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Chromogranin A Fragments Modulate Cell Adhesion

IDENTIFICATION AND CHARACTERIZATION OF A PRO-ADHESIVE DOMAIN*

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Although several functions have been suggested for chromogranin A, a glycoprotein secreted by many neuroendocrine cells, the physiological role of this protein and of its proteolytic fragments has not been established. We have found that mixtures of chromogranin A fragments can inhibit fibroblast adhesion. The anti-adhesive activity was converted into pro-adhesive activity by limited trypsin treatment. Pro-adhesive effects were observed also with recombinant N-terminal fragments corresponding to residues 1–78 and 1–115 and with a synthetic peptide encompassing the residues 7–57. These fragments induced adhesion and spreading of fibroblasts on plates coated with collagen I or IV, laminin, fetal calf serum (FCS) but not on bovine serum albumin. The long incubation time required for adhesion assays (4 h) and the FCS requirements for optimal adhesion suggest that the adhesive activity is likely indirect and requires other proteins present in the FCS or made by the cells.

These findings suggest that chromogranin A and its fragments could play a role in the regulation of cell adhesion. Since chromogranin A is concentrated and stored within granules and rapidly released by neuroendocrine cells and neurons after an appropriate stimulus, this protein could be important for the local control of cell adhesion by stimulated cells.

Chromogranin A (CgA)[1] was originally discovered as the major soluble protein of adrenal medullary chromaffin granules. This protein was found later to be a member of a family of regulated secretory proteins present in the electron-dense granules of many other neuroendocrine tissues and of the nervous system (1, 2).

The physicochemical properties and tissue distribution of CgA have been extensively characterized (2–5). For instance, CgA is co-stored with various hormones in the secretory vesicles of cells of the gastrointestinal tract (4), the adeno- and neurohypophysis (5), the parathyroid (6), the endocrine pancreas (7), the thyroid C-cells (8), the immune system (9), and the atrial myocardium (10). In addition, it is a component of dense core synaptic granules in many areas of the central nervous system (11, 12). Human CgA is a hydrophilic protein of 439 amino acids, characterized by low isoelectric point and by several post-translational modifications, including glycosylation, sulfation, and phosphorylation (3, 13, 14). Within the secretory vesicles, CgA may form dimers and tetramers as a function of pH and Ca^{2+} concentration (15–17). A large body of evidence suggests that tissue-specific patterns of proteolytic processing generate different fragments of CgA (18–21). Moreover, multiple forms having different hydrodynamic sizes of 600, 100, and 55 kDa have been detected in the serum of cancer patients (22).

As far as the physiological role is concerned, CgA has been suggested to be involved in hormone packaging within secretory granules and in modulating the secretory granule functions by binding Ca^{2+} and ATP. Moreover, it has been proposed that CgA represents a precursor of biologically active peptides with endocrine, paracrine, and autocrine functions (23). For instance, CgA residues 248–293 were found to be homologous to pancreastatin, a pancreatic peptide that inhibits insulin secretion (24), whereas chromostatin, a peptide corresponding to residues 124–143, inhibits secretion of catecholamines from chromaffin cells (25). Retrogradely perfused and nerve-stimulated bovine adrenal medullae release the CgA fragments corresponding to amino acids 1–76 and 1–113. These fragments have been named vasostatin I and II (VS-1 and VS-2), respectively, because of their inhibitory effects on vascular tension (26–28).

Despite the above proposed functions, the physiological roles of this protein remain largely undefined. In this work, we show that CgA fragments from natural and recombinant sources can modulate the adhesion of fibroblasts on various substrates and that a pro-adhesive domain is present in the N-terminal portion of the molecule.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—96-Well polyvinyl chloride microtiter plates (Falcon Micro Test III flexible assay plates) were obtained from Becton Dickinson and Co. (Oxnard, CA). Bovine serum albumin (BSA, fraction V), polyoxyethylene sorbitan monolaurate (Tween 20), paraformaldehyde, goat anti-mouse IgG horseradish peroxidase conjugate, goat anti-rabbit IgG-peroxidase conjugate, normal goat serum, *o*-phenylenediamine dihydrochloride and streptavidin-peroxidase, laminin and collagen type IV (isolated from Englebreth-Holm-Swarm mouse sarcoma) and poly-L-lysine were from Sigma. Crystal violet was

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[1] The abbreviations used are: CgA, chromogranin A; CgB, chromogranin B; mAb, monoclonal antibody; HSF, heat stable fraction; ECM, extracellular matrix; VS-1, vasostatin-1, VS-2, vasostatin-2; PBS, 0.15 M sodium chloride, 0.05 M sodium phosphate buffer, pH 7.3; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; Fmoc, *N*-(9-fluorenyl)-methoxycarbonyl.

from Fluka-Chemica (Milan, Italy). Rat tail type I collagen was from ICN Biomedicals, Inc. (Costa Mesa, CA). D-Biotinyl-6-aminocaproic acid *N*-hydroxysuccinimide ester was from Societá Prodotti Antibiotici S.p.A (Milan, Italy). Enhanced chemiluminescence (ECL™) Western blotting kit was from Amersham Italia SRL (Milan, Italy). Milk "Humana 3" was from Humana Italia S.p.A (Milan, Italy). Nitrocellulose membranes were from Schleicher & Schuell (D-3354, Dassel, Germany). Mouse monoclonal antibodies (mAb) A11 and B4E11 (anti-CgA) were described previously (29, 30).

SK-N-BE and CHP-134 neuroblastoma cells (obtained from Dr. G. Della Valle, University of Pavia, Italy) were cultured in RPMI, 20% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, at 37 °C, 5% CO_2; PC-12 (obtained from American Tissue Culture Collection, ATCC CRL1721), LB6 and NIH-3T3 mouse fibroblasts (obtained from Prof. F. Blasi, San Raffaele Scientific Institute), and human foreskin fibroblasts HFSF-132 (obtained from Dr. J. Bizik, San Raffaele Scientific Institute) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 1 mM sodium pyruvate (DMEM, 1 ×) and 10% FCS, at 37 °C, 5% CO_2.

Purification of Natural CgA—Natural human CgA was purified from pheochromocytoma tissues, as follows. The tumor was frozen in liquid nitrogen immediately after surgical excision, lyophilized, and homogenized in distilled water. The homogenate was boiled for 6 min and centrifuged at 120,000 × *g* for 30 min. The supernatant containing CgA, which is heat stable (1), was kept at −20 °C until use. CgA was then purified from the pheochromocytoma heat stable fraction (HSF) by immunoaffinity chromatography on mAb A11-Sepharose as follows: 3 mg of mAb A11 was coupled to 0.5 g of activated CH-Sepharose (Pharmacia Biotech Inc.), using 0.1 M sodium carbonate, pH 8.0, as coupling buffer (overnight at 4 °C), and 0.1 M Tris-HCl, pH 8.0, as blocking agent (2 h at room temperature). After column washings (three times with 0.5 M sodium chloride, 0.1 M Tris-HCl buffer, pH 8.0, and with 0.5 M sodium chloride, 0.1 M sodium acetate buffer, pH 4.0) pheochromocytoma HSF, 1 mg in 1 ml of sodium chloride, sodium phosphate buffer, pH 7.3 (PBS), was loaded onto the mAb A11-agarose column and washed with PBS until the absorbance of the effluent reached the base line. The column was then eluted with 3 ml of 0.5 M sodium chloride, 0.2 M glycine, pH 2.0 (fraction I), and with 10 ml of PBS (fraction A). The protein content in the starting material and in the purified fractions was quantified using the "Bio-Rad Protein Assay" kit. After pH neutralization, the CgA fractions were mixed with 2 volumes of acetone, incubated overnight at −20 °C, and centrifuged 45 min at 2500 × *g*. The pellet was dried using a SpeedVac concentrator, resuspended in water, extensively dialyzed against distilled water (three changes), and kept at −20 °C.

