Structure-Activity Relationships of Chromogranin A in Cell Adhesion

IDENTIFICATION OF AN ADHESION SITE FOR FIBROBLASTS AND SMOOTH MUSCLE CELLS*

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Previous studies showed that chromogranin A (CgA), a glycoprotein stored and co-released with various hormones by neuroendocrine cells and neurons, can modulate cell adhesion. We have investigated the structure-activity relationships of CgA using fibroblasts and coronary artery smooth muscle cells in adhesion assays. A recombinant CgA fragment 1–78 and a peptide 7–57 containing reduced and alkylated cysteines (Cys[17] and Cys[38]) induced cell adhesion after adsorption onto solid phases at 50–100 nM. Peptides lacking the disulfide loop region, including residues 47–68, 39–59, and 39–68, induced cell adhesion, either bound to solid phases at 200–400 nM or added to the liquid phase at 5–10 μM, whereas peptide 60–68 was inactive, suggesting that residues 47–57 are important for activity. The effect of CgA-(1–78) was blocked by anti-CgA antibodies against epitopes including residues Arg[53], His[54], and Leu[57]. Substitutions of residues His[54], Gln[55], and Asn[56] with alanine decreased the cell adhesion activity of peptide 47–68. These results suggest that the region 47–57 (RILSILRHQNL) contains a cell adhesion site and that the disulfide bridge is not necessary for the proadhesive activity. The ability of soluble peptides to elicit proadhesive effects suggests an indirect mechanism. The high sequence conservation and accessibility to antibodies suggest that this region is important for the physiological role of CgA.

Chromogranin A (CgA)[1] is an acidic and hydrophilic glycoprotein present in the secretory vesicles of many endocrine and neuroendocrine cells and neurons (1–5). It is concentrated and stored within secretory granules and is released in the extracellular environment together with the co-resident hormones after an appropriate stimulus. Human CgA is a polypeptide of 439 amino acids that may form dimers or tetramers depending on pH and Ca^{2+} concentration (6–8). The unique Cys[17]–Cys[38] disulfide bridge included in the N-terminal region is important for sorting and homodimerization (9). Tissue-specific patterns of proteolytic processing and formation of different fragments have been observed by many investigators (10–13). N-terminal fragments of CgA may also form dimers that can rapidly dissociate to monomers upon dilution (14).

The intracellular and extracellular functions of CgA and its proteolytic fragments are still unclear. It is believed that CgA is involved in hormone packaging and in modulating the secretory granule functions by buffering hydrogen ions and binding Ca^{2+}, ATP, and co-resident hormones (15). Moreover, it has been proposed that CgA is a precursor of biologically active peptides with endocrine, paracrine, and autocrine functions. For instance, CgA residues 248–293 were found to be homologous to pancreastatin, a pancreatic peptide that inhibits insulin secretion (16), whereas catestatin, a peptide corresponding to residues 344–364 of bovine CgA, inhibits secretion of catecholamines from chromaffin cells and noradrenergic neurites (17). Fragments corresponding to amino acids 1–76 and 1–113, named vasostatin-1 and vasostatin-2, inhibit vascular tension (18–20). These N-terminal fragments are released from the adrenal medulla (20) and from sympathetic nerve terminals in response to stimulation (21). Vasostatin-1 can also inhibit parathyroid hormone secretion (22), is neurotoxic in neuronal/microglial cell cultures (23), and can induce cell adhesion (24, 25).

In the attempt to identify the molecular determinants of CgA cell adhesion activity, we have investigated the structure-activity relationships of vasostatin-1 using several recombinant and synthetic peptides and anti-CgA antibodies. We show that the region 47–57 of CgA N-terminal fragments contains an accessible site that induces adhesion and spreading of fibroblasts and smooth muscle cells on solid phases.

EXPERIMENTAL PROCEDURES

Materials and Cell Lines—Mouse anti-CgA monoclonal antibodies (mAbs) 4D5, 7D1, 5A8, 5B12, B4E11, and A11 were described previously (14, 26, 27). NIH 3T3 mouse fibroblasts (obtained from F. Blasi, San Raffaele H Scientific Institute, Milan) were cultured in T80 flasks (Nunc, Roskild, Denmark, catalog no. 178891) using Dulbecco's modified Eagle's medium (DMEM) (catalog no. 12-604F, BioWhittaker Italia) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (DMEM-GPS), and 10% FBS (DMEM-GPSF), at 37 °C, 5% CO_2. The cell line was routinely checked for mycoplasma contamination. Human coronary artery smooth muscle cells (CASMC; obtained from Clonetics-BioWhittaker Italia) were cultured in smooth muscle cell basal medium, 5% FBS, 0.5 ng/ml recombinant human epidermal growth factor, 5 μg/ml insulin, 1 ng/ml human foreskin fibroblast growth factor, 50 μg/ml gentamicin, 50 ng/ml amphotericin B (smooth muscle cell basal medium-FBS). All cell culture reagents were

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[1] The abbreviations used are: CgA, chromogranin A; mAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; CASMC, coronary artery smooth muscle cell(s); HPLC, high pressure liquid chromatography.

from Clonetics-BioWhittaker Italia.

