Prohormone convertase and autocrine growth factor mRNAs are coexpressed in small cell lung carcinoma

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ABSTRACT

A hallmark of small cell lung carcinoma (SCLC) is the expression of autocrine growth factors such as neurotensin and gastrin-releasing peptide, which bind to cellular receptors and stimulate cell division. The biological activity of autocrine growth factors requires the concurrent expression of prohormone convertases that cleave the growth factors to their active form, suggesting the expression of these genes is linked in SCLCs. RNase protection assays were used to detect the expression of autocrine growth factor and prohormone convertase mRNAs in a panel of lung cancer cell lines. These mRNAs

are coexpressed in SCLC and lung carcinoid cell lines, but not in normal lung epithelium or in non-small cell lung cancers. These findings, together with earlier results from our laboratory, suggest the expression of prohormone convertases has an important role in the development and maintenance of the SCLC phenotype and that autocrine growth factor and prohormone convertase genes respond to a common transcriptional activator in SCLC.

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INTRODUCTION

A hallmark of small cell lung carcinoma (SCLC) is the expression of autocrine growth factors such as β -endorphin (Davis *et al.* 1989), neurotensin (Davis *et al.* 1991), insulin-like growth factors (Nakanishi *et al.* 1988), and gastrin-releasing peptide (GRP; Cuttitta *et al.* 1985, Moody *et al.* 1981), which bind to cellular receptors and stimulate cell division. These peptide hormones are initially synthesized as biologically inert prohormones which are subsequently cleaved to their active form by a family of enzymes called the prohormone convertases. Prohormone convertases are endoproteases that cleave proproteins and prohormones at specific pairs of basic amino acids (Barr 1991, Steiner *et al.* 1992). Seven mammalian prohormone convertases have been identified and categorized according to tissue distribution. Among these, PC1 (also known as PC3) and PC2, are expressed exclusively in endocrine and neuroendocrine tissues, where they process precursor proteins that are in transit through regulated secretory pathways (Smeekens *et al.* 1991, Day *et al.* 1992, Seidah *et al.* 1993). Recent studies indicate that pro-neurotensin is processed by PC1 in intestinal cells and by PC2 in

neuronal cells (Rovère *et al.* 1996). The prohormone convertase that processes pro-GRP has not been identified, however, pro-GRP is cleaved at a dibasic site (Lys-Lys) which is consistent with its processing by either PC1 or PC2.

In addition to the prohormone convertases, the biological activity of an autocrine growth factor requires the concurrent activity of several other gene products, including the amidating enzyme peptidylglycine α -amidating monooxygenase (Saldise *et al.* 1996), the molecular chaperone 7B2 (Braks & Martens 1995, Zhu & Lindberg 1995), and growth factor receptors, suggesting that the expression of these functionally related genes is linked. The broad goal of our research is to elucidate the mechanisms that regulate the expression of autocrine growth factors in neuroendocrine tumors. Our initial step has been to establish whether the genes for autocrine growth factors and the prohormone convertases that process them are coordinately transcribed in SCLCs. To this end, we screened a panel of human lung cancer cell lines for the expression of two autocrine growth factors (neurotensin and gastrin-releasing peptide) and two neuroendocrine-specific processing enzymes (PC1 and PC2). It is hoped that an improved

understanding of the cellular factors and mechanisms that lead to SCLC will facilitate the development of novel drugs and strategies for inhibiting the growth of SCLCs and other neuroendocrine tumors.

MATERIALS AND METHODS

Cell culture

The following NCI series of lung cell types was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA): H209, H345, H510, H69, H146, N592 (classical SCLCs), N417 (variant SCLC), H23 (adenocarcinoma, NSCLC), H157 (squamous cell carcinoma, NSCLC) and H727 (carcinoid). The growth and biochemical characteristics of these cell types, including bombesin-like immunoreactivity, have been described (Carney *et al.* 1985). The cells were grown in antibiotic-free RPMI 1640 (JRH Biosciences, Lenexa, KS, USA) supplemented with 10% heat-inactivated bovine calf serum (HyClone Laboratories, Inc., Logan, UT, USA) at 37 °C in a humidified atmosphere of 5% CO₂. The normal human lung fibroblast line CCD-19 Lu was also obtained from ATCC and grown in Eagle's minimal essential medium (Hazleton Biologics, Inc., Lenexa, KS, USA) supplemented with non-essential amino acids (Sigma, St Louis, MO, USA) and 10% heat-inactivated bovine calf serum (HyClone Laboratories, Inc.). Normal human small airway epithelial cells (SAECs) were purchased from Clonetics Corporation (San Diego, CA, USA) and grown in serum-free small airway epithelial cell media according to the supplier's instructions.

