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RESEARCH ARTICLE

Role of vasostatin-1 C-terminal region in fibroblast cell adhesion

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Abstract Fibroblast adhesion can be modulated by proteins released by neuroendocrine cells and neurons, such as chromogranin A (CgA) and its N-terminal fragment vasostatin-1 (VS-1, $CgA_{1–78}$). We have investigated the mechanisms of the interaction of VS-1 with fibroblasts and of its pro-adhesive activity and have found that the pro-adhesive activity of VS-1 relies on its interaction with the fibroblast membrane via a phospholipid-binding amphipathic α-helix located within residues 47–66, as well as on the interaction of the adjacent C-terminal region 67–78, which is structurally similar to ezrin–radixin–moesin-binding phosphoprotein 50 (a membrane-cytoskeleton adapter protein), with other cellular components critical for the regulation of cell cytoskeleton.

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Keywords Chromogranin A · Vasostatin-I · Ezrin · Ezrin–radixin–moesin-binding phosphoprotein 50 · Cytoskeleton · Cell adhesion

Abbreviations

CgA	Chromogranin A
VS-1	Recombinant Ser-Thr-Ala-$CgA_{1–78}$
mAb	Monoclonal antibody
ERM	Ezrin–radixin–moesin
EBP50	ERM-binding phosphoprotein 50

Introduction

Changes in fibroblast-extracellular matrix and endothelial cell–cell adhesion are critical for tissue homeostasis, organ development, and remodeling, as well as in many pathological events, including cancer development and host defense. Studies on the mechanisms that regulate cell shape, adhesion, and motility are therefore of great experimental and clinical interest.

Previous studies have shown that the adhesion and migration of fibroblasts and endothelial cells can be modulated by proteins of the regulated secretory pathway after their release in the extracellular space [1–3]. In particular, the release of chromogranin A (CgA), a well-established member of the granin family of proteins stored in secretory granules of neuroendocrine cells, neurons, granulocytes, and cardiomyocytes [3], has been proposed to be mechanistically involved in local control of fibroblasts and endothelial cell adhesion [1, 4–6]. Proteolytic processing of CgA by intra-granular and/or extracellular proteases, such as prohormone convertase 1 and 2 [7, 8], furin [7, 8], plasmin [9, 10], and cathepsin L [11], is

believed to be important for regulating its biological activity. For example, intact CgA can inhibit fibroblast adhesion to various extracellular-matrix proteins whereas its N-terminal fragment, called vasostatin-I ($CgA_{1–76}$), promotes their adhesion and spreading either when added to the liquid-phase or bound to a solid-phase [1, 12]. CgA can give rise upon cleavage also to other fragments, such as pancreastatin, a $CgA_{250–301}$ fragment involved in the regulation of carbohydrate and lipid metabolism [13, 14] and catestatin, a $CgA_{352–372}$ fragment that regulates catecholamine secretion and plays a role as a regulator of the cardiovascular system [15–18]. The recombinant fragment corresponding to $CgA_{1–78}$ (VS-1), which can be cleaved from CgA by plasmin [9], can also inhibit changes of endothelial cell adhesion and barrier function induced by tumor necrosis factor α (TNF) in blood vessels [6, 19], as well as endothelial cell proliferation, migration, and capillary formation induced by vascular endothelial growth factor (VEGF) in various experimental systems [20]. In endothelial cells, VS-1 can inhibit TNF-induced gap formation and phosphorylation of p38 MAPK by a pertussis toxin sensitive mechanism, suggesting a role for VS-1 in protection of endothelial barrier via a G-protein regulation of the stress-activated MAPK pathway [21]. Chronic production of CgA by neoplastic cells can affect tumor growth and morphogenesis in animal models by affecting the tumor cell microenvironment [9]. In addition to these biological effects, VS-1 can decrease the myogenic tone in isolated vessels [22, 23] and exerts negative inotropic effects in isolated beating hearts from different species [24–26]. Based on these findings, and on the observation that circulating levels of CgA are increased in patients with neuroendocrine tumors or with heart failure or sepsis [27–32] we have postulated a significant role for the natural vasostatin-I in the regulation of tissue homeostasis and repair, vascular biology, tumor growth, and cardiovascular physiology [3].

The mechanism of action of VS-1 is still unclear. Although we have previously shown that VS-1 can interact with artificial monolayers of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine [33, 34], the interaction with natural cell membranes and the molecular mechanisms underlying the various activities of VS-1 remain largely unexplored. The present study was undertaken to elucidate the mechanisms of the interaction of VS-1 with fibroblasts and of its pro-adhesive activity. We provide evidence to support a model of interaction of VS-1 with membrane phospholipids, via C-terminal domain. Furthermore, we show that this domain contains a hydrophilic region (residues 67–78) that shares strong sequence and structural similarity with ezrin–radixin–moesin-binding phosphoprotein 50, a membrane-cytoskeleton adapter protein [35], and that this region, although is dispensable for binding of VS-1 to the cell surface, is critical for its pro-adhesive activity.

Materials and methods

Cell lines, antibodies, and reagents

HDFa (normal human dermal fibroblasts) and NIH-3T3 mouse fibroblasts were cultured using Dulbecco's modified Eagle medium (DMEM, BioWittaker) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (DMEM-GPSF). Dulbecco's phosphate buffered saline with Ca^{2+} and Mg^{2+} (DPBS) was from BioWittaker. Anti-CgA monoclonal antibodies (mAbs) B4E11, 5A8, and A11 (all IgG_1), were generated in our laboratory and characterized as described previously [4, 36]. 4′-6-Diamidino-2-phenylindole (DAPI) and GAM-Alexafluor 546 antibody was from Invitrogen (San Giuliano Milanese, Italy). Phalloidin-FITC, SB-203580, LY-294002, actinomycin D, cycloheximide, wortmannin, latrunculin A, cytochalasin B, nocodazole and taxol were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody against residues $CgA_{1–16}$ (called α16) was from Primm (Milan, Italy). Sulfo-succinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido) hexanoamido] ethyl-1,3′ dithiopropionate (Sulfo-SBED) was from Pierce (Rockford, IL, USA). The peptide ACDCRGDCFCG (RGD-4C) was prepared and characterized as described previously [37].

Preparation of CgA and CgA fragments

Human natural CgA and synthetic $CgA_{47–76}$, $CgA_{47–65}$, $CgA_{1–40}$ and $CgA_{62–76}$ peptides were produced as described previously [4]. Ser-Thr-Ala-$CgA_{1–78}$ (VS-1) was prepared by recombinant DNA technology [38]. The product was 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) [4]. The product was homogeneous and its purity was >95% by SDS-PAGE analysis.

