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The interaction between ADAM 22 and 14-3-3ζ: regulation of cell adhesion and spreading☆,☆☆

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Abstract

The ADAM family consists of a number of transmembrane proteins that contain disintegrin-like and metalloproteinase-like domains. Therefore, ADAMs potentially have cell adhesion and protease activities. 14-3-3 proteins are a highly conserved family of cytoplasmic proteins that associate with several intracellular signaling molecules in the regulation of various cellular functions. Here we report the identification of a novel interaction between the ADAM 22 cytoplasmic tail and the 14-3-3ζ isoform by a yeast two-hybrid screen. The interaction between the ADAM 22 cytoplasmic tail and 14-3-3ζ was confirmed by an in vitro protein pull-down assay as well as by co-immunoprecipitation, and the binding sites were mapped to the 28 amino acid residues of the C-terminus of the ADAM 22 cytoplasmic tail. Furthermore, we found that overexpression of the ADAM 22 cytoplasmic tail in human SGH44 cells inhibited cell adhesion and spreading and that deletion or mutation of the binding site for 14-3-3ζ within the ADAM 22 cytoplasmic tail abolished the ability of the overexpressed cytoplasmic tail to alter cell adhesion and spreading. Taken together, these results for the first time demonstrate an association between ADAM 22 and a 14-3-3 protein and suggest a potential role for the 14-3-3ζ/ADAM 22 association in the regulation of cell adhesion and related signaling events.

ADAM (a disintegrin and metalloproteinase) proteins are a family of membrane-anchored glycoproteins. All family member proteins of ADAM have a pro-domain, a metalloproteinase-like domain, a disintegrin-like domain, a cysteine-rich region, an epidermal growth factor-like domain, a transmembrane domain, and a cytoplasmic tail [1,2]. Although the functions of most members of this family are unclear, their domain organization suggests that they may play roles in surface proteolysis, cell–cell or cell–matrix interactions, and cell signaling [3,4]. Of the 40 members of the ADAM family proteins identified so far, some have been shown to play an important role in diverse biological processes such as fertilization [1,5–7], myogenesis [8], neurogenesis [9], cell signaling [10,11], inflammatory response [3,12], and cell–cell/cell–matrix interaction [13–15]. In addition, ADAMs have been implicated in tumor metastasis. Many ADAMs are highly expressed in human tumors [13,16].

One important role of ADAMs relates to their metalloprotease activity. A well-studied example is ADAM 17, which functions in cell surface shedding of a number of growth factors, including tumor necrosis factor-α, transforming growth factor-α, and Notch [3,10,12,17]. Another important role of the ADAMs relates to cellular interactions, as shown for ADAM 1 and 2 in sperm–egg binding and fusion during fertilization [5,7]. ADAM 22 is a protein that is primarily expressed in brain [18].

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☆☆ *Abbreviations:* adam22cyt, cytoplasmic tail of adam 22; ADAM, a disintegrin and metalloproteinase; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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ADAM 22 lacks a zinc-binding motif in its metalloproteinase-like domain and therefore is unlikely to possess proteinase potency [18,19]. The structure of the ADAM 22 protein suggests that it could function as an integrin ligand and play a role in cell–cell adhesion. The disintegrin-like domain of ADAM 22 contains highly conserved cysteine residues and exhibits a high degree of sequence similarity to ADAM 2 (fertilin-β) and ADAM 15, which are known to be potential integrin ligands. Control of cell adhesion is important in many biological processes including embryonic-development, tumor cell invasion, and metastasis. Understanding the molecular mechanisms of these processes not only requires information about a variety of extracellular ligands and their interaction with cell membrane receptors, but also the subsequent downstream signal cascade.

14-3-3 proteins are a highly conserved family of proteins ubiquitously expressed in all eukaryotic cells. There are seven identified 14-3-3 isoforms encoded by separate genes (β, γ, ε, η, σ, τ, and ζ) in mammals [20,21]. It has been shown that members of the 14-3-3 family bind and regulate key molecules involved in various physiological processes such as intracellular signaling, cell cycle control, apoptosis, and transcription regulation [20,22,23]. 14-3-3 proteins can form homodimers or heterodimers that allow them to function as an adapter, a linker, a scaffold or a coordinator in assembling signaling complexes [24]. 14-3-3 proteins associate with a number of different signaling proteins, including Raf-1, MEKK1, and PI-3 kinase [25–27]; apoptosis regulatory protein BAD, ASK-1, FKHLR1, and tumor suppressor p53 [23,28–30]; transcription regulatory protein DAF-16, and histone deacetylase [31,32]. Although the biological consequence of the binding of 14-3-3 proteins is still poorly understood, the importance of 14-3-3 proteins in controlling signal transduction pathways is beginning to emerge. In many cases 14-3-3 proteins alter the function of the target protein thus allowing them to serve as direct regulators of their targets.

To get insight into the function of ADAM 22 and to identify molecules involved in the regulation of ADAM 22 function or in the mediation of ADAM 22 associated signaling events, we performed yeast two-hybrid screening using the cytoplasmic tail of ADAM 22 [ADAM22cyt] as bait. In the present study, we have demonstrated that ADAM22cyt interacts with 14-3-3ζ. We further show that the binding sites for 14-3-3ζ are located within the 28 amino acid residues of the C-terminus of the ADAM22cyt and that disrupting the interaction between the endogenous ADAM22cyt and 14-3-3ζ by introducing overexpressed ADAM22cyt inhibits cell adhesion and spreading. Taken together, we have identified for the first time a novel interaction between ADAM 22 and 14-3-3ζ and demonstrated that ADAM 22 plays a role in cell adhesion and that its interaction with 14-3-3ζ is essential for its function.

