

# The Tyrosine Kinase Substrate p120<sup>CAS</sup> Binds Directly to E-Cadherin but Not to the Adenomatous Polyposis Coli Protein or $\alpha$ -Catenin

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**The tyrosine kinase substrate p120<sup>CAS</sup> (CAS), which is structurally similar to the cell adhesion proteins  $\beta$ -catenin and plakoglobin, was recently shown to associate with the E-cadherin–catenin cell adhesion complex.  $\beta$ -catenin, plakoglobin, and CAS all have an Arm domain that consists of 10 to 13 repeats of a 42-amino-acid motif originally described in the *Drosophila* Armadillo protein. To determine if the association of CAS with the cadherin cell adhesion machinery is similar to that of  $\beta$ -catenin and plakoglobin, we examined the CAS–cadherin–catenin interactions in a number of cell lines and in the yeast two-hybrid system. In the prostate carcinoma cell line PC3, CAS associated normally with cadherin complexes despite the specific absence of  $\alpha$ -catenin in these cells. However, in the colon carcinoma cell line SW480, which has negligible E-cadherin expression, CAS did not associate with  $\beta$ -catenin, plakoglobin, or  $\alpha$ -catenin, suggesting that E-cadherin is the protein which bridges CAS to the rest of the complex. In addition, CAS did not associate with the adenomatous polyposis coli (APC) tumor suppressor protein in any of the cell lines analyzed. Interestingly, expression of the various CAS isoforms was quite heterogeneous in these tumor cell lines, and in the colon carcinoma cell line HCT116, which expresses normal levels of E-cadherin and the catenins, the CAS1 isoforms were completely absent. By using the yeast two-hybrid system, we confirmed the direct interaction between CAS and E-cadherin and determined that CAS Arm repeats 1 to 10 are necessary and sufficient for this interaction. Hence, like  $\beta$ -catenin and plakoglobin, CAS interacts directly with E-cadherin *in vivo*; however, unlike  $\beta$ -catenin and plakoglobin, CAS does not interact with APC or  $\alpha$ -catenin.**

The tyrosine kinase substrate p120<sup>CAS</sup> (CAS) is tyrosine phosphorylated in cells transformed by Src (38) and in response to ligand-induced signaling by the platelet-derived growth factor, epidermal growth factor, and colony-stimulating factor 1 receptors (3, 15). However, unlike most tyrosine kinase substrates involved in signal transduction, CAS lacks structural motifs such as SH2 and SH3 domains that would clarify its involvement in mitogenic signaling. Instead, CAS shows a high degree of structural similarity to the *Drosophila* segment polarity gene product Armadillo, its vertebrate homolog  $\beta$ -catenin, and the closely related protein plakoglobin/ $\gamma$ -catenin (37). CAS,  $\beta$ -catenin, and plakoglobin all possess an Arm domain (20, 32, 37) which consists of 10 to 13 copies of a degenerate 42-amino-acid motif originally described in the Armadillo protein (39). Recent evidence indicates that like Armadillo,  $\beta$ -catenin is involved in pattern formation and signal transduction (6, 8, 21, 34). Although the exact role of  $\beta$ -catenin in pattern formation and mitogenic signaling remains unknown, it is clear that  $\beta$ -catenin and plakoglobin are key players in cadherin-mediated cell adhesion. Hence, the structural similarity between CAS and  $\beta$ -catenin and the physical association of CAS with cadherin complexes (36, 43) imply that CAS also plays a role in cadherin-mediated cell-cell adhesion.

The cadherins are a family of transmembrane glycoproteins which mediate Ca<sup>2+</sup>-dependent cell-cell adhesion through the homophilic interactions of their extracellular domains (for a review, see reference 49). Their conserved intracellular domains bind directly to  $\beta$ -catenin and plakoglobin, which in turn bind to the vinculin-like  $\alpha$ -catenin protein that is thought to interact directly with actin filaments and anchor the cadherin

complex to the actin cytoskeleton (9, 13, 25, 26, 30, 42). Reduced E-cadherin expression correlates with increased invasiveness of normal and cancer cells (1, 5, 28, 50), a behavior which can be suppressed by enforced expression of E-cadherin (5, 50). However, metastasis has also been linked to defects in catenin function. For example, cell lines expressing E-cadherin but lacking  $\alpha$ -catenin exhibit a decreased cell-cell adhesion that can be reversed by transfection of the cells with  $\alpha$ -catenin cDNA (11, 22). Recent evidence suggests that the physical juxtaposition of  $\alpha$ -catenin with E-cadherin, as mediated by  $\beta$ -catenin or plakoglobin, is a crucial factor in cadherin-mediated cell adhesion (17, 23, 29). Hence, defective interactions between any of the components of these cadherin complexes appear to perturb cell-cell adhesion and promote metastasis (1, 17, 29, 31, 45).