$CgA_{1–65}$, a degradation product of VS-1, was recovered from the reverse-phase HPLC of VS-1 as a by-product. $CgA_{1–65}$ contained 3% of VS-1, as estimated by mAb B4E11/5A8 sandwich ELISA. SDS-PAGE, Western blotting, mass spectrometry, and ELISA assays showed that the purified product was homogeneous, had a mass of 9,065.94 Da (expected 9,065.70 Da), and was recognized by mAb 5A8 (against residues 54–57) but not by mAb B4E11 (against residues 68–71), as expected.

Labeling of VS-1

VS-1 was labeled with ^{125}I by GE Healthcare Amersham (Buckinghamshire, UK). Iodination of histidine residues with sodium [^{125}I] iodide was obtained by using the chloramine-T method. The product (^{125}I-VS-1) had a specific activity of 138 μCi/μg.

Preparation of recombinant EBP50

EBP50 was prepared by recombinant DNA technology using the Champion pET Directional TOPO Expression Kit (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence assay

Immunofluorescence experiments were performed as follows: HDFa fibroblasts were incubated with 3 μM VS-1 or $CgA_{1–65}$ in complete DMEM for 1.5 h at 37°C and washed two times with DPBS. Cells were fixed with 3% paraformaldehyde, 2% sucrose in DPBS (20 min at room temperature), washed twice with DPBS, then permeabilized with 0.5% Triton X-100 in DPBS. After two washes in DPBS, cells were incubated with mAb 5A8 in 3% normal goat serum, 0.5% BSA in DPBS (NBD) for 1 h at room temperature. Cells were washed twice in DPBS, incubated with GAM Alexafluor 546 and phalloidin-FITC in NBD for 1 h at room temperature, then with DAPI in DPBS (5 min at room temperature) and washed twice in DPBS. Cells were then analyzed by fluorescence microscopy.

Western blotting

Western-blot analysis of cell lysates and purified products was carried out with anti-CgA mAbs as follows: after SDS-PAGE (12.5% gel) and electro blotting on nitrocellulose, the membrane was treated with 2% blocking solution (Amersham) for 2 h, incubated with anti-CgA mAbs (1 μg/ml) in 2% blocking solution (1.5 h), and washed for 30 min with 100 mM sodium chloride, 20 mM Tris–HCl, pH 7.6 (TBS), containing 0.1% Tween (TBS-T). The membrane was then incubated for 1.5 h with GAM-HRP (1:10,000) in 2% blocking solution, and washed for 30 min with TBS-T (three times). Peroxidase activity was detected using the ECL Advance Western Blotting kit (Amersham).

NIH-3T3 cell adhesion assay

NIH-3T3 cell adhesion assays were performed as described previously [4] using microtiterplates coated with various amounts of VS-1, synthetic or recombinant CgA derived fragment ($CgA_{1–65}$, $CgA_{1–48}$, $CgA_{1–40}$, $CgA_{47–76}$, $CgA_{47–65}$, $CgA_{62–76}$) in the presence or in the absence of signaling and cytoskeleton inhibitors.

Binding assay of VS-1-SSBED to β1-integrins

VS-1-SSBED, an activated biotin-VS-1 conjugate, was prepared by coupling VS-1 with the trifunctional cross-linking reagent sulfo-succinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido) hexano-amido] ethyl-1,3′ dithiopropionate, according to the manufacturer's instructions (Pierce, Rockford, IL, USA). The conjugate contains a phenyl azide group, which upon photoactivation can react with amino-groups of interacting proteins, and a biotin group for detection. The binding of VS-1 to integrins on the surface of fibroblasts was studied by incubating 30 nM VS-1-SSBED with 150,000 cells for 1 h in DPBS, in ice. Cells were then washed with DPBS, scrapered, resuspended in 5 mM EDTA, 2 mM EDTA, 0.5% Triton X-100 in DPBS and lysed by sonication. Biotinylated complexed were precipitated from cell lysates with streptavidin–agarose beads. Bound material was eluted by boiling the beads with SDS-PAGE loading buffer. The presence or absence of VS-1/-β1 integrin complexes in the product was checked by Western blotting with anti-β1 integrin antibodies.

Results

The C-terminal domain of VS-1 (residues 47–76) is critical for fibroblast adhesion

To investigate the mechanism of VS-1-mediated fibroblast adhesion, we performed various cell-adhesion and binding experiments with immortalized NIH-3T3 fibroblasts and normal human dermal fibroblasts (HDFa). First, cell adhesion experiments showed that the $CgA_{1–40}$ fragment is less active than $CgA_{1–78}$ (VS-1) in promoting NIH-3T3 and HDFa cell adhesion (Fig. 1a), suggesting that the region 41–78 was crucial for activity. This region contains an RGD site (residues 43–45), a sequence that in other proteins can work as an integrin-binding site [39]. To dissect the contribution of this and other sites potentially present in VS-1, we have tested $CgA_{1–48}$ and $CgA_{47–76}$. $CgA_{47–76}$ (lacking RGD) was sufficient to induce fibroblast adhesion (Fig. 1b). In contrast, $CgA_{1–48}$ (containing RGD) was inactive. These results suggest that the region 47–76 (but not the region containing the RGD) is necessary for the activity.

The C-terminal domain of VS-1 contains a cell-binding site

We then analyzed the interaction of VS-1 and $CgA_{47–76}$ peptide with NIH-3T3 cells. To this aim, cells were