Experimental procedures

Materials and reagents. Protein A–Sepharose 4B, glutathione–agarose beads were purchased from Amersham Pharmacia Biotech. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, stock antibiotic/antimycotic mixture (10,000 U/ml of penicillin base, 10,000 μg/ml of streptomycin base, and 25 μg/ml Fungizone), Zeocin, and LipofectAMINE were products of Life Technologies. Antibodies against LexA, HA, and c-Myc were obtained from Clontech. Poly-L-lysine and human plasma fibronectin were purchased from Sigma Biochemical.

DNA constructs and mutagenesis. The yeast two-hybrid cloning vector pEG202, the human fetal brain cDNA library constructed in pJG4.5 vector and the yeast strain EGY48Z (leu2, his3, trp1, and ura3), which was derived from the original yeast strain EGY48 [33] by transforming with a LexAop-LacZ reporter, were provided by Dr. T. Wang, University of Washington. The bait construct used for the two-hybrid screening, pEG202-adam22cyt, contained the cytoplasmic domain of the ADAM 22 encompassing the amino acids 761–859 (Fig. 1B) and was generated by cloning the corresponding cDNA fragment in frame with the LexA coding sequence into the vector pEG202. The cDNA fragment encoding the amino acids 761–859 of the ADAM22cyt was amplified by polymerase chain reaction (PCR) using sense 5′-GGAATTCAAAAACTATCGAGAACAG and antisense 5′-CGCTCGAGTTACTCAGTTGAATTAG oligonucleotides and a human MDC2 cDNA (gifts from Dr. Koji Sagane, Tsukuba Research Laboratories, Eisai Co., Ltd.) as template. The cDNA of ADAM22cyt and ADAM 22 was also cloned into the mammalian expression vectors pCMV-Myc and pcDNA4.1/His (Invitrogen). The pcDNA4.1/His-adam22cytΔ plasmid containing a cDNA encoding a truncated ADAM22cyt lacking the 28 amino acid residues of the C-terminus was constructed similarly. The pcDNA4.1/His-myr-adam22cyt and pcDNA4.1/His-myr-adam22cytΔ plasmids were constructed by adding a myristoylation site to the N-terminus of adam22cyt and adam22cytΔ according to the described protocol [34]. Full-length 14-3-3ζ cDNA was amplified by PCR using sense 5′-GGAATTCATGGATAA AAATGAGCTG and antisense 5′-GACAAGCCGACAACCTTGA TTGGAG oligonucleotides and pJG4-5-14-3-3ζ plasmid as template which was screened out from a human fetal brain cDNA library by a yeast two-hybrid screening using ADAM22cyt as bait, and cloned into the mammalian expression vector pCMV-HA (Clontech) and into the *Escherichia coli* expression vector pGEX-4T-1 (Amersham Pharmacia Biotech). All DNA constructs were verified by DNA sequencing. Single point mutation and deletion constructs were prepared using PCR technique.

Yeast two-hybrid analysis. Yeast two-hybrid screens were performed essentially as described by Brent and Finley [35]. The plasmid pEG202-adam22cyt was introduced into the yeast strain EGY48Z, total protein extracts were prepared from individual colonies, and the expression of the fusion protein LexA-ADAM22cyt was confirmed by immunoblotting with a monoclonal antibody against LexA. An EGY48Z yeast clone expressing LexA-ADAM22cyt was transformed with a human fetal brain cDNA library constructed in the pJG4.5 vector [35]. Transformants (1.2×10^6) were selected on uracil-minus, histidine-minus, tryptophan-minus, leucine-minus galactose medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) [35]. Plasmid DNA from positive clones was isolated and re-tested for their interaction with ADAM22cyt in yeast. After the interaction was confirmed, plasmid DNA from the positive clones was sequenced and compared with the nucleotide database at GenBank, NIH.

Cell culture and transfection. HEK293 and glioma SHG44 cells were obtained from the Institute of Biochemistry and Cell Biology,

