

Cleavage of Chromogranin A N-terminal Domain by Plasmin Provides a New Mechanism for Regulating Cell Adhesion*

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Barbara Colombo‡, Renato Longhi§, Chiara Marini§, Fulvio Magni‡, Angela Cattaneo‡, Seung Hyun Yoo¶, Flavio Curnis‡, and Angelo Corti‡¶

From the ‡Department of Biological and Technological Research, San Raffaele H. Scientific Institute, via Olgettina 58, 20132 Milan, Italy, the §Consiglio Nazionale delle Ricerche ICRM, via M. Bianco 9, 20132 Milan, Italy, and the ¶National Creative Research Initiative Center for Secretory Granule Research, Department of Biochemistry, College of Medicine, Inha University, Nam Gu, Incheon 402-751, Korea

It has been proposed that chromogranin A (CgA), a protein secreted by many normal and neoplastic neuroendocrine cells, can play a role as a positive or a negative modulator of cell adhesion. The mechanisms that regulate these extracellular functions of CgA are unknown. We show here that plasmin can regulate the anti/pro-adhesive activity of CgA by proteolytic cleavage of the N-terminal domain. Limited proteolytic processing decreased its anti-adhesive activity and induced pro-adhesive effects in fibronectin or serum-dependent fibroblast adhesion assays. Cleavage of Lys⁷⁷-Lys⁷⁸ dibasic site in CgA^{1–115} was relatively rapid and associated with an increase of pro-adhesive effect. In contrast, antibodies against the region 53–90 enhanced the anti-adhesive activity of CgA and CgA^{1–115}. Structure-activity relationship studies showed that the conserved region 47–64 (RILSILRHQNLKELQDL) is critical for both pro- and anti-adhesive activity. These findings suggest that CgA might work on one hand as a negative modulator of cell adhesion and on the other hand as a precursor of positive modulators, the latter requiring proteolytic processing for activation. Given the importance of plasminogen activation in tissue invasion and remodeling, the interplay between CgA and plasmin could provide a novel mechanism for regulating fibroblast adhesion and function in neuroendocrine tumors.

Chromogranin A (CgA)¹ is a protein belonging to the “granin” family present in the diffuse neuroendocrine system (1–3). It is co-stored with amine, nucleotides, calcium, and other peptide hormones in the secretory granules of a variety endocrine and neuronal cells and is released in the extracellular environment by exocytosis (1). Elevated levels of circulating CgA have been detected in the blood of patients with endocrine

and neuroendocrine tumors (4–6), renal failure (7), and heart failure (8).

Human CgA is synthesized as a single polypeptide of 439 amino acids, is modified by post-translational glycosylation, sulfation, and phosphorylation (9), and can be further processed by intracellular proteolytic cleavage (10–13). The levels of post-translational modification and proteolytic processing may differ from tissue to tissue. Structure studies showed that intact CgA is a highly acidic and hydrophilic protein with large hydrodynamic volume, mostly in random coil (60–65%) and α -helix (25–40%) conformations (14). CgA may undergo pH- and Ca²⁺-dependent conformational changes causing exposure of hydrophobic residues and formation of dimers or tetramers (14–16). Evidence was obtained to suggest that the C-terminal region is important for dimer-tetramer equilibrium (17, 18). N-terminal fragments (residues 1–78) may also form dimers at micromolar concentrations that rapidly dissociate upon dilution (19).

The extracellular function of CgA is still unclear. The presence of several dibasic sites, potentially cleaved by proteases, and the observation of tissue-specific proteolytic processing led to the hypothesis that CgA is a precursor of various biologically active peptides (9). In particular, fragments corresponding to residues 1–76 and 1–113, named vasostatin-1 and vasostatin-2, are released from the adrenal medulla (20) and from sympathetic nerve terminals in response to stimulation (21). These fragments suppress vasoconstriction in isolated blood vessels (22–24). Vasostatin-1 can also inhibit parathyroid hormone secretion (25), is neurotoxic in neuronal/microglial cell cultures (26), and induces antibacterial and anti-fungal effects (27). The structural determinants of these activities are located in different regions of the N-terminal domain. For example, peptide 1–40, containing the Cys¹⁷-Cys³⁸ disulfide bridge, induces vasodilator effects and inhibition of parathormone secretion, whereas peptide 47–60 can kill a variety of filamentous fungi (27, 28).

We have shown previously that CgA, when abnormally expressed in tumors, can alter tumor growth and morphogenesis (29). Different fragments of CgA can increase or decrease fibroblast and smooth muscle cell adhesion to solid phases, suggesting a role as positive or negative modulators of cell adhesion (30). The long incubation time required for optimal adhesion suggests that the pro-adhesive activity is indirect. Other studies showed that CgA, at nanomolar concentrations, 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* (31), reinforcing the hypothesis of a role of CgA in cell adhesion and tissue morphogenesis.

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¶ To whom correspondence should be addressed: Dept. of Biological and Technological Research, San Raffaele H. Scientific Institute, via Olgettina 58, 20132 Milan, Italy. Tel.: 39-02-26434802; Fax: 39-02-26434786; E-mail: corti.angelo@hsr.it.

¹ The abbreviations used are: CgA, chromogranin A; hCgA, human CgA; mAb, monoclonal antibody; PBS, phosphate-buffered saline; FBS, fetal bovine serum; ESI-MS, electrospray ionization mass spectrometry; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high pressure liquid chromatography; DSS, disuccinimidyl suberate.

Structural determinants of the pro-adhesive activity were found to be located within the conserved residues 47–57 (RIL-SILRHQNL) (32), whereas the location of the anti-adhesive site has not been identified yet. In this work we have studied the structural determinants of the anti-adhesive activity of CgA using natural, recombinant, and synthetic fragments and fibroblast adhesion assays. We show here that structural determinants of both pro- and anti-adhesive activities are located in the N-terminal region and that the plasminogen/plasmin system can regulate these activities of CgA by limited proteolytic cleavage.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—NIH 3T3 mouse fibroblasts (obtained from F. Blasi, San Raffaele H. Scientific Institute, Milan) were cultured in T80 flasks (Nunc, Roskilde, Denmark) using Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker Italia) supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin (DMEM-GPS), and 10% FBS (DMEM-GPSF) at 37 °C, 5% CO₂. The cell line was routinely checked for mycoplasma contamination. Mouse anti-CgA monoclonal antibodies (mAbs) 4D5, 7D1, 5A8, B4E11, and A11 were described previously (19, 32, 33).