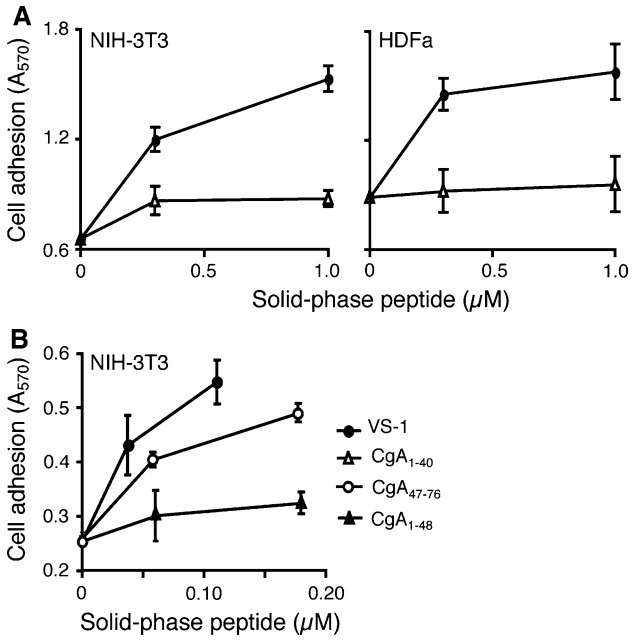

Fig. 1 Adhesion of NIH-3T3 and HDFa fibroblasts to CgA 1–78 (VS-1), $CgA_{1–48}$, $CgA_{1–40}$, and $CgA_{47–76}$. NIH-3T3 or HDFa cells were seeded on microtiterplates coated with various amounts of VS-1, $CgA_{1–40}$, $CgA_{1–48}$, and $CgA_{47–76}$, and left to adhere for 3 h at 37°C, 5% CO_2. Adherent cells were stained with crystal violet as described [42–49]. *Error bars* represent SD from a representative experiment repeated three times (each in triplicate)

incubated with 3 μM VS-1 or 10 μM $CgA_{47–76}$ peptide for 1.5 h at 4°C, in the presence of sodium azide to prevent endocytosis. Bound peptides were analyzed by Western blotting of cell lysates with the anti-CgA mAb B4E11, an antibody against residues 68–71 [4, 36]. The results showed that both VS-1 and $CgA_{47–76}$ peptide could bind these cells (Fig. 2a). Comparable levels of VS-1 binding were observed also with normal fibroblasts (data not shown). Preincubation of a 1/100-fold lower dose of VS-1 (30 nM) with mAb B4E11, but not with the polyclonal antibody α16 or the monoclonal antibody A11 (directed against CgA residues 1–16 and 81–90, respectively), could inhibit the binding of VS-1 to NIH-3T3 fibroblasts (Fig. 2b). These results suggest that the C-terminal domain 47–78 of VS-1 contains a cell-binding site.

To investigate the location of VS-1 after binding to NIH-3T3 fibroblasts, we treated cell-bound VS-1 with proteinase K, a protease with a very broad cleavage specificity that cannot penetrate into cells. Proteolytic digestion of VS-1 was monitored by Western-blot analysis of cell lysates with mAb B4E11. Cell-bound VS-1 was efficiently digested by proteinase K, as indicated by the marked decrease of VS-1 band intensity (Fig. 2c). This suggests that VS-1 was bound to the cell surface in a manner that was accessible to the protease. Of note, in certain experiments, a weak band corresponding to a short fragment of VS-1 immunoreactive with mAb B4E11 was observed after degradation (Fig. 2c). This result, together with the results of binding inhibition experiments with mAb B4E11, suggest that the C-terminal region is less accessible to antibodies and proteases when bound to cells, possibly because of being involved in cell binding.

VS-1 binding sites on the fibroblast cell membrane are resistant to proteinase K

To characterize the putative receptors or binding sites of VS-1 on fibroblasts we then analyzed the interaction of ^{125}I-VS-1 with NIH-3T3 cells by radio-binding assay. The assay was carried out in the presence of sodium azide to prevent endocytosis. Binding of ^{125}I-VS-1 occurred at concentrations greater than 1 nM in an apparently non-saturable manner (Fig. 3a). Noteworthy, the binding curve was not modified by co-incubation with an excess of unlabeled VS-1 (3 μM). These results suggest that NIH-3T3 cells express non-competitive binding sites for VS-1, different from canonical membrane receptors. Non-competitive and non-saturable binding of VS-1 was also observed with normal fibroblasts (not shown).

To assess the role of surface proteins in VS-1/fibroblast interactions, we analyzed the binding of VS-1 to cells pretreated with proteinase K. This experiment relies on the broad specificity of this enzyme, which can degrade almost every protein. The results showed that most binding sites on cells were resistant to this treatment, as VS-1 could bind protease-treated cells to the same extent of untreated cells (Fig. 3b). The lack of degradation was not due to lack of proteolytic activity, as the same amount of protease could completely degrade cell-bound VS-1 when added after VS-1/cell incubation (Fig. 3b). This suggests that the domain 47–78 of VS-1 can interact with proteinase K-resistant structures. Since we previously showed that the region 47–66 of VS-1 contains an amphipathic α-helix that can interact with artificial monolayers of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine [33, 34], it is likely that these structures correspond to membrane phospholipids. These results do not rule out the possibility that VS-1 can also interact with membrane-associated proteins or other components after interactions with lipids in untreated cells.

The C-terminal domain of VS-1 contains a region structurally similar to ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) adjacent to the amphipathic α-helix

Sequence analysis and alignment with proteins present in the NCBI database showed that the 47–78 C-terminal domain of VS-1 contains a hydrophilic region sharing a