Production of Recombinant CgA, VS-1 and VS-2, and RGE Mutant—Various recombinant fragments of human CgA (rCgA) were prepared including the following: (*a*) rCgA-(7–439), (*b*) NH_2-Ser-Thr-Ala-rCgA-(1–78) (VS-1); (*c*) NH_2-Ser-Thr-Ala-rCgA-(1–115) (VS-2); (*d*) a variant of the rCgA-(7–439) in which residues 46–48 (RGD) were replaced with RGE (RGE-rCgA-(7–439)) (numbering according to Konecki *et al.* (13)). Cloning, purification, and characterization of all these recombinant polypeptides are described elsewhere.[2] Since all products contain the residues 68–70 recognized by mAb B4E11 (30) and were able to bind to B4E11-agarose, all products were purified from recombinant *Escherichia coli* strains using this immunoadsorbent, essentially as described for natural CgA.

Preparation of CgA Synthetic Peptides—Various 23–25-mer peptides spanning most of the CgA sequence were simultaneously synthesized on pre-loaded NovaSyn TGA resin (Novabiochem) using an apparatus for manual multiple peptide synthesis and a solid phase method based on Fmoc chemistry (31). Tyrosine and glycine spacers were added at the N or the C termini to those peptides not containing chromophoric amino acids, to allow spectrophotometric detection. Each peptide was purified by reverse-phase HPLC and lyophilized.

The peptide 7–57 was synthesized by the solid phase Fmoc method using an Applied Biosystems model 433A peptide synthesizer. After peptide assembly the side chain protected peptidyl resin was de-blocked as described (32) and purified to apparent homogeneity by reverse-phase chromatography. The peptide, containing two cysteines, was oxidatively folded by overnight treatment with 5-fold excess of oxidized glutathione (33) and purified by ion exchange followed by reverse-phase chromatography.

[2] A. Corti, L. Perez-Sanchez, A. Gasparri, F. Curnis, R. Longhi, A. Brandazza, A. G. Siccardi, and A. Sidoli, manuscript in preparation.

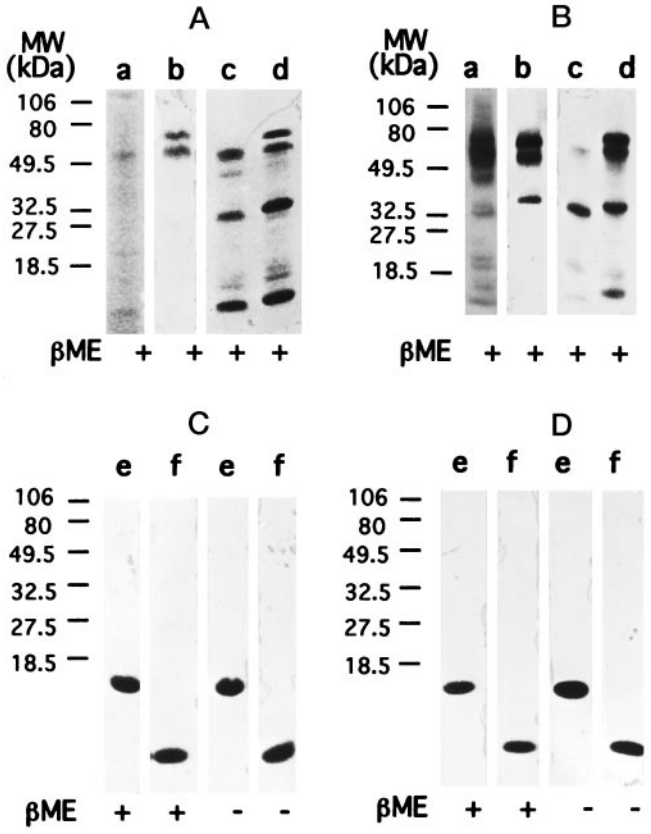

FIG. 1. **SDS-PAGE (*panels A* and *C*) and Western blot analyses (*panels B* and *D*) of natural and recombinant CgA, VS-1, and VS-2 under reducing (+*βME*) and nonreducing (−*βME*) conditions.** CgA fraction A and fraction I (*lanes a* and *b*); rCgA-(7–439) fraction A and fraction I (*lanes c* and *d*); VS-2 (*lanes e*); VS-1 (*lanes f*). SDS-PAGE was carried out in a Phast System apparatus (Pharmacia) using ready-made PhastGels 12.5% (*panels A* and *B*) and PhastGels 20% (*panels C* and *B*).

Mass Spectrometry—Electrospray ionization mass spectrometry of VS-2 was carried out by PRIMM s.r.l. Laboratories (Milan, Italy).

CgA-Enzyme-linked Immunosorbent Assay—Polyvinylchloride microtiter plates were coated with B4E11 (10 μg/ml in PBS, 50 μl/well, overnight at 4 °C). All subsequent steps were carried out at room temperature. After washing three times with PBS, the plates were blocked with 3% BSA in PBS (200 μl/well, 2 h at room temperature) and washed with PBS again. CgA standard or sample solutions, diluted 1:2 in PBS containing 0.5% BSA, 0.05% (v/v) Tween 20, and 2.5% normal goat serum ("assay buffer") were added (50 μl/well) and incubated for 1.5 h at 37 °C. The plates were washed eight times by emptying and filling with PBS containing 0.05% (v/v) Tween 20 and incubated with rabbit anti-rCgA-(7–439) antiserum, 1:1000 in assay buffer (50 μl/well, 1.5 h at room temperature). The plates were washed again as above and further incubated with goat anti-rabbit IgG-peroxidase conjugate (1:3000 in assay buffer, 50 μl/well, 1 h at room temperature). After the final wash, the plates were incubated with 0.4 mg/ml *o*-phenylenediamine solution in 0.05 M phosphate-citrate buffer, pH 5.0, containing 3.5 mM hydrogen peroxide (100 μl/well, 30 min). The reaction was stopped by adding 10% (v/v) sulfuric acid (100 μl/well), and the absorbance of each well was read at 492 nm. Each assay was calibrated with eight rCgA-(7–439) solutions at various concentrations.