Plasmid constructs

The human PC1 construct (hPC1 15–5) was a gift of R Day (DeSève Laboratories of Biochemical Neuroendocrinology, Montreal, Quebec, Canada). Linearization with BglII and transcription with T7 RNA polymerase generates an antisense RNA probe of ~300 bases; ~210 bases are protected from RNase digestion by hybridization to PC1 mRNA. Plasmid hPC2 was obtained from M Mbikay (Institut de Recherches Cliniques de Montreal, Université de Montreal, Quebec, Canada). phPC2 contains 193 bases of human PC2 cDNA cloned into the PstI/BamHI site of pSP72. Cleavage with BamHI and transcription with SP6 RNA polymerase generates an antisense probe of 220 bases, 193 of which are protected from RNase degradation by hybridization to PC2 mRNA. The human neurotensin plasmid, phNTE0·9, was a generous gift of

P Dobner (Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Center, Worcester, MA, USA). phNTE0·9 consists of an 806-base fragment containing exon 1 and portions of the 5'-flanking region and intron 1 cloned into EcoRI site of pGEM4 as described by Bean *et al.* (1992). Linearization with SmaI and transcription with SP6 RNA polymerase generates a 834 base antisense RNA probe; 184 bases are protected from RNase degradation by hybridization to neurotensin mRNA. ppGRP was provided by James Battey (NIH, Bethesda, MD, USA). The plasmid contains 1 kb human GRP cDNA cloned into the HindIII site of pSP64 (Promega, Madison, WI, USA). A 300-base antisense probe was generated by cutting with PvuII and transcribing with SP6 RNA polymerase. The probe migrates as a doublet, presumably because of strong secondary structure. pTRI- β -actin-human and pTRI- β -actin-125-human were purchased from Ambion (Austin, TX, USA) in linearized form. pTRI- β -actin antisense RNA protects 245 bases of β -actin mRNA, whereas pTRI- β -actin-125 probe protects 127 bases of β -actin mRNA.

In vitro transcription

³²P-labeled antisense RNA probes were generated using an RNA transcription kit from Stratagene (La Jolla, CA, USA). For the prohormone convertase and growth factor probes, 5 μ l [α -³²P]UTP (800 Ci/mmol; NEN Life Science Products, Boston, MA, USA) and 1 μ l unlabeled 250 M UTP were included in a 20 μ l reaction volume. For the β -actin probes, 1 μ l [α -³²P]UTP and 1 μ l unlabeled 1 mM UTP were included in a 20 μ l reaction volume. Reactions were incubated at 37 °C for 45 min. Plasmid DNA was removed by digestion with 1 unit RNase-free DNase 1 for 15 min at 37 °C. Two microliters of the reaction were diluted into 500 μ l water and counted in a scintillation counter. Generally, 2 μ l diluted probe (~20 000 c.p.m.) were used for each sample in the RNase protection assay.

RNase protection assay

Total RNA was isolated using TRI REAGEN LS (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the supplier's instructions. RNase protection assays were performed using an RPA II kit from Ambion as previously described (Waters *et al.* 1997). Briefly, ~20 000 c.p.m. antisense RNA probe was precipitated with 10 μ g total RNA from each cell type. The RNA pellet was

FIGURE 1. RNase protection assay of PC1 mRNA expression in human lung cell lines.

resuspended in 20 μ l hybridization buffer, heated at 90 °C for 3 min, then incubated overnight at 40–45 °C. Unhybridized RNA was digested with 200 l of 1:100 dilution of stock RNase A/T1 in RNase digestion buffer for 30 min at 37 °C. The reaction was stopped by the addition of 300 l RNase inactivation/precipitation mixture and the products were precipitated by chilling at –20 °C for 20 min, followed by centrifugation at 12 000 *g*. The samples were resuspended in 10 μ l loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA, and 0.025% SDS), heated at 90 °C for 3 min, then loaded on a 7 M urea–4% polyacrylamide gel and run at 300 V until the bromophenol blue migrated to the bottom of the gel. The gel was dried and then imaged by electronic autoradiography on a Packard Instant-Imager (Meriden, CT, USA).