Production of Recombinant CgA Fragments—Recombinant human CgA-(1–439) and CgA-(7–439) were described previously (24). Recombinant NH$_2$-Ser-Thr-Ala-CgA-(1–78), hereafter termed (STA)1–78, was obtained by expression in *Escherichia coli* cells (14). The cell extract was boiled for 15 min and centrifuged at $12,000 \times g$ (20 min). (STA)1–78 was purified from the supernatant by reverse-phase HPLC using a SOURCE 15 RPC column (Amersham Pharmacia Biotech) as described (14), followed by gel filtration chromatography on a Sephacryl S-200 HR column, pre-equilibrated and eluted with 0.9% sodium chloride. 12 mg of purified protein, by the BCA protein assay kit (Pierce), was recovered from 1 liter of culture. SDS-polyacrylamide gel electrophoresis analysis of the product showed a single band of about 9 kDa under reducing and nonreducing conditions. Endotoxin content was 0.008–0.016 units/μg by the Lymulus Amoebocyte Lysate Pyrotest (Difco).

Recombinant NH$_2$-Ser-Thr-Ala-CgA-(1–48) ((STA)1–48) was prepared as follows. The plasmid pET12a/VS-2 (14), containing the sequence coding for (STA)1–115, was digested with *Bam*HI. The fragment obtained, coding for (STA)1–46, was ligated with the palindromic adapter 5′-G ATC TAA TGA GTC GAC TCA TTA-3′ to insert the codons for residues 47 and 48, two stop codons, and a *Sal*I site. The plasmid obtained, named pET12a/CgA1–48, was used for periplasmic expression of (STA)1–48 in *E. coli* cells. The product was purified by reverse-phase chromatography as described for (STA)1–78 (14). Since the purified (STA)1–48 still contained disulfide-aggregated material, as indicated by SDS-polyacrylamide gel electrophoresis analysis under reducing and nonreducing conditions, the product was reduced with β-mercaptoethanol (50 mM in 0.15 M sodium chloride, 0.05 M sodium phosphate buffer, pH 7.3 (PBS), 30 min on ice) and dialyzed against PBS. The product was then mixed with oxidized L-glutathione (0.065 mg/ml, final concentration) and incubated for 2 h at room temperature. The refolded material was purified by reverse-phase HPLC using a Protein C4 column (1×25 cm) (Vydac, Hesperia, CA) as follows: buffer A, 0.1% (v/v) trifluoroacetic acid, 3% acetonitrile in water; buffer B, 0.1% trifluoroacetic acid, 80% acetonitrile in water; 0% B for 4 min; linear gradient 14 to 60% B in 26 min; 100% B for 4 min (flow rate, 9 ml/min). Fractions containing immunoreactive material, by ELISA, and corresponding to a 6-kDa monomeric protein by SDS-polyacrylamide gel electrophoresis, were pooled and lyophilized. Part of the product, reconstituted with water, was hydrolyzed with 6 M hydrochloric acid (24 h at 110 °C) and analyzed using an amino acid analyzer. The amino acid composition of (STA)1–48 was in good agreement with that expected (not shown). The protein content was calculated from the amount of recovered amino acids and expected amino acid compositions.

Preparation of CgA Synthetic Peptides—Various peptides spanning most of the CgA sequence were prepared manually by chemical synthesis as described previously (26). Each product was purified by reverse-phase chromatography. The peptide (Y)7–57SS was synthesized using an Applied Biosystems model 433A peptide synthesizer. This peptide, containing two cysteines, was oxidized by overnight incubation with a 5-fold excess of oxidized glutathione (28) and purified by ion exchange and reverse-phase chromatography. The peptide 7–57SEtM, corresponding to CgA-(7–57) with alkylated Cys^{17} and Cys^{38}, was prepared as follows. A peptide encompassing the region 7–57 was synthesized, as above, and reduced with 50 mM β-mercaptoethanol (30 min). The product was then alkylated with 0.15 M *N*-ethylmaleimide (2 h) and purified by reverse-phase chromatography.

Mass Spectrometry—Electrospray ionization mass spectrometry of recombinant and synthetic peptides was carried out as described (29).

CgA-ELISA—Epitope mapping of mouse antibodies was carried out by direct and sandwich ELISAs. Direct ELISA was performed using various CgA peptides adsorbed onto polyvinylchloride microtiter plates and a goat anti-mouse IgG-peroxidase conjugate, as detecting reagent, as described previously (14). Sandwich ELISAs of (STA)1–78 were performed using various anti-CgA mAbs adsorbed onto polyvinyl chloride microtiter plates, biotinylated mAbs, and streptavidin-peroxidase conjugate as described (30).

Adhesion Assays—Adhesion assays were carried out using 96-well polystyrene cell culture plates (Costar, catalog no. 3595). Before each assay 2×10^6 NIH 3T3 cells were seeded in T80 culture flasks (Nunc) and cultured for 72 h in DMEM-GPSF, at 37 °C, 5% CO$_2$. Confluent cells were detached from culture flasks by treatment with a prewarmed trypsin-EDTA solution (0.5 mg/ml trypsin, 0.2 mg/ml EDTA) for 4–5 min at 37 °C and mixed with DMEM-GPSF. About $6–7 \times 10^6$ cells were usually recovered from one flask. The cells were washed one time with 20 ml of 0.9% sodium chloride, centrifuged at $200 \times g$ for 10 min, and resuspended in DMEM-GPS containing 0–0.2% FBS and 3% BSA (10^6 cell/ml).

Adhesion assays with solid-phase bound peptides were carried out as follows. 96-Well cell culture plates were coated with peptide solutions in PBS (50 μl/well, 90 min at 37 °C). After coating, the plates were washed with 0.9% sodium chloride, filled with 3% BSA (Sigma) in DMEM (200 μl/well) and left to incubate for 1 h at 37 °C. The plates were washed again as above and filled with the cell suspension (50 μl/well). After incubation in a 5% CO$_2$ incubator at 37 °C (3–4 h), the medium was aspirated using a multichannel pipette. The cells were washed twice with DMEM and fixed by adding 100 μl/well of a solution containing 3% paraformaldehyde and 2% sucrose in PBS (15 min at room temperature). The solution was then aspirated using a needle connected to a vacuum pump. Fixed cells were stained by adding 50 μl/well of 0.5% crystal violet, in 20% methanol (10 min) and washed with water. The absorbance at 540 nm was read with a microplate reader.