Western Blot Analysis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Phast System apparatus (Pharmacia) using ready made polyacrylamide gels (Phast Gels 12.5% or 20%, Pharmacia). Samples were 2-fold diluted with 20 mM Tris-HCl, pH 8.0, containing 2 mM EDTA, 5% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.02% (w/v) bromphenol blue and boiled for 5 min prior to electrophoresis. Western blot analysis was carried out essentially as follows: proteins, after SDS-PAGE, were electrophoretically transferred to nitrocellulose membranes using 20 mA for 25 min and 25 mM Tris, 150 mM glycine, 20% (v/v) methanol, pH 8.9, as transfer buffer. The nitrocellulose membranes were rinsed twice with PBS and were

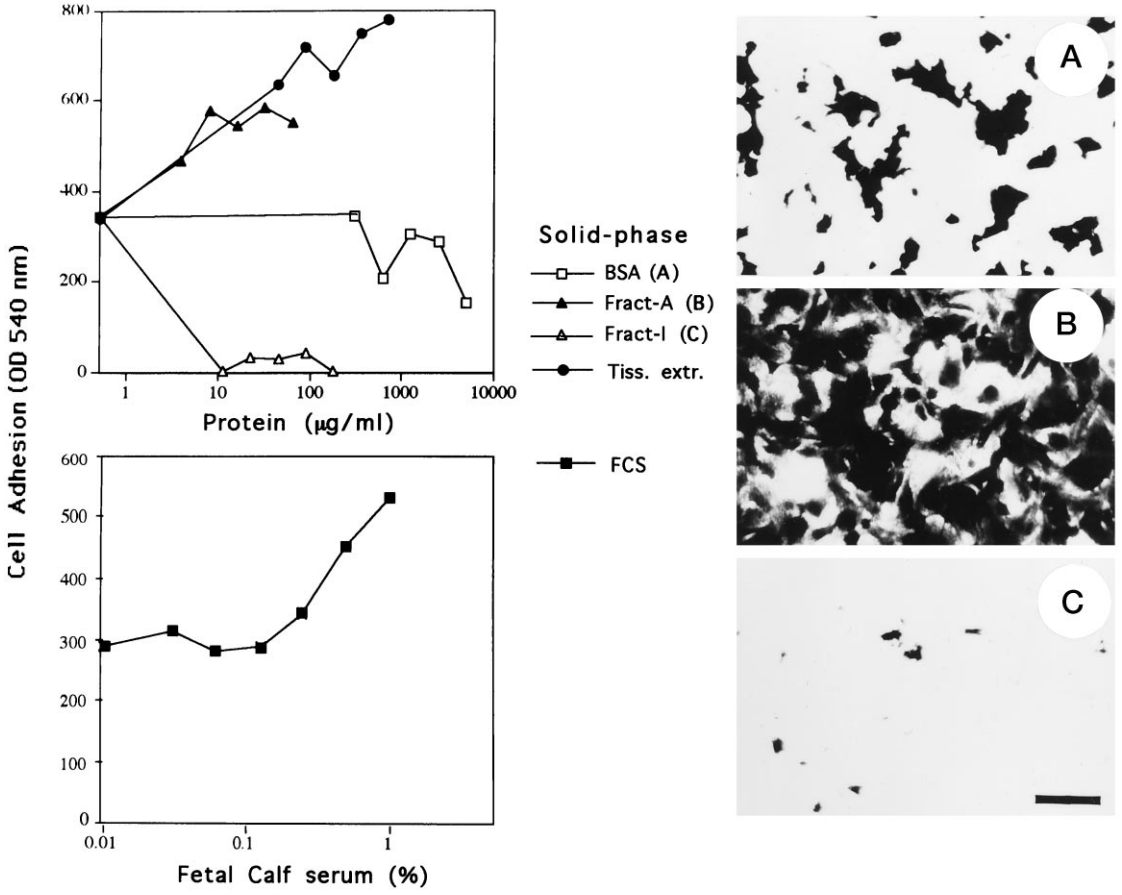

FIG. 2. **Adhesion of HFSF-132 fibroblasts to solid phases coated with BSA (*panel A*), fraction A (*panel B*), fraction I (*panel C*) (*left and right panels*), or pheochromocytoma HSF (*tiss. extr.*) or FCS (*left panels*).** The assay was carried out according to the assay 1 protocol (see "Experimental Procedures"). *Bar*, 30 μm.

incubated overnight at 4 °C in PBS containing 1% (w/v) BSA and 3% (w/v) milk. The membranes were then incubated for 2 h with anti-CgA or anti-CgB mAbs (2 μg/ml) in PBS containing 1% (w/v) BSA, 3% (w/v) milk, 1% (v/v) normal goat serum ("binding buffer"). After washing with PBS containing 0.02% (v/v) Tween 20, the membranes were further incubated for 2 h with goat anti-mouse IgG horseradish peroxidase conjugate (1:1000) in binding buffer. After the final wash, the visualization reaction was carried out with "ECL™-Western blotting" kit (Amersham Corp.), based on chemiluminescence of a luminol substrate.

Adhesion "Assay 1"—HSFS-132 cells were detached by treatment with a prewarmed trypsin/EDTA solution (0.2 μg/ml trypsin, 0.2 mg/ml EDTA) for 5 min at 37 °C, and mixed with DMEM 1 × supplemented with 10% FCS. After three washings with 0.9% (w/v) sodium chloride to remove residual FCS, by centrifugation at 200 × *g* for 10 min, the cells were resuspended at 3×10^5 cell/ml in DMEM 1 ×, containing 1% FCS. Ninety-six-well polystyrene cell culture plates (Nunc) were coated with 50 μl/well of CgA fraction solutions in PBS (90 min at 37 °C). After coating, 100-μl aliquots of a 3% (w/v) BSA solution in DMEM was added to each well and left to incubate for 30 min at 37 °C. The plates were then washed two times by emptying and filling with 0.9% (w/v) sodium chloride and filled with 50 μl/well cell suspension. After 4 h incubation in a 5% CO_2 incubator at 37 °C, 200-μl aliquots of DMEM were added to each well and aspirated using a glass pipette connected to a vacuum pump. The cells were washed again with DMEM and fixed by adding 100 μl/well of 3% (w/v) paraformaldehyde (5 min at room temperature). The solution was then removed, and the fixed cells were stained by adding 50 μl/well of 0.5% (w/v) crystal violet for 10 min.

Adhesion "Assay 2"—This assay was performed using solid phases coated with various solutions containing collagen type I (10 μg/ml) or type IV (10 μg/ml), or laminin (10 μg/ml), or 1% FCS, or poly-L-lysine (50 μg/ml) in PBS (90 min at 37 °C). After coating, the plates were washed and blocked with BSA as described for assay 1. Cells were resuspended at 3×10^5 cell/ml in DMEM containing 4 mM glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, 0.5 μg/ml amphotericin B, 2 mM sodium pyruvate solution (DMEM, 2 ×) and 2% (w/v) BSA. Twenty-five μl of cell suspension was then added to each well and mixed with 25 μl of CgA fractions at various concentrations in 0.9% (w/v) sodium chloride. After 4 h incubation, adherent cells were washed, fixed, and stained as described for assay 1.

RESULTS

Natural and Recombinant CgA Affect Human Fibroblast Adhesion—Human CgA was isolated from the heat stable fraction (HSF) of homogenized pheochromocytomas by immunoaffinity chromatography, using a Sepharose-bound monoclonal antibody directed against the CgA-(81–90) epitope (mAb A11) (30). Two sequential fractions containing immunoreactive CgA material were recovered by eluting the column with a pH 2.0 buffer (fraction I) and then with PBS (fraction A). Reducing SDS-PAGE and Western blotting analyses were then performed using an antibody that recognizes an epitope located within residues 68–70 of CgA (mAb B4E11) (30). These studies showed that both fractions consist of heterogeneous mixtures of CgA fragments (Fig. 1, *panels A* and *B*).

