Cloning and Characterization of Cbl-associated Protein Splicing Isoforms

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Cbl-associated protein (CAP) is an adaptor protein that plays important roles in both signal transduction and cytoskeleton rearrangement. Alternative splicing of the gene *SORBS1* results in multiple isoforms of CAP protein. We report here the cloning of 3 new CAP isoforms, CAP2, CAP3, and CAP4, from mouse adipose tissue. RT-PCR analyses reveal that the isoform mRNAs are differentially expressed. CAP2, CAP3, and CAP4 contain a coiled-coil domain. In addition, CAP4 contains a proline-rich region, part of which exists in CAP3. Coimmunoprecipitation experiments show that CAP4 forms a homodimeric complex. While these new isoforms similarly interact with Cbl, they exhibit varied binding specificity toward vinculin. In contrast to CAP1 and CAP2, CAP4 does not interact with vinculin, and CAP3 binds with low affinity. Immunofluorescence analysis demonstrates differential subcellular localization of Myc-tagged CAP isoforms in 3T3-L1 adipocytes. These results suggest that these new isoforms of CAP might play different signaling roles.

INTRODUCTION

Cbl-associated protein (CAP) is a widely expressed, multifunctional adaptor protein involved in intracellular signaling (1,2). CAP belongs to the sorbin homology (SoHo) family of proteins, including vinexin and ArgBP2, which contain 1 or 2 SoHo domains in their amino terminus and 3 adjacent SH3 domains in their carboxyl terminus (3). Accumulating evidence suggests that the proteins in this family are involved in the organization of both signal transduction and the cytoskeleton. CAP has been shown to play a critical role in insulin signaling (1). Cbl constitutively associates with CAP, and upon insulin stimulation, both are recruited to the insulin receptor by the adapter protein APS (4). Cbl is then phosphorylated, and the Cbl-CAP complex accumulates in lipid raft subdomains in the plasma membrane through the interaction of CAP with the raft-associated protein, flotillin (3). Phosphorylated Cbl recruits CrkII to the lipid raft, which initiates downstream signaling (5).

The CAP gene is highly expressed in insulin-sensitive tissues and is markedly induced during adipocyte differentiation (6). CAP transcription is increased by insulin-sensitizing thiazolidinedione drugs, by means of activation of the nuclear receptor, PPARγ, through a PPAR response element in its promoter (7). CAP interacts directly with Cbl through its C-terminal SH3 domain. Overexpression of dominant-negative CAP mutants with deletion of the SH3 or SoHo domains interfere with the localization of Cbl to lipid rafts and specifically block insulin-stimulated Glut4 translocation and glucose uptake (1). In addition to its role in insulin action in adipocyte, CAP also has been implicated in ephrin reverse signaling in neuronal cells (8,9).

The SoHo family of proteins also regulates cell adhesion and cytoskeletal organization. Previous studies have shown that CAP and ArgBP2 are associated with actin stress fibers (2,10), whereas CAP, vinexin, and ArgBP2 bind to vinculin through their 2 N-terminal SH3 domains (11-13). Vinculin is an actin-binding cytoskeletal protein, localized at cell-extracellular matrix and cell-cell adhesion sites. Exogenous expression of vinexin and CAP in fibroblasts stimulates formation of actin stress fibers and focal adhesions (2,12). This effect was observed with vinculin overexpression (14), suggesting that vinculin and the SoHo family proteins may work cooperatively to regulate actin cytoskeleton rearrangement.

CAP is the product of the sorbin and SH3 domain containing 1 gene (formally designated as *SORBS1*). The cDNA was first cloned by screening a mouse cDNA expression library with an SH3 binding ligand and named SH3P12 (15). It was subsequently identified in a yeast 2-hybrid screen of a 3T3-L1 adipocyte cDNA library using Cbl as bait (6), therefore designated as CAP for Cbl-associated protein. Previous studies on CAP suggested the existence of various isoforms or alternatively spliced variants of this gene. At least 13 isoforms have been identified as alternative splicing products of the human *SORBS1* gene (16). Northern blot analyses on multiple mouse tissues identified several CAP mRNA transcripts of different sizes. An anti-CAP antibody, which recognizes the C-terminal portion of CAP, was able to detect multiple proteins in Western Blot analyses, suggesting multiple splicing products of the mouse gene as well (6). Two splicing variants have been identified by a blot overlay method on subcellular fractions of rat liver with labeled 1-afadin and denoted as ponsin for interaction with 1-afadin and vinculin (11). Since CAP is an important adaptor protein in multiple signaling pathways, these splicing variants are potentially interesting in the effort to further delineate these pathways. We report here the cloning of 3 new splicing isoforms of CAP from mouse adipose tissue and the characterization of their protein interaction domains and subcellular localization in adipocytes.