RESULTS

To determine whether prohormone convertase genes are coexpressed with autocrine growth factor genes in SCLC, we used RNase protection assays to examine the level of PC1, PC2, neurotensin,

and GRP mRNA expression in a panel of human lung cancer cell lines. We found that PC1 mRNA is not expressed in normal SAECs (Fig. 1, lane 4) or in normal lung fibroblasts (19 Lu; lane 5). In contrast, PC1 is abundantly transcribed in five of the six classical SCLC cell lines tested (H209, H345, H510, H69, and H146; lanes 6–10) and in the lung carcinoid tumor cell line H727 (lane 15). PC1 mRNA was not expressed in the classic SCLC cell line N592 (lane 11) or in the variant SCLC cell line N417 (lane 12). These two cell types were initially established by passage through nude mice (hence the N prefix); whether this has any effect on the expression of PC1 mRNA is unknown. Neither of the two non-SCLC cell lines – H23 (adenocarcinoma; lane 13) and H157 (squamous cell carcinoma; lane 14) – expressed detectable levels of PC1 mRNA. The β -actin signal shows that equal quantities of RNA were tested in each sample.

The transcriptional activity of the neurotensin gene was examined in the same panel of lung cancer cells. The expression of neurotensin mRNA parallels the expression of PC1 mRNA in that neurotensin mRNA is found in five of six classical SCLCs and in the lung carcinoid cell line H727, but

FIGURE 2. RNase protection assay of neurotensin mRNA expression in human lung cell lines.

not in the normal lung cells or in the non-SCLCs (Fig. 2).

Next, the expression of PC2 mRNA in these cell types was examined (Fig. 3). As with PC1, PC2 was expressed in five of the six classical SCLC cell types (H209, H345, H510, H69, and H146; lanes 6–10), but not in the normal lung cells (SAEC and 19 Lu; lanes 4 and 5) or in the non-SCLCs (H23 and H157; lanes 13 and 14). In contrast to PC1 and neurotensin, PC2 gene expression was absent in the lung carcinoid cell line H727 (Fig. 3, lane 15).

The expression of GRP mRNA (Fig. 4) among this panel of lung cancer cells parallels the expression of PC2 in that GRP mRNA is expressed in the classical SCLCs (lanes 6–10), yet is absent from the non-SCLCs (H23 and H157; lanes 13 and 14) and lung carcinoid cell line H727 (lane 15). The GRP probe migrates as a doublet, because of its strong secondary structure.

DISCUSSION

Essentially all known peptide hormones are initially synthesized as inactive precursors that require cleavage and post-translational processing for activity. The functional expression of an autocrine growth factor therefore requires the concurrent expression of several other gene products, including the prohormone convertases. This information led us to hypothesize that prohormone convertase genes would be expressed along with autocrine growth factor genes in small cell lung cancer. To examine this possibility, we isolated RNA from a panel of lung cancer cell lines and assayed for the expression of prohormone convertase and autocrine growth factor mRNAs.

The expression of PC1 mRNA in the SCLCs and carcinoid cell line, and its absence in normal lung and non-SCLCs (Fig. 1), suggest that PC1 is

FIGURE 3. RNase protection assay of PC2 mRNA expression in human lung cell lines.

processing the autocrine growth factors found in these cell types and has an important role in the development of SCLC and carcinoid tumors of the lung. As the expression of neurotensin mRNA parallels the expression of PC1 mRNA (Fig. 2), we reason that PC1 is processing neurotensin in these cell types. This finding is consistent with an earlier study that shows that PC1 processes proneurotensin in neuroendocrine cells (Rovère *et al.* 1996).

PC2 and GRP are also expressed in five of the SCLC cell lines tested. However, whereas PC1 and neurotensin mRNA are expressed in the lung carcinoid cell line (H727), PC2 and GRP mRNA are not. This finding corroborates the lack of bombesin-like immunoreactivity reported for H727 (Moody *et al.* 1990) and suggests that, in this cell line, neurotensin is processed by PC1 rather than PC2. Overall, these studies support the idea that, in SCLCs and lung carcinoids, PC1 is expressed along with neurotensin, and PC2 is expressed with GRP. These findings are consistent with an earlier study

by Mbikay *et al.* (1997), who found that PC1 and PC2 are overexpressed in primary SCLC tumors.

