Chromogranin A Peptide-Specific Antisera and High-Performance Size Exclusion Chromatography Demonstrate Amino-Terminal and Carboxy-Terminal Fragments of the Native Molecule in Human Cell Lines (43712)

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Abstract. **We have studied the pattern of amino-terminal and carboxy-terminal protein processing of chromogranin A (CgA) in five human cell lines, four derived from lung cancers: NCI-H478, NCI-H1011, NCI-H727, and BEN; and one derived from human medullary thyroid carcinoma: TT. This was accomplished by fractionation of cell extracts by high-performance size exclusion chromatography (HPSEC) and measurement of CgA in the fractions by radioimmunoassay (RIA). Three RIA's were used, one specific for the amino-terminus of CgA, one specific for the carboxy-terminal region, and a monoclonal antibody-based assay that recognizes only the native CgA molecule. We demonstrated the presence of different amino- and carboxy-terminal immunoreactive species of CgA in the different cell lines. The amino-terminal assay demonstrated distinct low-molecular-size species in the NCI-H478 and NCI-H1011 cell lines, and a similar peak in the TT cells. The amino-terminal assay did not recognize any distinct species in BEN and NCI-H727 cell lines. The carboxy-regional assay demonstrated distinct low-molecular-size species in the NCI-H478 and NCI-H101 cell lines and high-molecular-size species in the NCI-H727 and BEN cells. Our studies demonstrate with region-specific RIA's the presence of both amino- and carboxy- forms of CgA in human cells that secrete this protein. These results provide direct evidence that CgA-producing cells produce, probably through endoproteolytic processing of the native molecule, amino- and carboxy-terminal forms of the protein.**

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Chromogranin A (CgA) is a 439 amino acid protein that is produced by a variety of human endocrine and neuroendocrine cells (1). It has consequently become a serum and tissue marker for a wide variety of endocrine and neuroendocrine neoplasia (2). Although the function of CgA remains unknown, it appears to be processed into peptides that can regulate the production of its coresident hormones (3, 4). For example, amino-, carboxy-, and midregion CgA peptides can regulate the production of coresident insulin, catecholamines, calcitonin (CT), parathyroid hormone (PTH), PTH-like protein (PLP), and proopiomelanocortin (POMC) (5–15). The processing of CgA into peptides has been demonstrated by several immunochemical and biochemical procedures (1–16). However, the presence of such peptides has not been demonstrated by region-specific radioimmunoassays (RIA's) for the molecule. We developed such re-

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gion specific RIA's for CgA in order to directly measure CgA peptides in human cells. We chose the amino- and carboxy-regions of CgA for these studies, because they are highly conserved and because there is indirect evidence that processing occurs at these sites (1, 14, 16).

Material and Methods

The following CgA-producing human cell lines were grown as previously described: TT, derived from a medullary thyroid carcinoma; and BEN, NCI-H727, NCI-H478, and NCI-H1011, all derived from human lung cancers (16, 17). These cells were chosen because of their high CgA production and their favorable growth characteristics (17). The cells were extracted in the presence of protease inhibitors and the extracts were fractionated by high-performance size exclusion chromatography (HPSEC) as previously described (16, 18). In brief, cells at various densities were washed twice in PBS, scraped in RPMI media, and pelleted by centrifugation at 200*g* for 5 min. Cells were resuspended in 1 *M* guanidine HCl, 0.1 *N* acetic acid, 10 m*M* dithiothreitol, 0.25 mg/ml protamine sulfate, 1 m*M* EDTA, 0.1 mg/ml bacitracin, 0.2 m*M* phenylmethylsulfony-fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.1% Tween-20 (wt/vol). Cells were homogenized by two 30-sec bursts of sonication at 4°C. Homogenates were clarified by centrifugation for 30 min at 135,000*g* using a TL-100.3 rotor in a TL-100 ultracentrifuge (Beckman, Palo Alto, CA).

Chromatography was performed using a Waters (Milford, CT) model 6000A HPLC system. Cell extracts (0.5 ml) were loaded on to a TSK3000SW (75 × 600 mm) size exclusion column at a flow rate of 0.3 ml/min and 0.7-min fractions were collected and assayed directly by radioimmunoassay (RIA). The assay buffers in the standard curves and samples were identical. The running buffer was composed of 0.1 *M* sodium phosphate, pH 7.5, and 0.5% bovine serum albumin (BSA, Pentex). Recoveries from HPSEC chromatography were calculated by comparing the total amount of CgA applied to the total amount recovered; they varied between 70% and 80%. Void and total volumes were determined by exclusion of Blue Dextran and retention of $Na^{125}I$, respectively, and radioiodinated native CgA and PTHrP 1–141 were used as additional elution standards (16, 18).