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MATERIALS AND METHODS

Antibodies

The antibodies to hemagglutinin (HA) (F-7), Myc (9E10), c-Cbl (C-15) were purchased from Santa Cruz Inc. The FLAG (M2) monoclonal antibody was obtained from Stratagene. The antivinculin monoclonal antibody was from Upstate Biotechnology Inc. Horseradish peroxidase–linked secondary antibodies were from Pierce Chemical Co. The Alexa Fluor secondary antibody was from Molecular Probes.

RT-PCR

Total cellular RNA was purified from 3T3-L1 fibroblasts, adipocytes, and various mouse tissues using the TRIzol reagent (Invitrogen). The synthesis of the first-strand cDNA was performed using SuperScript system from Invitrogen. For the full-length cloning of CAP variants, the white adipose tissue cDNA was amplified with PCR using the Advantage2 DNA polymerase (BD Biosciences), with the primer pair: 5′-ATCGGATTCATGAGTTCT GAATGTGATGTTGGAAGC-3′ (forward) and 5′-ATCGCGGCCG CTTATAGATGTAAAGGTTTTACATAG-3′ (reverse). The PCR product was separated by agarose gel electrophoresis, subcloned into the TA vector (Stratagene), and the full sequences were obtained by automated DNA sequencing.

For the multitissue RT-PCR analysis of CAP variants, cDNA from fibroblasts, adipocytes, and various mouse tissues were amplified using Taq DNA polymerase (Invitrogen) with different primer sets as follows: 5′-TGGGCTCAAGCGACTTTC-3′ (forward) and 5′-CAGTTCTGGTCAATCTGTCTGTAG-3′ (reverse); 5′-CCGTCTGAGGTAATAGTTGTTCC-3′ (forward) and 5′-GAG CACAATGGTAGGGTTGACG-3′ (reverse).

Expression Constructs

Triple-HA-tagged or Myc-tagged CAPs were constructed by subcloning CAP cDNAs in frame in the *Bam*HI site of pKH3 or pKMyc vectors. Flag-tagged CAP constructs were made by subcloning CAP cDNAs in frame in the *Bam*HI site of pcDNA-F2 vector. Flag-CAP1ΔSH3 was made as described previously (6).

Cell Culture and Transfection

COS-1 cells were grown in Dulbecco's Modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum. 3T3-L1 fibroblasts were maintained in DMEM supplemented with 10% calf serum, 100 U of penicillin G sodium per milliliter, and 100 μg of streptomycin sulfate per milliliter. Differentiation to adipocytes was induced as previously described (17). The cells then were cultured in DMEM containing 10% fetal bovine serum. 3T3-L1 adipocytes were transfected by electroporation as described previously (1,18). COS-1 cells in 60-mm-dia dishes were transfected by using FuGene 6 reagent (Roche Diagnostics).

Immunoprecipitation and Immunoblotting

Cells in 60-mm-dia dishes were washed twice with ice-cold phosphate-buffered saline and were lysed for 30 min at 4 °C with buffer containing 50 mM Tris HCl (pH 8.0), 135 mM NaCl, 1% Triton X-100, 1.0 mM EDTA, 1.0 mM sodium pyrophosphate, 1.0 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors (1 tablet per 10 mL buffer) (Roche Diagnostics). The clarified lysates were incubated with the indicated antibodies for 1 h at 4 °C. The immune complexes were precipitated with protein A/G agarose (Santa Cruz Inc) for 1 h at 4 °C and were washed extensively with lysis buffer before solubilization in sample buffer. For antiFLAG immunoprecipitation, FLAG antibody conjugated on agarose beads (Sigma) was incubated with lysates for 2 h at 4 °C. Bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Individual proteins were detected with the specific antibodies and visualized by blotting with horseradish peroxidase–conjugated secondary antibodies.

Fluorescence Microscopy

Electroporated 3T3-L1 adipocytes were grown on glass cover slips in 6-well dishes. Cells were fixed with 10% formalin for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with 3% bovine serum albumin and 1% ovalbumin for 1 h. Primary and Alexa Fluor secondary antibodies were used at 2 μg/μL in blocking solution, and samples were mounted on glass slides with Vectashield (Vector Laboratories). Cells were imaged using confocal fluorescence microscopy. Images were then imported into Adobe Photoshop (Adobe Systems Inc) for processing.