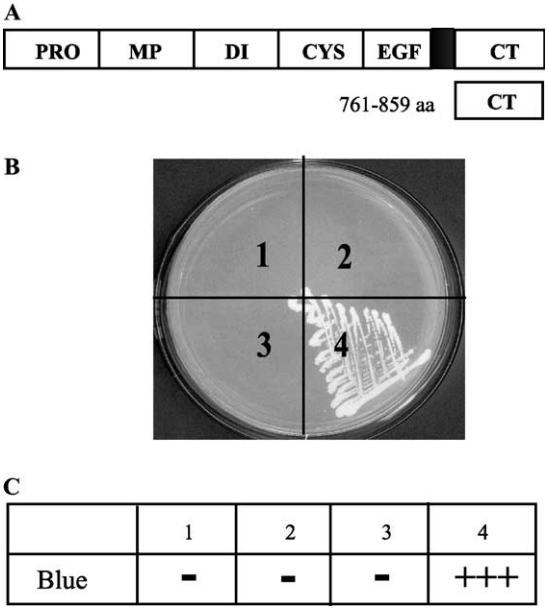

Fig. 1. Interaction between ADAM 22 cytoplasmic tail and 14-3-3ζ in yeast two-hybrid assay. (A) Schematic representation of the domain structure of ADAM 22 and the cytoplasmic tail (amino acids 761–859) used in the yeast two-hybrid screen (pEG202-adam22cyt). The white box represents a domain and the black box indicates a putative transmembrane domain. Abbreviations of the domains are as follows: PRO, pro-domain; MP, metalloproteinase-like domain; DIS, disintegrin-like domain; CYS, cysteine-rich domain; EGF, EGF-like repeat domain; CT, cytoplasmic domain (cytoplasmic tail). (B) Growth of yeast transformants. The yeast strain EGY48Z was cotransformed with the combination of plasmids pJG4.5 and pEG202-adam22cyt, pJG4.5-14-3-3ζ and pEG202, pJG4.5-14-3-3ζ and pEG202-adam19cyt, pJG4.5-14-3-3ζ and pEG202-adam22cyt and selected on uracil-minus, histidine-minus, tryptophan-minus glucose yeast medium for the presence of all plasmids. Independent transformants (six colonies from each sample) were restreaked onto six uracil-minus, histidine-minus, tryptophan-minus, leucine-minus galactose plates to evaluate the activation of the LEU2 reporters. Pictures were taken 72 h after the colonies were streaked (area 1, yeast transformed by pJG4.5 and pEG202-adam22cyt; area 2, by pJG4.5-14-3-3ζ and pEG202; area 3, by pJG4.5-14-3-3ζ and pEG202-adam19cyt; and area 4, or by pJG4.5-14-3-3ζ and pEG202-adam22cyt). The photo is representative of six plates. (C) Table of β-galactosidase activity. Independent transformants (six colonies from each sample) were re-spotted onto uracil-minus, histidine-minus, tryptophan-minus galactose medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to score activation of the LacZ reporter (+++ indicates deep blue, – indicates white). Column 1, the yeast strain EGY48 cotransformed with the combination of plasmids pJG4.5 and pEG202-adam22cyt; Column 2, with the combination of plasmids pJG4.5-14-3-3ζ and pEG202; Column 3, with the combination of plasmids pJG4.5-14-3-3ζ and pEG202-adaml9cyt; and Column 4, with the combination of plasmids pJG4.5-14-3-3ζ and pEG202-adam22cyt.

Chinese Academy of Science, maintained in DMEM containing 10% fetal calf serum, and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cell transfections were performed using LipofectAMINE according to manufacturer's instructions (Invitrogen). SHG44 cells transfected with pcDNA4.1/His-adam22cyt, pcDNA4.1/His-myr-adam22cyt, and pcDNA4.1/His-myr-adam22cytΔ were selected in DMEM containing 10% serum and 400 μg/ml Zeocin and then maintained in DMEM containing 10% serum and 200 μg/ml Zeocin.

GST pull-down assay. In vitro association experiments were carried out with GST fusion protein containing full-length 14-3-3ζ protein and lysates from yeast transfected with pEG202-adam22cyt and pEG202-adam22cyt-mutant plasmids. The fusion protein GST-14-3-3ζ was expressed in *E. coli* strain BL21 and purified according to manufacturer's instructions (Amersham Pharmacia Biotech). Yeast lysates were incubated with 10 μg GST alone or GST-14-3-3ζ immobilized on glutathione beads (Amersham Pharmacia Biotech) at 4 °C for 2 h. Protein complexes were recovered by centrifugation, washed three times with washing buffer (25 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 0.5 mM dithiothreitol), and analyzed by standard SDS–PAGE and immunoblotting techniques. Immunoblotting was performed using a monoclonal antibody against LexA. Lysate prepared from the yeast transfected with pEG202 without insert was used as a negative control.

Immunoprecipitation assays. Immunoprecipitations were performed with lysates prepared from HEK293 cells cotransfected with pCMV-Myc-adam22cyt and pCMV-HA-14-3-3ζ. Briefly, 48 h post-transfection, cells were lysed on ice with lysis buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 10 mg/ml each of pepstatin A, leupeptin, and aprotinin). Cell lysates were cleared by centrifugation at 4 °C for 15 min and normalized for protein content. Immunoprecipitations were carried out with a polyclonal anti-HA antibody and protein A–Sepharose, followed by SDS–PAGE. Immunoblotting was performed with a monoclonal anti-c-Myc antibody. Immunoprecipitations using lysates prepared from cells cotransfected with the plasmid pCMV-Myc-adam22cyt together with pCMV-HA or pCMV-Myc together with HA-14-3-3ζ were performed as controls.

Cell adhesion assay. Cell adhesion assays were performed as described [36]. Briefly, fibronectin (5 μg/ml) or poly-L-lysine (50 μg/ml) was used to coat 96-well plates (100 μl/well) at 37 °C for 30 min. The plates were then washed twice with PBS and blocked with 100 μl of 1% BSA. SHG44 cells stably transfected with pcDNA4.1/His-adam22cyt, pcDNA4.1/His-myr-adam22cyt, and pcDNA4.1/His-myr-adam22cytΔ and the mock-transfected cells were trypsinized, washed, plated at 10^4 cells/well in 100 μl DMEM/0.25% BSA, and then incubated at 37 °C for 1 h. Nonadherent cells were removed by gentle washing with PBS three times. The number of attached cells was assessed by MTT assay [36].

Cell spreading assay. Cell spreading assays were performed according to the procedure as described by Han et al. [37] with minor modifications. Briefly, fibronectin (10 μg/ml) or poly-L-lysine (50 μg/ml) was used to coat 24-well plates (500 μl/well) at 37 °C for 1 h. The plates were then washed twice with PBS and blocked with 500 μl of 1% BSA. SHG44 cells stably transfected with pcDNA4.1/His-adam22cyt, pcDNA4.1/His-myr-adam22cyt, and pcDNA4.1/His-myr-adam22cytΔ and the mock transfected cells were collected by trypsinization, washed three times with DMEM, and then replated (10^5 cells/well) on the 24-well plate coated with fibronectin or poly-L-lysine. Cells were allowed to spread at 37 °C for 10–40 min and then photographed. Spread cells were defined as cells with irregular morphology and lacking phase-brightness and nonspread cells were round and phase-bright under the microscope.