Each of the chromatography fractions was evaluated by three RIAs for CgA. One RIA has been previously described and recognizes only native CgA (16, 17). It is based on a monoclonal antibody that was raised against crude human CgA preparations (9, 16, 17). Although it recognizes CgA in tissues, conditioned culture medium, and serum, it fails to recognize any of the synthetic CgA peptides that span the linear molecule, namely, 1–40, 79–113, 250–301, 316–329, 332–364, 367–391, and 403–428 (16). This observation indicates the limitation of antisera in identifying CgA forms of unknown structure. The second and third RIA's were respectively based on synthetic amino- and carboxy-terminal peptides of bCgA, CgA 1–40 and CgA 367–391, respectively. The antibodies to CgA 1–40 and CgA 367–391 were raised in rabbits by immunizing them with the corresponding unconjugated tyrosinated peptides (19). Using standard protocols previously described by us, these peptides were radiodinated by chloramine T for use as RIA tracers and in their unlabeled form for use as assay standards (16, 19). These two assays were conducted under nonequilibrium conditions and did not recognize any of the other aforementioned CgA peptides (19). All assays were performed according to the same protocol (17, 19). Figure 1 illustrates typical assay standard curves and its legend provides additional experimental details.

Results

Figure 2 displays the CgA elution profile from each of the cell lines for each of the RIA's: Panel A for

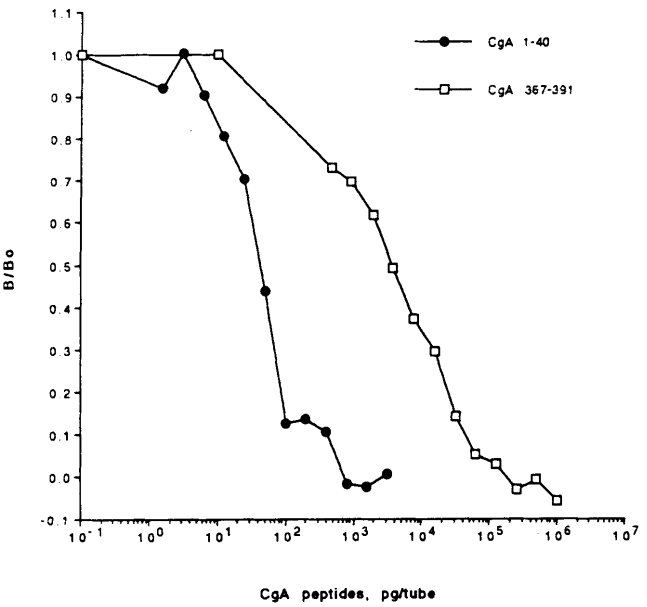

Figure 1. Radioimmunoassay standard curves for CgA 1–40 and CgA 367–391. Rabbit antibodies at respective dilutions of 1:1000 and 1:2000 were used in four-day nonequilibrium assay incubations. Each of the peptides was used as standards. Radioiodinated peptides were used as tracers. Inter- and intrassay-variations were 7% and 15%, respectively. There was no cross-reactivity between the peptides in the two assays and no reactivity with any CgA peptides that span the linear sequence of the molecule, including chromostatin (Deftos Galindo *et al.*, 1991; Deftos *et al.*, 1990). Each of the column fractions was measured in both of these assays as described in the text. See text for experimental details.

