Communication

Chromogranin A-like Proteins in the Secretory Granules of a Protozoan, *Paramecium tetraurelia*¹

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Joan B. Peterson and David L. Nelson²

From the Department of Biochemistry, The University of Wisconsin-Madison, Madison, Wisconsin 53706-1589

Eva Ling and Ruth Hogue Angeletti

From the Division of Neuropathology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

The ciliate protozoan *Paramecium tetraurelia* produces secretory granules (trichocysts) which release needle-like structures composed of small, acidic proteins. Using antibodies against isolated chromogranin A (CGA) and against trichocyst proteins, we found cross-reactive proteins in chromaffin granules and trichocysts. Four independently derived sera against isolated CGA stained bands of the Mr 15,000–25,000 family of trichocyst proteins on immunoblots. A positive response was also obtained with antiserum against chemically synthesized peptides (PL26 and GE25) corresponding to defined regions of the CGA amino acid sequence. In extracts of whole *Paramecium*, larger proteins (Mr 53,000 and 49,000) also reacted with antibodies against CGA and the related synthetic peptides. These larger proteins may represent unprocessed precursors to the smaller proteins of mature trichocysts. Antiserum to trichocysts recognized CGA in chromaffin granule lysates. Further evidence of a *Paramecium* protein related to CGA was provided by hybridization of *Paramecium* mRNA with cloned cDNA for bovine CGA. Our results suggest striking conservation in evolution of CGA-like proteins that may play some role, as yet unknown, in secretion.

The ciliate protozoan *Paramecium tetraurelia* contains about a thousand trichocysts, secretory granules lying in regular arrays beneath the cytoplasmic membrane. Upon stimulation, the highly condensed and crystalline protein contents of the trichocysts are simultaneously rearranged into another highly regular form and are discharged into the medium (1).

Analysis of discharged trichocysts by SDS-PAGE² reveals a family of acidic, low molecular weight proteins (Mr 15,000–25,000) (2, 3). Greater complexity is apparent with two-dimensional PAGE: over 100 species with acidic pI values between 4.4 and 6.2 are evident (3, 4). Mature trichocyst proteins are derived from precursors (Mr 40,000–45,000) which undergo post-translational proteolytic cleavage. This processing correlates with the conversion of amorphous material in the immature vesicle to the crystalline structure of the mature trichocyst, and is defective in certain mutants of *Paramecium* (5).

No biological activity is as yet associated with any of the components of the trichocyst vesicle. The molecular properties of trichocyst proteins are reminiscent of those of the chromogranins, also of unknown function, which are packaged with the contents of neuroendocrine secretory granules in higher organisms. Chromaffin granules, the catecholamine-storing vesicles of the adrenal medulla, contain a set of acidic proteins, chromogranins A, B, and C, which are secreted with the catecholamines and other neuropeptides (6).

In bovine chromaffin granules, chromogranin A (CGA) is the most abundant protein present, comprising about 40% of the soluble matrix protein (7). It has a pI of 5.0 and migrates on SDS-PAGE with an apparent Mr, of 70,000–75,000 (6). However, its electrophoretic behavior is anomalous: it has an apparent Mr of 48,000 as determined from its amino acid sequence, deduced from cDNA sequences independently by two groups (8, 9). CGA undergoes partial processing by proteases within chromaffin granules (10). The presence of CGA has been demonstrated in many endocrine tissues and neurons since its initial detection in the adrenal medulla (11–15). Interest in elucidating the function of CGA has been intensified by evidence suggesting that a similar protein may be a prohormone precursor of pancrestatin, a polypeptide recently isolated from rat endocrine pancreas which inhibits glucose-induced insulin secretion (16). The 49-amino acid peptide pancrestatin exhibits greater than 70% homology with a portion of the CGA molecule (17, 18).