Production of CgA and CgA Fragments—Natural human CgA (hCgA) was purified from pheochromocytoma tissues, as follows. The tumor was frozen in liquid nitrogen immediately after surgical excision and homogenized in distilled water. The homogenate was boiled for 6 min and centrifuged at 120,000 \times g for 30 min. CgA was then purified from the supernatant (heat stable fraction) by immunoaffinity chromatography on mAb A11-Sepharose (30). Bovine CgA was isolated from bovine adrenal glands as described (14). Recombinant hCgA 1–439, 7–439, and 47–439 were prepared as described previously (30). Recombinant NH₂-Ser-Thr-Ala-hCgA^{1–78}, NH₂-Ser-Thr-Ala-hCgA^{1–115}, hereinafter termed (STA)1–78 and (STA)1–115, were obtained by expression in *Escherichia coli* cells (19). The products were purified by reverse phase HPLC using a SOURCE 15 RPC column (Pharmacia-Upjohn), followed by gel filtration chromatography on a Sephacryl S-200 HR column (Pharmacia-Upjohn), as described (32). The molecular masses of (STA)1–78 and (STA)1–115 were 9067.72 \pm 0.5 and 13,247.4 \pm 0.5, respectively, by electrospray mass spectrometry (ESI-MS) analysis. Endotoxin content was < 0.03 units/ μ g by the Lymulus Amoebocyte Lysate Pyrotest (Difco Laboratories, Detroit, MI).

Preparation of CgA Synthetic Peptides—Various peptides spanning most of the hCgA N-terminal sequence were prepared by chemical synthesis using an Applied Biosystems model 433A peptide synthesizer, as described previously (26). Tyrosine was added at the N or C terminus to those peptides not containing chromophoric amino acids to enable spectrophotometric quantitation. Each peptide is indicated hereinafter by the number of the first and last residue (numbering according to Koneki *et al.* (34)). N- and C-terminal extra-sequence residues are indicated with a single code letter in parentheses. Each product was purified by reverse phase chromatography and lyophilized. The cysteines of peptide 7–57 (with amidated C terminus) were oxidized by overnight incubation with 5-fold excess of oxidized glutathione (35) and purified by ion exchange and reverse phase chromatography. The molecular mass of each peptide was checked by ESI-MS.

Enzyme-linked Immunosorbent Assay—Binding of mouse antibodies to CgA peptides was checked by enzyme-linked immunosorbent assay using the peptides adsorbed onto polyvinylchloride microtiter plates and a goat anti-mouse IgG-peroxidase conjugate as a detecting reagent, as described previously (19).

Adhesion Assays—Solid phase and liquid phase adhesion assays were carried out using 96-well polystyrene cell culture plates as we described previously in detail (32). Briefly, serum-dependent adhesion assays with solid phase bound peptides were carried out by: (a) coating plates with peptide solutions in PBS (0.15 M sodium chloride, 0.05 M sodium phosphate buffer, pH 7.3; 50 μ l/well, 90 min at 37 °C); (b) blocking with 3% bovine serum albumin (Sigma) in DMEM (200 μ l/well, 1 h at 37 °C); and (c) seeding NIH3T3 cell suspension (50 μ l/well in DMEM-GPS) containing 0.1–0.6% FBS. After 3–4 h at 37 °C, nonadherent cells were removed by washing twice with DMEM, whereas adherent cells were fixed by adding 100 μ l/well of a solution containing 3% paraformaldehyde and 2% sucrose in PBS (15 min at room temperature). Fixed cells were stained by adding 50 μ l/well of 0.5% crystal violet in 20% methanol (10 min) and washed with water. The absorbance at 570 nm was read with a microplate reader.

Adhesion assays with liquid phase peptides were carried out essen-

tially as described above except that peptide solutions were prepared in DMEM containing 4 mM glutamine, 200 units/ml penicillin, 200 μ g/ml streptomycin (DMEM-GPS2x), 0–0.3% FBS, and 6% bovine serum albumin. Peptide solutions (50 μ l/well) and NIH 3T3 cell suspension in DMEM (50 μ l/well) were sequentially added to microtiter plates, incubated for 3 h, and stained as described above. Adhesion assays with solid phase bound fibronectin were carried out as described previously (30).

Peptide Cross-linking and SDS-PAGE—CgA peptide were cross-linked with disuccinimidyl suberate (DSS) (Sigma) as follows. Aliquots (8 μ l) of each product, 2.5 mg/ml in PBS, were mixed with 1.6 μ l of 2 mM DSS in dimethyl sulfoxide and left to incubate for 40 min at 22 °C. The concentration of each peptide in the reaction mixture was as follows: (STA)1–115, 156 μ M; (STA)1–78, 228 μ M; 7–57, 358 μ M; 7–53, 391 μ M; 7–47, 450 μ M; 47–68(Y), 759 μ M; and 52–68(Y), 964 μ M. The reaction was blocked by adding 3 μ l of 1 M ammonium acetate. Each product was then analyzed by SDS-PAGE under nonreducing conditions.

SDS-PAGE was carried out in a Phast System apparatus using high density polyacrylamide Phast gels (Pharmacia Corp.). After separation, the gels were fixed with 2.5% glutaraldehyde (5 min, 60 °C) and stained with Coomassie Blue, according to standard procedures.

Digestion of CgA and CgA Fragments with Plasmin—Plasmin (2 mg) was coupled to 1 ml of Activated-CH-Sepharose (Pharmacia Corp.), using 0.5 M sodium chloride, 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). The gel was washed 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, and stored at 4 °C.

Protein digestion was carried out by using plasmin-CH-Sepharose suspension (1:6) in PBS solutions containing CgA (3.2 μ M) or (STA)1–115 (4.5 μ M). Each tube was incubated at room temperature under gentle agitation. At various times during digestion the samples were rapidly centrifuged, and aliquots of the supernatants were withdrawn. The adhesive properties of each sample were analyzed using the solid phase NIH 3T3 cell adhesion assay. The fragments present in the digested samples were identified by liquid chromatography-ESI-MS as follows. Approximately 20 μ l of each mixture was injected into a Wacocil C18 column (150 \times 1 mm; 5 μ m, SGE, Rome Italy), directly connected to the ion source. The column was eluted with 5% acetonitrile, containing 0.025% trifluoroacetic acid (2 min), followed by a linear gradient from 5 to 60% acetonitrile (30 min, flow rate, 40 μ l/min).

RESULTS

CgA Inhibits Fibroblast Adhesion and Spreading—We have shown previously that natural human CgA isolated from the heat stable fraction of pheochromocytoma inhibits fibroblast adhesion and spreading to solid phases coated with collagen or fibronectin (30). To assess whether protein denaturation, potentially caused by heat treatment, was necessary for the anti-adhesive activity of CgA, we studied the activity of natural CgA prepared using a procedure that does not involve boiling steps. To this aim, we tested the activity of nondenatured CgA isolated from bovine adrenal glands in the NIH 3T3 fibroblast adhesion assay using fibronectin-coated plates or FBS as a source of adhesion molecules. When bovine CgA was added to cell culture plates at concentrations ranging from 6 to 200 nM, we observed inhibition of adhesion to fibronectin-coated plates as well as inhibition of the serum-dependent cell adhesion (Fig. 1). This suggests that heat treatment is not necessary for the anti-adhesive activity of CgA.

Similar results were obtained with human CgA. Of note, human CgA inhibited cell adhesion also in a 24-h incubation assay, but it did not inhibit cell viability, as checked by staining cells with a vital dye, after 72 h of incubation (data not shown). This indicates that inhibition of cell adhesion was not related to cytotoxicity.