Results

Identification of ADAM22cyt interaction with 14-3-3ζ

We employed a yeast two-hybrid interaction screen to identify proteins that interact with ADAM22cyt, the cytoplasmic portion of the ADAM 22. The domain structure of ADAM 22 is given in Fig. 1A. When screening a human fetal brain cDNA library using ADAM22cyt as bait several positive clones were isolated

from 2×10^6 transformants. Among those positive clones, a clone encoding 14-3-3ζ, was identified. To reconfirm the interaction between ADAM22cyt and 14-3-3ζ, we transformed the yeast strain EGY48Z with the combination of plasmids pJG4.5 and pEG202-adam22cyt, pJG4.5-14-3-3ζ and pEG202, pJG4.5-14-3-3ζ and pEG202-adam19cyt, or pJG4.5-14-3-3ζ and pEG202-adam22cyt. As shown in Fig. 1, only the yeast co-expressing ADAM22cyt and 14-3-3ζ grew on uracil-minus, histidine-minus, tryptophan-minus, and leucine-minus galactose media (Fig. 1B) and had LacZ protein (Fig. 1C, table) expressed. The yeast cotransfected with the combination of pEG202-adam22cyt and the empty vector pJG4.5, pJG4.5-14-3-3ζ and the empty bait vector pEG202, or pJG4.5-14-3-3ζ and a bait vector pEG202-adam19 did not grow on the selective media. These results confirmed a specific association between ADAM22cyt and 14-3-3ζ.

Confirmation of the ADAM22cyt interaction with 14-3-3ζ in vitro and in mammalian cells

Biochemical interactions between ADAM22cyt and 14-3-3ζ were then tested by a GST pull-down assay. Cell lysates from yeast transfected with HA-tagged ADAM22cyt were incubated with GST or GST-14-3-3ζ that had been immobilized on glutathione–agarose beads. After washing, the bound proteins were analyzed by immunoblotting with a monoclonal antibody against LexA. As shown in Fig. 2A, the LexA-ADAM22cyt fusion protein was captured by the immobilized GST-14-3-3ζ (lane 3) but not by the immobilized GST (lane 4). In addition, no interaction between LexA and GST-14-3-3ζ was detected (lane 5). These results indicated that the fusion protein LexA-ADAM22cyt was captured by the immobilized GST-14-3-3ζ through a direct interaction between 14-3-3ζ and ADAM22cyt. An aliquot of the lysate from yeast transfected with pEG202-adam22cyt or the parent vector pEG202 was also analyzed directly as positive markers (lanes 1 and 2).

To confirm the association between ADAM22cyt and 14-3-3ζ in mammalian cells, immunoprecipitations were performed using cell lysates prepared from HEK293 cells cotransfected with pCMV-Myc-adam22cyt and pCMV-HA, pCMV-Myc and pCMV-HA-14-3-3ζ, or pCMV-Myc-adam22cyt and pCMV-HA-14-3-3ζ. As shown in Fig. 2B, the Myc-ADAM22cyt was immunoprecipitated together with HA-14-3-3ζ (lane 4). The interaction between Myc-ADAM22cyt and HA-14-3-3ζ was specific to ADAM22cyt and 14-3-3ζ. No immunoactive bands were detected using lysates from cells cotransfected with pCMV-Myc-adam22cyt and pCMV-HA (lane 2) or pCMV-Myc and pCMV-HA-14-3-3ζ (lane 3). An aliquot of the lysates from cells cotransfected with pCMV-Myc-adam22cyt and pCMV-HA-14-3-3ζ or pCMV-Myc-adam22cyt and pCMV-HA was also analyzed directly as a positive marker (lanes 1 and 7). Myc-ADAM22cyt could be easily detected in the lysate prepared from HEK 293 cells cotransfected with pCMV-HA and pCMV-Myc-adam22cyt (lane 7). This excluded the possibility that the negative detection (lane 2) was due to a lack of Myc-adam22cyt expression and that the positive detection (lane 4) was due to an incomplete washing of the protein A–Sepharose beads during immunoprecipitation. Lysates from HEK 293 cells transfected with pCMV-HA-14-3-3ζ or pCMV-HA-14-3-3ζ, plus pCMV-Myc were also analyzed (lanes 5 and 6). It can be seen that Myc-tagged protein was expressed only in cells transfected with pCMV-Myc-adam22cyt (compare lanes 5 and 6 with lanes 1 and 7), which confirmed the identify of the protein detected (lane 4).