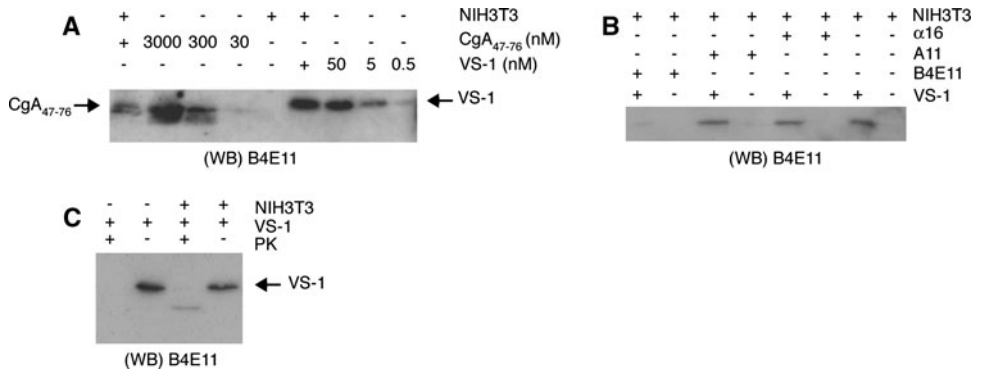

Fig. 2 Binding of VS-1 and $CgA_{47–76}$ to NIH-3T3 cells. **a** Western-blot analysis of cell lysates and of various amount of $CgA_{47–76}$ or VS-1 (as reference standard) with mAb B4E11. The cells were cultured in 24-well plate (2×10^5/well) and incubated with 3 μM VS-1 or 10 μM $CgA_{47–76}$ in DMEM-GPSF containing 0.02% sodium azide (1.5 h, 4°C). After washing, the cells were lysed with reducing SDS-PAGE loading buffer (7 min, 95°C). **b** Competition of the binding of VS-1 to NIH-3T3 with anti-CgA antibodies. MAb B4E11, mAb A11, and polyclonal antibody α16 (200 nM, directed against CgA residues 68–71, 81–90, and 1–16, respectively) were pre-complexed with VS-1 (30 nM), in DMEM-GPSF containing 0.02% sodium azide (10 min, r.t.) and incubated with NIH-3T3 cells (1 h, 4°C). Cells were washed with DPBS and lysed with SDS-PAGE loading buffer. The products were analyzed by Western blotting with mAb B4E11. **c** Effect of proteinase K on soluble VS-1 or cell-bound VS-1, as measured by Western blotting with mAb B4E11. VS-1 and cell-bound VS-1 were incubated with 50 μg/ml of proteinase K in 50 mM Tris–HCl, pH 8.0 (15 min, r.t.). Cells were washed with DPBS and lysed with SDS-PAGE loading buffer before analysis. Results of representative experiments repeated at least three times (**a**, **b**) or twice (**c**)

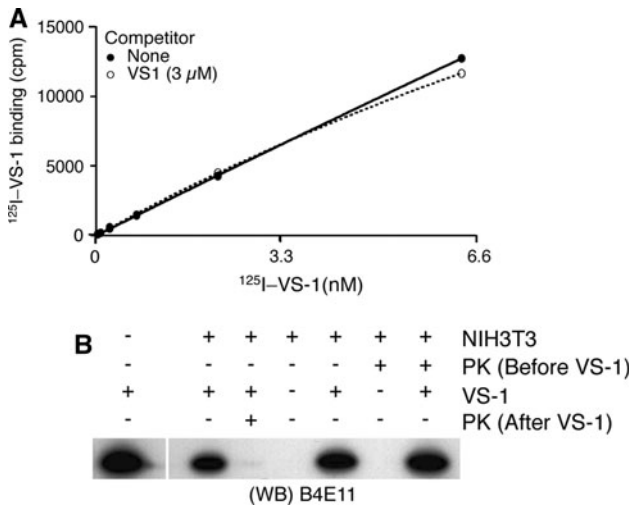

Fig. 3 The VS-1 binding sites of NIH-3T3 cells are non-saturable, non-compatible, and are not affected by proteinase K. **a** Binding of ^{125}I-VS-1 to NIH-3T3 cells in the presence or in the absence of VS-1. NIH-3T3 cell suspensions (1×10^6 cells/0.1 ml) were incubated with various amounts of ^{125}I-VS-1 in DPBS containing 0.02% sodium azide, in the presence or in the absence of 3 μM VS-1 (1 h at r.t.). The cells were washed with DPBS and cell-bound radioactivity was measured using a gamma counter. **b** Binding of VS-1 to NIH-3T3 cells pre-treated with proteinase K. Cells were treated with 100 μg/ml proteinase K in 50 mM Tris–HCl, pH 8.0, 0.02% sodium azide (25 min, r.t.) before adding VS-1 (3 μM). In a parallel experiment, the same treatment with proteinase K was carried out after incubation with VS-1 as described above

conserved sequence with human ezrin-radixin-moesin (ERM)-binding phosphoprotein 50 (EBP50), a 50-kDa membrane-cytoskeleton adapter protein. This region corresponds to CgA residues 67–80 and to EBP50 residues 329–339 (Fig. 4a). In particular, the region 69–75 (AKERAHQ) is 100 and 86% conserved between EBP50 and CgA of different species, respectively. Furthermore, a conserved dibasic site follows this sequence, in human, bovine, and swine CgA/EBP50. Remarkably, this CgA region includes the epitope of mAb B4E11 [36].

To assess whether the corresponding regions have similar structures in the two proteins, we evaluated the cross-reactivity of mAb B4E11 with EBP50. Western-blotting experiments showed that mAb B4E11 can recognize a 50-kDa band in extracts of recombinant *E. Coli* cell genetically engineered to express EBP50 and of NIH-3T3 cells (Fig. 4b, c). No bands were detected by the control mAb A11 (Fig. 4c). Furthermore, immunoprecipitation experiments of NIH-3T3 cell lysates with B4E11–Sepharose showed a band of 50 kDa (Fig. 4d). Immunoaffinity purification of the 50-kDa product (Fig. 4e) and peptide mass fingerprinting by MALDI-TOF mass spectrometry showed that the sequence of this product correspond to that of EBP50 (14 matching peptides out of 25; 51% sequence coverage). Remarkably, binding studies of recombinant EBP50 and VS-1 to mAb B4E11 showed that the affinities were very similar, the concentration of proteins giving 50% of the maximal binding being 1 nM for both EBP50 and CgA (Fig. 4f). These results strongly suggest that the C-terminal hydrophilic region of VS-1 (residues 69–75) is structurally similar to the cognate EBP50 region, and that these regions are exposed and accessible to solvent in both molecules. Notably, a fragment corresponding to residues 65–76 has been identified in the chromaffin cell secretions [40], suggesting that a cleavage site is present between residues 64–65.