The molecular similarities between chromogranins and trichocyst proteins and the occurrence of both protein families within secretory granules led us to investigate the possibility that CGA and trichocyst proteins might be related structurally and perhaps functionally. Using antibodies against bovine CGA and trichocyst proteins, we have found cross-reactive proteins in bovine chromaffin granules and trichocysts. Further evidence of a *Paramecium* protein related to CGA was provided by hybridization of *Paramecium* mRNA with cloned cDNA for bovine CGA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Trichocyst Isolation**—Methods for the isolation and purification of extended trichocysts from axenically grown cultures of *Paramecium tetraurelia* stock 515 are described (19). Heat treatment of trichocysts under defined conditions to generate partial structural breakdown was as in Ref. 19. Suspensions of heated trichocysts were centrifuged 4 min in a Beckman Model B Microfuge (11,000 × g). The protein remaining in the supernatant fraction represents protein released in the heat step which did not pellet with the residual structure after low speed centrifugation.

**Chromaffin Granule Lysates**—Chromaffin granule lysates were prepared from granules isolated by sucrose gradient centrifugation as described (20).

**One-dimensional SDS-PAGE**—The procedures used for electrophoretic separation of proteins have been described (14, 19).

**Gel Transfers**—Gel slabs containing separated proteins were electrophoretically transferred to nitrocellulose paper (Schleicher &
Staining, Fixing, and Immunoblotting—Proteins transferred to nitrocellulose paper were visualized by pre-staining in 0.2% (v/v) Ponceau S (22). The nitrocellulose paper was then incubated in 0.25% (v/v) Ponceau S (20) in phosphate-buffered saline (10 mM Na2HPO4, 150 mM NaCl, pH 7) to increase retention of bound protein, rinsed in TBS (10 mM Tris, 150 mM NaCl, pH 8), and then incubated in blocking solution (3% (w/v) BSA in TBS) for 2 h at 37 °C, followed by incubation in primary antibody (1:100 to 1:1000) in TBS containing 0.3% (w/v) BSA for 4–16 h. Subsequent rinses and incubation in alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:1000) were in TBS containing 0.3% (w/v) BSA and 0.05% Tween 20 (v/v) followed by incubation in substrate (0.25 mg/ml bichloro-indole-phosphate in 0.7 M Tris, pH 9.5) (24).

Preparation of Antiseras—Rabbit polyclonal antibodies to denatured bovine CGA were as described in Ref. 20. Antibodies were also prepared against synthetic peptides corresponding to defined regions of the bovine CGA amino acid sequence. PL26 is a peptide of 26 residues representing residues 403–429 of CGA: Pro-Glu-Asp-Gln-Glu-Leu-Glu-Ser-Leu-Ser-Ala-Ile-Glu-Ala-Glu-Leu-Lys-Val-Ala-His-Leu-Leu-Leu-Glu-Leu. Peptide GE25 represents residues 367–391: Gly-Leu-Pro-Leu-Gln-Val-Arg-Gly-Tyr-Pro-Glu-Glu. Peptides were synthesized on Applied Biosystems 430A peptide synthesizer by solid phase peptide synthesis (25), and characterized by amino acid analysis on a Beckman 6300 amino acid analyzer and by sequencing of the bovine CGA amino acid sequence. PL26 is a peptide of 26 residues representing residues 403–429 of CGA: Pro-Glu-Asp-Gln-Glu-Leu-Glu-Ser-Leu-Ser-Ala-Ile-Glu-Ala-Glu-Leu-Lys-Val-Ala-His-Leu-Leu-Leu-Glu-Leu. Peptide GE25 represents residues 367–391: Gly-Leu-Pro-Leu-Gln-Val-Arg-Gly-Tyr-Pro-Glu-Glu. Peptides were synthesized on Applied Biosystems 430A peptide synthesizer by solid phase peptide synthesis (25), and characterized by amino acid analysis on a Beckman 6300 amino acid analyzer and by sequencing on an Applied Biosystems 470A microsequencer with on-line detection. The rabbit polyclonal antiseras to these peptides react with bovine, human, and rat CGA.

For the production of antisera to trichocysts, rabbits were injected subcutaneously with a preparation of 0.5 mg of intact, gradient-purified trichocysts in Freund’s complete adjuvant and then boosted with injections of 0.2 mg in Freund’s incomplete adjuvant.