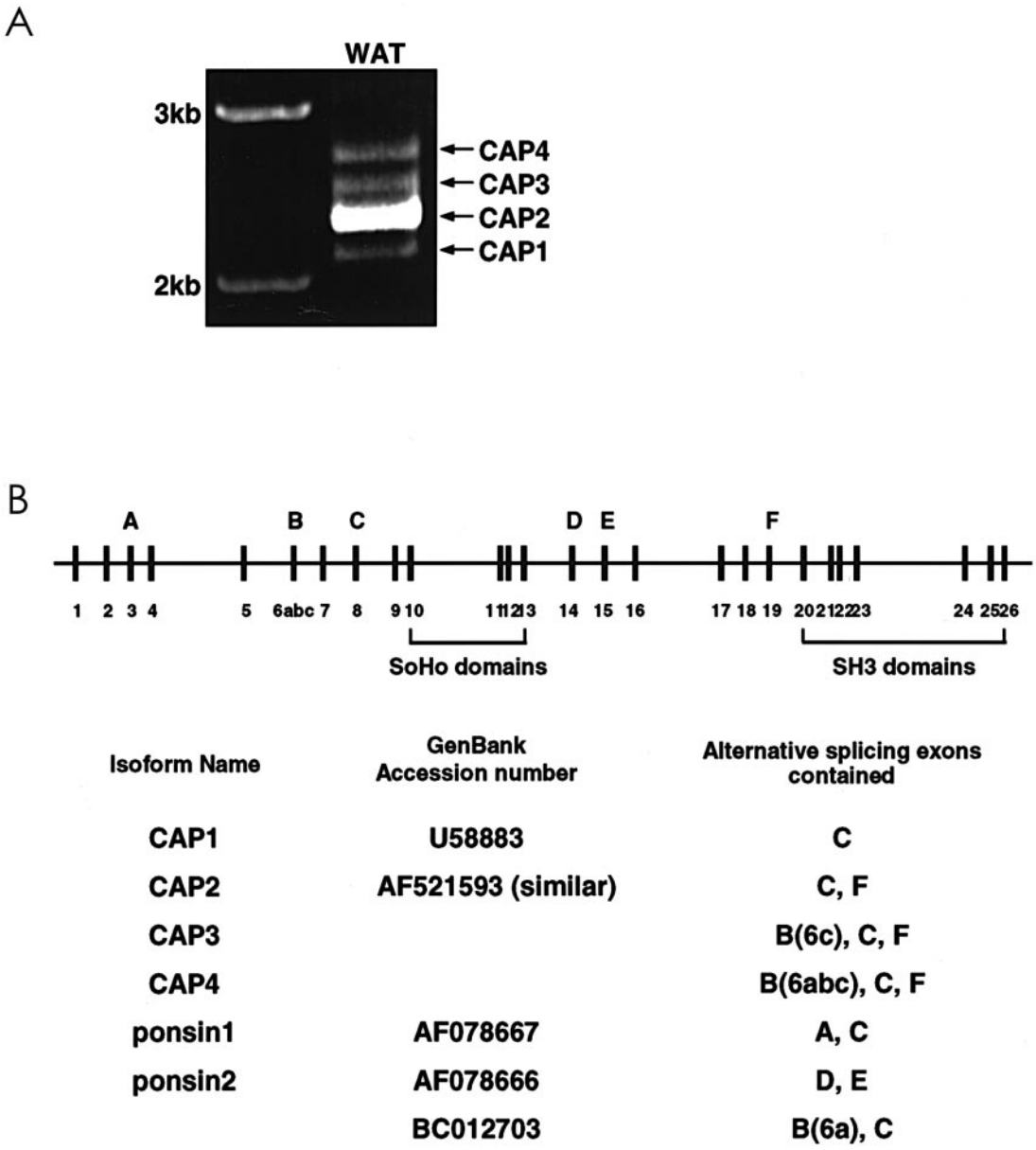
RESULTS

Cloning of CAP Splicing Isoforms

Total RNA was extracted from mouse white adipose tissue (WAT), and RT-PCR was performed to amplify the full-length CAP variants, using a primer set flanking the coding region of the originally cloned CAP, which is designated CAP1. Four major products were cloned and sequenced (Figure 1A). The shortest one was identical to CAP1, with the exception of 2 nucleotide substitutions, which resulted in amino acid changes of $T^{206}A$ and $P^{309}L$. This might represent a polymorphism between mouse tissue and the 3T3-L1 adipocytes from which CAP1 was originally cloned. The other 3 were named CAP2, CAP3, and CAP4. Analysis in GenBank indicated these 3 cDNAs were novel, although there is 1 cDNA sequence (AF521593) similar to the CAP2 cDNA (with 3 nucleotides different). These isoforms appear to be alternatively spliced products of the same gene, *SORBS1*, with identical amino acid compositions except for the insertion regions.

To further delineate the genomic organization of the mouse *SORBS1* gene, CAP1 through CAP4 cDNAs, together with several other cDNAs obtained from GenBank that appear to be products of this same gene, were used to blast against the NCBI mouse genomic sequence databank. The mouse *SORBS1* gene has been mapped to chromosome 19 36.5 cM. Based on the search and alignment, the coding region of this gene is encoded by 26 known exons spanning a region of 110 kb (see Figure 1B). CAP1 through CAP4, ponsin1 and ponsin2, and another GenBank sequence (BC012703) are likely to be generated by alternative splicing of 6 exons (A to F) and 2 additional donor sites in exon 6abc. Comparison to the human *SORBS1* gene suggests the potential existence of up to 7 more alternatively spliced exons (16).