The effect of fraction I and fraction A on the adhesive properties of HFSF-132 human foreskin fibroblasts was examined using these fractions adsorbed onto a solid phase (adhesion assay 1). In the presence of 1% FCS in the culture medium, the pattern of cell adhesion to fraction I- or fraction A-coated plates was opposite, being enhanced by fraction A and inhibited by fraction I (Fig. 2, *left panels*). Interestingly, microscopic inspection revealed that in fraction A-coated wells both cell adhesion and spreading were increased, whereas virtually no cells were found in fraction I-coated wells (Fig. 2, *right panels*). In the absence of FCS, HFSF-132 fibroblasts were still able to adhere to fraction A which were totally unable to adhere to wells coated with BSA or with fraction I. Similar results were ob-

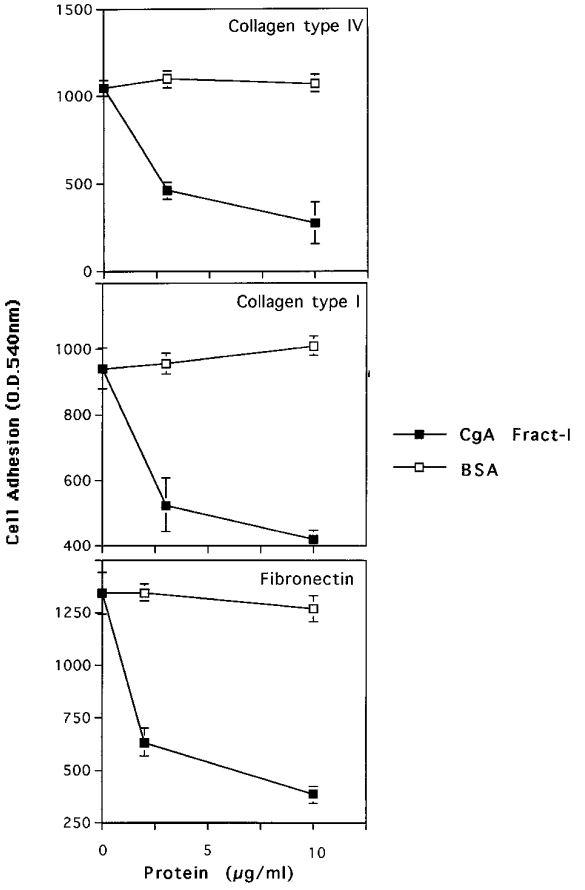

FIG. 3. **Adhesion of mouse NIH-3T3 fibroblasts to solid phases coated with non-saturating amounts of collagen I (0.3 μg/ml), collagen IV (3 μg/ml), or fibronectin (0.2 μg/ml) and overcoated with CgA fraction I or with BSA.** Microtiter wells were coated with solutions of each adhesive protein in PBS (50 μl, 1.5 h at 37 °C) and further incubated with CgA fraction A or BSA at the concentration indicated in the abscissa (50 μl, 1.5 h, 37 °C). The plates were further blocked with 3% BSA in DMEM (200 μl, 0.5 h, 37 °C). NIH-3T3 cells (40,000/well, in DMEM 1 × without FCS) were then added and left to adhere for 3 h at 37 °C, 5% CO_2. The plates were then washed and stained as described for assay 1 (see "Experimental Procedures").

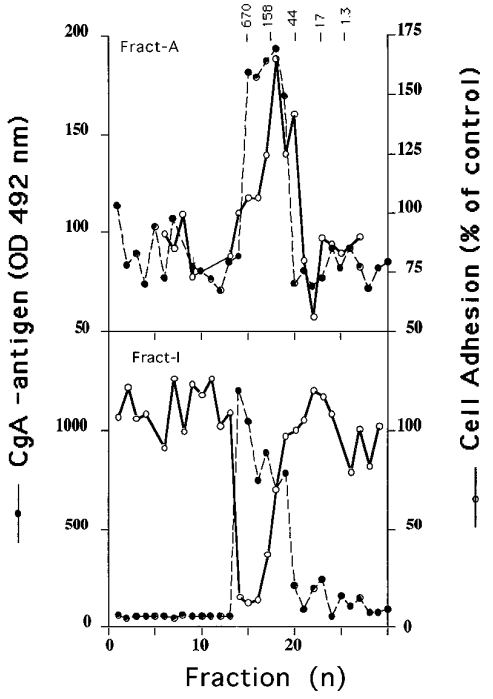

FIG. 4. **Size-exclusion HPLC of fraction A (*upper panel*) and fraction I (*lower panel*).** Size-exclusion HPLC was carried out at room temperature using a Bio-Sil 250 Guard column joined to a Bio-Sil SEC-250 column (Bio-Rad) as follows. The column was equilibrated and eluted with PBS (flow rate 0.6 ml/min). Fractions (0.3 ml) were collected and stored at −20 °C until analysis. The column was calibrated using thyroglobulin (670 kDa), IgG (158 kDa), bovine serum albumin (66 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and cyanocobalamin (1.3 kDa) as molecular markers. Fractions were analyzed by CgA-enzyme-linked immunosorbent assay and adhesion assay 1.

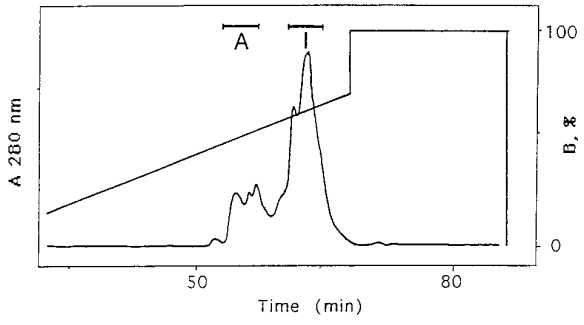

FIG. 5. **Reverse-phase HPLC of rCgA-(7–439).** rCgA-(7–439), purified by immunoaffinity chromatography on mAb B4E11-agarose, was loaded onto a reverse-phase Pro-RPC10/15 column (Pharmacia) and eluted as follows: buffer A, 0.1% trifluoroacetic acid in water, buffer B, 0.1% trifluoroacetic in acetonitrile; 0% B for 20 min; linear gradient 0–70% B for 20 min; flow rate 0.3 ml/h. Fractions corresponding to *A* and *I bars* were collected and termed rCgA-fraction A and rCgA-fraction I, respectively.

served also with mouse NIH-3T3 fibroblasts, indicating that these effects are not species-specific (not shown).

The inhibitory effect of fraction I was further tested using a different type of assay, in the absence of FCS. Plates were coated with nonsaturating amounts of various adhesive proteins, such as type I or type IV collagen or fibronectin, further incubated with various amounts of fraction I, and then blocked with a large excess of BSA. As shown in Fig. 3, 10 μg/ml fraction I was sufficient to inhibit NIH-3T3 fibroblast adhesion to these substrates.

Size-exclusion HPLC of fraction I and fraction A, followed by detection of CgA antigen and adhesion-promoting activity in the chromatographic fractions, indicated that the hydrodynamic properties of the pro-adhesive and anti-adhesive materials were different, being eluted at M_r 50–150 × 10^3 and M_r 300–700 × 10^3, respectively (Fig. 4).