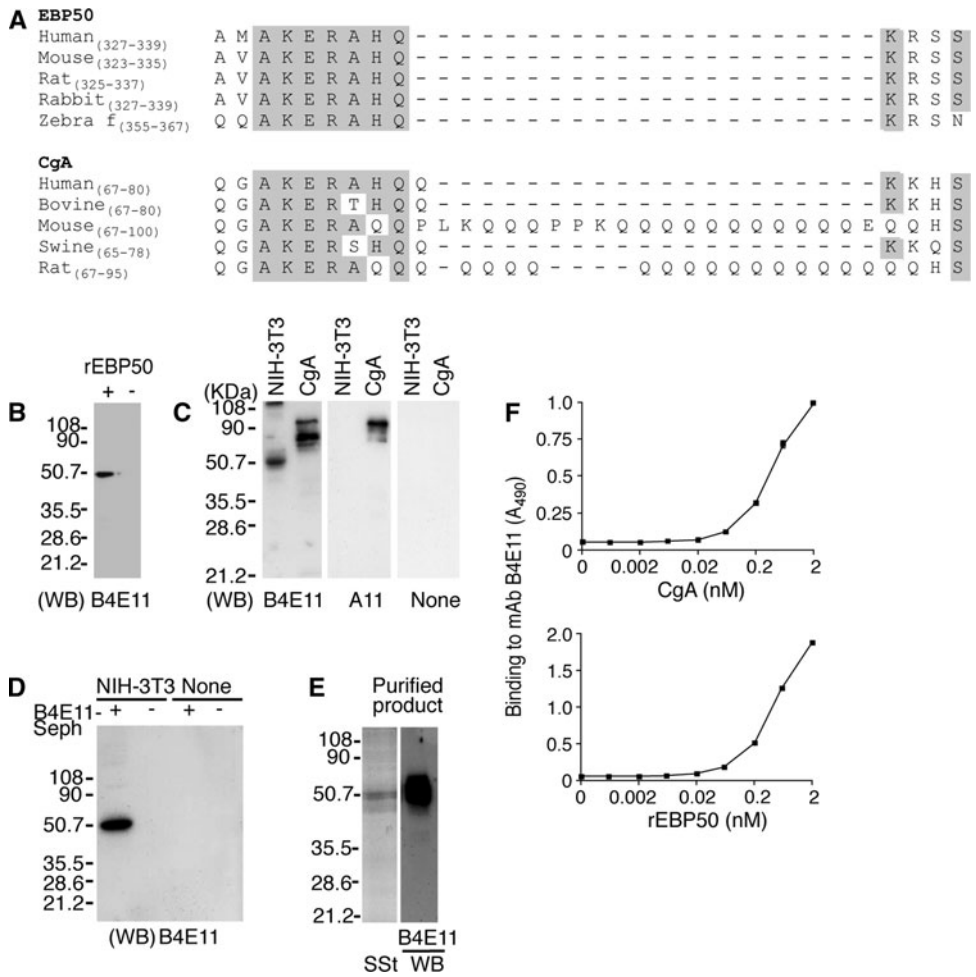

Fig. 4 Sequence alignment of EBP50 and CgA from different species and cross-reactivity of mAb B4E11 with both proteins. **a** Sequence alignment of EBP50 and CgA (human, *Homo Sapiens*; mouse, *Mus Musculus*; rat, *Rattus Norvegicus*; rabbit, *Oryctolagus cuniculus*; zebra fish, *Danio rerio*; bovin, *Bos taurus*; swine, *Sus scrofa*) reveals the presence of conserved regions shared between the two proteins. *Boxed regions* indicate identical residues. **b** Western-blot analysis of recombinant EBP50 crude extract with mAb B4E11. **c** Western-blot analysis of NIH-3T3 cell lysates and of CgA (purified from pheocromocytoma) with anti-CgA mAbs B4E11 and A11. **d** Western-blot analysis of material immunoprecipitated with B4E11–Sepharose beads from cell lysates. The beads were incubated with cell lysate (30 min, r.t.), washed with DPBS, resuspended in SDS-PAGE loading buffer (5 min, 95°C) and analyzed by Western blotting with mAb B4E11. **e** *Left* SDS-PAGE and silver staining (SSt) of the material purified from cell lysates by affinity chromatography on B4E11–Sepharose. *Right* Western-blot analysis of the immunopurified product with mAb B4E11. A band corresponding to a 50-kDa B4E11 cross-reactive product was observed in all assays. **f** Binding of recombinant EBP50 or CgA to B4E11-coated plates as measured by sandwich ELISA with anti-EBP50 or anti-CgA antibodies, respectively. Each panel from **b** to **f** shows the results of representative experiments repeated at least three times

The amphipathic α-helix, but not the EBP50-like region, is necessary for binding

The observations that the 47–78 C-terminal domain contains an amphipathic α-helix (residues 47–66) followed by the hydrophilic EBP50-like region (residues 67–78 (see Fig. 5a) for sequences and for a schematic representation) prompted us to dissect their role in cell binding and in cell adhesion activity.

To this aim, we first prepared a recombinant $CgA_{1–65}$ fragment, i.e., a deletion mutant lacking the EBP50-like region. Western-blot analysis of $CgA_{1–65}$ showed, as expected, that this fragment lacks the mAb B4E11 epitope (Fig. 5b). Since this precluded the possibility to investigate the binding by Western-blot analysis with this antibody, a different approach was used. HDFa cells were incubated with VS-1 or $CgA_{1–65}$ for 1.5 h at 37°C, then fixed with paraformaldehyde and immunostained with mAb 5A8, directed to residues 54–57. A dot-like staining was observed with both molecules (Fig 5c). The dots were observed both in the absence and presence of sodium azide, suggesting that they do not correspond to endocytotic