The coordinated transcription of prohormone convertase and autocrine growth factor genes in SCLCs, and their lack of expression in non-SCLCs and normal lung cells, indicates these genes are responding to the same transcriptional signals. What features of the promoters account for this apparently linked gene expression? The promoter regions for each of these genes have been cloned and partially characterized by deletion and mutational analysis (PC2, Ohagi *et al.* 1992; PC1, Jansen *et al.* 1995; GRP, Nagalla & Spindel 1994; neurotensin, Evers *et al.* 1995). A cursory inspection of the promoter sequences for these genes reveals a diversity of promoter types. For example, the GRP promoter contains a TATA box, a CAAT sequence, and a single transcription start site (Nagalla & Spindel 1994), whereas the PC1 promoter has multiple transcription start sites and no TATA box or CAAT sequence (Jansen *et al.* 1995). A common

FIGURE 4. RNase protection assay of GRP mRNA expression in human lung cell lines.

feature among these genes is the presence of a consensus cyclic AMP response element (CRE), which may confer hormonal responsiveness to each promoter. We and others (Mania-Farnell *et al.* 1996, Jansen *et al.* 1995) have demonstrated that the PC1 promoter is transcriptionally activated by second messenger activators that stimulate the cAMP pathway, and that this regulation is directed through the cyclic AMP response elements.

Although the CRE is functional (to varying degrees) in these genes, it is unlikely that this feature alone is responsible for the tissue- and stage-specific expression of these genes. The GRP promoter, for example, is not particularly sensitive to increased levels of cAMP in SCLCs (Nagalla & Spindel 1994). Moreover, whereas PC1 and neurotensin are expressed in H727 cells, PC2 and GRP are not. Hence, factors other than cAMP activators, are likely also to be involved in regulating the

transcriptional activity of these genes. Nagalla & Spindel (1994) have identified an upstream element in the GRP promoter that allows expression in neuroendocrine-differentiated SCLC cell lines and binds to nuclear proteins from classic (GRP-expressing) SCLCs but not variant SCLCs. In addition, Jansen *et al.* (1997) have shown that a novel, yet unidentified, nuclear protein, present in AtT-20 and β-Tc3 cells but not in COS-1 cells, forms a heterodimer with c-Jun and binds to a CRE in the PC1 promoter. A purine-rich region of the neurotensin promoter, which is essential for promoter activity, also binds to nuclear proteins in neurotensin-producing cells (Evers *et al.* 1995). Taken together, these studies suggest that neuroendocrine-specific transcription factors work in concert with activated CRE-binding proteins to increase transcription from these genes. However, apart from the CREs, there is no striking homology

between these promoters. Therefore, the mechanism by which these genes are coordinately expressed remains unknown. In order to understand better the activation of these SCLC-associated genes, it will be necessary to identify the transcription factors that bind to their promoter regions and regulate their expression.

The differential expression of prohormone convertases in neuroendocrine tumors may provide the basis for new treatments for these tumors. The specific inhibition of PCs may permit the selective inhibition of autocrine growth factor expression and thereby diminish the rate of cancer cell proliferation. As autocrine growth factors and prohormone convertase are expressed in SCLCs but not in normal lung tissue, they present an attractive target for chemotherapeutic inhibition. In an earlier report we showed that the proliferation of SCLC lines is differentially inhibited by the Bowman-Birk protease inhibitor (BBI; Clark *et al.* 1993). In that study the *in vitro* growth of NCI-H345 was significantly inhibited by BBI, whereas the growth of NCI-N592 was unaffected. Western blot analysis with antibodies to GRP showed that the processing of prepro-GRP in NCI-H345 cells is inhibited by treatment with BBI. As NCI-H345 expresses PC2 (the processing enzyme for preproGRP), we propose that BBI is inhibiting PC2 activity, which in turn reduces the expression of GRP and thereby reduces the growth of the cell. As NCI-N592 does not express PC2 (Fig. 3), its growth rate is not dependent upon the processing of growth factors by PC2 and is therefore unaffected by treatment with BBI.

In summary, these observations, together with earlier findings from our laboratory and others, suggest the expression of PC1 and PC2 has an important role in the development and maintenance of the SCLC phenotype: prohormone convertase and autocrine growth factor gene expression are linked at the transcriptional level in SCLCs; novel, neuroendocrine-specific transcription factors are involved in the *de novo* expression of prohormone convertases and autocrine growth factors in SCLCs; and the inhibition of prohormone convertase activity by specific protease inhibitors may reduce the processing of autocrine growth factors and thereby reduce the rate of SCLC proliferation. Subjects for further study include identifying the neuroendocrine-specific transcription factors responsible for the transcriptional activation of prohormone convertase and autocrine growth factor genes in SCLCs. These studies will enable us to define better the genetic and biochemical events that lead to the development of SCLC, and may reveal novel targets and strategies for the treatment of these tumors.

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