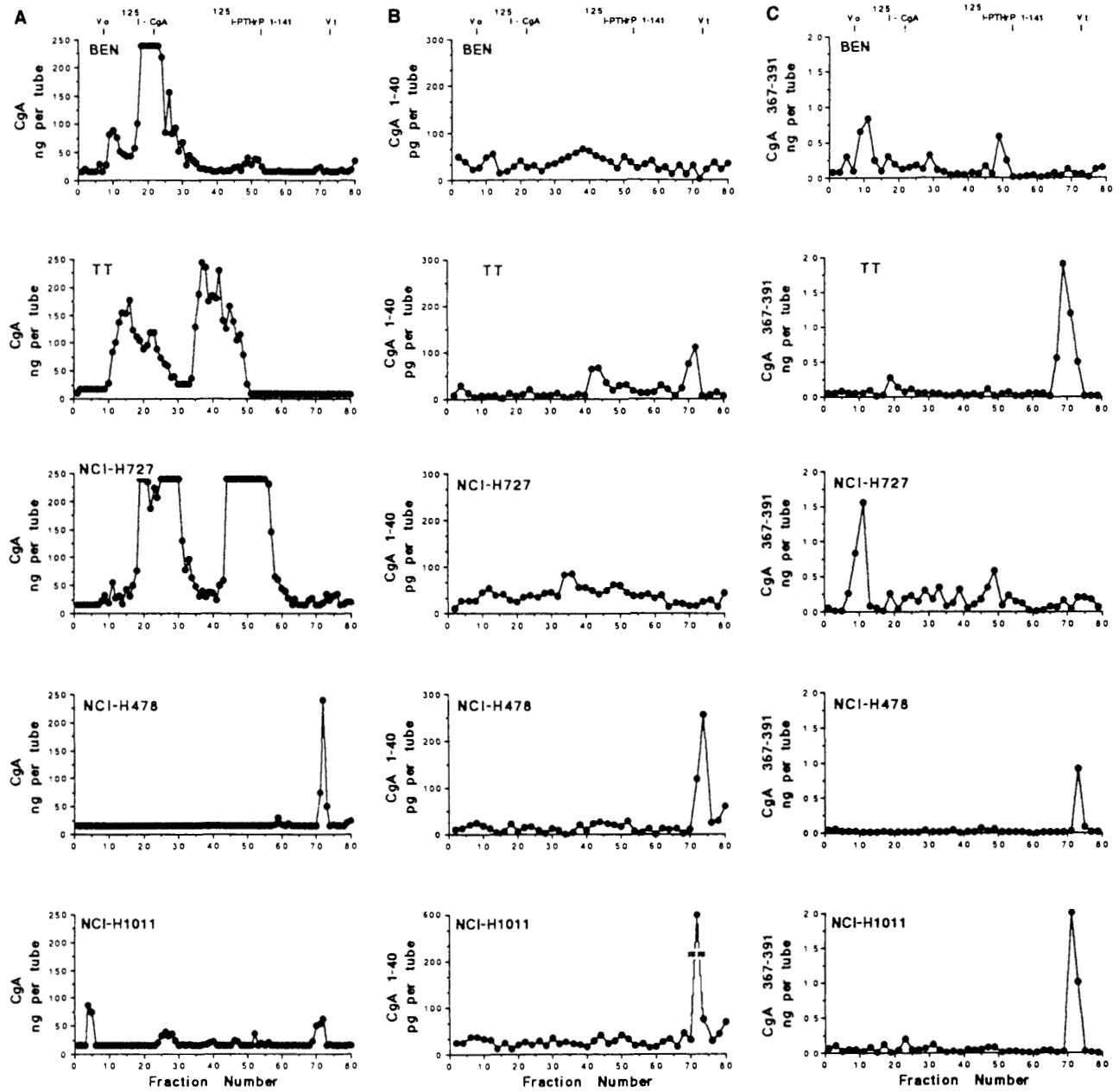

Figure 2. CgA heterogeneity demonstrated by region-specific radioimmunoassays for CgA using high-pressure size exclusion chromatography (TSK 3000SW, 75 × 600 mm column) in extracts from cell lines derived from medullary thyroid carcinoma (TT), and the lung cancers BEN, NCI 727, NCI 478, and NCI 1011 (26). The cell extracts were prepared in 1 *M* guanidine, 0.1 *N* acetic acid, 10 m*M* dithiothreitol, 0.1% Tween-20, and various protease inhibitors (see Materials and Methods) and the extracts were fractionated in 0.1 *M* phosphate buffer pH 7.5 supplemented with 0.5% BSA. The specificities of the antibodies (also see Fig. 1) are as follows: Panel A—native CgA (26), Panel B—CgA 1–40, Panel C—CgA 367–391. Also indicated are the void volume (*Vo*, Blue dextran), the elution positions of ^{125}I-CgA and ^{125}I-PTHrP1–141, and the total volume (*Vt*, Na^{125}I).

the native CgA assay, Panel B for the amino-regional CgA assay, and Panel C for the carboxy-regional CgA assay. The native CgA assay (Fig. 2A) demonstrates multiple peaks of immunoreactivity in each cell line. Although the CgA immunoreactivity eluting with ^{125}I-CgA is a major species in the BEN, TT, and NCI-H727 cells, also present are smaller and larger species, the latter presumably representing aggregates. The amino-terminal assay (Fig. 2B) demonstrates distinct low-molecular-size CgA species at the column total volume (*Vt*) in the NCI-H478 and NCI-H1011 cells and a similar peak of immunoreactivity in the TT cells; in the BEN and NCI-H727 cells, only broad peaks were present. The carboxy-regional assay (Fig. 2C) demonstrates distinct low-molecular-size species at *Vt* in the TT, NCI-H478, and NCI-1011 cells; high-molecular-size species at the column void volume (*Vo*) are present in the NCI-H727 and BEN cells, with a broad

peak also present at the elution position of the PLP marker in these two cell lines.