The amino acid sequences of CAP1 through CAP4 are shown in Figure 2. Sequence analysis revealed distinct domain structures in the newly cloned isoforms. All 4 isoforms contain 2 N-terminal



Isoform Name	GenBank Accession number	Alternative splicing exons contained
CAP1	U58883	C
CAP2	AF521593 (similar)	C, F
CAP3		B(6c), C, F
CAP4		B(6abc), C, F
ponsin1	AF078667	A, C
ponsin2	AF078666	D, E
	BC012703	B(6a), C

Figure 1. The splicing variants of the mouse *SORBS1* gene. A: RT-PCR amplification of full-length CAP from total RNA extracted from mouse WAT. B: Genomic organization of mouse *SORBS1* gene and the exon composition of cloned CAP isoforms. Exons are depicted as black lines and numbered underneath. The alternative splicing exons are indicated as A to F. For all cloned isoforms, their names, GenBank accession numbers, and the alternative splicing exons that they contain are listed.

SoHo domains and 3 C-terminal SH3 domains. In addition, CAP2 through CAP4 contain an extra coiled-coil domain before the N-terminal SH3 domain, identified using the COILS algorithm (19). The InterPro analysis revealed an additional proline-rich region N-terminal to the SoHo domain in CAP4, part of the proline-rich region that exists in CAP3. Coiled-coil domains are superhelix structures that can interact with each other, thus mediating protein interactions (20). A proline-rich region may serve as a binding motif for SH3 or WW domains (21). Therefore, these additional domains in the newly identified CAP isoforms may provide important binding sites to recruit other signaling molecules, and/or modulate protein complex formation in CAP-mediated signaling pathways.

Tissue Distribution of CAP Isoforms

As shown in Figure 1A, CAP2 represents the major isoform in mouse WAT. To determine the expression pattern of the newly cloned CAP isoforms, RT-PCR analyses were performed on RNA isolated from 3T3-L1 fibroblasts, fully differentiated adipocytes, and various mouse tissues. RT-PCR analysis with primers flanking the coiled-coil domain revealed that although they are detectable in every tissue, the coiled-coil domain-containing isoforms are present predominantly in the heart and WAT, and highly induced in 3T3-L1 adipocytes compared with fibroblasts (Figure 3A). CAP1 also is detectable in most tissues, with high-level expression in the kidney and WAT, and induced in the adipocytes also. With primers flanking the proline-rich region of