Adhesion assays with liquid-phase peptides were carried out essentially as described above except that peptide solutions were prepared in DMEM containing 4 mM glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin (DMEM-GPS2×), 0–0.4% FBS, and 6% BSA. Peptide solutions (50 μl/well) and NIH 3T3 cell suspension in DMEM (50 μl/well) were sequentially added to microtiter plates, incubated for 2–3 h, and stained as described above.

CASMC adhesion assays were carried out as described for NIH 3T3 cells, except that cells were cultured in smooth muscle cell basal medium-FBS medium (see above).

RESULTS

Preparation and Characterization of Recombinant and Synthetic CgA N-terminal Fragments—To investigate the structure-activity relationships of CgA, we have prepared various recombinant and synthetic fragments. Each fragment is indicated with its sequence numbers (according to Konecki *et al.* (31)); extrasequence amino acids, derived from the cloning procedures or added to enable spectrophotometric detection, are indicated with the single letter codes in parenthesis. (STA)1–78, containing the vasostatin sequence, and (STA)1–48 were obtained by recombinant DNA technology (14), whereas the other peptides used in this work were prepared by chemical synthesis. The molecular mass of (STA)1–78 and (STA)1–48, as measured by electrospray mass spectrometry, was 9069.7 and 5579 Da, respectively (expected mass, 9069.3 and 5579 Da). To study the function of the disulfide loop, we have also prepared a peptide encompassing residues 7–57 with an oxidized disulfide bridge (peptide (Y)7–57-SS) and another one with the cysteines reduced and alkylated with *N*-ethylmaleimide (peptide 7–57-SEtM). The molecular mass of (Y)7–57-SS and 7–57-SEtM, were 5949 and 6038 Da, respectively (expected 5950 and 6039 Da) by electrospray mass spectrometry. SDS-polyacrylamide gel electrophoresis of recombinant (STA)1–78 and (STA)1–48 showed that both products were homogeneous under reducing and nonreducing conditions (data not shown), indicating that these products did not contain disulfide-related aggregated material. ELISAs carried out with various mAbs against the N-terminal region of CgA (14) showed that (STA)1–48 was recognized by antibodies against the N-terminal region (mAbs 4D5 and 7D1), whereas (STA)1–78 was recognized by antibodies against epitopes located in the N terminus and C-terminal region of vasostatin, as expected (Table I).

CgA Fragments Containing Residues 47–57 Increase NIH 3T3 Fibroblast Adhesion and Spreading—The effect of (STA)1–78 on NIH 3T3 cell adhesion was first investigated using this peptide bound to a solid phase. Preliminary experiments showed that 1 μg/ml (STA)1–78 induces proadhesive effects in the presence of 0.1–0.2% FBS. With 0.5–1% FBS, cell adhesion occurred even in the absence of (STA)1–78, while in the absence of FBS the effect of (STA)1–78 was weaker (not shown). Thus, subsequent experiments were carried out using 0.1–0.2% FBS. Under these conditions, coating with 0.3–1 μg/ml (STA)1–78 was sufficient to increase adhesion and spreading of NIH 3T3 cells (Fig. 1, *A* and *B*). Also, the synthetic peptide 7–57-SEtM increased cell adhesion, indicating that the

TABLE I

Epitope mapping of anti-CgA mAbs by ELISA with recombinant and synthetic CgA fragments

Fragment	Immunoreactivity with anti-CgA mAbs[a]					
	4D5	7D1	5A8	5B12	B4E11	A11
Recombinant						
1–439	++	+++	++++	ND	++++	+++
7–439	+	++	++++	ND	++++	
(STA)1–78	++	++++	++++	++++	++++	+++
(STA)1–48	+++	+/−	−	−	−	ND
Synthetic						
(TA)1–20	+++	−	−	−	−	−
(Y)7–57-SS	+/−	++	+	+/−	−	−
7–57-SEtM	++	−	+/−	−	−	−
25–46(GY)	−	+/−	−	−	−	−
(Y)34–57	−	+/−	+/−	−	−	ND
39–59	−	−	++	+/−	−	ND
39–68(Y)	−	−	++++	+++	−	ND
47–68(Y)	−	−	++++	++	−	−
60–68(Y)	−	−	−	−	−	ND
(Y)68–91	−	−	−	−	+++	+++
Epitope-containing region[b]	7–20	34–46	47–57	47–60	68–70[c]	81–90[c]

[a] The immunoreactivity has been determined by ELISA using solid-phase bound peptides and a goat anti-mouse (IgG)-peroxidase conjugate as detecting reagent, as described (14). Values for the absorbance at 492 nm are given as ranges as follows: $++++$, >2.500 units; $+++$, 1.500–2.500 units; $++$, 800–1500 units; $+$, 300–800 units; $+/−$, 200–300 units; $−$, <200 units. ND, not determined with these peptides.

[b] As deduced by comparing the reactivity with different fragments.

[c] Epitope location within (Y)68–91 was identified in previous work (26).

sequence 7–57 contains a proadhesive site and that the Cys^{17}–Cys^{38} disulfide bridge is not necessary for activity. Of note, a "bell-shaped" dose-response curve was obtained with (STA)1–78 but not with 7–57-SEtM (Fig. 1A).