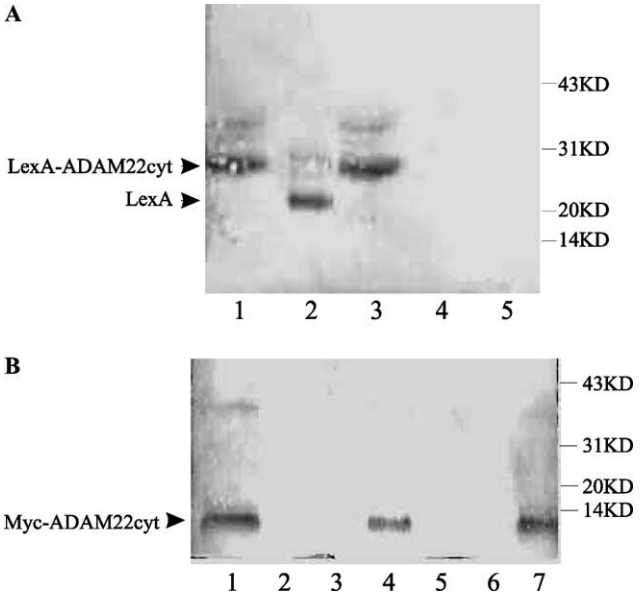

Fig. 2. Confirmation of the interaction between ADAM 22cyt and 14-3-3ζ by in vitro protein pull-down assay and in vivo co-immunoprecipitation. (A) Immobilized GST-14-3-3ζ (lane 3) or GST (lane 4) was incubated with lysate from yeast transfected with pEG202-LexA-adam22cyt. Immobilized GST-14-3-3ζ was also incubated with lysate from yeast transfected with the parent vector pEG202-LexA (lane 5). After washing, the bound proteins were analyzed by Western blotting with a monoclonal anti-LexA antibody. Lysates from yeast transfected with pEG202-LexA-adam22cyt (lane 1) and the parent vector pEG202-LexA (lane 2) were also analyzed directly as positive markers. (B) HEK293 cells were cotransfected with pCMV-Myc-adam22cyt and pCMV-HA (lane 2), pCMV-Myc and pCMV-HA-14-3-3ζ (lane 3) or pCMV-Myc-adam22cyt and pCMV-HA-14-3-3ζ (lane 4). Two days after transfection, HA-14-3-3ζ or HA tag was immunoprecipitated from the lysates using a polyclonal anti-HA antibody and protein A–Sepharose. Immunoblotting was performed with a monoclonal anti-Myc antibody. Lysates from HEK 293 cells transfected with pCMV-Myc-adam22cyt and pCMV-HA-14-3-3ζ (lane 1), pCMV-Myc-adam22cyt and pCMV-HA (lane 7), pCMV-HA-14-3-3ζ (lane 5) or pCMV-HA-14-3-3ζ plus pCMV-Myc (lane 6) were also analyzed directly as positive markers and to show that Myc-tagged protein expressed only in cells transfected with pCMV-Myc-adam22cyt.

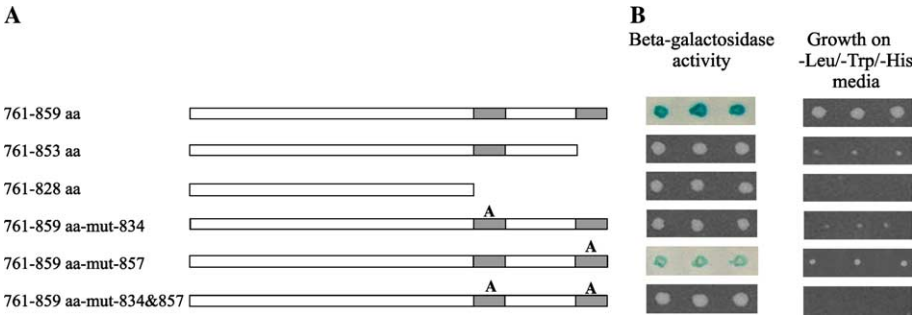

Fig. 3. 14-3-3ζ binds to specific motifs in the C-terminal of the cytoplasmic tail of ADAM 22. (A) Diagram of the constructs with the full-length, truncated or mutated cytoplasmic tail of adam22 in the pEG202 vector. (B) β-galactosidase activity and growth of yeast transformants. The yeast strain EGY48Z was cotransformed with the combination of plasmids pJG4.5-14-3-3ζ, and one of the six adam22cyt expression plasmids as indicated in Fig. 3A and selected on uracil-minus, histidine-minus, tryptophan-minus glucose yeast medium for the presence of all plasmids. Independent transformants (10 colonies from each sample) were re-spotted either onto uracil-minus, histidine-minus, tryptophan-minus galactose medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to evaluate the activation of the LacZ reporter or onto uracil-minus, histidine-minus, tryptophan-minus, leucine-minus galactose plates to evaluate the growth of the yeast transformants. Pictures were taken 72 h after the colonies were re-spotted. Protein interactions were indicated either by the intensity of the blue color or by the growth rate of the yeast transformants. Three colonies for each sample are shown.

Localization of the 14-3-3ζ binding site in ADAM22cyt

To map the 14-3-3ζ binding site in ADAM22cyt, a series of adam22cyt deletion or mutation constructs in the pEG202 vector were prepared (Fig. 3A) and co-transfected into the yeast strain EGY48Z with pJG4.5-14-3-3ζ. As shown in Fig. 3B, a substantial loss of growth in selection media and complete loss of β-galactosidase activity occurred when the six amino acid residues of the C-terminus of ADAM22cyt were deleted, indicating that the six amino acid residues of the C-terminus of ADAM22cyt were involved in 14-3-3ζ binding. When deletion extended to 31 amino acid residues of the C-terminus of ADAM22cyt, both the growth on selective media and the β-galactosidase activity were completely lost. These results suggested that besides the six amino acid residues of the C-terminus there might be another 14-3-3ζ binding site located between the amino acid residues 829 and 852. Examining the amino acid sequence of the C-terminus of ADAM22cyt, we found two RSxPSxP sites (Fig. 3A, indicated by the shade areas) similar to the consensus binding motifs of 14-3-3 proteins [38]. One of the sites consists of the six amino acid residues of the C-terminus mentioned above. Another one was located at amino acid residues 831 and 836. 14-3-3 proteins are adapter molecules that often bind to phosphorylated Ser motifs. To investigate whether the amino acid Ser^{857} within the putative 14-3-3 binding site located at the C-terminus tip of ADAM22cyt was critical for the binding of 14-3-3ζ, we mutated the Ser^{857} to alanine. As shown in Fig. 3B, mutation at Ser^{857} resulted in a substantial loss of the β-galactosidase activity and a decrease in the growth of yeast transformants on selective media, confirming that this putative 14-3-3 protein binding motif was indeed a 14-3-3ζ binding site and that the Ser^{857} residue is important for 14-3-3ζ binding. Next, using the same strategy, we examined the possibility that the second putative 14-3-3 protein binding motif upstream from the first one was also involved in 14-3-3ζ binding. Mutation of the Ser^{834} within the second putative 14-3-3 protein binding motif to alanine resulted in a complete loss of the β-galactosidase activity and a substantial decrease in the growth of yeast transformants on selective media. These results indicated that the second putative 14-3-3 protein binding motif was also a 14-3-3ζ binding site and that the amino acid residue Ser^{834} was critical for 14-3-3ζ binding. Double mutation at both Ser^{834} and Ser^{857} within the two 14-3-3ζ binding sites abolished the interaction between ADAM22cyt and 14-3-3ζ completely. The results of the mutation study were consistent with the results from deletion analysis.