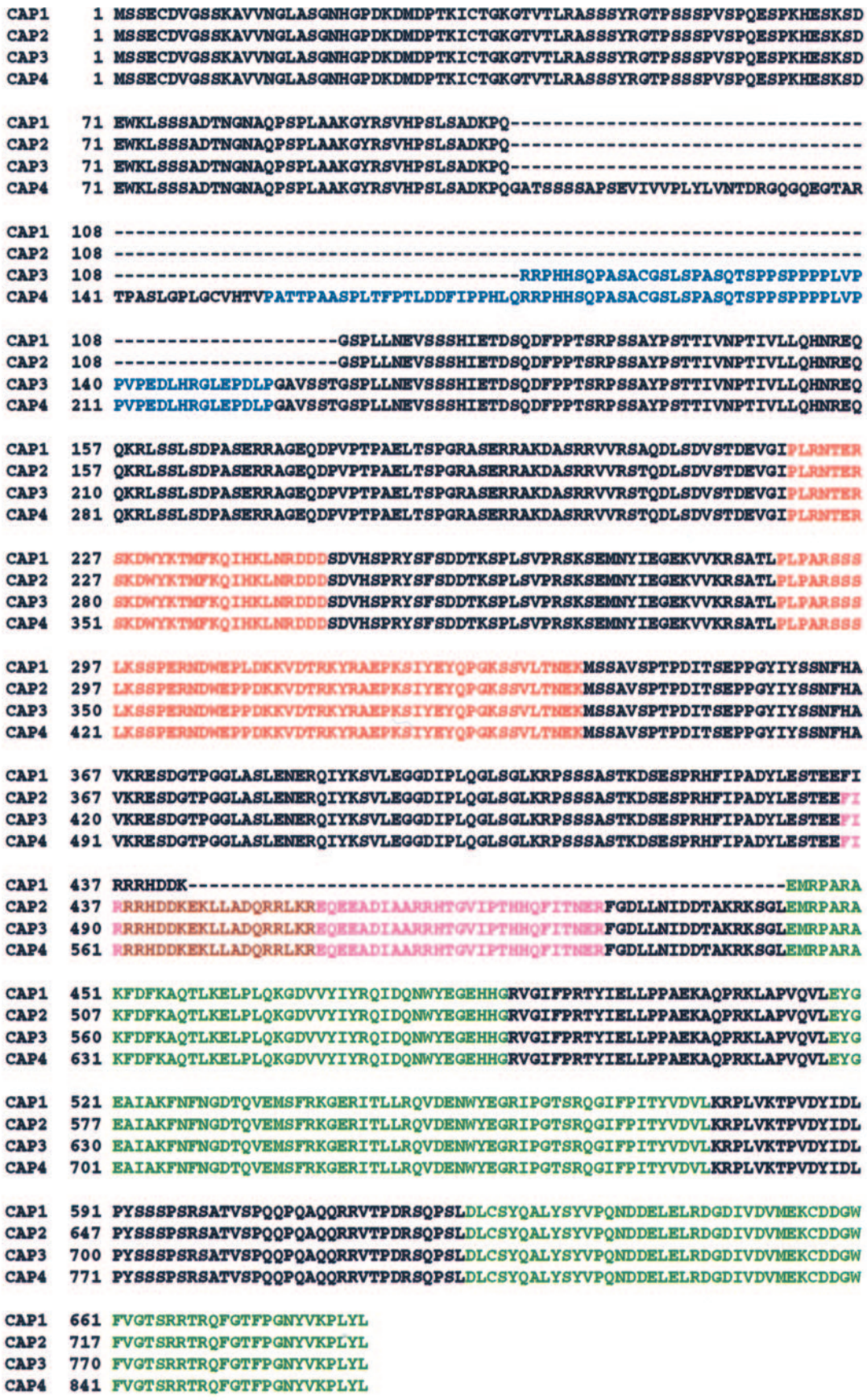


Figure 2. Amino acid sequences of CAP isoforms and domain composition. The deduced amino acid sequences of CAP1 through CAP4 were aligned by ClustalW and Boxshade. The proline-rich region is indicated in blue; the 2 SoHo domains are in red; the coiled-coil domain is in pink; the bipartite nuclear localization signal is in brown; and the 3 SH3 domains are in green.

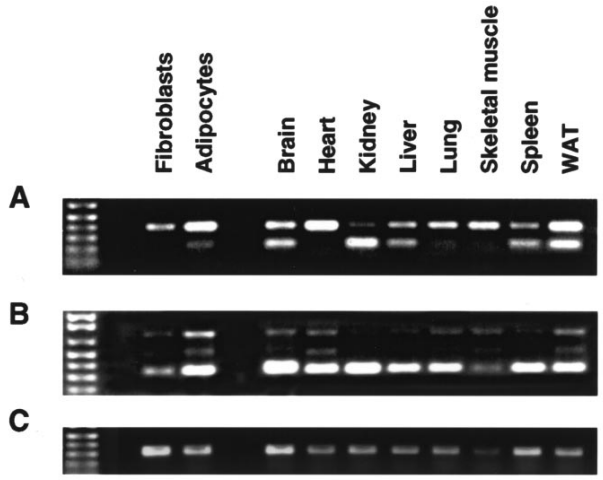


Figure 3. Tissue distribution of the new CAP isoform mRNAs. A: RT-PCR analysis using primers flanking the coiled-coil domain. Top band, isoforms contain the coiled-coil domain; bottom band, isoforms do not have the coiled-coil domain. B: RT-PCR analysis using primers flanking the proline-rich region (PRR). Top band, isoform(s) contain the PRR (CAP4); middle band, isoform(s) contain a partial PRR (CAP3); bottom band, isoforms do not contain the PRR. C: RT-PCR amplification of Rac1 as control.

CAP4, CAP3, and CAP4 were found in most of the tissues examined, with relatively high levels in heart and WAT, and were also induced in differentiated 3T3-L1 adipocytes (see Figure 3B). These data suggest that the CAP isoforms are expressed in a tissue-specific manner with potential distinct physiological functions. The fact that all CAP isoforms are significantly induced in 3T3-L1 adipocytes compared with fibroblasts indicates a restricted signaling role in these cells.

Dimerization between CAP Isoforms

To study the role of the newly discovered domains in mediating protein interactions, we first examined the interaction among CAP isoforms. To test the hypothesis that CAP can form homo- and heterodimers through the interaction between 2 coiled-coiled domains or between the proline-rich region and the SH3 domains, Myc-tagged and HA-tagged CAPs were cotransfected into 3T3-L1 adipocytes by electroporation, and coimmunoprecipitation was performed to evaluate dimerization between any 2 isoforms. As shown in Figure 4A, CAP4 forms a homodimeric complex, and it also forms heterodimers with CAP1 at a much lower affinity. CAP1 does not show self-interaction, nor does it interact with CAP2. Similar experiments were done to explore the other possibilities of dimerization. The only strong interaction detected was the self-interaction of CAP4 (see Figure 4B). These data imply that intermolecular interactions between coiled-coil domains, the SH3 and proline-rich regions, or both are not sufficient to induced dimerization. Furthermore, the homodimerization of CAP4 might result from a conformational change caused by the formation of an intramolecular SH3-proline complex. This in turn, results in the exposure of the coiled-coil domain in 2 molecules that can subsequently interact with each other. It seems that the short proline-rich sequence in CAP3 is not enough to induce such a structural change.