RNA Hybridization—Total cellular RNA isolated from Paramecium by extraction with guanidinium thiocyanate (26, 27) was used for hybridizations. The pChPr4A plasmid, containing the entire coding region for bovine CGA, was a generous gift of Dr. Lee Eiden, National Institute of Mental Health, Bethesda, MD (8). The plasmid was cut with EcoRI endonuclease (a gift of Dr. Paul Modrich, Duke Univ. Medical Center) and end-labeled as in Ref. 26. Total RNA was serially diluted, lyophilized, and denatured before application to GeneScreen nylon membrane (Du Pont-New England Nuclear) (29).

The membranes were prehybridized overnight at 37 °C in the presence of various amounts of formamide. The labeled probe was added, and hybridization was allowed to proceed for an additional 18 h at 37 °C. Autoradiographic detection was performed using Kodak XAR5 film and DuPont Cronex enhancing screens at -60 °C. Dextran sulfate, formamide, and hybridization solutions were purchased from Oncor-probe (Gaithersburg, MD).

RESULTS

Proteins of Isolated Trichocysts: Immunoreaction with Anti-trichocyst Serum—The general pattern of disulfide-reduced trichocyst proteins resolved on SDS-PAGE is depicted in Fig. 1, lane a. About 10 polypeptides appear in a tight cluster at M, 15,000–25,000. Antiserum to whole trichocysts stains this set of proteins and detects a higher molecular weight group at M, 33,000–39,000, not visible after protein staining with Ponceau S, which probably represents highly antigenic, minor trichocyst components (Fig. 1, lane b). Unreduced samples show the closely migrating polypeptides at M, 30,000–35,000 with a corresponding reduction in intensity of protein at M, 17,000 so prominent in reduced samples (Fig. 1, lane c).

Immunoblots of unreduced proteins (Fig. 1, lane d) demonstrate intense staining at M, 30,000–35,000 and a marked loss of signal at M, 17,000. The immunoreactive proteins at M, 45,000–50,000 and the doublet at M, 68,000 were not detected with Ponceau S and they may represent some of the minor proteins at M, 33,000–39,000 seen on blots of unreduced samples which are now disulfide-linked (cf. Fig. 1, lane b).

The trichocyst proteins after heat treatment and fractionation by centrifugation are shown in Fig. 1, lanes e and f. The supernatant fraction has proteins separated from the pellet fraction containing the residual organelle. This subset of proteins was of special interest since it can be isolated with relative ease on a preparative scale. It had 2–3 prominent bands at M, 20,000–22,000 and a minor band at M, 25,000 (Fig. 1, lane e). The proteins of the pellet fraction are shown in Fig. 1, lane f. Immunoblots of these two fractions appear in Fig. 1, lanes g and h.

Reaction of Bovine CGA Antiserum with Trichocyst Proteins—Four independently derived antisera raised against natural CGA detected trichocyst proteins on immunoblots. All sera reacted with bands in the M, 15,000–25,000 cluster. On blots using reduced trichocyst proteins, anti-CGA serum labeled several proteins in the M, 15,000–25,000 range (Fig. 1, lane i). The region of M, 17,000, which reacted strongly with antiserum against trichocysts, was not as strikingly labeled by antiserum against CGA. Furthermore, polypeptides at M, 33,000–39,000 which had been recognized by anti-trichocyst serum were not stained. Samples with unreduced disulfide bonds, which stain intensely for protein in the M, 30,000–35,000 dimer cluster, were immunostained at this region by the anti-CGA serum (data not shown). Heat treatment of supernatant and pellet fractions (Fig. 1, lanes j and k) showed that most of the supernatant proteins visible after Ponceau S staining were immunoreactive (cf. Fig. 1, lane e). These same supernatant fractions run on isoelectric focusing gels contained 2 of the 24 trichocyst bands resolved by isoelectric focusing (19). The more acidic band (PI 4.8) reacted strongly with anti-CGA serum (data not shown).

Immunoreactivity of Bovine Chromaffin Granule Lysozyme with Antiserum to Trichocysts—Chromaffin granule lysozyme run on SDS-PAGE has a complex protein pattern with the major staining at M, 75,000 attributable to CGA (data not shown). Antiserum against trichocyst proteins stained CGA and two other proteins of lower Mr (Fig. 2, lane a). Anti-CGA serum recognized CGA in the lysate as well as several smaller poly-
peptides, possibly breakdown products (Fig. 2, lane b). Preimmune serum gave no response (Fig. 2, lane c). Immunoblots stained with antisera raised against the chemically synthesized peptides PL26 and GE25 specific to CGA (see “Experimental Procedures”) had staining profiles similar to those seen with antiserum to purified CGA (data not shown).