To characterize the structural correlates of the adhesive and anti-adhesive effects, CgA was then produced by recombinant DNA technology. The cDNA coding for residues 7–439 of human CgA was cloned and expressed in *E. coli*. The product was isolated from the heat stable fraction of *E. coli* extract by immunoaffinity chromatography on mAb B4E11-agarose, followed by reverse-phase HPLC (Fig. 5). Again, two fractions were obtained, named rCgA-fraction A and rCgA-fraction I, characterized by adhesive and anti-adhesive effects. SDS-PAGE and Western blot analysis of the products (Fig. 1, *panels A* and *B*) revealed various immunoreactive bands of 70, 60, 45, 32, and 30 kDa and several other <20-kDa bands in both products. The N-terminal sequence of the 70- and 60-kDa bands was identical to that of residues 7–20 of CgA (not shown). This pattern suggested that extensive proteolysis occurred also in rCgA, probably starting from the C terminus, and that both the isolated fractions are highly heterogeneous. Since rCgA-fraction A was lacking the 70-kDa band and was apparently more degraded than rCgA-fraction I, we assume that proteolysis was a critical event in the generation of the pro-adhesive activity.

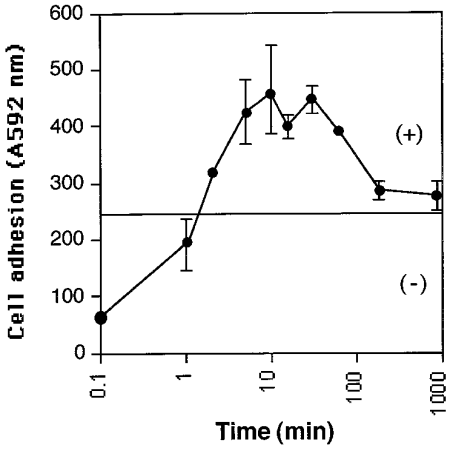

FIG. 6. **Adhesion of HSFS-132 cells to solid phases coated with rCgA-fraction I pretreated for various times with trypsin-agarose.** The *horizontal line* indicates the adhesion level to BSA-coated solid phase. Trypsin-agarose was prepared by coupling 1 mg of trypsin to 0.3 g of activated CH-Sepharose (Pharmacia), using 0.1 M sodium carbonate, pH 8.0, as coupling buffer (overnight at 4 °C), and 0.1 M Tris-HCl as blocking agent (2 h at room temperature). After washing (three times with 0.5 M sodium chloride, 0.1 M Tris-HCl buffer, pH 8.0, and with 0.5 M sodium chloride, 0.1 M sodium acetate buffer, pH 4.0), 0.1 ml of gel was mixed with 0.1 ml of rCgA-fraction I (0.73 mg/ml) and incubated at room temperature. The supernatant was withdrawn at various times and tested by adhesion assay 1.

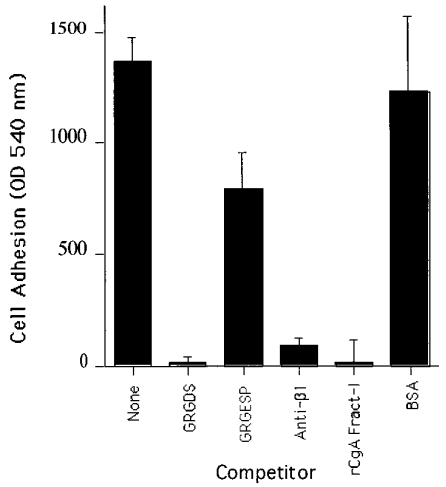

FIG. 7. **Adhesive effects of rCgA-fraction A in the presence of soluble competitors.** The assay was carried out by coating plates with rCgA-fraction A (50 μg/ml in PBS, 90 min at 37 °C) and, after washing three times with 0.15 M sodium chloride, by plating HFSF-132 cells in DMEM 1 × (without FCS) in the presence of various competitors, including GRGDS peptide, GRGESP peptide, rCgA-fraction I; BSA (all at 500 μg/ml) and goat anti-β1 integrin subunit antiserum (1/100). Cells were incubated for 4 h at 37 °C (5% CO_2) and stained as described for assay 1.

To verify this hypothesis, we carried out various digestions of rCgA-fraction I with trypsin-agarose, followed by removal of the immobilized enzyme and analysis of the adhesion activity. Limited trypsin treatment (5–10 min) was accompanied by a change from anti-adhesive to pro-adhesive effects, whereas extensive treatment (1–2 h) completely abolished both activities (Fig. 6). Various bands corresponding to <20-kDa products were observed by SDS-PAGE and Western blotting after limited digestion (not shown). These results strengthen the assumption that increased CgA fragmentation is associated with a change from anti-adhesive to pro-adhesive activity.

In conclusion, both natural and recombinant CgA behave like precursors that generate polypeptides endowed with opposite adhesive activities.

Anti-β1-integrin Antiserum Inhibits rCgA-Fraction A-mediated Cell Adhesion—To assess the potential role of integrins in rCgA-fraction A-mediated cell adhesion, we performed competition experiments using a GRGDS-peptide, commonly used to compete the interaction with integrin and RGD-containing substrates, and an anti-β1 integrin antiserum. As shown in Fig. 7, the adhesion of HFSF-132 fibroblasts to rCgA-fraction A-coated plates, was efficiently competed by GRGDS and by the anti-β1 antiserum and poorly or not at all by control GRGESP and BSA. Interestingly, cell adhesion to fraction A was virtually abolished by fraction I suggesting that these CgA mixtures contain materials that compete with each other in cell adhesion.

These results indicate that the rCgA-fraction A-mediated cell adhesion can be inhibited by affecting the β1-integrin function, hinting at a specific adhesion mechanism. However, we are unable from these data to speculate whether β1-integrin interact directly or indirectly with CgA fragments. Considering the long incubation time required for this adhesion assay (4 h), it is possible that the adhesive activity is indirect and requires other proteins made by the cells.

Recombinant CgA N-terminal Fragments (Vasostatins) Promote Fibroblast Adhesion and Spreading on Various Substrates—Three N-terminal fragments were prepared by recombinant DNA technology and peptide synthesis. In particular, we produced (*a*) the recombinant NH_2-Ser-Thr-Ala-rCgA-(1–78) fragment (VS-1); (*b*) the recombinant NH_2-Ser-Thr-Ala-rCgA-(1–115) fragment (VS-2); and (*c*) the synthetic CgA-(7–57) peptide.

The homogeneity and authenticity of recombinant products were assessed by SDS-PAGE, Western blotting, and in the case of VS-2, also by mass spectrometry.

Reducing SDS-PAGE of the purified VS-1 and VS-2 showed bands of about 15 and 10 kDa (Fig. 1, *panels C* and *D*). Moreover, electrospray ionization mass spectrometry of VS-2 indicated a molecular mass of 13247.38 ± 0.54 daltons, which differs of only 0.33 daltons from the calculated mass. These results are consistent with the expected sequences of VS-1 and VS-2.

The effects of VS-1 and VS-2 and the synthetic CgA-(7–57) peptide on the adhesive properties of various cell lines were assessed by two adhesion assays in which these products were either adsorbed onto a solid phase (assay 1) or in the liquid phase (assay 2). The cell lines tested included both CgA-producing and non-producing cell lines. The results, shown in Fig. 8, can be summarized as follows. (*a*) VS-1 and VS-2 adsorbed onto the solid phase have little or no effect on the adhesion of human HFSF-132 in the absence of FCS in the medium (*panel A*), while these polypeptides can significantly increase adhesion and spreading of these cells in the presence of 1% FCS (*panel B*). (*b*) All soluble polypeptides, including CgA-(7–57), at concentrations greater than 3 μM, increase human and mouse fibroblast adhesion to solid phases coated with 1% FCS or with poly-L-lysine, even in the absence of FCS in the liquid phase (*panels C* and *D*, and *E* and *F*). (*c*) None of the polypeptides are effective on the adhesion of human CgA-producing SK-N-BE and CHP-134 neuroblastoma or rat PC12 cells (*panels G–N*). (*d*) similar amounts of control proteins such as BSA, *E. coli* protein extract, and invertase had no effects. No effects were observed also with other control proteins spanning a wide range of isoelectric points such as the mouse IgG1 B4E11, transferrin, and avidin (not shown). Of note, FCS was apparently necessary for VS-1 and VS-2 adhesive activity in assay 1 (Fig. 8, *panels A* and *B*) while it was not necessary for that of fraction A (Fig. 7). Possibly, VS-1 and VS-2 peptides stick to the plastic less efficiently than CgA or these peptides need a FCS component to work.