Limited Proteolytic Processing with Plasmin Converts the Anti-adhesive Activity of CgA into Pro-adhesive Activity—The mechanism that regulates the anti/pro-adhesive activity of CgA was then investigated. Because proteolytic processing is believed to be a general mechanism for the regulation of CgA, we investigated the effect of plasmin on the anti-adhesive activity of bovine CgA. Limited treatment with this enzyme decreased

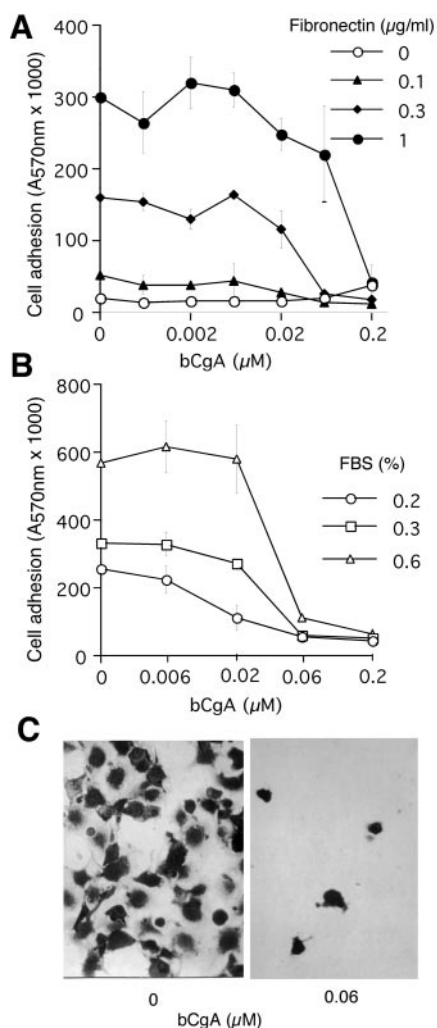


FIG. 1. Effect of bovine CgA on NIH 3T3 fibroblast adhesion. *A*, adhesion of fibroblasts to microtiter plates coated with various doses of fibronectin and coated with bovine CgA. *B*, adhesion of fibroblasts to microtiter plates coated with bovine CgA in the presence of various amounts of FBS in cell culture medium. *C*, microphotographs of wells coated with PBS (*left panel*) or bovine CgA in PBS (*right panel*).

the anti-adhesive activity and increased the pro-adhesive activity of CgA in fibronectin- or FBS-dependent adhesion assays (Fig. 2, *A* and *B*, *left panels*). This suggests that plasmin can destroy the anti-adhesive activity and activate a pro-adhesive effect. The pro-adhesive effect declined after extensive treatment (>16–32 h), indicating that both pro- and anti-adhesive sites can be degraded by plasmin, although with different kinetics.

We have shown previously that a pro-adhesive site is present in the N-terminal domain of CgA (30). Thus, we tested, in parallel to CgA, the effect of plasmin on recombinant (STA)1–78 and (STA)1–115. Limited digestion of (STA)1–115 (1–4 h), but not of (STA)1–78, was accompanied by an increase in cell adhesion (Fig. 2, *A* and *B*, *right panels*). These results suggest that the transient increase of cell adhesion observed with (STA)1–115 is related to removal of residues 79–115 or to cleavage within these residues.

To verify these hypotheses we studied the sites and the kinetics of (STA)1–115 cleavage. The fragments obtained after various times of digestion of (STA)1–115 were separated by reverse phase HPLC and identified using ESI-MS (Fig. 3*A* for a schematic representation). The digestion pattern suggests that plasmin cleaves the following sites: Lys⁹, Lys¹⁶, Arg⁴⁷, Arg⁵³, Lys⁵⁹, Lys⁷⁰, Lys⁷², Lys⁷⁷, Lys⁷⁸, and Lys¹⁰⁰. However,

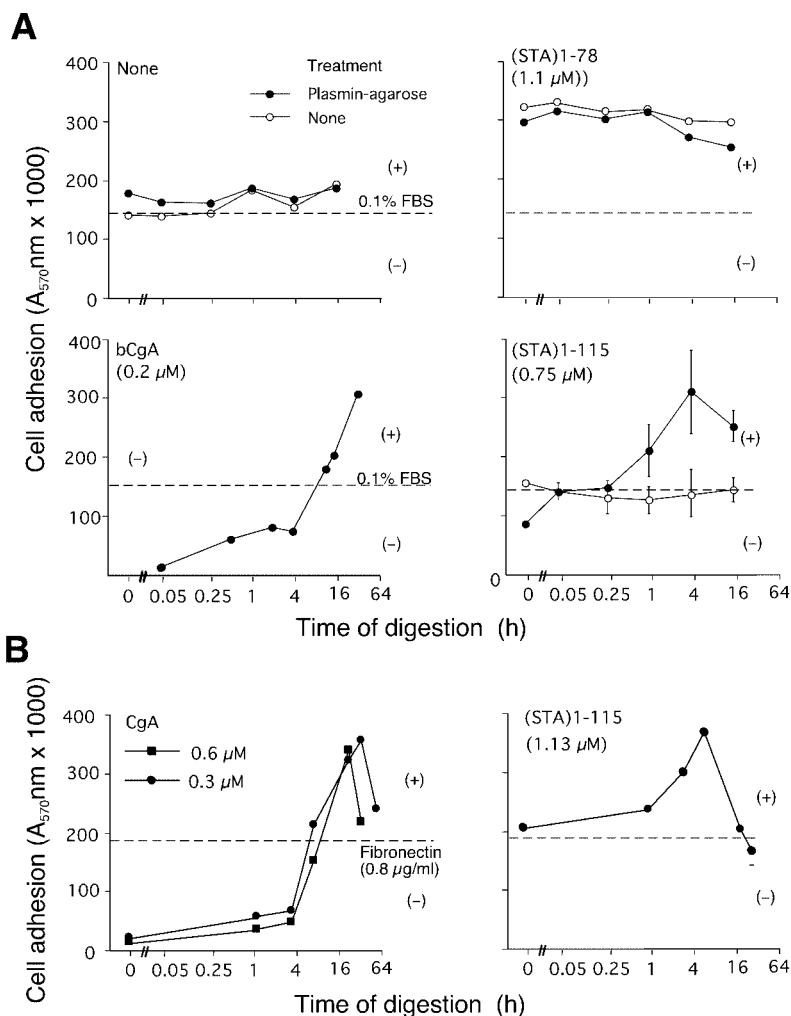
the kinetics of cleavage at each site is markedly different and decreased as follows: Lys⁷⁷, Lys⁷⁸ > Lys⁹, Lys¹⁰⁰ > Lys¹⁶, Arg⁴⁷, Arg⁵³ > Lys⁵⁹, Lys⁷⁰, Lys⁷² (Fig. 3). Of note, cleavage of Lys⁷⁷ and Lys⁷⁸ was relatively rapid, (STA)1–78, 79–115, (STA)1–77, and 78–115 fragments being detected in the reaction mixture after 1–4 h of digestion. Cleavage of Lys¹⁰⁰ and the other sites required >16 h. Thus, the increase in cell adhesion (Fig. 2, *A* and *B*, *right panels*) correlates with the cleavage of Lys⁷⁷ and Lys⁷⁸. These results suggest that processing of the CgA N-terminal domain by plasmin could provide a novel mechanism for regulating the anti/pro-adhesive activity of this protein and its fragments.