CAP Interaction with Cbl and Vinculin

The presence of protein interaction domains, as well as the dimerization of the new CAP isoforms, suggests the possibility of altered ligand binding specificity of CAP proteins. CAP1 has been shown to bind to Cbl through its C-terminal SH3 domain. This interaction is critical to the CAP/Cbl pathway in insulin-stimulated glucose transport, since the mutant with SH3 domains deleted (CAP1ΔSH3) does not bind to Cbl and behaves as an dominant negative inhibitor (1,6). To see whether the CAP isoforms interact with Cbl differently, FLAG-tagged CAPs were transfected into COS cells. Immunoprecipitation was performed with an anti-FLAG antibody, and followed by anti-Cbl immunoblotting. As shown in Figure 5, endogenous Cbl was coprecipitated with all

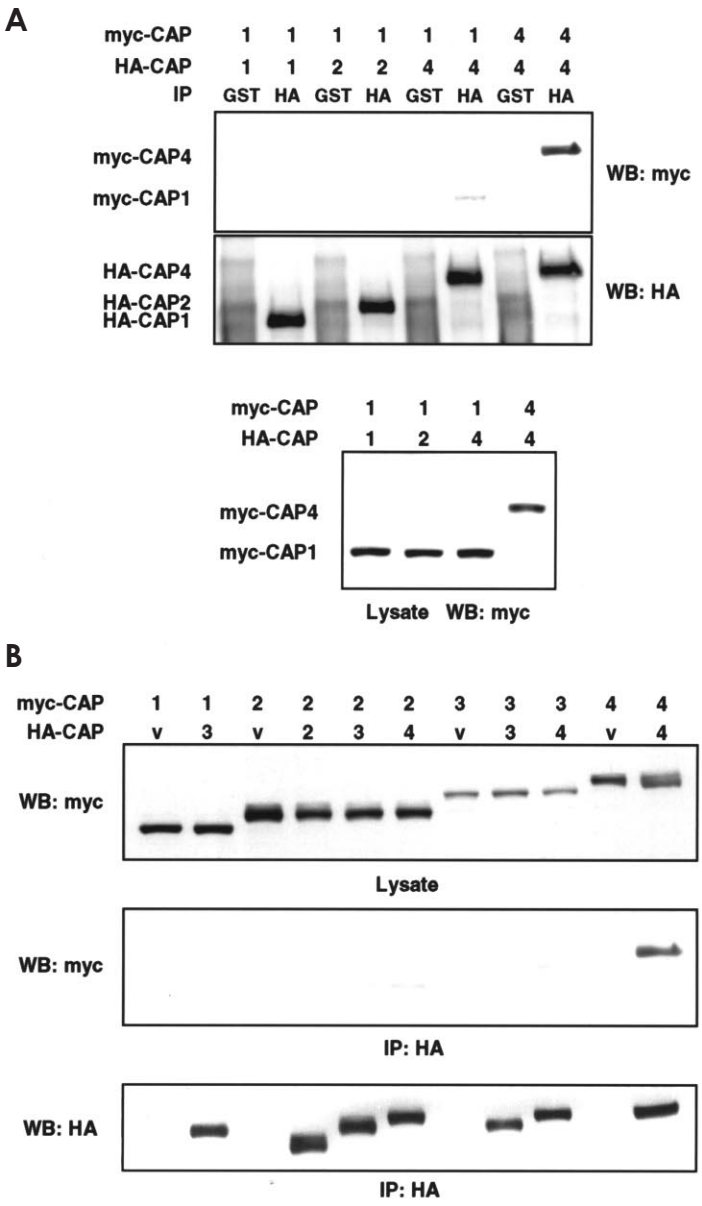


Figure 4. CAP4 forms a homodimeric complex. Myc-tagged CAP isoforms were cotransfected into 3T3-L1 adipocytes with HA-CAP isoforms (A and B) or HA vector (B). Cell lysates were immunoprecipitated with anti-HA antibody (A and B) or anti-glutathione S-transferase antibody as control (A) and blotted with anti-Myc or anti-HA antibody. Immunoblots also were performed on whole cell lysates with anti-Myc antibody.

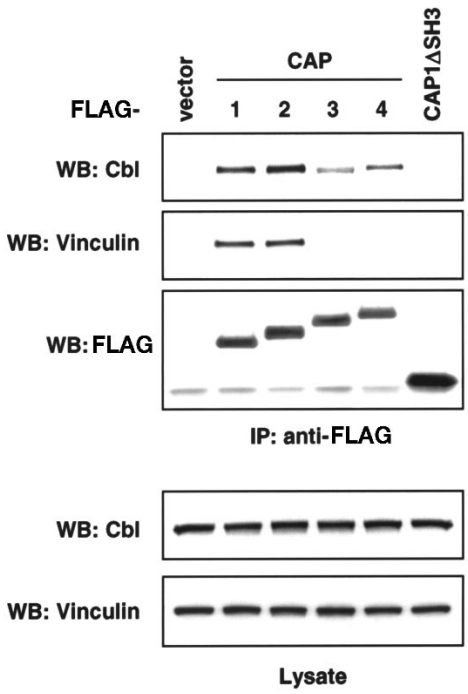


Figure 5. Interaction of CAP isoforms with Cbl and vinculin. Flag-tagged CAPs were transfected into COS cells and immunoprecipitated with antiFLAG antibody. The coprecipitated endogenous Cbl or vinculin was detected with anti-Cbl or antivinculin antibody. The total Cbl or vinculin in whole cell lysates is shown at the bottom.