To further confirm the above results, we performed in vitro protein pull-down assay using the same set of constructs and an in vivo immunoprecipitation assay using the same set of constructs prepared in pcDNA4.1 vector. As shown in Fig. 4A, only the wild type ADAM22cyt and the peptide ADAM22cyt-mut-857 were captured by the fusion protein GST-14-3-3ζ (top panel, compare lanes 1 and 5 with lanes 2, 3, 4, and 6), which was consistent with the results obtained from a yeast two-hybrid assay as shown in Fig. 3B. None of the six peptides examined was captured by GST (data not show). Lysates prepared from yeast were also analyzed directly by immunoblotting. It shows that all peptides were expressed in the yeast transfected with wild type or mutant ADAM22cyt expression plasmids (bottom panel). Similarly, as shown in Fig. 4B only the Myc-ADAM22cyt and ADAM22cyt-mut-857 were immunoprecipitated together with HA-14-3-3ζ (top panel, lanes 1 and 5). In addition, the full length of ADAM22 was also immunoprecipitated together with HA-14-3-3ζ (top

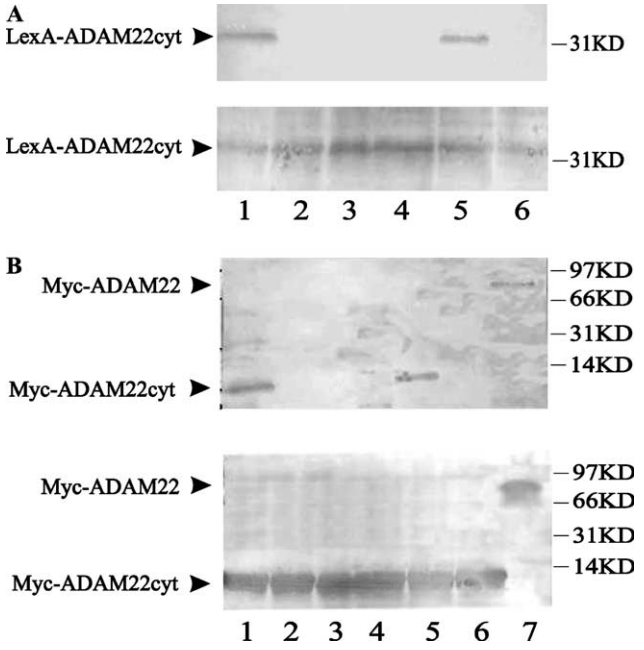

Fig. 4. Interaction between the different peptides of ADAM22cyt and 14-3-3ζ. (A, Top panel) In vitro protein pull-down assay. Immobilized GST-14-3-3ζ was incubated with the lysates prepared from yeast transfected with one of the six ADAM22cyt expression plasmids as indicated in Fig. 3A (respectively, lane 1, adam22cyt; lane 2, 761–853 aa; lane 3, 761–828 aa; lane 4, 761–859 aa-mut-834; lane 5, 761–859 aa-mut-857; and lane 6, 761–859 aa-mut-834 and 857). After washing, the bound proteins were analyzed by Western blot with a monoclonal anti-LexA antibody. (A, Bottom panel) Lysates prepared from yeast transfected were also analyzed directly by Western blot. (B, Top panel) In vivo co-immunoprecipitation assay. HEK293 cells were cotransfected with pCMV-Myc-adam22cyt, pCMV-Myc-adam22cyt-mutant (as indicated in Fig. 3A) or the full length of ADAM22 expression plasmids pCMV-Myc-adam22 together with pCMV-HA-14-3-3ζ (respectively, lane 1, adam22cyt; lane 2, 761–853 aa; lane 3, 761–828 aa; lane 4, 761–859 aa-mut-834; lane 5, 761–859 aa-mut-857; lane 6, 761–859 aa-mut-834 and 857; and lane 7, full length of adam 22). Two days after transfection, HA-14-3-3ζ was immunoprecipitated from the lysates using a polyclonal anti-HA antibody and protein A–Sepharose. Immunoblotting was performed with a monoclonal anti-Myc antibody. (B, Bottom panel) Lysates prepared from HEK 293 cells transfected were also analyzed directly by Western blot.

panel, lane 7). Lysates prepared from HEK 293 cells were also analyzed directly by immunoblotting. It shows that all peptides and the full length of ADAM22 were expressed in the cells transfected (bottom panel).