Proadhesive effects were also obtained with peptides lacking the disulfide loop region, such as 47–68(Y), 39–59, 39–68(Y), but not 60–68(Y), either bound to a solid phase (Fig. 1, A and B) or added to the liquid phase (Fig. 2A). Significant effects were obtained also in the absence of FBS (Fig. 2, *upper panels*). Other 22–24-mer peptides spanning most of the regions 1–46, 68–244, and 416–439, the latter corresponding to the CgA C-terminal region, were inactive (Fig. 2, B and C). Thus, peptides that included residues 47–57 increased adhesion and spreading of NIH 3T3 cells, suggesting that this region of vasostatin contains a cell adhesion site. Of note, (STA)1–48 was poorly active or not active, suggesting that the contribution of this region to the adhesive activity of (STA)1–78 is of little or no importance (data not shown).

To investigate the contribution of each residue in the region 47–57 (RILSILRHQNL), we prepared various 47–68(Y) peptides in which single residues were substituted with alanine. Interestingly, the activity was diminished, but not completely abolished, by some substitutions (Fig. 3). In particular, replacement of residues His^{54}, Gln^{55}, or Asn^{56} resulted in a marked decrease of the activity at high concentrations but not at lower levels. This behavior was observed in several assays. Although these results indicate that these residues are critical for the activity of 47–68(Y) at high doses, we cannot conclude that they are directly involved in the interaction with a target molecule. Given that changes with alanine may decrease the polarity of the peptide, these results could be also explained by changes in the peptide physico-chemical properties.

CgA Fragments Containing Residues 47–57 Increase Coronary Artery Smooth Muscle Cell Adhesion and Spreading—The effect of CgA fragments on cell adhesion was also investigated using primary CASMC. As shown in Fig. 4, (STA)1–78 and 7–57-SEtM increased adhesion of CASMC to solid phases, whereas (STA)1–48 was less efficient. These results confirm the importance of the region 47–57 in the proadhesive activity of (STA)1–78.

Topographic Relationships between Adhesion Site and Epitopes—In previous reports, we have described several mAbs directed to epitopes located within the N-terminal region of CgA (14, 26). To investigate the topographic relationships between epitopes and adhesion sites, we studied the epitope location and the capability of each antibody to inhibit cell adhesion induced by (STA)1–78. The epitope location was studied by measuring antibody binding to recombinant and synthetic fragments. It is important to note that these studies, although they can provide information on the regions of CgA that contain residues critical for the antibody binding, do not necessarily define the epitope boundaries. The results (Table I) suggest that the region 7–20 contains some residues recognized by mAb 4D5. The disulfide bridge is not necessary for 4D5 binding, since the 7–57SEtM peptide was recognized efficiently. In contrast, 7D1 binds an epitope located within residues 34–46, and the disulfide bridge is critical for the binding. mAb 5A8, 5B12, and B4E11 are directed to epitopes located within the regions 47–57, 47–60, and 68–70, respectively (Table I), whereas A11 is directed to residues 81–90 (26). In cell adhesion inhibition assays, mAb 5A8, 5B12, and B4E11 inhibited NIH 3T3 cell adhesion to plates coated with (STA)1–78, whereas 4D5, 7D1, and A11 were poorly active or not active, even at concentrations higher than those necessary to saturate (STA)1–78 (Fig. 5, A and C). None of the antibodies affected NIH 3T3 cell adhesion to FBS-coated plates (Fig. 5B). Thus, antibodies against epitopes located within the region 47–70 neutralized specifically the adhesive activity of (STA)1–78.

Interestingly, the epitopes recognized by mAbs 5A8 and 5B12 appear to colocalize with the adhesion site. In an attempt to better define the epitope boundaries of these mAbs, we investigated their reactivity using the peptides encompassing the region 47–68 in which single residues were replaced with alanine. The results of direct binding assays, with solid-phase peptides, showed that residues Arg^{53}, His^{54}, and Leu^{57} are critical for antibody binding (Fig. 6). The loss of binding was not related to solid-phase artifacts, since similar results were obtained by competitive binding assays in which each peptide was added to the liquid phase (not shown). These data strengthen the hypothesis that an adhesion site is located within the region 47–57 and suggest that mAbs 5A8 and 5B12 inhibit (STA)1–78-induced adhesion by blocking this site.

In contrast, B4E11 recognizes residues 68–70 (26) outside the adhesion region. Possibly, B4E11 inhibits cell adhesion by

FIG. 1. **Adhesion of NIH 3T3 fibroblasts to solid phases coated with recombinant (STA)1–78 and synthetic peptides at various doses.** *A*, the assay was carried out in the presence of 0.1% FBS, as described under "Experimental Procedures." *B*, microscopy inspection of wells coated with nothing (*none*), 0.3 μg/ml (STA)1–78, or 1 μg/ml synthetic peptide.