Monoclonal Antibodies Directed to Region 47–90 Inhibit the Pro-adhesive Activity of CgA N-terminal Fragments and Increase Their Anti-adhesive Activity—The location of the anti-adhesive site was then investigated. In previous work we showed that a recombinant CgA N-terminal fragment, called (STA)1–78, increases cell adhesion and spreading of fibroblasts to solid phases and that a pro-adhesive site is located within residues 47–57 (RILSILRHQNL) (32). To identify the anti-adhesive sites of CgA, we tested the effect of several monoclonal antibodies on the anti/pro-adhesive activity of human CgA, (STA)1–115, and (STA)1–78, using the NIH 3T3 fibroblast adhesion assay. In particular, we tested the effect of mAb 4D5 (epitope located within residues 7–20), mAb 7D1 (epitope 34–46), mAb 5A8 (epitope 53–57), mAb B4E11 (epitope 68–70), and mAb A11 (epitope 81–90) (19, 32, 33) (Fig. 3*B* for a schematic representation of epitopes). Although none of these antibodies inhibited the anti-adhesive activity of natural human CgA, antibodies directed against the region 53–90, namely 5A8, B4E11, and A11, increased its anti-adhesive activity (Fig. 4, *B* and *F*). This effect was observed also with recombinant (STA)1–115 (Fig. 4, *C* and *G*) and (STA)1–78 (Fig. 4, *D* and *H*). The (STA)1–115-mAb A11 complex inhibited cell adhesion also in a 24-h incubation assay (Fig. 5, *A*), but it did not inhibit cell viability, as checked by staining cells with a vital dye, after 72 h of incubation (Fig. 5*B*). This indicates that inhibition of cell adhesion was not related to cytotoxicity. Noteworthy, the (STA)1–78-mAb B4E11 complex was sufficient to induce anti-adhesive effects, suggesting that an anti-adhesive site is located within residues 1–78 (Fig. 4, *D* and *H*). The lack of anti-adhesive effects with (STA)1–78-mAb A11 mixtures (Fig. 4*D*) indicates that the effect of (STA)1–115-mAb A11 is specific and related to antigen-antibody binding, because (STA)1–78 lacks the A11 epitope (33).

Because removal of residues 79–115 from (STA)1–115, by plasmin, is accompanied by an increase in cell adhesion, we performed further experiments to test the hypothesis that these residues contain an anti-adhesive site. Peptides spanning the C-terminal domain of vasostatin-2, such as peptides 68–91, 91–113, and 91–115, were unable to exert anti-adhesive or pro-adhesive effects even at very high concentrations (data not shown). Although we cannot totally exclude the possibility that an anti-adhesive site is located within residues 79–115, these and the above data suggest that these residues contribute to the anti-adhesive activity in an indirect manner, *e.g.* by regulating an anti-adhesive site located within residues 1–78. In summary, these results indicate that the N-terminal region of CgA (residues 1–78) contains pro- and anti-adhesive sites and that monoclonal antibodies against region 47–90 can block the pro-adhesive effect and enhance the anti-adhesive activity.

The Region 47–68 Contains Pro- and Anti-adhesive Sites—To assess the role of N-terminal residues in cell adhesion, we studied the activity of recombinant CgA 1–439, 7–439, and 47–439 expressed in *E. coli* cells. All of these compounds inhibited cell adhesion (data not shown), indicating that N-

FIG. 2. Effect of plasmin on the adhesive properties of bovine bCgA, (STA)1-115, or (STA)1-78. A, FBS-dependent (0.1%) adhesion of NIH 3T3 fibroblasts to microtiter plates coated with bovine CgA, (STA)1-115, or (STA)1-78 treated with plasmin-CH-Sepharose for various times. B, adhesion of fibroblasts to microtiter plates coated with fibronectin and overcoated with digested bovine CgA or (STA)1-115.



terminal residues 1–46 are not necessary for the anti-adhesive activity.

In attempt to identify the location of the anti-adhesive site, we studied the activity of various synthetic peptides encompassing the region 47–90. Because the pro-adhesive activity could mask the anti-adhesive effect of some peptides, we decided to investigate the activity of each product either in the absence or in the presence of mAb 5A8 or B4E11, taking advantage of the fact that these antibodies block the pro-adhesive activity but not the anti-adhesive activity. Interestingly, peptide 47–68(Y), shown previously to be pro-adhesive and to contain the 5A8 epitope (32), behaved as an anti-adhesive molecule in the presence of this antibody (Fig. 6A). In contrast, peptide (Y)68–91, lacking the 5A8 epitope and containing the B4E11 epitope (33), was unable to affect cell adhesion either in the absence or in the presence of 5A8 or B4E11 (Fig. 6A). Noteworthy, the dose-response curve of peptide 47–68(Y), added alone to the cell supernatant, was bell-shaped, because this peptide promoted cell adhesion when added at 1–11 μM and inhibited cell adhesion when added at 110 μM (Fig. 6B). Thus, this peptide can exert anti-adhesive effects, either when it is bound to mAb 5A8 on a solid phase or when it is present at high concentrations in the liquid phase. No effects were observed with liquid phase peptide 60–68 either at high or low concentrations. These results suggest that the region 47–68 contains pro- and anti-adhesive sites.

The Pro-adhesive Site Is Distinct from the Anti-adhesive Site—Previous studies suggested that region 47–66 adopts a helical structure (28). Moreover, other studies showed that the

5A8 epitope (RHQNL) includes residues critical for antibody binding (Arg⁵³, His⁵⁴, and Leu⁵⁷) and residues that are less important (Gln⁵⁵ and Asn⁵⁶), the latter being replaced with alanine without loss of binding (32). The helical structure of this epitope may explain the differential importance of each residue in the RHQNL sequence for antibody binding, the epitope likely being located on one side of the helix. To investigate the importance of these residues for the anti/pro-adhesive activity of CgA, we tested various 47–68(Y) peptides in which residues RHQNL were substituted with alanine. Interestingly, peptides with changes in residue Gln⁵⁵ inhibited cell adhesion in a liquid phase assay (Fig. 7). Thus, perturbation of the region RHQNL, either by changing residue Gln⁵⁵ with alanine or by steric hindrance with mAb 5A8, blocked the pro-adhesive activity of peptide 47–68(Y) and increased its anti-adhesive activity. These results suggest that the pro- and the anti-adhesive sites are somehow distinct.