Recently, two groups reported that  $\beta$ -catenin interacts with the adenomatous polyposis coli (APC) tumor suppressor protein (41, 48). The physiological function of the APC protein is unknown, but mutations in the APC protein-encoding gene appear to be the first step in the development of colorectal tumors (4, 7, 18, 35). Antibodies to E-cadherin do not coprecipitate APC, or vice versa, indicating that  $\beta$ -catenin forms distinct, independent complexes with either the APC protein or E-cadherin (42, 48). Although different Arm repeats in  $\beta$ -catenin mediate its binding to E-cadherin (repeats 4 to 13) and to the APC protein (repeats 1 to 10), the binding regions overlap significantly, and this results in competition between E-cadherin and APC for exclusive binding to  $\beta$ -catenin (13). Interestingly, APC also possesses an Arm domain (32); however, the interaction between  $\beta$ -catenin and APC appears to be mediated not by the Arm domains of the two proteins but rather via the Arm domain of  $\beta$ -catenin and several 15- or 20-amino-acid repeats in APC for which a consensus binding sequence has been proposed (42, 48). Thus, while the Arm domain of  $\beta$ -catenin mediates a protein-protein interaction

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with short peptide sequences of APC or E-cadherin (30, 31), the ligands for the Arm domain of APC and CAS are unknown. The Arm domains of CAS and APC may therefore mediate similar protein-protein interactions with as yet unidentified proteins.

Although we and others have demonstrated that CAS associates with E-cadherin complexes (36, 43), the direct binding partner(s) of CAS within the complex remains unknown. The structural similarity of CAS to  $\beta$ -catenin and plakoglobin implies some conservation of function and, possibly, binding partners. However, while  $\beta$ -catenin and plakoglobin are  $\sim 70\%$  identical in the Arm domain (34), this region of CAS shares only  $\sim 20\%$  identity with either protein. Consequently, it is not clear whether CAS mediates interactions similar to or different from those of  $\beta$ -catenin and plakoglobin. Furthermore, unlike  $\beta$ -catenin and plakoglobin, for which isoforms have not been described, CAS is present in most cells as multiple isoforms (usually four) that vary quantitatively from one cell type to another (36; unpublished observations). In murine cells, we have termed the more slowly migrating isoforms ( $\sim 115$  and  $112$  kDa) CAS1A and CAS1B and the faster migrating isoforms ( $\sim 105$  and  $90$  kDa) CAS2A and CAS2B (36). CAS1A is an alternatively spliced variant of CAS1B that contains an additional 21 amino acids near the carboxy terminus. The CAS2 isoforms likewise differ from each other at the carboxy terminus, but in addition, they both lack an amino-terminal sequence defined by the epitope for CAS monoclonal antibody (MAb) 2B12 (36). The functional significance of these multiple isoforms and their association with cadherin complexes is unknown.

In this study, we examined the interactions between CAS and the different components of the cadherin-catenin complex in three tumor cell lines and in the yeast two-hybrid system. In addition, to determine if the functional similarity of CAS to  $\beta$ -catenin extends beyond its association with cadherin complexes, we also looked for an association between CAS and APC. Our results show that CAS interacts directly with E-cadherin but not with  $\alpha$ -catenin or APC. These data indicate that the role of CAS in cadherin function is likely to be fundamentally different from the role(s) of  $\beta$ -catenin and plakoglobin.

## MATERIALS AND METHODS

**Cells and tissue culture.** PC3 human prostate cancer cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2% L-glutamine, penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Colon carcinoma cell lines HCT116 and SW480 were cultured in McCoy's 5A medium (supplemented with 10% fetal calf serum, penicillin [100 U/ml], and streptomycin [100  $\mu$ g/ml]) and Leibovitz's L-15 medium (supplemented with 10% fetal calf serum, 2% L-glutamine, penicillin [100 U/ml], and streptomycin [100  $\mu$ g/ml]), respectively.