FIG. 2. **Effect of various peptides spanning CgA region 1–244 (*A* and *B*) and 416–439 (*C*) on the adhesion of NIH 3T3 fibroblasts.** The assay was carried out in the absence (*upper panels*) or in the presence (*lower panel*) of 0.2% FBS and with soluble peptides (50 μg/ml) added to the cell culture medium.
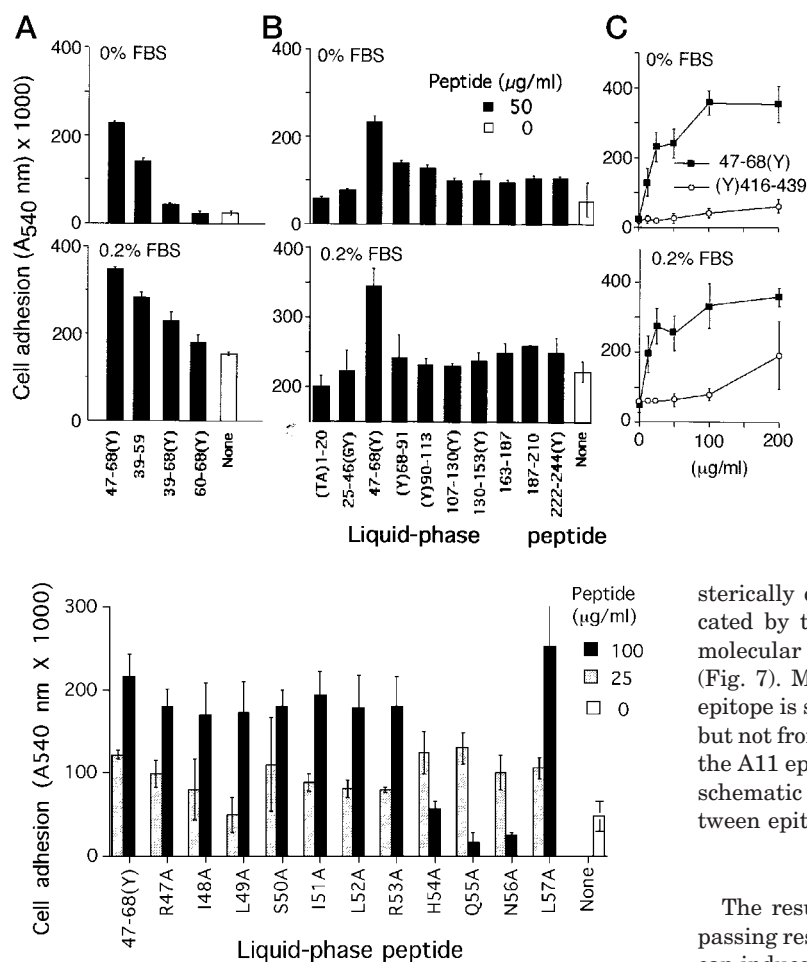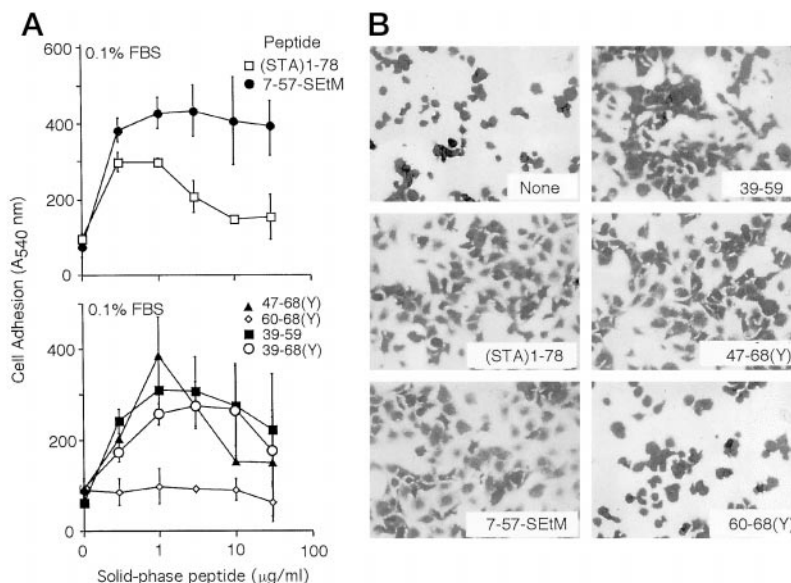

FIG. 3. **Effect of peptides encompassing the sequence 47–68(Y) in which single residues were replaced with alanine.** For each peptide, the residue changed and its position are indicated in the *abscissa*. The assay was carried out in the presence of 0.2% FBS and with soluble peptides added to the cell culture medium.

steric hindrance or by inducing structural changes in (STA)1–78.

The epitope/adhesion site topography was further investigated by measuring the capability of these mAbs to form molecular sandwiches with (STA)1–78. The epitope of B4E11 is sterically distinct from those of 7D1, 5A8, and 5B12 as indicated by the capability of a biotin-B4E11 conjugate to form molecular sandwiches with (STA)1–78 and these antibodies (Fig. 7). Moreover, similar experiments showed that the 5A8 epitope is sterically distinct from that of 4D5, 7D1, and B4E11, but not from that of 5B12 (Fig. 7). A previous study showed that the A11 epitope is sterically distinct from that of B4E11 (26). A schematic representation of the topographic relationships between epitopes and adhesion site is shown in Fig. 8.