Discussion

Several studies have indicated that the processing of CgA to peptides occurs in a tissue specific manner (1). Our observations demonstrate the presence of different patterns of amino- and carboxy-terminal forms of CgA in various cell lines that produce this protein (Fig. 2). These observations, along with other similar studies by us and others, are consistent with the hypothesis that CgA is processed to smaller peptides (1, 3, 4, 16). Our studies demonstrate this phenomenon by region-specific RIA's used in concert with HPSEC. In the BEN, TT, and NCI-H727 cells studies, large immunoreactive species that eluted with native CgA (Fig. 2A) were not recognized by either the amino- or carboxy-regional assays, whereas low-molecular species of peptidic size were present in these cells (Fig. 2B and 2C). The monoclonal antibody-based assay did recognize relatively small amounts of low-molecular-weight forms of CgA in the NCI-478 and NCI-1011 cells. It is possible that these small CgA forms revealed unique antigenic sites for this antibody that were weakly recognized. It should also be noted that the amounts of low-molecular-weight forms detected by the peptide-specific assays was small. It is also possible that CgA is further processed or degraded to reveal the epitopes that these antibodies recognized. However, even at low concentrations, these peptides could exert the paracrine and autocrine effects that have been attributed to CgA (9–15). It is also likely that there were molecular species of CgA present that were better recognized by the monoclonal-based assay than by the amino- or carboxy-terminal assays. The relatively low or absent amount of CgA detected by the monoclonal-based assay in the NCI-478 and NCI-1011 cells suggest that the native molecule is rapidly processed in these cells. These considerations notwithstanding, our observations are consistent with other studies indicating that CgA is processed at its amino- and carboxy-terminal regions (1, 4, 5, 17). These regions and their dibasic processing sites are well conserved among all of the CgA's (1). In the NCI-H478 and NCI-H1011 cells, primarily low-molecular-weight forms of CgA with both amino- and carboxy-regional specificity were measured. These observations also suggest that the terminal regions of CgA are important sites for processing (16). The presence in the TT cells of only low molecular size amino- and carboxy-species of CgA also supports this view (Fig. 2B and 2C). Thus, three of these cell lines BEN, NCI-H727, and TT, appear to process CgA primarily at its amino- and carboxy-regions. By contrast, the cell lines NCI-H478 and H1011 appear to process CgA further to small peptides (Fig. 2A). These observations suggest the presence of different protein processing pathways for CgA in different cell types.

Although we cannot attribute specific molecular sizes to the CgA forms present in the different cell lines, their elution position and immunoreactivity are consistent with processing of CgA at the lys-lys site at residue 77 and at one of the several dibasic sites that include or are distal to the arg-lys site at residue 325 (1). The evidence that CgA serves as precursor for biologically active peptides is furthered by observations about potential processing of the protein (8–15). Amino- and carboxy-peptides of CgA such as those identified in these cell lines have been shown to regulate the production of their coresident hormones, specifically the calcium-regulatory peptides (1–13, 14). Furthermore, the CgA peptides named pancreastatin and chromostatin inhibit the production of their coresident hormones (5, 15). These biologically active CgA peptides may be derived from endoproteolytic processing of native CgA (1).

Recent advances have been made in the understanding of the mechanism of protein processing into peptides (21). The most common processing sites in mammals are simple pairs of the basic residues of arginine (R) and lysine (K) and related motifs, including KK and RR (proopromelanocortin), KTRR (proinsulin), RKRR (insulin proreceptors), and RKKR (TGF beta) (21), sequences that are well represented in CgA (1). Two enzymes have been recently identified from mammalian tissues that can serve this function, PC2 and PC3 (22–24). PC2 is a 638 residue protein cloned from a human insulinoma and PC3 residue protein cloned from a mouse pituitary cell lines, AtT20 (22, 23). These two proteins are structurally related to the prokaryotic subtilisin family of serine proteases, to the yeast endoprotease KEX2, and to the product of human C-fur/PACE (paired basic amino acid cleavage site gene (21). PC2 and PC3 are expressed in a variety of neuroendocrine tissues: rat pancreatic islets and brains; mouse and human insulinomas; and mouse ATt20 pituitary cells, but not in liver, kidney, skeletal muscle, spleen, heart, or intestine (21–25). In preliminary studies, we have demonstrated the presence of PC2 and PC3 in CgA-producing cell lines, so these enzymes are candidates for contribution to the processing that occurs in CgA (26).

While our studies are consistent with the hypothesis the CgA takes place primarily at the amino- and carboxy-termini of the protein, they do not provide conclusive evidence for this view (25–27). Although we attempted to control for nonspecific proteolytic activity, such activity could still contribute to our results. Furthermore, post-translational modifications like glycosylation could influence immunoreactivity and elution positions (28). Since our studies were limited to tumor cell lines, their findings are not neces-

sarily applicable to normal cells. Nevertheless, our observations, along with these from other laboratories, provide an impetus for more conclusive studies of CgA processing, such as pulse-chase and labeling experiments, immunoprecipitation studies, microsequencing, *in vitro* mutagenesis, anti-sense studies, and cotransfection studies with endoproteolytic peptidases (25–32).

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