Overexpression of ADAM22cyt inhibits cell adhesion and spreading

To examine the potential cellular function of ADAM22cyt association with 14-3-3ζ, we performed cell adhesion and spreading assays using SHG44 cells stably transfected with pcDNA4.1, pcDNA4.1-adam22cyt, pcDNA4.1-myr-adam22cyt or pcDNA4.1-myr-adam22cytΔ. We chose SHG44 cells because ADAM22 is highly expressed in SHG44 cells (data not shown), which makes it suitable for inhibition study. We reasoned that the overexpressed ADAM22cyt would compete with the endogenous ADAM22cyt for 14-3-3ζ binding and in so doing disrupt the interaction between the endogenous ADAM22cyt and 14-3-3ζ. To optimize the competition between the overexpressed ADAM22-cyt and the endogenous ADAM22cyt, we added a myristoylation site to the N-terminus of the adam22cyt cDNA to target the overexpressed myr-ADAM22cyt to the plasma membrane. As shown in Fig. 5A, overexpression of the ADAM22cyt in human SHG44 cells inhibited cell adhesion (compare bars 1 and 2). Introduction of a myristoylation to the N-terminus of the overexpressed ADAM22cyt inhibited cell adhesion further (compare bars 1, 2, and 3). More significantly, the inhibition was lost when the 14-3-3ζ binding sites within the ADAM22cyt were deleted (bar 4). These results indicated that the association between the ADAM22cyt and 14-3-3ζ, was essential for the cell adhesion mediated by ADAM 22. Since cell adhesion is intrinsically linked with cell spreading, we further examined cell spreading on both fibronectin and poly-L-lysine coated plates. At 10 min after attachment, little cell spreading was observed for either pCDNA4.1/His-adam22cyt stably transfected cells or the mock cells (data not shown). At 30 min after attachment, a significantly higher fraction of mock cells was spread compared with the adam22cyt stably transfected cells. Similar to cell adhesion, overexpression of ADAM22cyt inhibited cell spreading on fibronectin-coated plates (Fig. 5B, compare bars 1 and 2; Fig. 5C, photographs 1 and 2). Introduction of a myristoylation to the N-terminus of the overexpressed ADAM22cyt inhibited cell spreading almost completely (Fig. 5B, compare bars 1 and 3; Fig. 5C, photographs 1 and 3). Again, the inhibition was lost when the binding site for 14-3-3ζ, was deleted (Fig. 5B, compare bars 1, 3, and 4; Fig. 5C, photographs 1, 3, and 4). When the cell spreading assays were performed on poly-L-lysine coated plates no significant difference was observed between these cells (data not shown).

Discussion

ADAM22/MDC2 was originally identified from a human brain RNA [39] and later again from a human genomic library [19]. At almost the same time, it was cloned in mouse together with ADAM 11 and ADAM 23 [18]. The importance of ADAM 22 in biological processes was demonstrated in a gene knockout experiment [18]. Adam22 knockout mice presented severe ataxia and died before weaning while no detectable abnormalities were observed in the adam11 knockout mice although these two proteins share a high degree of homology in their external domains [18,39]. The mRNA distributions of ADAM 11 and ADAM 22 are nearly identical, with transcripts most abundant in the