DISCUSSION

The results show that a CgA N-terminal fragment encompassing residues 1–78 adsorbed onto solid phases at 50–100 nM can induce adhesion and spreading of fibroblasts and coronary artery smooth muscle cells. This fragment contains at least three sites characterized by sequence similarity with other adhesive molecules and integrin binding motifs, including (*a*) a KGD sequence (residues 9–11) present in barbourin, a snake venom disintegrin (32); (*b*) a sequence characterized by 32% identity and 64% similarity with that of a portion of the fibronectin type III-9 domain of tenascin (residues 19–37) (33, 34); (*c*) an RGD sequence (residues 43–45) often present in extracellular matrix adhesive proteins (35). However, the results of the present study suggest that the main structural
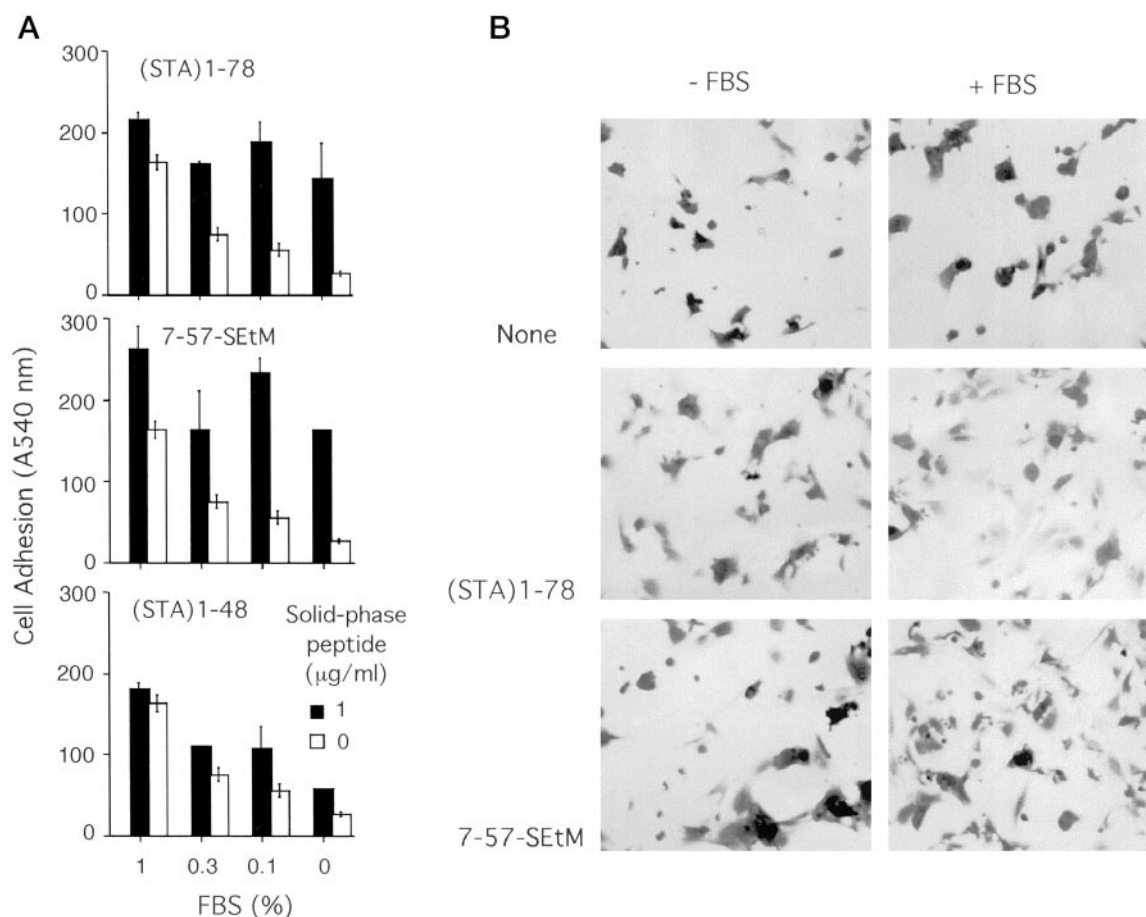
FIG. 4. **Adhesion of CASMC to solid phases coated with (STA)1–78, 7–57-SEtM, and (STA)1–48.** *A*, the assay was carried out in the presence of various amounts of FBS. *B*, microscopy inspection of wells coated with 1 μg/ml peptide and incubated with CASMC in the presence of 0.1% FBS.
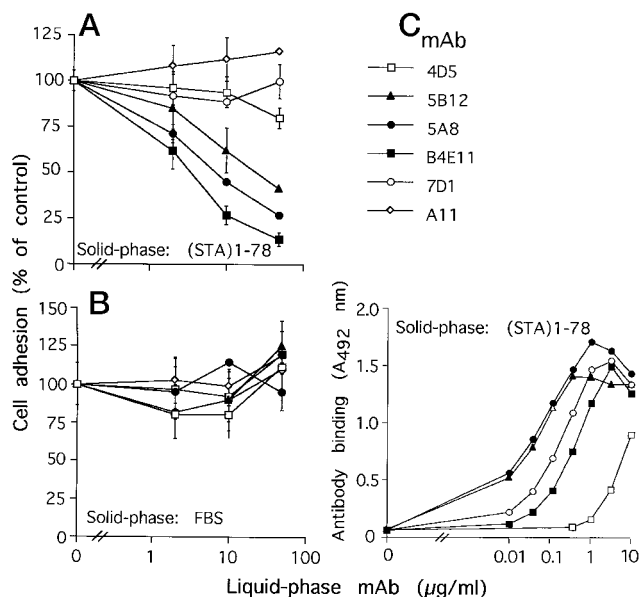

FIG. 5. **Effect of mAs, directed to the N-terminal sequence of CgA, on the adhesion of NIH 3T3 cells to solid phases coated with 1 μg/ml (STA)1–78 (A) or with 0.1% FBS (B).** Both assays were carried out using 0.1% FBS in the cell culture medium. Binding of antibodies to solid phases coated with 1 μg/ml (STA)1–78, as measured by direct ELISA (*C*).

determinant of CgA fragment proadhesive activity resides within residues 47–57, *i.e.* in a region adjacent to the KGD-loop-RGD domain (Fig. 8). Indeed, the proadhesive activity of

fragment (STA)1–48 was markedly lower than that of (STA)1–78, whereas fragments containing residues 7–57 were highly effective. Moreover, synthetic peptides lacking the KGD-loop-RGD domain, such as peptide (Y)47–68, were sufficient to induce fibroblast adhesion either bound to solid phases at 200–400 nM or added to the liquid phase at 5–10 μM, indicating that this domain is not necessary for the adhesion activity.