**Immunoreactivity of Paramecium Whole Cell Extract to Serum against Trichocysts, CGA, and the Synthetic Peptides PL26 and GE25**—To test for immunoreactivity of the unprocessed trichocyst precursor proteins, we performed immunoblots using extracts of whole cells. Mature trichocysts, obtained after exocytosis, contain little or no unprocessed precursor (5), and immature trichocysts are not capable of being discharged. Immunoblots of Paramecium extract after incubation in antiserum to CGA (Fig. 3, lane a) stained intensely at Mr 53,000, less intensely at Mr 49,000, and weakly at bands of lower Mr. Antiserum to the synthetic peptide PL26 generated a similar profile (Fig. 3, lane b). Immunoblots of Paramecium extract after incubation in anti-trichocyst serum showed the expected immunoreactive bands at Mr 15,000-25,000 (Fig. 3, lane c). Also stained were larger polypeptides at Mr 53,000, 49,000, 39,000, and 37,000 which have similar Mr to those of the trichocyst precursor protein cluster (5). Antiserum against the synthetic peptide GE25 reacted with a band at Mr 49,000 and other smaller polypeptides (Fig. 3, lane d). Although immunoreactivity of bands in the region where mature trichocyst proteins are found (Mr 15,000-25,000) was weak in the extracts, mature trichocyst proteins clearly are immunoreactive (cf. Fig. 1, lanes i-k; data not shown for antisera to PL26 and GE25). Preimmune serum and antiserum to synthetic peptides preabsorbed with the appropriate peptide gave no response (data not shown).

**Hybridization of RNA from Paramecium with CGA cDNA on Northern Blots**—The cDNA for the entire coding region of bovine CGA was inserted into a pUC13 plasmid, now termed pChrg4A (8). Hybridization of labeled pChrg4A to mRNA from Paramecium was tested by dot blot and Northern analysis. Dot blots showed hybridization at two different formamide concentrations (Fig. 4, lanes a and b). A positive

**FIG. 2. Immunoblot of chromaffin granule lysate with antisera against CGA and trichocyst proteins, and with preimmune serum.** Chromaffin granule lysate (see "Experimental Procedures") was run on SDS-PAGE after disulfide reduction and then transferred to nitrocellulose filters. Blots were stained with: anti-CGA serum (1:100); anti-trichocyst serum (1:200); and preimmune serum.

**FIG. 3. Immunoblot of Paramecium cell extract with antisera to trichocysts, isolated CGA, and the synthetic peptides PL26 and GE25.** Whole cell extract of Paramecium (see "Experimental Procedures") was subjected to SDS-PAGE after disulfide reduction (10% gel for lanes a and b and 8-12% gradient gel for lanes c and d) and then transferred to nitrocellulose filters and stained with: lane a, anti-CGA serum (1:100); lane b, anti-PL26 (1:100); lane c, anti-trichocyst serum (1:1000); lane d, anti-GE25 serum (1:500).

**FIG. 4. Hybridization of pChrg4A to Paramecium mRNA.** Hybridization conditions are as indicated under "Experimental Procedures." Dot blots were hybridized in the presence of 20% formamide (lane a) and 30% formamide (lane b). The amount of total cellular RNA applied is, from left to right: 1, 0.1, 0.01, and 0.001 µg. Blots were exposed to film for 2 weeks.

**FIG. 5. Northern blot hybridization of pChrg4A to Paramecium mRNA.** Northern blot hybridized as for the dot blots in Fig. 4 at 37°C in the presence of 20% formamide; 20 µg of total cellular RNA was used. The transfer membrane was exposed to film for 7 days. The positions of 28S and 18S rRNA are shown.
signal was demonstrated only with the pChrg4A; hybridization with the labeled pUC13 vector was negative (data not shown). Northern hybridization using the same conditions as for the dot blots is shown in Fig. 5. The position of the stained rRNA markers indicates that the cross-hybridizing message size is similar to that of bovine CGA.