**Immunoprecipitation and immunoblot analysis.** Cells were lysed at 0°C in a buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.1 mM sodium vanadate, 0.1 trypsin inhibitor unit of aprotinin, and 5  $\mu$ g of leupeptin per ml. The proteins were immunoprecipitated from cell lysates with the appropriate antibodies, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19), and transferred to nitrocellulose membranes as previously described (38). Blots were briefly blocked at room temperature with Tris-buffered saline (TBS), pH 7.4, that contained 3% nonfat dry milk before being incubated with the primary antibody (0.2 to 2.0  $\mu$ g/ml) in TBS-3% milk at 4°C overnight. The membranes were then washed five times with TBS before incubation with the secondary peroxidase-conjugated donkey anti-mouse, anti-rabbit, or anti-rat antibody in TBS-3% milk for 1 to 2 h at room temperature. The immunoblots were subsequently washed five times with TBS and then processed with the enhanced chemiluminescence immunodetection system (Amersham) in accordance with the manufacturer's protocols.

Antibodies against CAS (MAb 2B12; 16),  $\beta$ -catenin (polyclonal sera; 21, 33), plakoglobin (MAb 5F11; 14),  $\alpha$ -catenin (MAb 1G5; 14), and E-cadherin (MAb E9; 14) have been described previously. Anti-p120 MAb pp120 was purchased

from Transduction Laboratories (Lexington, Ky.), whereas the anti-APC protein antibody, MAb Fe9, was obtained from Oncogene Science (Uniondale, N.Y.).

To achieve optimal resolution and transfer of the high-molecular-weight APC protein to a polyvinylidene difluoride membrane for Western blot (immunoblot) analysis, immunoprecipitates were electrophoresed on a 3% low-melting-point agarose gel and Western blotted in accordance with the Oncogene Science protocol.

**Yeast two-hybrid system.** pGBT9 is a DNA-binding domain cloning vector that contains the *trp1* gene and is used to generate fusions of the bait protein with the GAL4 DNA-binding domain (residues 1 to 147). pGAD424 is an activation domain cloning vector that contains the *leu2* gene and is used to generate fusions of the target protein with the GAL4 transcriptional activation domain (residues 768 to 881). Fusion constructs of the proteins of interest were generated as follows. A cDNA encoding full-length murine CAS was cloned in frame into the pGBT9 vector to generate fusion proteins containing the GAL4 DNA-binding domain. cDNAs encoding a panel of CAS deletion mutants were similarly cloned into pGBT9. This strategy was also used to clone full-length human  $\alpha$ -catenin (40) and cDNA restriction fragments of murine  $\beta$ -catenin (spanning amino acid residues 58 to 781) (2) and murine plakoglobin (residues 125 to 621) (2) in frame into the pGBT9 or pGAD424 vector. A cDNA encoding a cytoplasmic region of murine E-cadherin (residues 634 to 884) (24) was also cloned in frame into the pGAD424 vector.

To test for potential protein-protein interactions, the appropriate pair of plasmids were cotransformed into yeast strain Y190 by the lithium acetate-polyethylene glycol procedure as outlined by the Clontech MATCHMAKER Two-Hybrid System protocol. Transformants were grown at 30°C for 3 to 4 days on synthetic medium lacking histidine, tryptophan, and leucine and then assayed for  $\beta$ -galactosidase activity as follows. Yeast colonies were lifted off the plates onto nitrocellulose filters and permeabilized by two freeze-thaw treatments at  $-70^\circ\text{C}$ . The filters were then placed colony side up on Whatman no. 1 paper presoaked with 0.33 mg of X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) per ml and incubated at 30°C until the blue color developed (30 min to overnight). Each plasmid construct was also tested to verify that it could not transactivate the reporter genes by itself.

## RESULTS

**CAS expression in human tumor cell lines.** Whole-cell lysates from the PC3, HCT116, and SW480 cell lines were used for immunoprecipitation and Western blot analysis with antibodies to CAS,  $\beta$ -catenin, plakoglobin,  $\alpha$ -catenin, E-cadherin, and APC. First, we confirmed previous reports and found that (i) PC3 cells do not express  $\alpha$ -catenin (22) but HCT116 and SW480 cells do (Fig. 1A), (ii) SW480 cells express a truncated APC protein (27, 46) while HCT116 (47) and PC3 cells appear to express a full-length APC protein (Fig. 1G), and (iii) HCT116 and PC3 cells express E-cadherin at apparently wild-type levels (Fig. 1D). Although SW480 cells have been reported to lack E-cadherin (48), we observed low but detectable levels of E-cadherin in some experiments (Fig. 1D, lane 3). In addition, all three cell lines expressed approximately equivalent amounts of  $\beta$ -catenin and plakoglobin (Fig. 1B and C, respectively). Western blot analysis of CAS immunoprecipitates from these cell lines further revealed a heterogeneous and distinct pattern of expression of the CAS isoforms in each cell line (Fig. 1F), suggesting that these different isoforms have precise roles in different cell types. It is interesting that although all three cell lines expressed the faster-migrating CAS2 isoforms, the CAS1 isoforms, which are recognized by CAS MAb 2B12, were completely absent from HCT116 cells (Fig. 1E, lane 2), raising the possibility that the lack of CAS1 expression is one of the underlying genetic defects in these cells.