Interestingly, the sequence 50–57 (SILRHQNL) is 100% conserved within human, mouse, rat, porcine, bovine, ostrich, and frog CgA (3, 37, 38),[2] pointing to a functional importance. In contrast, the RGD sequence is replaced with QGD in the mouse and rat (39, 40), whereas the KGD is replaced with TGD or EDN in horse and frog CgA, respectively (38).[2] The poor conservation and the observation that a CgA mutein with RGE in place of RGD can still induce fibroblast adhesion after limited trypsinization (24) suggest that both RGD and KGD domains are not critical for CgA adhesion activity. Also the disulfide bridge does not appear to be necessary for activity, since a peptide with reduced and alkylated cysteines (7–57-SEtM) induced fibroblast and CASMC proadhesive effects. Interestingly, the activity of this peptide was even stronger than that of (STA)1–78, particularly at relatively high concentrations considering that the dose-response curve of (STA)1–78 is "bell-shaped." Possibly, the disulfide bridge, although not necessary for the adhesive activity, is critical for the structure of (STA)1–78 at different doses. For instance, monomer-dimer transitions might occur at higher concentrations (9, 14) and

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[2] F. Sato, N. Ishida, T. Hasegawa, and H. Mukoyama, GenBank™ accession number AB025570.

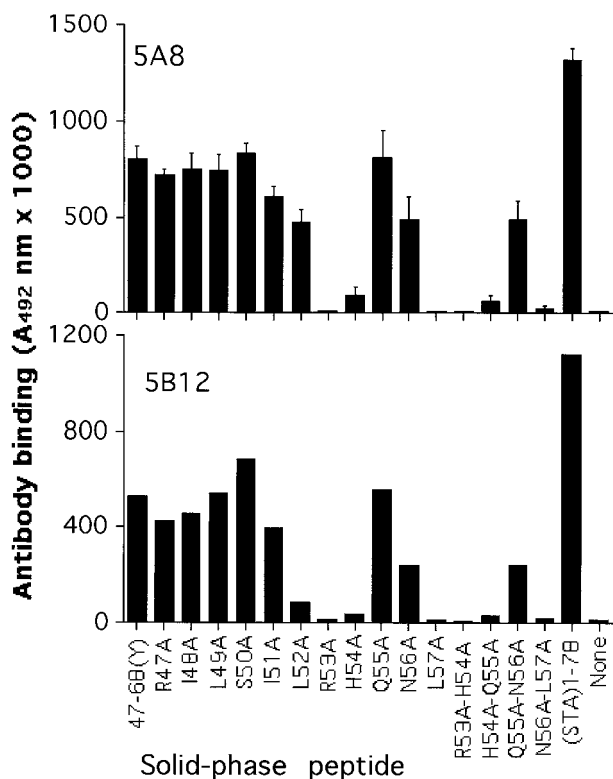*Structure-Activity Relationships of Chromogranin A*

FIG. 6. **Binding of mAb 5A8 and 5B12 to peptides encompassing the sequence 47–68(Y) in which single residues were replaced with alanine.** The residues changed with alanine and their positions are indicated in the *abscissa*. In some peptides, two substitutions were made, as indicated. Antibody binding was assessed by direct ELISA using synthetic peptides adsorbed onto microtiter plates, 3 μg/ml mAb solutions in PBS containing 1% BSA, and a goat anti-mouse IgG-peroxidase conjugate, as detecting reagent.
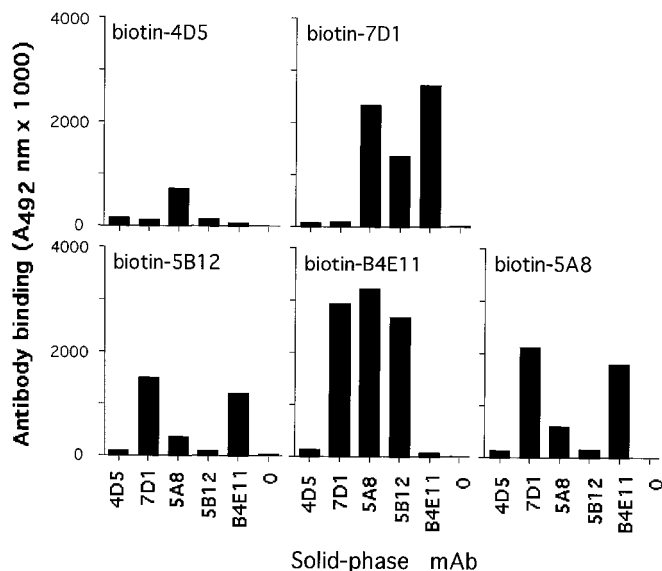

FIG. 8. **Schematic representation of epitope and cell adhesion site topography.** Sterically overlapping epitopes are represented with *overlapping circles*. The primary sequence of the N-terminal region of CgA is also shown. The *arrows* indicate the region recognized by the relevant antibody. The region 47–57, containing a cell adhesion site, is *boxed*.

FIG. 7. **(STA)1–78 sandwich ELISAs.** Sandwich ELISAs of 0.5 μg/ml (STA)1–78 were performed using various anti-CgA mAbs adsorbed onto microtiter plates and 2 μg/ml biotinylated mAbs followed by streptavidin-peroxidase as detecting reagent.

change the adsorption onto the solid phase and/or the activity. However, other interpretations are possible, and further studies are necessary to investigate the quaternary structure/activity relationships.