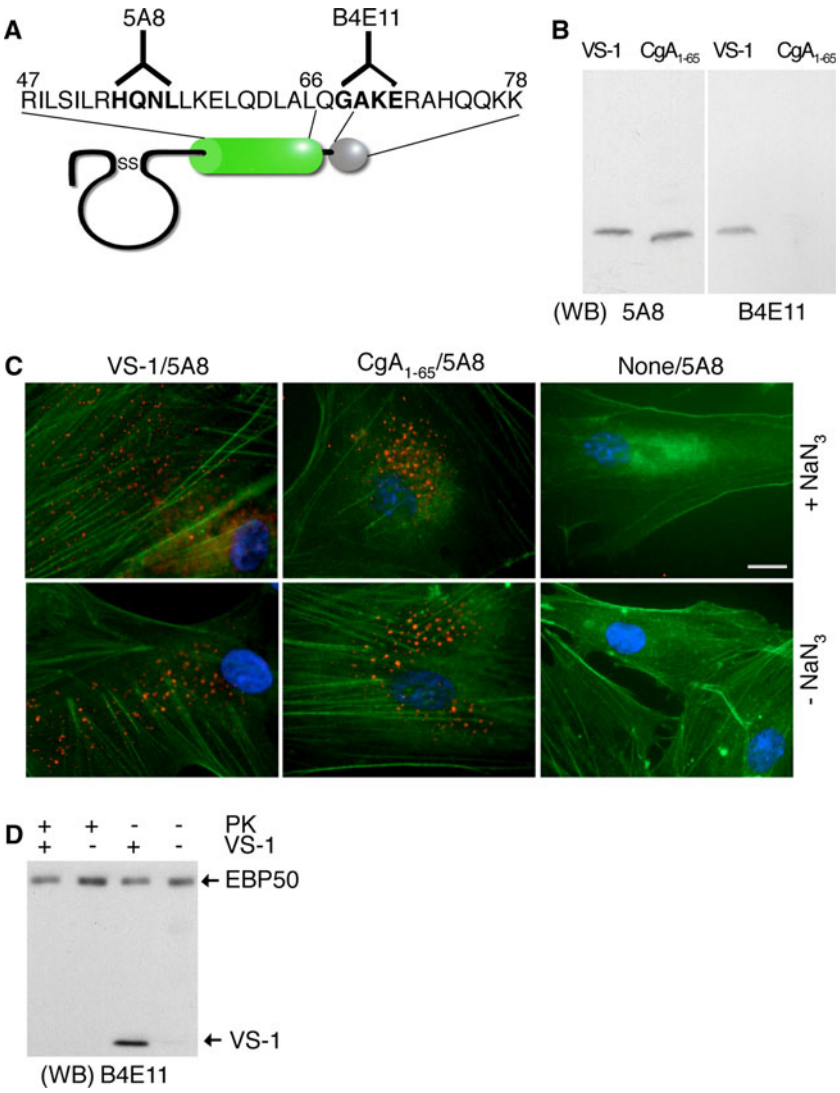

Fig. 5 Detection of VS-1 and $CgA_{1–65}$ binding to fibroblasts with mAb 5A8. **a** Schematic representation of VS-1 and C-terminal sequence with 5A8 and B4E11 epitopes (*bold*) [59]. **b** Characterization of VS-1 and $CgA_{1–65}$ (300 nM) by Western blotting with mAbs B4E11 and 5A8 using ECL standard developing method. **c** Binding of VS-1 and $CgA_{1–65}$ to HDFa fibroblasts. HDFa cells were incubated with 3 μM VS-1 or $CgA_{1–65}$ for 1.5 h. After fixation with 3% paraformaldehyde, 2% sucrose in DPBS, cells were permeabilized with 0.5% Triton X-100 in PBS and incubated with mAb 5A8, followed by GAM Alexafluor 546 (*red*), phalloidin-FITC (*green*) and DAPI. Cells were then analyzed by fluorescence microscopy. *Bar* 10 μm. **d** Effect of proteinase K digestion on cell bound VS-1 and EBP50. HDFa cells were treated with 3 μM VS-1 for 1.5 h, incubated with 50 μg/ml of proteinase K (as described in the legend of Fig. 2), and lysed with SDS-PAGE loading buffer before Western-blot analysis with B4E11. Panels **c** and **d** show the results of representative experiments repeated more than four times

vesicles. More likely, the dots correspond to clusters of molecules bound to the cell surface. These results suggest that the EBP50-like region 67–78 of VS-1 is not necessary for binding to the cell surface, and that the main structural determinants of cell binding are located within the amphipathic α-helix region 47–66.

To gain further information on VS-1 and EBP50 location after binding, degradation experiments with proteinase K were undertaken. Remarkably, cell-bound VS-1, but not EBP50, was completely degraded by proteinase K (Fig. 5d). This suggests that the locations of VS-1 and EBP50 are different, i.e., on the cell surface and within the cells, respectively. Of note, the EBP50 band was observed also in the other Western-blot assays of cell lysates reported above (not shown), and represented a good internal control of antibody reactivity and sample loading.

The entire C-terminal domain of VS-1, including the EBP50-like region, is critical for its pro-adhesive activity

To assess whether region 67–78 can play a role in the biological activity of VS-1, we performed cell-adhesion assays with VS-1 and $CgA_{1–65}$ bound to solid phases. Deletion of residues 66–78 caused significant loss, albeit not complete, of pro-adhesive activity (Fig 6a, b). The lower activity was not due to lower adsorption to solid phase, as checked by ELISA with mAb 5A8 (data not shown). Similarly, cell-adhesion assay carried out with synthetic fragments $CgA_{47–76}$ and $CgA_{47–65}$ on HDFa cells showed again that deletion of residues 66–78 impaired cell adhesion (Fig 6b). Thus, although the EBP50-like region is dispensable for binding, it appears to be important for the

pro-adhesive activity. Of note, a peptide $CgA_{62–76}$ encompassing the EBP50-like region but lacking the amphipathic α-helix region could not induce cell adhesion (data not shown). These results suggest that the entire C-terminal region encompassing both amphipathic α-helix and EBP50-like regions is critical for the activity of VS-1.

The C-terminal domain of VS-1 promotes integrin-mediated cell adhesion in an indirect manner

We next investigated the mechanism of action of VS-1 dependent cell-adhesion. In a previous study we showed that the VS-1-mediated cell adhesion was completely inhibited by an anti-β1 integrin antibody [1]. To assess whether VS-1 can interact with β1-integrins after interaction with the cell membrane, we studied the formation of complexes between VS-1-SSBED, an activated biotin-VS-1 conjugate (see "Materials and methods") and β1-integrins on NIH3T3 cell surface. Biotinylated complexed were precipitated from cell lysates with streptavidin–agarose beads and analyzed by Western blotting with anti-β1 integrin antibodies. No bands corresponding to VS-1/β1-integrin complexes were observed (data not shown). Furthermore, no binding of VS-1 to purified $\alpha5\beta1$ integrin adsorbed onto microtiter plates was observed by ELISA. These results argue against the hypothesis that VS-1 directly interacts with β1-integrin and suggest that this integrin is involved, or is activated, in an indirect manner. Accordingly, fibroblast adhesion to plates coated with VS-1 or $CgA_{47–76}$ was completely inhibited by a peptide containing the RGD sequence (Fig 7a, b), a well-known integrin-binding motif, whereas soluble $CgA_{47–76}$ could not inhibit RGD-mediated cell adhesion (data not shown).

A series of inhibitors were also tested in the attempt to gain further insights into the mechanisms. No inhibition of VS-1 mediated cell adhesion was observed with actinomycin D, a transcription inhibitor, and with cycloheximide, an inhibitor of protein biosynthesis (Fig. 7b), suggesting that VS-1-mediated cell adhesion did not require protein synthesis. Furthermore, LY-294002/wortmannin and SB203580, specific inhibitors of phosphatidylinositol 3-kinase and p38-MAPKs (which may play a role in actin reorganization) failed to inhibit VS-1-mediated cell adhesion (Fig. 7b), suggesting that it was independent from these kinases. Of note, no adhesion occurred to plates coated with VS-1 or $CgA_{47–76}$ in the absence of serum (not shown), further supporting the hypothesis of an indirect mechanism.