**DISCUSSION**

Our results represent the first evidence for the presence of chromogranin-like proteins in cells other than secretory cells of vertebrate organisms. We found proteins in *Paramecium* which reacted with six antisera recognizing CGA or portions of CGA; four different, independently derived antisera against natural CGA and sera raised against two synthetic peptides corresponding to C-terminal portions of CGA. Natural CGA is known to be post-translationally modified by glycosylation and phosphorylation (6). The possibility that the cross-reactation of *Paramecium* proteins with the anti-CGA serum represented the recognition of similar modifying groups on otherwise unrelated proteins is ruled out by the results with antibodies against synthetic chromogranin peptides, which do not have such modifying groups; these antibodies reacted at least as well with *Paramecium* proteins as did the anti-CGA antisera. It appears therefore that the *Paramecium* proteins have sufficient primary sequence homology with CGA to give a strong immunological cross-reaction.

This conclusion was further supported by the reciprocal experiment, in which antisera raised against a group of *Paramecium* secretory proteins (trichocysts) recognized CGA on immunoblots. A third line of evidence for CGA-like sequences in *Paramecium* was the specific hybridization of CGA cDNA with a single species of *Paramecium* RNA on Northern blots. Taken together, these consider results strong evidence for the conservation of some portion of the CGA sequence between *Paramecium* and vertebrates, across a wide distance in evolution.

The cross-reactive proteins in *Paramecium* were detected in cell extracts and in the purified secretory products (the extruded trichocysts), suggesting some localization in secretory granules. Proteins of the trichocysts share other similarities with CGA. Both are synthesized as larger precursors and are proteolytically processed to smaller peptides during maturation (5, 6). Both are acidic: most have pI values between 4.5 and 5.5 (3, 4). The heat-soluble fraction of isolated trichocysts is enriched for cross-reactive protein; we are purifying these to determine amino acid sequences for comparison with CGA.

Although CGA-like proteins may exist elsewhere in the cell than in secretory granules, it is equally plausible that cross-reactive protein is localized in immature granules whose contents we cannot isolate. It may also be released as a soluble protein during exocytotic discharge of trichocysts and therefore not be present in the insoluble secretory product we isolate. We expect to resolve this question by electron microscopic immunocytochemical localization of the cross-reactive proteins.

From the *M* of the cross-reactive proteins in *Paramecium*, it appears likely that the anti-CGA sera recognize both the high *M* precursor and the mature product of trichocyst development. The largest protein recognized in *Paramecium* extracts by any of the sera was of about the same *M* as the largest trichocyst precursor identified (5), and the major cross-reactive proteins in isolated trichocysts were of the same size as the fully processed proteins of mature trichocysts. It may be significant that the largest *Paramecium* protein stained by anti-CGA antiseraum (M, 53,000) is about the same size as unglycosylated CGA (M, 48,000, deduced from the cDNA sequence) and that the *Paramecium* mRNA detected with CGA cDNA is about the same size as the CGA mRNA (8, 9). The ephorophoretic mobility of glycosylated CGA on SDS-PAGE is known to be anomalous; we do not know whether the *M* of the trichocyst precursors or of the proteins of *Paramecium* extracts that cross-react with anti-CGA is a true reflection of the protein's size.

Conservation of sequence in secretory proteins of *Paramecium* and vertebrates predicts conservation of function(s) related to secretion. The function of chromogranins is not known, but recent evidence that pancreastatin (16) has >70% homology to a specific portion of CGA indicates a possible role as precursor(s) to smaller, biologically active peptides destined for secretion (17, 18). The acidic pI of chromogranins suggests that these proteins may act to bind Ca2+ in secretory granules or to buffer the pH at the acidic level characteristic of secretory granules (6). The pH within mature trichocysts is also low (32), and Ca2+ is known to trigger the extension of the trichocyst contents associated with exocytosis (30-32).

We hope to gain insight into the function of the CGA-like protein in *Paramecium* by studying the many mutants defective in exocytosis (33, 34).

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