Residues 47–51 (RILSI) Are Critical for Anti-adhesive Activity—To identify the residues critical for the anti-adhesive activity, we tested shorter peptides. Deletion of residues 47–51 from peptide 47–68(Y) abolished or largely reduced the anti-adhesive effects of peptide/mAb 5A8 complexes (Fig. 8A). Deletion of these residues did not affect the binding of mAb 5A8, as indicated by the strong reactivity with peptides 50–69(Y), 51–68(Y), and 52–68(Y) (Fig. 8B). Thus, the lack of anti-adhesive activity was not related to lack of antigen-antibody complex formation. These results suggest that N-terminal residues 47–51 are critical for the anti-adhesive activity. In contrast, C-terminal residues 64–68 are not critical, because the 47–64/

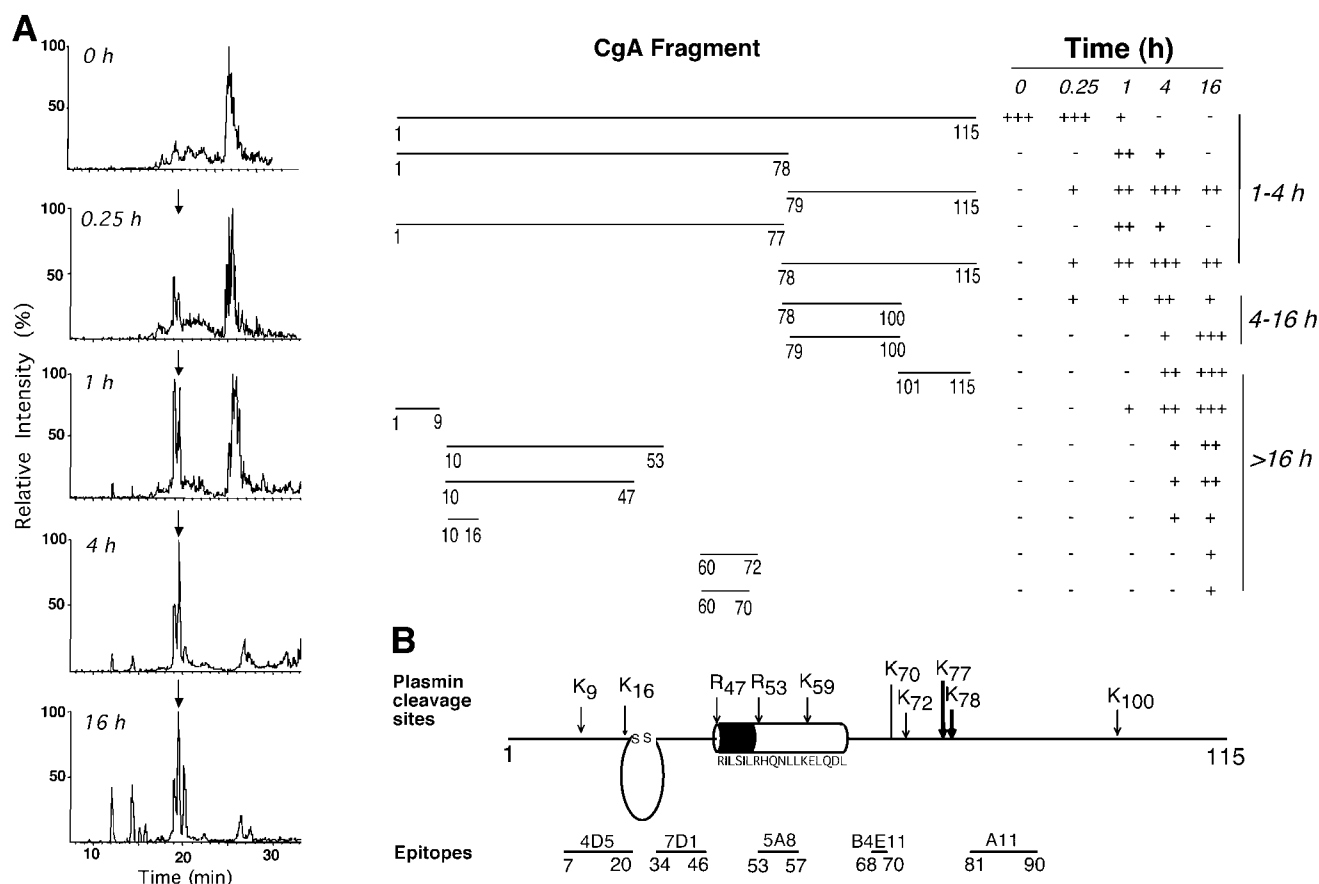


FIG. 3. Time course of (STA)1-115 fragmentation by plasmin-agarose. The fragments present in the reaction mixture were identified by liquid chromatography-ESI-MS. A, liquid chromatography-ESI-MS chromatograms of aliquots of the reaction mixture taken at different times. The arrows indicate fragment 79-115, as identified by ESI-MS. The relative proportion of each fragment (indicated by minus and plus signs) was estimated taking into account the change of signal intensity generated by each peptide. B, schematic representation of plasmin cleavage sites and epitopes of (STA)1-115.

5A8 complex was still active (Fig. 8A). The importance of residues 47-51 for the anti-adhesive activity is further supported by the observation that peptide 47-68(Y), but not peptide 52-68(Y), inhibits cell adhesion at high doses (Fig. 8A). In conclusion, the results indicate that (a) the region 47-64 (RILSIL-RHQ NLLKELQDL) includes pro-adhesive and anti-adhesive sites; (b) residues 47-51 (RILSI) are critical for the anti-adhesive activity; and (c) the anti-adhesive activity of CgA and CgA fragments is enhanced by blocking the 5A8 epitope (RHQNL) or by replacing Gln⁵⁵ with alanine.

Residues 47-51 (RILSI) Are Critical for Peptide Oligomerization—We showed previously that (STA)1-115 is almost entirely dimeric at concentrations $>4 \mu\text{M}$ and that it rapidly dissociates after dilution (19). CgA, at pH 7.5, also form dimers (15). Because the quaternary structure could be critical for cell adhesion activity, we decided to investigate the structural determinants of dimer formation. SDS-PAGE analysis of (STA)1-115, (STA)1-78, (Y)7-57-SS, and 7-53, before and after cross-linking with DSS, showed that these peptides form dimers at high concentrations (Fig. 9). In contrast, peptide 7-47 formed few or no dimers. These results suggest that residues 47-51 (RILSI) are critical for dimer formation.

Interestingly, SDS-PAGE of peptide 47-68(Y) after cross-linking, showed a “ladder” of bands consistent with formation of dimers, trimers, tetramers, and other multimeric forms in different proportions. Replacement of Gln⁵⁵ and Asn⁵⁶ with alanine did not prevent oligomerization (data not shown). In contrast, no oligomers were observed with peptide 52-68(Y). This and the above results suggest that the sequence 47-51

(RILSI) is critical for peptide oligomerization as well as for their anti-adhesive activity.

DISCUSSION

In previous work we showed that solid phase bound CgA exerts anti-adhesive effects in fibroblast adhesion assays, whereas the N-terminal fragment 1-78 exerts pro-adhesive effects (30). The results of the present work show that proteolytic processing of natural CgA with plasmin decreases its anti-adhesive activity and induces pro-adhesive effects in fibronectin- or serum-dependent fibroblast adhesion assays. Limited digestion of a recombinant fragment spanning residues 1-115 (called (STA)1-115) was accompanied by an increase in the pro-adhesive activity, concomitant with the generation of (STA)1-78 and (STA)1-77 fragments. The anti-adhesive activity of CgA and the pro-adhesive effects of its fragments suggest that this protein might work on one hand as a negative modulator of cell adhesion and on the other hand as a precursor of positive modulators.