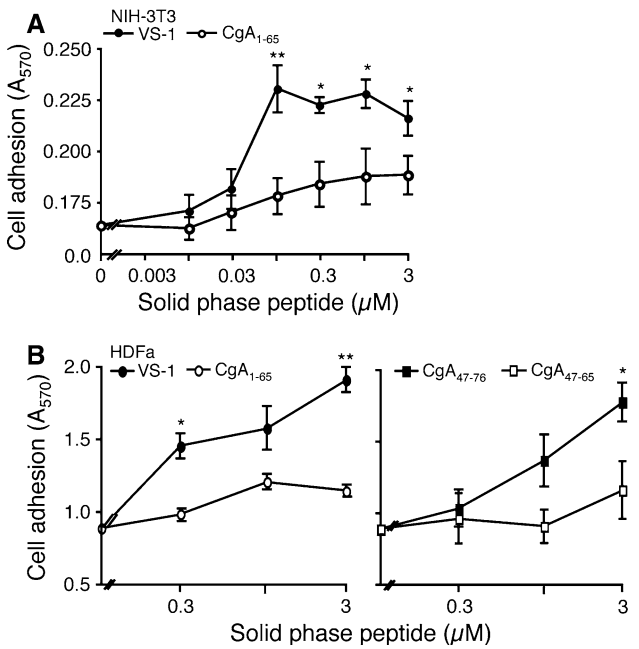

Fig. 6 Effect of VS-1 and VS-1 fragments on NIH-3T3 and HDFa fibroblasts adhesion. **a** NIH-3T3 cells were seeded on microtiterplates coated with various amounts of VS-1 or $CgA_{1–65}$ and left to adhere for 3 h at 37°C, 5% CO_2. Adherent cells were stained with crystal violet as described [4]. Each *curve* represents the mean ± SEM of three independent experiments carried out in quadruplicate. $^*p < 0.05$; $^{**}p < 0.01$ (two-tailed *t* test). **b** HDFa cell adhesion to VS-1, $CgA_{1–65}$, $CgA_{47–76}$, and $CgA_{47–65}$ was performed in a similar manner

The proadhesive activity of VS-1 requires cytoskeleton reorganization

To assess the role of cytoskeleton reorganization in the pro-adhesive activity of VS-1 we studied the effect of cytochalasin B and latrunculin A, two inhibitors of actin polymerization, on VS-1 binding and fibroblast adhesion. VS-1-mediated cell adhesion as well as VS-1 dot-like staining of cells was completely inhibited by these agents (Fig. 7c–e). No inhibition was observed with taxol and nocodazole, two agents that interfere with the polymerization of microtubules (Fig. 7c–e). These results suggest that VS-1-mediated cell adhesion requires actin-cytoskeleton reorganization.

Discussion

The results of this study support a model of interaction of VS-1 with fibroblasts based on binding to cell membrane via C-terminal domain (residues 47–78). This domain contains an amphipathic α-helix (residues 47–66), previously shown to bind phospholipids, and a hydrophilic region (residue 67–78) sharing strong sequence and structural similarity with ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50). Furthermore, the results suggest that the amphipathic α-helix is critical for both binding and activity, whereas the EBP50-like region is dispensable for binding,

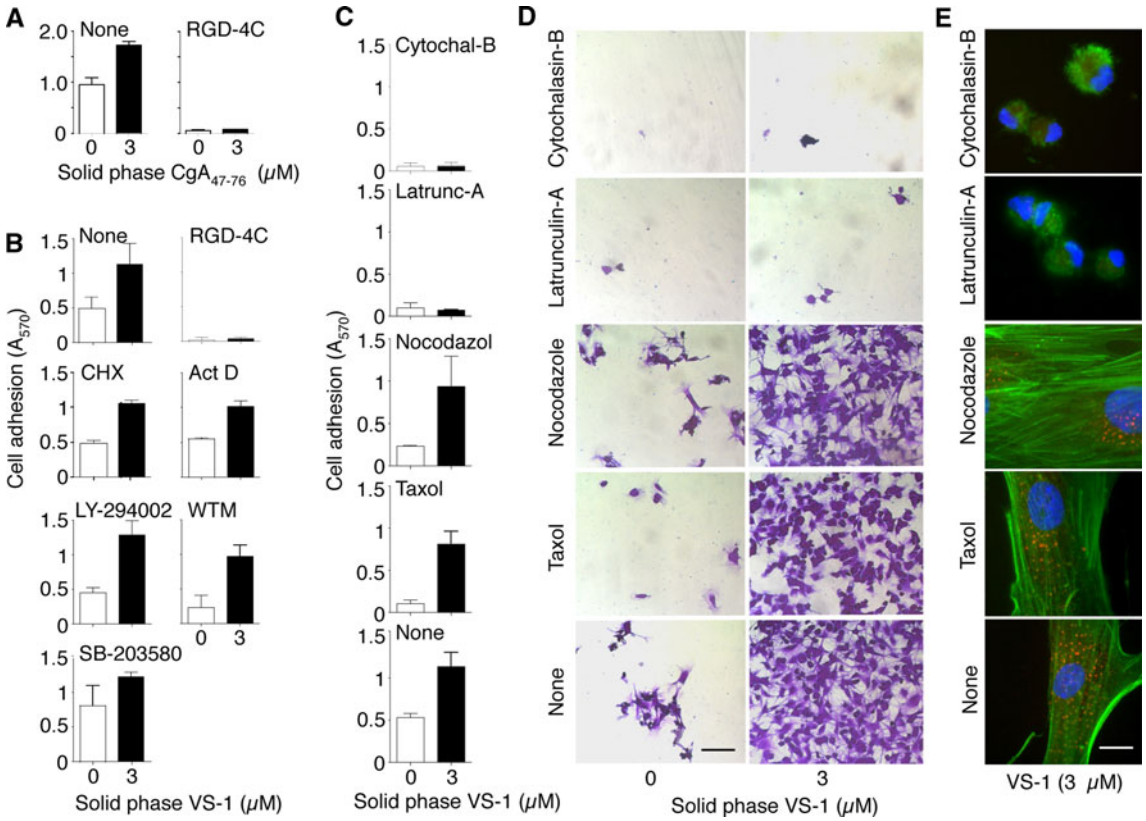

Fig. 7 Effect of various compounds on fibroblast adhesion to VS-1 coated plates (**a**–**d**) and on VS-1 localization after binding (**e**). **a**–**d** HDFa cells were seeded on microtiterplates coated with 3 μM of VS-1 or $CgA_{47–76}$ in the presence of 0.3 μg/ml ACDCRGDCFC peptide (RGD-4C), 10 μM SB-203580, 1 μg/ml cycloheximide (CHX), 2 μg/ml actinomycin D (Act D), 20 μM LY-294002, 25 nM wortmannin (WTM), 5 μM cytochalasin B, 5 μM latrunculin A, 150 nM nocodazole, 1 μM taxol in DMEM containing 1% FCS, 3% BSA, and incubated for 3 h at 37°C, 5% CO_2. Cells were stained with crystal violet and quantified by measuring the absorbance at 570 nM as previously described [4]. *Bar* 100 μm. **e** Binding of 3 μM VS-1 to HDFa cells in the presence (or absence of) cytoskeleton inhibitors. *Bar* 10 μm. Panels **a**–**e** show the results of representative experiments repeated three times

but is critical for the activity. Finally, the results provide evidence for a cell adhesion mechanism that involves integrins in an indirect manner and requires cytoskeleton reorganization.