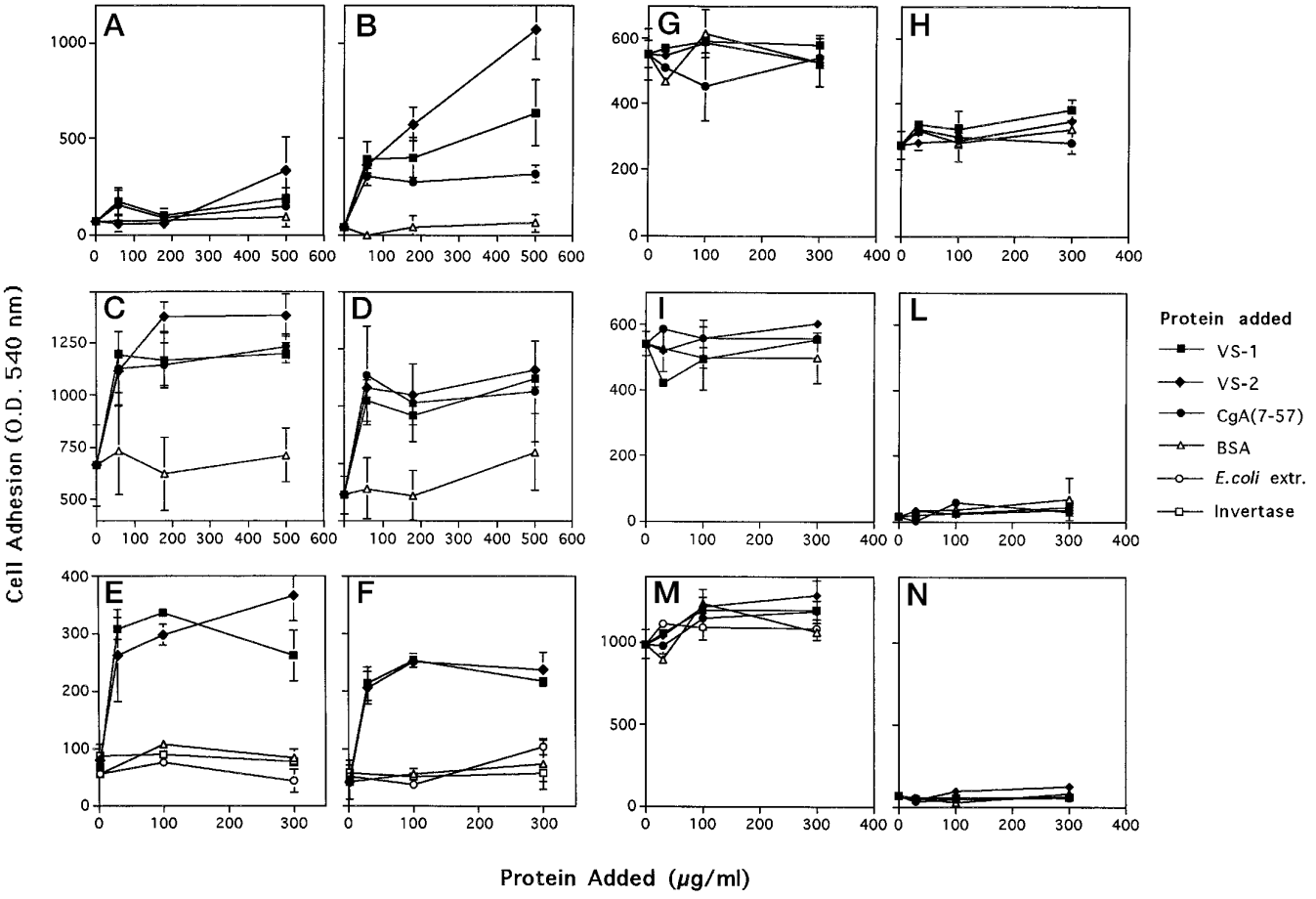

FIG. 8. **Effects of CgA N-terminal fragments on adhesion of human and mouse fibroblasts and other CgA-producing cells, as measured by assay 1 and assay 2.** Assay 1 (*panels A* and *B*), HFSF-132 human fibroblasts in DMEM 1 × containing 1% FCS (*panel B*) or in DMEM 1 × without FCS (*panel A*) were plated in wells coated with various amounts of CgA N-terminal fragment or with control proteins and analyzed as described under "Experimental Procedures." Assay 2 (*panels C–N*), cell suspensions in DMEM 1 × containing various amounts of soluble proteins (without FCS) were plated for 4 h onto solid phases coated with poly-L-lysine (*panels C, E, G, I*, and *M*) or FCS (*panels D, F, H, L*, and *N*) (see "Experimental Procedures"). Cells tested included human HFSF-132 fibroblasts (*panels C* and *D*), mouse LB6 fibroblasts (*panels E* and *F*), human SK-N-BE neuroblastoma cells (*panels G* and *H*), human CHP-134 neuroblastoma cells (*panels I-L*), and rat PC-12 pheochromocytoma cells (*panels M* and *N*).

The adhesive effects of N-terminal CgA polypeptides were not restricted to FCS or poly-L-lysine-coated plates. As shown in Fig. 9, the same effects were observed also on the adhesion of human fibroblasts to plates coated with types I and IV collagens or laminin but not with BSA.

To assess the capability of soluble N-terminal CgA fragments to interact with cell membrane components, we compared the adhesion of human fibroblasts after preincubation with or without VS-2 or CgA-(7–57) (30 min) and after washing out unbound ligands. As shown in Fig. 10, even in this case both CgA fragments were able to promote adhesion to various solid phase extracellular matrix (ECM) proteins, suggesting that CgA fragments can interact with cell surface molecules and trigger pro-adhesive effects.

Mapping of Vasostatin Adhesion Sites with Synthetic Peptides—Various 18–22-mer peptides spanning the entire CgA sequence were produced by chemical synthesis. These peptides included CgA 1–20, 25–46, 37–57, 47–68, 68–91, 91–113, 107–130, 130–153, 163–187, 187–210, 222–244, 231–255, 254–275, 275–297, 297–319, 315–337, 331–352, 353–375, 374–396, 395–417, and 416–439. None of these peptides, employed at 50 μM concentration, exhibited significant effects on the adhesion of LB6 and HFSF-132 cells. The fact that CgA-(7–57) was sufficient to exert pro-adhesive effects whereas no effects were observed with the 1–20, 25–46, 37–57, and 47–68 peptides suggests that conformational constraints in the N-terminal domain of CgA are necessary for activity.

DISCUSSION

In neuroendocrine cells and in neurons at least two secretory pathways are active: the regulated pathway, where the secreted products are concentrated and stored in secretory granules and released in response to external stimulation, and the constitutive pathway, where proteins are continuously transported to the cell surface without prior concentration or storage (34).