Extensive processing of CgA with plasmin caused further fragmentation. It is noteworthy that, although many basic residues are cleaved by plasmin, the kinetics of cleavage at each site are markedly different. For instance cleavage of the Lys⁷⁷-Lys⁷⁸ dibasic site was evident after 15 min, whereas cleavage of other sites started after 4-16 h. Remarkably, cleavage at this site matches the *in vivo* cleavage of CgA (20, 21) and may therefore be of physiological relevance. Although we cannot draw conclusions on the biological relevance of the cleavage at other sites, because we have no indication of *in vivo* forma-

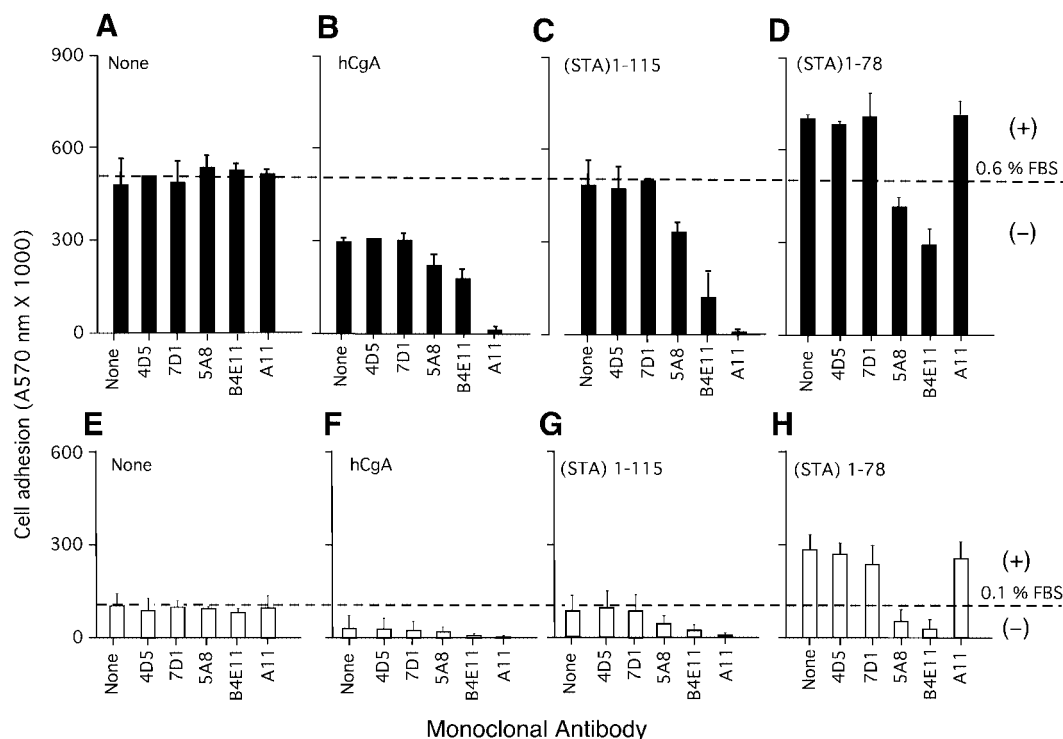


FIG. 4. Effect of anti-CgA monoclonal antibodies on the adhesion of NIH 3T3 fibroblast to culture plates coated with human CgA, (STA)1-115, (STA)1-78, or vehicle (None). NIH 3T3 fibroblasts were seeded in microtiter plate wells coated with 0.01 μM human CgA (B and F), 0.33 μM (STA)1-115 (C and G), 0.22 μM (STA)1-78 (D and H), in PBS, or with PBS alone (A and E). Each well was incubated for 1 h with anti-CgA monoclonal antibodies (50 $\mu\text{g}/\text{ml}$, in DMEM containing 6% bovine serum albumin, 50 $\mu\text{l}/\text{well}$). Cell adhesion assays were then performed in the presence of 0.6% FBS (A–D) or 0.1% FBS (E–H) as described under “Experimental Procedures.” The dashed lines indicate the basal cell adhesion to plates coated with PBS. + and – indicate pro-adhesive and anti-adhesive effects, respectively.

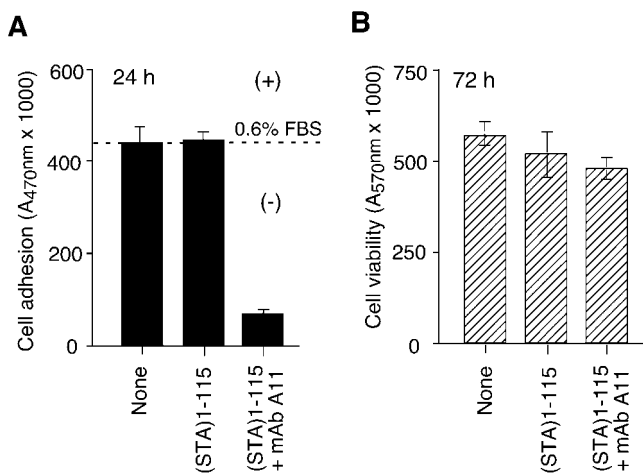


FIG. 5. Effect of (STA)1-115-mAb A11 complexes on NIH 3T3-fibroblast adhesion and viability. NIH 3T3 fibroblasts were seeded in two separate microtiter plates coated with (STA)1-115 and incubated in culture medium containing 0.6% FBS without or with 50 $\mu\text{g}/\text{ml}$ mAb A11 at 37 $^{\circ}\text{C}$. The adherent cells were stained after 24 h in one plate with crystal violet as described under “Experimental Procedures” (A). The viable cells were stained in the other plate after 72 h with 0.5 mg/ml 3(4,5-dimethylthiazolyl-2-yl)2,5-diphenyltetrazolium bromide (Calbiochem, San Diego, CA 92112) (B). See legend of Fig. 4 for the meanings of symbols and dashed line.

tion of shorter fragments, these results suggest that limited proteolytic processing of CgA and vasostatin-2 by plasmin could provide a mechanism for regulating their extracellular functions.

The results of structure-activity relationships studies show that structural determinants of both pro- and anti-adhesive activity are located within the region 1–78 and that the region 47–64 (RILSILRHQNLKELQDL) is critical for both activi-

ties. This suggests that plasmin induces structural changes in the N-terminal domain that could unmask and/or mask the sites involved in the pro-adhesive and anti-adhesive activity. Interestingly, the 47–64 region is 100% conserved in human, porcine, bovine, equine, and mouse CgA and is 89% conserved in frog CgA (36, 37), pointing to a functional importance.