Among a panel of different antibodies described previously (14, 26), we have identified three antibodies (mAb 5A8, 5B12,
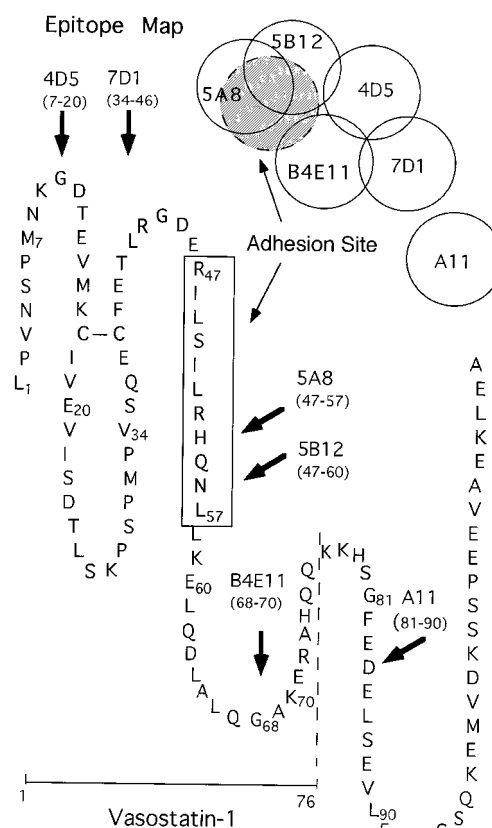
and B4E11) that neutralize the (STA)1–78 adhesion activity. Epitope mapping experiments, based on the alanine scanning method, showed that residues Arg[53]-His[54] and Leu[57], included in the RHQNL sequence, are very critical for 5A8 and 5B12 binding, the reactivity being markedly decreased when these residues were replaced with alanine. Thus, these antibodies bind epitopes that sterically overlap, at least partially, with the cell adhesion site. The fact that Gln[55]-Asn[56] can be replaced with alanine with minor effects on the antibody reactivity strongly suggests that these epitopes are conformational and not linear. Computer-aided secondary structure prediction suggested that the 47–57 region contains an amphiphilic α-helix (26). Accordingly, circular dichroism analysis of peptide 47–68(Y) showed that this peptide tends to form α-helix in solution (data not shown). Thus, given that there are 3.6 residues per turn in α-helixes, it is very likely that the Arg[53]-His[54] and Leu[57] are accessible to the antibody, while Gln[55]-Asn[56] are located on the opposite side of the helix. No sequence similarity between the 47–57 region and other known adhesive motifs was found that might suggest a function. However, a previous study showed that the region 40–65 includes a calcium-dependent calmodulin-binding site (41). Although the biological meaning of this remains unknown, the binding of calmodulin and antibodies to this region suggests that this site is accessible and may therefore interact with other biological structures. The high sequence conservation and accessibility strongly suggest that this region plays an important role.

Besides cell adhesion, CgA N-terminal fragments can exert other biological effects. For instance, vasostatin-1 can inhibit vascular tension (18–20) and parathyroid hormone secretion

(22). Furthermore, recent reports showed that vasostatin-1 is neurotoxic in neuronal/microglial cell cultures (23) and can kill Gram-positive bacteria as well as a variety of filamentous fungi at μM concentrations (36). It has been proposed that CgA fragments contribute, together with other antibacterial peptides from neural and neuroendocrine tissues, to innate immunity during stress situations. Interestingly, antifungal activity was observed with (STA)1–78, peptide 7–57 (with or without disulfide bridge) and peptide 47–60. In contrast, inhibition of parathormone secretion was obtained with peptide 1–40, but not after reduction and alkylation. It seems, therefore, that different activities require structural determinants contained in different regions of vasostatin and that the disulfide bridge may or may not be critical. Interestingly, both antifungal and proadhesive activities appear to rely on the region 47–60 and do not require the disulfide loop. The mechanisms of action underlying all these effects and their physiological significance still need to be investigated.

In addition to fibroblasts, we have found that also smooth muscle cells are affected by vasostatin. The long incubation time required for optimal adhesion of both cell types (3–4 h) suggests that the adhesive activity is probably indirect and requires the synthesis of other proteins. Moreover, the observation that FBS is critical for the effect of (STA)1–78 suggests that other proteins present in FBS are involved in the (STA)1–78 mechanism of cell adhesion. The hypothesis of an indirect mechanism is also supported by the finding that peptide 47–68(Y) can elicit proadhesive effects in our assays even when added in solution. Interestingly, recent work by Soriano *et al.* (25) has shown that CgA at nanomolar concentration may increase deposition of basement membrane components, such as collagen type IV, laminin, and perlecan, by mammary epithelial cells and alter ductal morphogenesis *in vitro*. Given the physiological importance of cell adhesion for fibroblasts and smooth muscle cells and for the production of extracellular matrix, one appealing possibility is that secretion of CgA might contribute to determining the architecture and remodeling of tissues where this protein is released, such as in normal and neoplastic neuroendocrine tissues or, in the case of smooth muscle cells, within the wall of vessels with sympathetic nerve endings. Moreover, since CgA immunoreactivity has been detected in polymorphonuclear cell secretions (41), the capability of its fragments to affect the physiology of fibroblast could be important in inflammatory reactions and tissue repair. The reagents identified in this study (peptides and antibodies) could provide useful tools for investigating these hypotheses.

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Structure-Activity Relationships of Chromogranin A in Cell Adhesion:
IDENTIFICATION OF AN ADHESION SITE FOR FIBROBLASTS AND
SMOOTH MUSCLE CELLS

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