Chromogranins/secretogranins are a family of regulated secretory proteins present in the electron-dense granules of a variety of endocrine and neuroendocrine cells. These proteins are released to the extracellular environment together with the co-resident hormones and reach the bloodstream via the capillaries or the lymph vessels (2). Although several functions have been proposed for chromogranins, the intracellular and extracellular functions of these proteins and of their proteolytic fragments are still unclear.

In this work we have shown that different mixtures of natural and recombinant chromogranin A fragments (CgA-fraction A and -fraction I) when adsorbed onto solid phases, can exert different effects on fibroblast adhesion: pro-adhesive (fraction A) or anti-adhesive (fraction I). These activities are related to

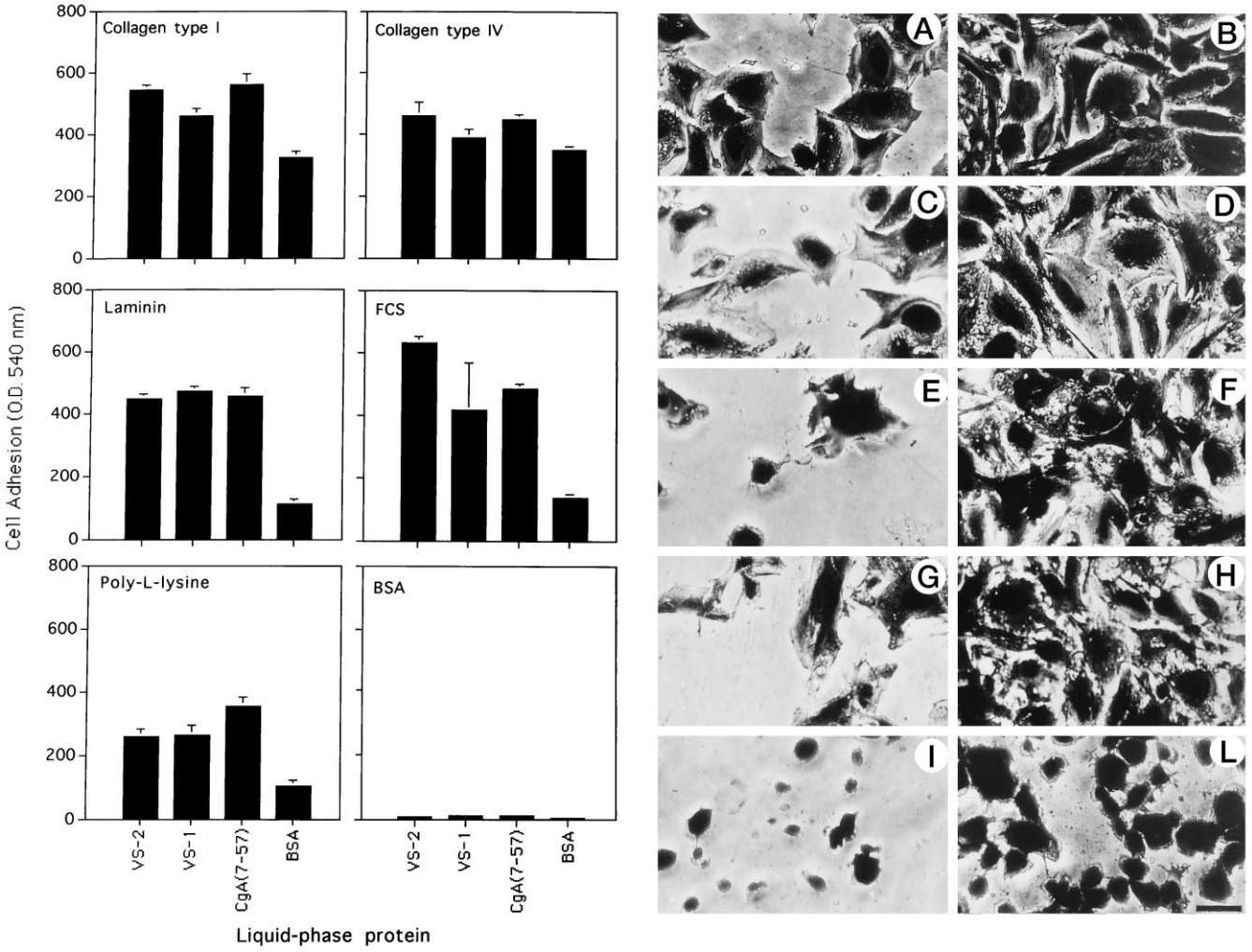

FIG. 9. **Pro-adhesive effects of CgA fragments on human HSFS-132 fibroblasts as measured by assay 2 (see "Experimental Procedures") using various substrates.** Solid phase proteins are indicated within the frame of each panel, and the proteins added in the liquid phase are indicated on the abscissa. Protein concentrations in the liquid phase were as follows: 70 μg/ml VS-2 or VS-1, 200 μg/ml CgA-(7–57), 500 μg/ml BSA. *Panels A–L* show the cell adhesion to the various substrates in the presence of BSA (*left panels*) or CgA-(7–57) peptide in the liquid phase, as observed by microscopy inspection. Solid phases were coated with type I collagen (*A* and *B*), type IV collagen (*C* and *D*), laminin (*E* and *F*), FCS (*G* and *H*), and poly-L-lysine (*I–L*). *Bar*, 10 μm.

antigenic forms with different hydrodynamic properties, since fraction I was eluted earlier then fraction A in gel filtration experiments. Moreover, limited proteolysis of the anti-adhesive fraction is associated with the appearance of a pro-adhesive effect. We also found that recombinant N-terminal fragments of CgA corresponding to VS-1 and VS-2 can exert pro-adhesive effects either when bound to a solid phase or when added in solution in various fibroblast adhesion assays. These results suggest that CgA and/or its N-terminal fragments may be involved in the regulation of cell adhesion.

Analysis of the primary structure of CgA has revealed in the N-terminal domain the presence of integrin binding motifs and sequence similarities with other adhesive molecules (Fig. 11). First, an RGD sequence, often present in ECM proteins involved in adhesive processes, *e.g.* fibronectin, vitronectin, and collagen (35), is present at residues 43–45. Second, a KGD sequence, also present in the integrin binding domain of barbourin, a snake venom disintegrin (36), is present at residues 9–11. Third, the sequence of the disulfide-loop located between the KGD and RGD regions (residues 19–37) is characterized by 32% identity and 64% similarity with that of a portion of the fibronectin type III-9 domain of tenascin, a tumor- and development-associated ECM protein (37, 38). These sequence similarities with other molecules involved in controlling adhesion make it attractive to speculate that the KGD-disulfide loop-RGD region (residues 8–45) plays a role in the adhesive effects observed with N-terminal fragments. Interestingly, we found that a synthetic peptide encompassing this region (peptide 7–57) is sufficient for mediating fibroblast pro-adhesive effects. The importance of this region in CgA is also suggested by the high degree of conservation (>82%) among various species (Fig. 11). However, while the KGD sequence is highly conserved within human, mouse, rat, porcine, bovine, and ostrich CgA (3, 39), the RGD sequence is replaced with QGD in the mouse and rat (40, 41), arguing against its functional importance. Accordingly, we found that an RGE-rCgA-(7–439) mutant was still able to induce anti-adhesive and pro-adhesive effects, after trypsin treatment, just as the wild type RGD-rCgA-(7–439) (not shown).