The existence of sites that can be masked or unmasked in the N-terminal domain of CgA after fragmentation is further supported by the results of antibody binding experiments showing that mAbs directed to epitopes located within the region 47–90 (5A8, B4E11, and A11), in contrast to plasmin, enhance the anti-adhesive activity of CgA and (STA)1-115, whereas they inhibit the pro-adhesive activity of (STA)1-78. This indicates that the pro- and anti-adhesive sites of this region are somehow distinct. Several possibilities can be considered to explain this observation. One possibility is that these antibodies inhibit, by steric hindrance, the interaction of CgA N-terminal fragments with a cellular component important for the pro-adhesive effect, leaving the anti-adhesive site free to act. Alternatively, it is possible that antibody binding interferes with the monomer-dimer equilibrium of these fragments by stabilizing or by blocking dimer formation (19). This could mask or unmask different functional sites. Another possibility is that antibody binding induces conformational changes leading to exposure of cryptic sites and masking of other sites. The pronounced anti-adhesive effect of mAb A11 (epitope 81–90), compared with that of saturating amounts of mAb 5A8 and B4E11 (epitopes 53–57 and 68–70), suggests that this mAb can efficiently inhibit the pro-adhesive activity without impairing the anti-adhesive effects.

The results of experiments aimed at investigating the quaternary structure-activity relationships showed a striking correlation between peptide oligomerization and anti-adhesive activity. For instance, we obtained evidence to suggest that both oligomerization and anti-adhesive activity require residues

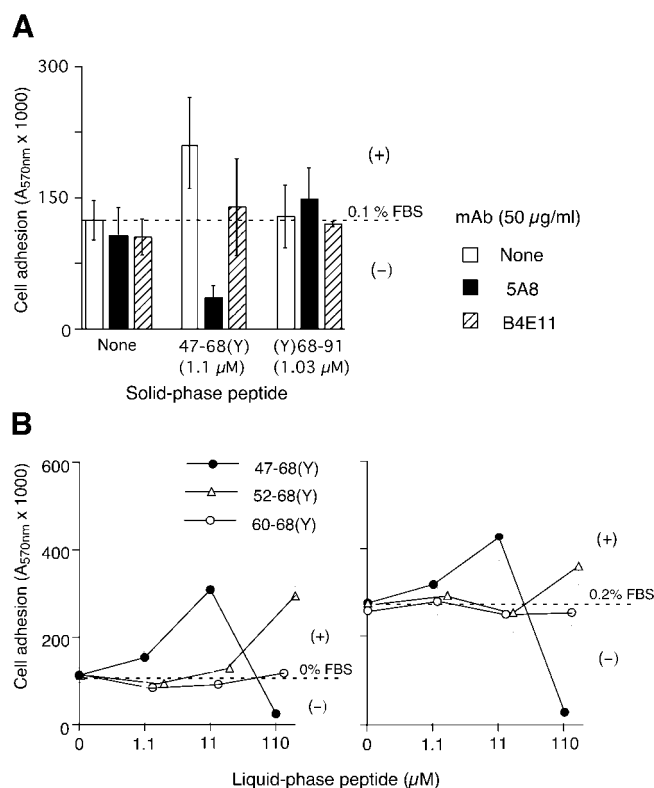


FIG. 6. Effect of synthetic CgA peptides in solid phase (A) and liquid phase adhesion assays (B). Solid phase and liquid phase NIH 3T3 fibroblast assays were carried out as described under "Experimental Procedures." A, microtiter plates were coated with peptide 47–68(Y) (1.1 μM) or (Y)68–91 (1.03 μM), blocked with bovine serum albumin, and further incubated with mAb 5A8 or B4E11 (50 $\mu\text{g}/\text{ml}$) in culture medium. B, each peptide was added at various concentrations to the culture medium.

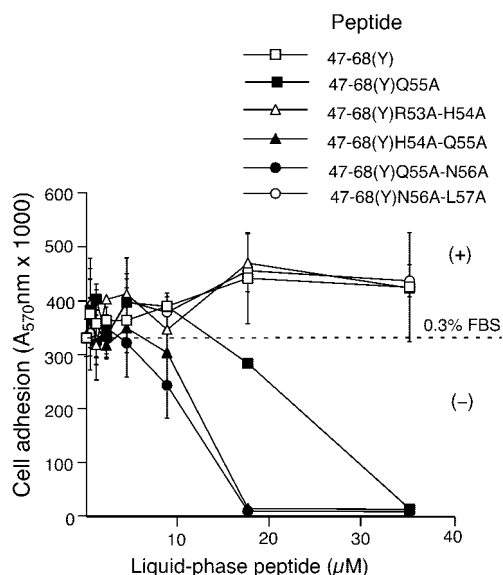


FIG. 7. Effect of amino acid substitutions in peptide 47–68(Y) on its pro/anti-adhesive properties. Amino acid substitutions and position of change are indicated with the single-letter code. For instance 47–68(Y)Q55A corresponds to a peptide 47–68(Y) with Gln⁵⁵ replaced with alanine. Each peptide was tested using the "liquid phase" adhesion assay.

47–51 (RILSI). Moreover, those factors that promote peptide oligomerization, such as an increase in peptide concentration, decreased the pro-adhesive activity and increased the anti-adhesive activity. Although these correlations suggest that

changes in quaternary structures could be important for the regulation of the pro/anti-adhesive activity of CgA-derived fragments, further experiments are necessary to provide direct evidence of this hypothesis.

Computer-aided secondary structure prediction of CgA (33), circular dichroism analysis of peptide 47–68(Y) (32), and ¹H NMR analysis of peptide 47–66 (28) suggest that region 47–51 forms a short hydrophobic helix, followed by an amphipathic helix (residues 53–66). The presence of several hydrophobic residues on one side of the helix, mainly leucines, and of positively charged residues on the other side could explain the tendency of peptides that contain the RILSILRHQNLKLDL region to form oligomers at high concentrations.

The 47–64 region, besides containing (a) an oligomerization site, (b) an antibody epitope (RHQNL), and (c) a pro-adhesive and an anti-adhesive site, also contains a Ca²⁺-dependent calmodulin-binding site (38). The affinities of calmodulin for CgA and peptide 40–65 were very similar in the presence of Ca²⁺ (38), indicating that this region and the cognate peptide can assume similar conformations. This may explain the capability of short peptides, such as 47–68(Y), to mimic CgA in antibody binding and cell adhesion assays albeit with different efficiency.

The receptors or the molecular targets of CgA are unknown. Analysis of the primary structure revealed that RGD an integrin-binding motif, often present in ECM proteins involved in adhesive processes, is present at residues 43–45. Although interaction of CgA with integrins cannot be totally excluded, the following observations suggest that this site is unlikely to play a major role in the pro/anti-adhesive activity of CgA and its fragments: (a) a recombinant RGE-CgA (7–439) mutant induced anti-adhesive and pro-adhesive effects after tryptic digestion, as the wild type recombinant RGD-CgA (7–439) (30); (b) peptide 47–68 (lacking the RGD motif) can induce pro-adhesive effects; and (c) RGD is replaced with QGD in the mouse and rat (39, 40), arguing against a functional importance. Thus, we believe that molecular targets different from RGD-binding receptors must be sought.