The proposed model of VS-1/fibroblast interaction is supported by the results of binding studies and cell-adhesion assays performed with normal dermal fibroblasts and NIH-3T3 fibroblasts, and a series of VS-1-deletion mutants and antibodies. Radio-binding assays showed the presence of a large number of non-saturable and non-competitive binding sites for the C-terminal domain (residues 47–78) of VS-1, most likely distinct from canonical membrane receptors. Previous studies showed that recombinant VS-1 and the synthetic $CgA_{47–66}$ peptide, at 5–10 nM concentrations, can penetrate into monolayers of phospholipids, inducing conspicuous increases in the surface area in phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine monolayers [34]. Of note, $CgA_{47–66}$ is a cationic peptide with an amphipathic α-helical structure [33] typical of antimicrobial peptides able to penetrate plasma membrane [41]. Based on these observations, we speculate that the 47–78 domain of VS-1 interacts with fibroblast membrane phospholipids through the hydrophobic-amphipathic 47–66 region. This may explain the observation that radiolabeled VS-1 could not be competed by cold VS-1 in radio-binding assays, considering the extremely large number of potential binding sites on cell membranes.

Noteworthy, the adjacent hydrophilic 67–78 region, sharing high sequence similarity with the region 329–339 of EBP50 (Fig. 4), is highly conserved in both proteins and contains the AKERAHQ sequence that is identical in human EBP50 and CgA. The good cross-reactivity of mAb B4E11 with both proteins and the similar binding affinity implies that the AKERAHQ regions in these molecules have similar conformations, this antibody being directed to an epitope overlapping with this sequence [36]. Human EBP50 is a 358-residue protein that can work as a molecular linkage between ezrin–radixin–moesin (ERM) proteins, which are membrane-cytoskeleton adapter molecules, and the cytoplasmic domain of many proteins and receptors, including Na/H exchanger (NHE)-3, β2-adrenergic

receptor, platelet-derived growth factor receptor, cystic fibrosis transmembrane conductance regulator, and PTEN [42–49]. Based on the evidence of structural similarity between VS-1 and EBP50, it is tempting to speculate that VS-1 can affect cell cytoskeleton and promote cell adhesion by interacting with EBP50-related membrane-cytoskeleton adapter molecules. Notably, it has been shown that fragments of VS-1 encompassing the 47–66 region can penetrate the plasma membrane and interact with calmodulin, another cytoplasmic protein [50–52]. Thus, it is possible that the C-terminal domain of VS-1 (or a fragment containing it) can somehow penetrate and protrude within the cytoplasm, where it could potentially interfere with EBP50 function and/or mimic some of its activities. Further work is necessary to prove this hypothesis and to identify the interactors of the EBP50-like region of VS-1. Nevertheless, the observation that deletion of this region caused significant loss of VS-1 pro-adhesive activity strongly suggests that indeed it is crucial for the VS-1 function. The observation that mAb 5A8 and B4E11, against residues 54–57 and 68–71, respectively, but not mAb 7D1 directed against residues 34–46, can inhibit the pro-adhesive activity of VS-1 [4], and that short peptides lacking the α-helix or the EBP50-like region are inactive, further support this concept and suggest that both 47–66 and 67–78 regions are necessary for efficient cell adhesion and spreading activity.

These results of adhesion experiments with various inhibitors suggest that adhesion requires activation of specific cellular mechanisms, including actin polymerization, and it is not purely related to mechanical or sticky effects. In particular, competition experiments with RGD peptides suggest that integrins are involved in an indirect manner. One possibility is that VS-1 affects integrin function indirectly, e.g., by altering intracellular components involved in "inside-out" integrins signaling [53, 54].

Regarding the physiological significance of our findings, it is important to note that pro-adhesive effects observed in our experimental systems were obtained with concentrations of VS-1 greater than 100–300 nM. These concentrations are markedly higher than the serum levels of CgA in normal subjects (0.5–2 nM) and even more than those of VS-1 (0.2–2 nM) [12, 55]. It is therefore unlikely that circulating CgA/VS-1 play significant roles as regulators of fibroblast adhesion after distribution in tissues. However, CgA and its fragments could play significant roles in the microenvironment of CgA-secreting cells where high concentrations can be reached. This could be particularly relevant for neuroendocrine tumors, considering that neoplastic cells in certain patients can release abnormal amounts of CgA, enhancing the circulating levels up to 0.1–1 μM [27, 56]. One might expect, therefore, that in the microenvironment of neuroendocrine tumor cells the levels of CgA and its fragments can reach the bioactive concentrations observed in our assays.

The concentration of VS-1 in cancer patients has not yet been reported. However, it is known that VS-1 is efficiently cleaved from CgA by plasmin [9], a proteolytic enzyme known to be produced in tumors and to play an important role in tumor tissue invasion [57]. It is therefore possible that a certain amount of VS-1 is locally produced in neuroendocrine tumors, at least in those areas with active plasmin. The observation that VS-1 can affect fibroblast physiology, together with our previous finding that CgA expression in cancer cells can affect tumor growth and tissue morphogenesis by affecting the tumor microenvironment [9], and the growing body of evidence implicating fibroblasts in the regulation of the tumor growth [9, 58], may suggest a role for CgA and its fragments in the tumor microenvironment pathophysiology.

In conclusion, the results of the present study provide evidence to suggest that the C-terminal region of VS-1 can activate a fibroblast adhesion mechanism that involves integrins in an indirect manner, does not require protein synthesis, and requires cytoskeleton reorganization. This mechanism requires the interaction of the C-terminal domain with the fibroblast membrane, likely via the phospholipid-binding amphipathic α-helix 47–66, as well as the interaction of the adjacent hydrophilic region 66–78, containing the AKERAHQ sequence, with other cellular components critical for the regulation of cell shape and adhesion. The high conservation of this sequence in VS-1 and EBP50 of different species and their good structural similarity could stimulate further work aimed at investigating potential EBP50-mimicry function of VS-1.

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