Another protein of the granin family, *i.e.* secretogranin I/chromogranin B (CgB), has been shown to be a heparin-binding adhesive protein (42). It has been proposed that CgB becomes associated with the ECM after secretion from endocrine cells or neurons and can thus mediate local cell-substrate adhesion. However, the adhesive properties of CgA and CgB are likely to rely on different mechanisms. Indeed, putative heparin binding sequences are not included in CgA. Although in a previous study an RGD-peptide was unable to block 3T3

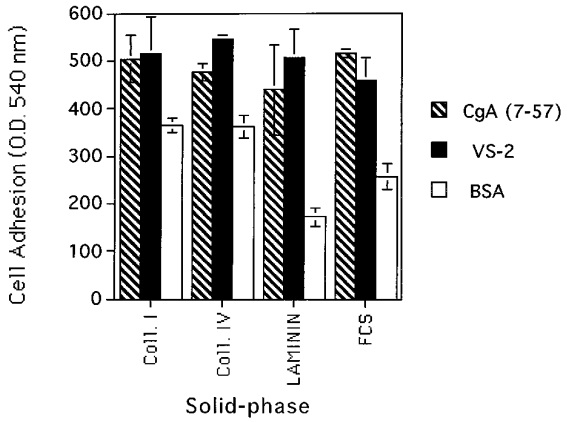

FIG. 10. **Adhesion of human HSFS-132 fibroblasts to various solid phase proteins after preincubation with CgA-(7–57), VS-2, BSA.** Cells were preincubated with 200 μg/ml CgA-(7–57), 100 μg/ml VS-2, 200 μg/ml BSA for 30 min at 37 °C, washed once with 0.15 M sodium chloride, and plated for 4 h at 37 °C in DMEM 1 × containing 1% (w/v) BSA in microtiter plates precoated with types I and IV collagen, laminin, and FCS as described in the assay 2 protocol.

cell adhesion to CgB (42), here we demonstrate that a similar peptide as well as an anti-β1 integrin antiserum block HFSF-132 adhesion to recombinant fraction A. These results suggest that CgA may modulate adhesion mechanisms by regulating either the binding capacity or the affinity of integrins for their ligands, possibly via inside-out or outside-in signals (43). Several hypotheses can be made on how this phenomenon occurs: for instance, CgA could interact with other adhesive molecules of the extracellular matrix and thus modulate their interaction with integrins. Alternatively, CgA could interact with membrane receptors that trigger regulatory signals that, in turn, affect either cytoskeletal organization or the interaction between integrins and ECM proteins. Further work is necessary to verify these hypotheses. The long incubation time for adhesion assays (4 h) and the FCS requirements suggest that the adhesive activity is more likely indirect and requires other proteins present in the FCS or made by the cells.

CgA and CgB are often co-stored in the secretory granules of neuroendocrine cells. Considering that CgA N-terminal fragments were found to induce adhesion and spreading of fibroblasts on various ECM proteins, it is possible that CgA fragments cooperatively regulate not only cell adhesion to ECM proteins but also the adhesive properties of ECM-bound CgB. Although we were unable to identify the structural correlates of the anti-adhesive activity of fraction I, the finding that it can be converted into pro-adhesive material by trypsin treatment suggests that proteolytic processing of CgA could be critical for its adhesive functions. Tissue-specific patterns of proteolytic processing and different fragments thereof have been observed by many investigators (18–21). Thus, the modulation of both the intra-granular and/or the extracellular proteases involved in these processes could represent a mechanism by which secretory cells control the adhesion of themselves and/or the adjacent cells.

The adhesive effects of CgA were observed with human and mouse fibroblasts, *i.e.* with cells that do not secrete CgA. When we added exogenous CgA fragments to SK-N-BE or CHP-134 neuroblastoma cells no pro-adhesive effects were observed. Several explanation are possible. For instance, it is possible that these cells do not express the proper receptors for CgA-mediated adhesion. Moreover, considering that these tumor cells continuously secrete CgA in the culture medium, as we monitored by enzyme-linked immunosorbent assay, it is possible that secreted CgA has saturated the receptor/target molecules and produced a maximum adhesive effect that cannot be further increased by an extra addition of CgA fragments. Although no clear conclusions can be drawn on these points, the lack of effects on these cell lines strengthens the concept that the positive effects observed with fibroblasts are dependent on specific mechanisms.

```
                7                                                   57
hCgA    MNKGDTEVMKCIVEVISDTLSKPSPMPVSQECFETLRGDERILSILRHQNL
bCgA    ----------------------------K-------------------
pCgA    ----------------------------------------------------
rCgA    -T----K----VL-----S----------P--L---Q---V---------
mCgA    -T----K----VL-----S----------P--L---Q---------------
oCgA    ------K---------------N-L-I-E--L---------I--------
```

FIG. 11. **Comparison of amino acid sequences of human (*h*), bovine (*b*), porcine (*p*), mouse (*m*), rat (*r*), and ostrich (*o*) CgA-(7–57) (3, 13, 39–41, 48).**

What is the physiological relevance of the modulation of fibroblast adhesion by CgA? In our assay systems, the adhesive and anti-adhesive effects on fibroblasts occurred in the 1–10 μM range, depending on the assay type. It is well known that CgA can reach a very high concentration in secretory granules, approaching the millimolar range (44), and that intact as well as proteolytic fragments can be released together with the co-stored hormones. For instance, N-terminal peptides are generated naturally in the adrenal medulla (45), the parathyroid glands (46), and the endocrine pancreas (47). It is also known that, after secretion, CgA can reach the bloodstream and that various forms of the CgA antigen circulate at nanomolar levels (2, 22). Micromolar concentration of CgA and its fragments are, therefore, likely reached in the extracellular environment, at least in close proximity to the secreting cells, and therefore may affect adhesion of bystander cells. Fibroblasts and fibroblast-like cells are present within endocrine tissues and the peripheral nervous system and are known to play a role in the organization of the extracellular matrix, which, in turn, is important not only for the physiological function of the neuroendocrine cells but also for the development and the tissue architecture. Since factors that change the adhesion of fibroblasts markedly change their physiology, the adhesive activity of CgA may be important for the regulation of neuroendocrine tissue development and remodeling. One attractive hypothesis is that modulation of fibroblast-like cell adhesion by CgA plays a role during embryogenesis, *e.g.* during axonal path finding. Another possibility is that the co-release of adhesion-effective molecules together with hormones represents a mechanism for the regulation of diffusion of the latter molecules from the site of production to their targets and, thus, for the regulation of hormone action, at least for large polypeptide hormones. The CgA-adhesive activity may also have implications in tumor pathology. Since CgA is abnormally produced by many endocrine and neuroendocrine tumor tissues (23), it is possible that the adhesive effects of CgA may play a role in the progression and metastatization of neuroendocrine tumors.

In conclusion, our findings have revealed that in addition to ECM and cell surface membrane proteins, which are released by constitutive secretion, also proteins stored in the secretory vesicles of the regulated pathway, such as CgA, can play major roles in cell adhesion. Since CgA is present in high amounts within secretory granules and is released in bulk after an appropriate stimulus, in contrast to constitutive secretory proteins that are continuously secreted without concentration, its effects might be more discretely delimited.

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Chromogranin A Fragments Modulate Cell Adhesion: IDENTIFICATION AND CHARACTERIZATION OF A PRO-ADHESIVE DOMAIN

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