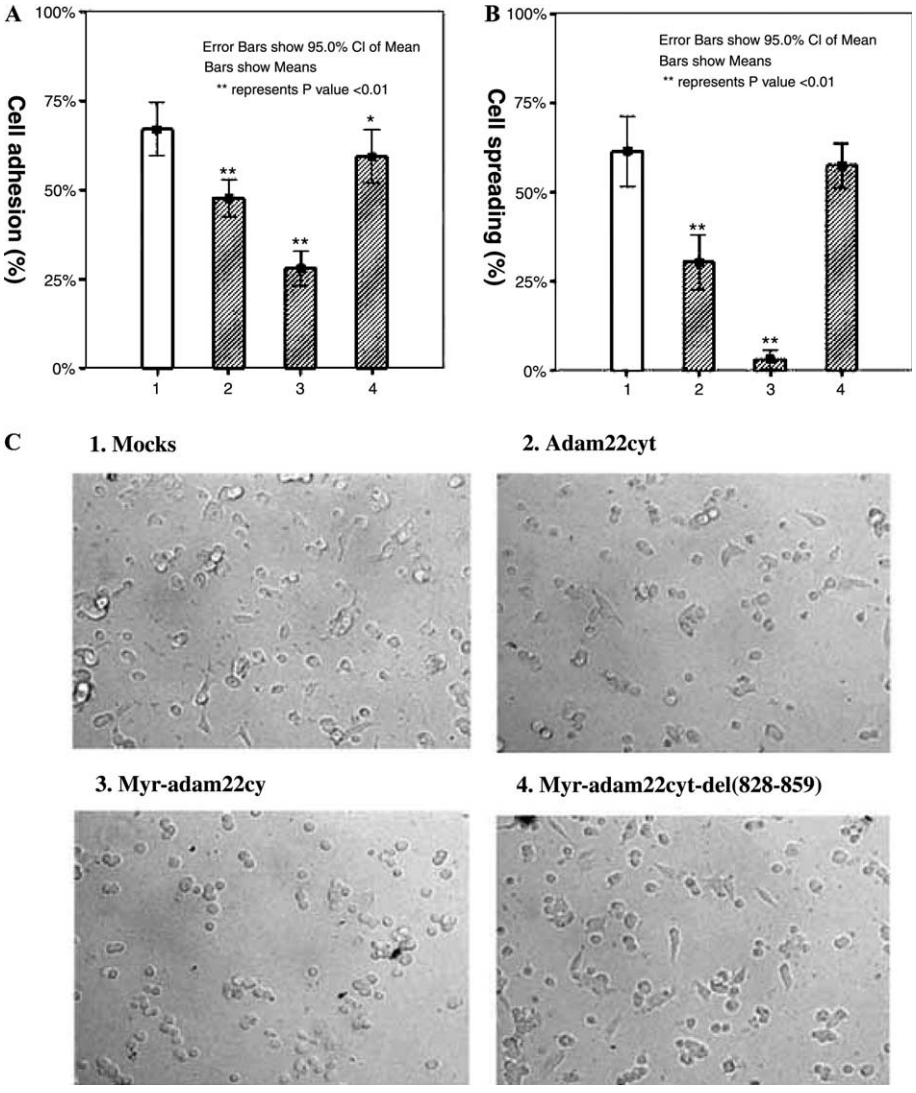

Fig. 5. Effects of overexpression of the cytoplasmic tail of ADAM 22 on cell adhesion and cell spreading. **(A)** SHG44 cells were transfected with the empty vector pcDNA4.1/His (bar 1), pcDNA4.1/His-adam22cyt (bar 2), pcDNA4.1/His-myr-adam22cyt (bar 3) or pcDNA4.1/His-myr-adam22-cytΔ (bar 4) and Zeocin-resistant clones were selected in DMEM with 10% serum containing 400 μg/ml Zeocin. The stably transfected cells were then subjected to cell adhesion assays as described in Experimental procedures. (B) The same set of cells was subjected to cell spreading assays as described in Experimental procedures. Average and standard deviations were derived from three independent experiments. (C) The morphology of cells 30 min after plating in a fibronectin coated plate. The majority of mock cells had an irregular and characteristic spread morphology (C1). ADAM22cyt, especially Myr-ADAM22cyt, reduced the number of cells with the irregular and characteristic spread morphology (compare C1 to C2 and C3). More than 90% of the Myr-ADAM22cyt transduced cells had a rounded morphology (C3). The number of cells with irregular and characteristic spread morphology increased when the 14-3-3ζ binding motif within the peptide of Myr-ADAM22cyt was deleted (compare the C3 to C4).

cerebellum. The major structural difference between the two proteins is seen in their cytoplasmic domains. ADAM 11 has a short intracellular domain of 12 residues. In contrast, the ADAM 22 contains a long intracellular domain of 99 residues [18,39]. The different fate of the adam22 knockout and the adam11 knockout mice implies the importance of the ADAM22cyt and the cytoplasmic proteins that interact with the ADAM22cyt in signal transduction related to cell growth and development. In the present study, we have identified a novel interaction between ADAM22cyt and 14-3-3ζ. This interaction was identified using yeast two-hybrid screening and was subsequently confirmed by an in vitro binding assay and in vivo co-immunoprecipitation. Through deletion and mutation analysis, we have identified two major 14-3-3ζ binding sites with the 28 amino acid residues of the C-terminus of ADAM22cyt, one of which is located at the C-terminus tip of ADAM22cyt and the second one is located at the amino acid residues 831–836. In addition, mutations to alanine of the Ser^{834} and the Ser^{857} amino acid residues within the two 14-3-3ζ binding sites abolished the ADAM22cyt association with 14-3-3ζ completely, indicating that the two Ser amino acid residues are critical for 14-3-3ζ binding.

Furthermore, we have demonstrated that overexpression of ADAM22cyt in human SHG44 cells inhibited cell-adhesion and spreading on fibronectin coated plates. It is conceivable that the overexpressed ADAM22cyt may act as a decoy (or competing molecule) and "deceive" the cellular protein that normally interacts with the endogenous ADAM22cyt. The mechanism of inhibition appears to be mediated through the disruption of the association between the endogenous 14-3-3ζ and ADAM22cyt and the normal signal transduction by the introduced ADAM22cyt. Consistent with this hypothesis, directing the overexpressed ADAM22cyt to the plasma membrane with myristoylation greatly enhanced the inhibitory effect of the overexpressed ADAM22cyt and deletion or mutation of the binding sites for 14-3-3ζ within ADAM22cyt abolished the inhibitory effect completely. Although the exact cellular function of the 14-3-3ζ association with ADAM 22 is not completely clear at present time, the inhibition of cell adhesion and spreading by overexpressed ADAM22cyt and the reversion of the ADAM 22 enhanced cell adhesion and spreading by deleting the 14-3-3ζ binding site suggest that the association of 14-3-3ζ with ADAM22cyt is critical for cell adhesion mediated by ADAM 22, and therefore implies a potential role of 14-3-3ζ protein in the regulation of cell adhesion associated with ADAM 22.

It is noteworthy that the cytoplasmic domains of both integrin α and β subunits have been recently implicated in the regulation of integrin affinity [40,41]. In addition, a recent study demonstrated that the cytoplasmic tail of integrin β1 interacted with 14-3-3β, suggesting that 14-3-3β regulated integrin-mediated cell adhesion and spreading [37]. Here we have demonstrated that a member of the 14-3-3 protein family interacts with ADAM22cyt and regulates the cell adhesion and spreading mediated by ADAM 22 on the integrin-containing substrate fibronectin but not on the non-integrin substrate poly-L-lysine (data not shown). Taken together, these results suggest that the 14-3-3 protein family plays an important role in the regulation of integrin/disintegrin mediated cell adhesion. Although the detailed mechanism of ADAM 22/14-3-3ζ mediated cell adhesion is currently unclear, it is possible that the disintegrin of ADAM 22 is also a ligand for αvβ3 integrin because of the high degree of structural similarity between the disintegrin domains of ADAM 22 and ADAM 23, both of which have a conserved AVN(E/D)CD integrin binding motif instead of RDG in their disintegrin domains [39,42]. The hypothesis that the binding of 14-3-3ζ to ADAM22cyt enhances cell adhesion by increasing the affinity of ADAM 22 disintegrin to its receptor, presumably an integrin, is currently under examination in our laboratory.

In summary, we have identified a novel interaction between ADAM 22 cytoplasmic domain and 14-3-3ζ and also provided evidence for a potential role of 14-3-3ζ in the regulation of ADAM 22 mediated cell adhesion. Although the biological significance of the ADAM 22 association with 14-3-3ζ is not clear at present time it warrants extensive further investigation considering the important role of ADAM 22 in cell growth and brain development [18,39] and the involvement of 14-3-3 proteins in many diverse biological processes including cell growth and brain development [20,22,23,43–45].

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