4 isoforms, and CAP3 seems to interact with a modified form of Cbl with a slightly lower molecular weight. As expected, CAP1ΔSH3 did not bind to Cbl.

CAP has been suggested to play a role in cytoskeleton rearrangement, especially the formation of focal adhesions, which may involve its binding with vinculin. This interaction is mediated through its 2 N-terminal SH3 domains (2,11). We examined the ability of CAP2, CAP3, and CAP4 to bind to vinculin in the same coimmunoprecipitation experiment, using an antivinculin antibody. Endogenous vinculin was coprecipitated with CAP1 and CAP2, but only bound with low affinity to CAP3 and not at all with CAP4 (see Figure 5). Taken together, these data indicate that the SH3 domains of CAP3 and CAP4 have altered protein-binding properties, possibly through conformational changes caused by the additional proline-rich region in these isoforms.

Subcellular Localization of CAPs in 3T3-L1 Adipocytes

Since CAP1 plays a critical role in the spatial compartmentalization of insulin signaling, we decided to examine the subcellular localization of all 4 isoforms in 3T3-L1 adipocytes. Myc-tagged CAP isoforms were overexpressed in fully differentiated 3T3-L1 adipocytes by electroporation, followed by immunofluorescence staining and microscopy. As shown in Figure 6, and consistent with previous studies (4), CAP1 was localized predominantly at the plasma membrane, with some found in the cytoplasm. Interestingly, CAP2 showed nuclear localization in more than half of the cells transfected, whereas CAP4 showed patchy membrane and cytoplasmic localization with vesicular staining of unidentified structures. The staining of CAP3 was less homogenous; membrane, nuclear and/or patchy and vesicular cytoplasm staining were all observed. These results suggest that CAP isoforms might localize at different cellular compartments, which may serve differential functions in signaling.

DISCUSSION

CAP is an adaptor protein that plays important roles in several signaling pathways, including insulin-stimulated glucose transport. Alternative splicing of the same gene, *SORBS1*, results in multiple isoforms of CAP protein. We have cloned 3 new splicing variants of mouse CAP, CAP2, CAP3, and CAP4. All 3 isoforms contain a coiled-coil domain that is not present in CAP1. CAP3 and CAP4 have an additional proline-rich region with different length in these 2 isoforms. Coiled-coil domains are composed of amphipathic α-helices that exhibit a heptad repeat pattern and align to form a hydrophobic interface between the supercoiled strands. Coiled-coils are found in many proteins and are often involved in mediating protein dimerization (19). A proline-rich region may represent SH3 or WW domain-binding motifs, which are often found in signaling proteins (21). The residues surrounding the PxxP core contribute to the optimal ligand preference, and thus help to determine the specificity of protein–protein interaction (22). The presence of these additional domains in the CAP isoforms implies novel or altered interactions.

CAP4 was found to form homodimers. The fact that CAP2 shows no self-interaction indicates that the coiled-coil domain alone is not sufficient. The interactions between CAP4 and other CAPs, all of which contain the SH3 domains, are weak, indicating that the potential proline-rich region in CAP4 also is not sufficient for dimerization. Moreover, no dimer formation was detected between CAP4 and CAP2, demonstrating that dimerization cannot be induced through intermolecular interactions between these isoforms. Thus, it is likely that the proline-rich region in CAP4 interacts with the SH3 domain(s) within the same molecule, which induces a conformational change of the protein, thus exposing the coiled-coil domain to form dimers. Another possibility is that CAP4 interacts with another protein that brings 2 molecules of CAP4 together. CAP3 contains the same PxxP cores as CAP4, but misses a segment of the sequence before the core region. It does not form a dimer with any other CAP isoforms, indicating that the region surrounding the PxxP cores is important in dimerization.

Previous studies showed that the 2 splicing isoforms of CAP, ponsin1 and ponsin2, bind to and colocalize with vinculin. This interaction is mediated through the binding of the first 2 SH3 domains of ponsin to the proline-rich hinge region of vinculin. We examined the interaction between vinculin and the newly cloned CAP isoforms. CAP2 binds to vinculin in a manner similar to CAP1, whereas CAP3 showed a low-level interaction. No interaction was detected between CAP4 and vinculin. Interestingly, CAP3 also exhibits a lower affinity for Cbl, although CAP4 binds in a manner similar to what is observed for CAP1 and CAP2. These data suggest that the additional proline-rich sequences in CAP3 and CAP4 may differentially restrict the availability of the first 2 SH3 domains, through induction of protein conformation changes and/or interactions with other proteins, thus modulating vinculin or Cbl binding to CAP.

