid conserved C terminus required for biological activity and that growth of BLP-dependent SCCa cell lines is potentiated by CD10/NEP inactivation and inhibited by CD10/NEP addition.

One mechanism for reducing cell surface CD10/NEP activity is free-radical-mediated damage of the enzyme by cigarette smoke. Recent reports suggest that CD10/NEP enzymatic activity in the lung may also be reduced by two other mechanisms. An alternatively spliced CD10/NEP cDNA that encodes enzyme with <4% wild-type enzymatic activity has recently been isolated from a human lung cancer cDNA library (C. Gerard, personal communication). Therefore, it is possible that, in certain lung cancers, reduced cell surface CD10/NEP enzymatic activity and unopposed BLP stimulation result from expression of a different CD10/NEP gene product. Whether such a product results from a mutation in the CD10/NEP gene or other unidentified mechanisms is unknown. Additional studies demonstrate that phorbol ester treatment of human bronchial epithelial cells and other CD10/NEP$^+$ cell types reduces CD10/NEP transcripts, protein, and enzymatic activity whereas glucocorticoid treatment increases CD10/NEP (44, 45). These data suggest that inflammatory stimuli may reduce CD10/NEP and thereby increase BLP levels. That certain fibrotic lung diseases are associated with neuroendocrine cell hyperplasia and increased BLP secretion is consistent with this hypothesis (16, 19). Earlier studies prompted by the mitogenic effects of BLP on SCCa of the lung indicated that these peptides also increased the clonal growth rate and colony-forming efficiency of bronchial epithelial cells (17). Therefore, reductions in bronchial epithelial cell surface CD10/NEP resulting from cigarette smoke, genetic alterations, or chronic inflammation may also contribute to the dysregulated BLP-mediated growth of non-small cell lung cancers.

BLP are potent mitogens for multiple normal CD10/NEP$^+$ cell types, implicating the enzyme in the regulation of normal cell growth. For this reason, data associating the levels of bronchial epithelial cell surface CD10/NEP enzymatic activity with cellular proliferation (44) are of particular interest. Increased CD10/NEP cell surface enzymatic activity may actually limit normal cell growth by controlling local concentrations of mitogenic peptides such as BLP that are CD10/NEP substrates. BLP, which are increased during the canalicular phase of fetal lung development, stimulate both the growth and maturation of this developing fetal organ (46, 47). Therefore, it will be of interest to determine whether CD10/NEP also plays a role in normal fetal lung development.

Our data suggest that an important mechanism for controlling cell growth is the catabolism of essential autocrine growth factors by cell surface enzymes such as CD10/NEP. CD10/NEP limits the proliferation of malignant pulmonary neuroendocrine cells that produce and respond to BLP. Therefore, in this context, the enzyme functions as a tumor suppressor, the loss of which may facilitate the development of small cell carcinoma of the lung.

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