CgA is an acidic protein with a large proportion of glutamic acid (20.5%). Interestingly, osteonectin, a protein known to inhibit spreading of fibroblasts and smooth muscle cells (also called SPARC, for secreted protein acidic and rich in cysteine), also contains a high proportion of acidic residues, mostly glutamic (41). This raises the question as to whether the anti-adhesive activity of CgA is related to a specific mechanism or is due to a net negative charge. Although it is possible that the negative charges of CgA contribute to its anti-adhesive activity, the fact that (STA)1–78, with a balanced content of negative and positive charges can work as a negative modulator in the presence of antibodies or as a positive modulator in their absence suggests that the anti-adhesive activity is more likely related to a specific mechanism.

Previous studies showed that a peptide encompassing residues 47–66, called chromofungin, could interact with lipids and penetrate into the cytoplasm of fungi (27, 28). In addition, it has been shown that chromofungin can inhibit calcineurin, a calmodulin-activated phosphatase (28). Whether the pro-adhesive activity of (STA)1–78 and 48–68(Y) requires internalization and inhibition of calmodulin-dependent enzymes or not remains to be investigated.

Concerning the physiological relevance of our findings, the capability of large and short fragments identified in this work to affect cell adhesion deserves further comment. CgA is present in neuroendocrine secretory vesicles at very high concentrations, approaching millimolar levels (15). CgA is also present in the blood of normal subjects at 0.5–2 nM (42, 43) and up

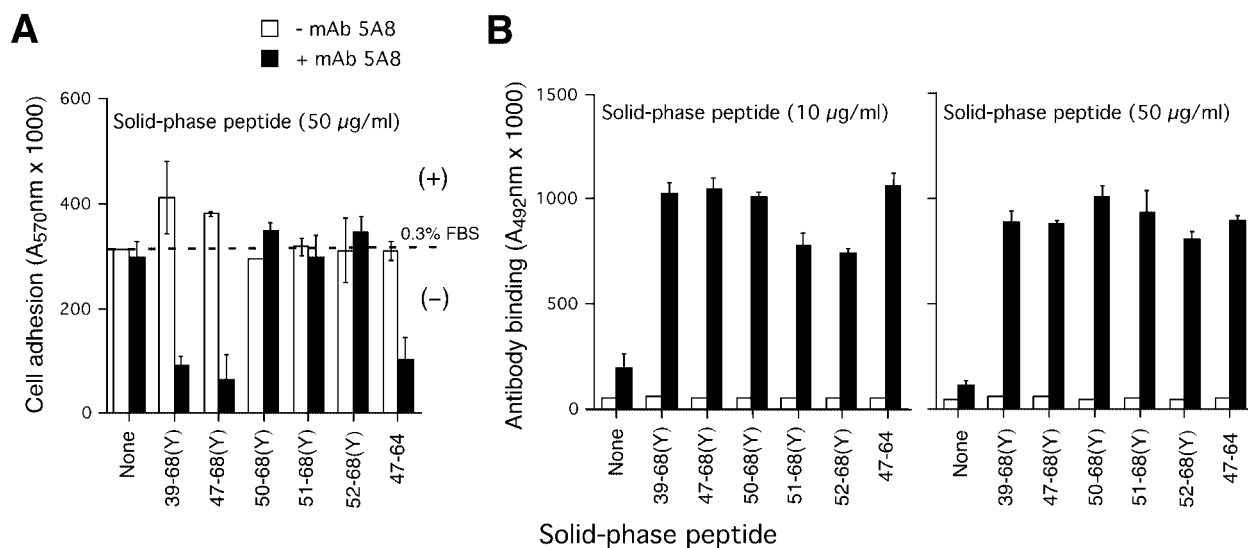


FIG. 8. *A*, effect of mAb 5A8 on the pro/adhesive properties of various solid phase peptides. *B*, the binding of mAb 5A8 to each peptide was checked, in parallel, by enzyme-linked immunosorbent assay.

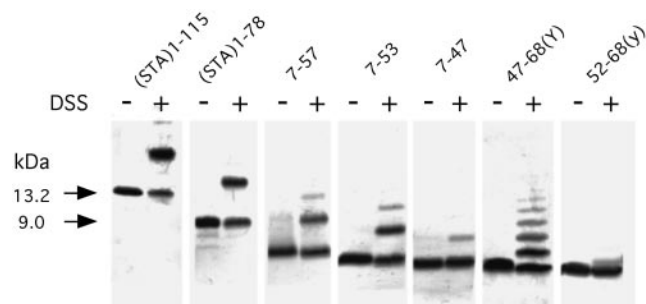


FIG. 9. SDS-PAGE analysis of various CgA N-terminal fragments before (-) and after (+) cross-linking with DSS. Each product was treated with DSS in dimethyl sulfoxide (+) or with dimethyl sulfoxide alone (-) as described under "Experimental Procedures" and analyzed by SDS-PAGE under nonreducing conditions.

to 20–40 nM in patients with heart failure (42). In addition, increased levels of CgA (up to 100–1000 nM) have been detected in the blood of patients with different neuroendocrine tumors (44, 45). Given that CgA and (STA)1–78 affect cell adhesion at concentration 7–70 and 30–300 nM, respectively (*i.e.* 0.3–3 $\mu\text{g/ml}$ on a weight basis), it is likely that CgA and its N-terminal fragments can reach sufficient levels to affect cell adhesion, at least in the secretory cell microenvironment and in pathological conditions. In contrast, peptide 47–68(Y) induced pro-adhesive effects at 11 μM (30 $\mu\text{g/ml}$) and anti-adhesive effects at 111 μM (300 $\mu\text{g/ml}$). These levels are markedly higher than the physiopathological levels of CgA, and it is therefore unlikely that this peptide and the other short peptides generated by plasmin, if formed *in vivo*, have a physiological relevance. Nevertheless, these small fragments can be useful tools for investigating the structure-activity relationships of CgA and may provide important information for the identification of the molecular determinants of CgA activity.

The effect of plasmin on CgA may also be physiologically relevant. It has been reported that tissue-type plasminogen activator is expressed in chromaffin cells, is sorted in the regulated secretory pathway, and is co-released with hormones and CgA after cell stimulation (46). In addition, it has been shown that tissue-type plasminogen activator and plasminogen bind to the cell surface (47). Plasmin generated on the cell surface can in turn selectively liberate a CgA-specific bioactive fragment that regulates catecholamine secretion (48). Thus, chromaffin cells possess in their microenvironment all of the

components necessary for exerting a tightly regulated proteolytic cleavage of CgA. Plasminogen activation and CgA processing may not be limited to the surface of chromaffin cells, because many other neuroendocrine and non-neuroendocrine cells can express these molecules (3, 27). Given the well recognized importance of fibroblasts and plasminogen activation in tissue invasion, remodeling, and repair (49–52), the interplay between CgA and the plasminogen/plasmin system could provide a novel mechanism for regulating fibroblast adhesion and function in physiopathological conditions characterized by CgA hyperproduction, such as neuroendocrine tumors and chronic heart failure.

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Cleavage of Chromogranin A N-terminal Domain by Plasmin Provides a New Mechanism for Regulating Cell Adhesion

Barbara Colombo, Renato Longhi, Chiara Marinzi, Fulvio Magni, Angela Cattaneo, Seung Hyun Yoo, Flavio Curnis and Angelo Corti

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