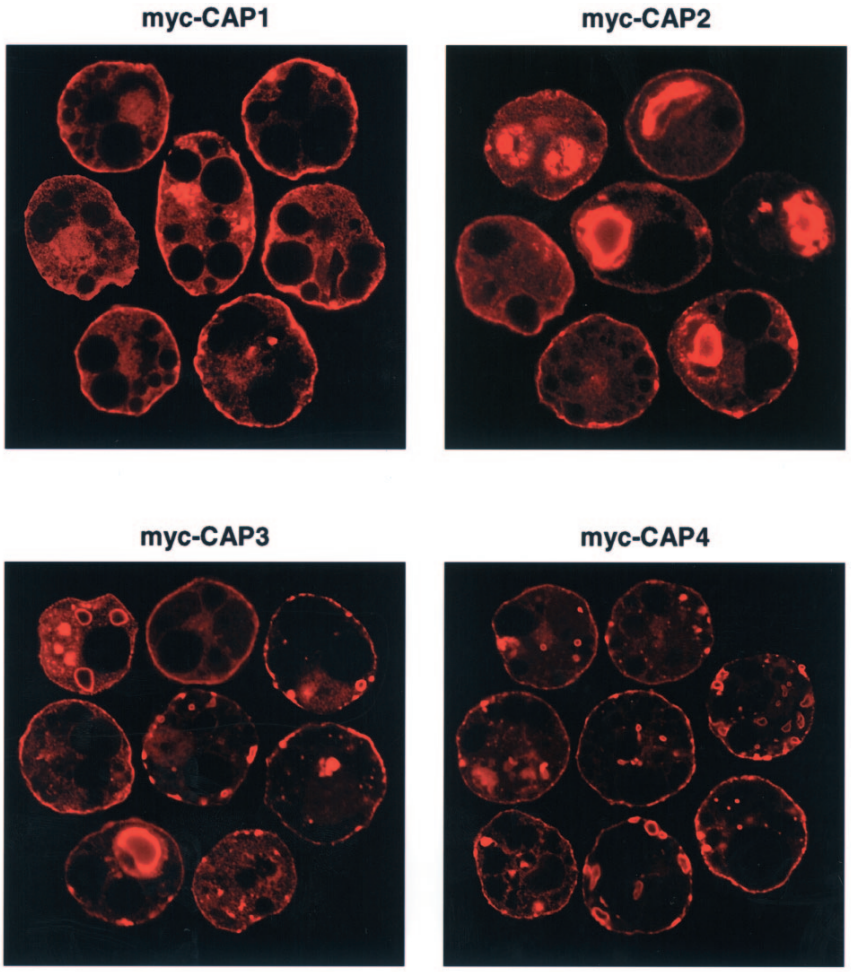


Figure 6. Subcellular localization of CAP isoforms in 3T3-L1 adipocytes. 3T3-L1 adipocytes were electroporated with 100 μg of Myc-tagged CAP isoforms. Cells were plated on cover slips and allowed to recover for 30 h. Myc-CAPs were visualized by indirect immunofluorescent staining.

The subcellular localization of adaptor proteins is important in the compartmentalization of signaling pathways, through the recruitment of proteins to specified subcellular domains. CAP1 plays such a role in insulin-stimulated glucose transport by bringing tyrosine phosphorylated Cbl to the lipid raft domain of the plasma membrane. We analyzed the localization of ectopically expressed CAP isoforms in 3T3-L1 adipocytes. While the plasma membrane localization of CAP2, CAP3, and CAP4 was similar to that exhibited by CAP1 in some cells, CAP2 showed strong nuclear staining in more than half of the cells, and CAP3 also exhibited nuclear localization. This suggested that the interactions of CAPs with vinculin were not an important determinant of localization in the adipocytes, although they may direct localization in other cell types. Previous immunostaining of fibroblasts using an anti-CAP antibody, which recognizes all the isoforms, showed some localization in the nucleus (9,11). Sequence analysis identified a bipartite nuclear localization signal in 1 of the alternatively spliced exon (F), which is present in CAP2, CAP3, and CAP4 (see Figure 2). The recently cloned human homologue of CAP2, R85, together with its binding partner, ataxin-7 (a protein responsible for the neurodegenerative disease spinocerebellar ataxia 7), is localized in the nucleus in transfected COS cells and the neuronal intranuclear inclusions in brain (23). In addition, the progesterone receptor has been shown to bind to the C-terminal SH3 domain of CAP (24). Furthermore, ArgBP2 is localized in the nucleus as well as its localization at stress fibers (10). The ArgBP2 binding partner c-Abl exhibits similar localization and has been shown to shuttle between stress fibers and the nucleus to integrate cell adhesion and cell cycle signals (25). Taken together, these data suggest potential nuclear functions of specific CAP isoforms.

The altered ligand binding specificity and differential subcellular localization of CAP isoforms suggest the possibility that these isoforms might carry out distinct functions. The varied expression pattern of each isoform in different tissues implies tissue-specificity of its functions. The significant induction of expression of all the isoforms in fully differentiated adipocytes indicates the overall importance of the CAP proteins in these cells. Whether these isoforms indeed serve distinct signaling functions needs further investigation. Moreover, the differences in domain structures and localization of CAP isoforms also imply potential novel ligand, which may be identified in future studies.

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