**CAS binds E-cadherin in vivo.** If CAS binds directly only to E-cadherin, it should not coprecipitate with other components of the cadherin complex from carcinoma cells that no longer express E-cadherin, e.g., SW480 cells. In HCT116 cells, which express all known components of the cadherin-catenin complex, except the above-noted CAS1 isoforms, the CAS2 isoforms coprecipitated with  $\alpha$ -catenin,  $\beta$ -catenin, plakoglobin, and E-cadherin (Fig. 2A, lane 2). In PC3 cells, which do not express  $\alpha$ -catenin, all four CAS isoforms coprecipitated with  $\beta$ -catenin, plakoglobin, and E-cadherin (Fig. 2A, lane 1). However, when immunoprecipitates from SW480 cell lysates were

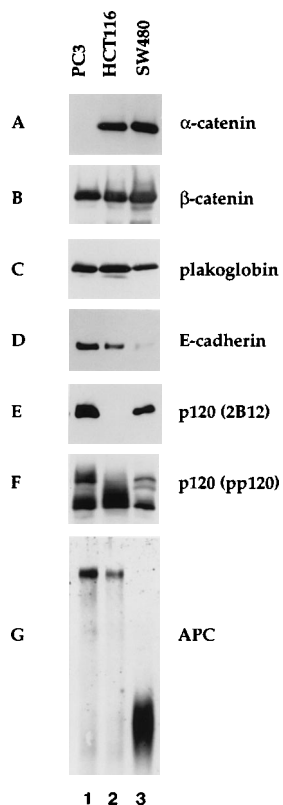


FIG. 1. Expression level comparison of APC and the different components of the cadherin-catenin cell adhesion complex in the PC3, HCT116, and SW480 cell lines. Whole-cell lysates of equal numbers of cells from each cell line were immunoprecipitated and Western blotted with the antibodies indicated on the right. The approximate molecular masses of the proteins detected are as follows:  $\alpha$ -catenin, 102 kDa;  $\beta$ -catenin, 94 kDa; plakoglobin, 85 kDa; E-cadherin, 120 kDa; p120 isoforms, 90 to 115 kDa; full-length APC, 300 kDa; truncated APC, 147 kDa.

examined by Western blot, CAS was not detected (Fig. 2A, lane 3), even though SW480 and HCT116 cells express similar levels of  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin (Fig. 1A, B, and C). Since E-cadherin was the only component of the complex known to be absent in these cells, these results suggested that CAS did not directly interact with  $\alpha$ -catenin,  $\beta$ -catenin, or plakoglobin but actually coprecipitated in the complex through its association with E-cadherin. In a parallel Western blot analysis using an anti- $\beta$ -catenin antibody, normal levels of  $\beta$ -catenin coprecipitated with CAS, plakoglobin, and E-cadherin from HCT116 cell lysates (Fig. 2B, lane 5). However, we detected significantly reduced levels of  $\beta$ -catenin in similar immunoprecipitates from SW480 cell lysates (Fig. 2B, lane 6). The low level of  $\beta$ -catenin which coprecipitated with CAS, plakoglobin, and E-cadherin from these cells (Fig. 2B, lane 6) correlates with the low level of E-cadherin expression which we detected in some experiments (Fig. 1D, lane 3). Like CAS,  $\beta$ -catenin coprecipitated with  $\alpha$ -catenin in lysates from HCT116, but not PC3, cells (Fig. 2B, lanes 4 and 5). However, unlike CAS,  $\beta$ -catenin associated with  $\alpha$ -catenin in SW480 cell lysates (Fig. 2B, lane 6), indicating that  $\beta$ -catenin can directly interact with  $\alpha$ -catenin in the absence of E-cadherin.

**CAS does not associate with  $\alpha$ -catenin.** In addition to binding E-cadherin,  $\beta$ -catenin and plakoglobin directly interact with the vinculin-like protein  $\alpha$ -catenin (13, 42), raising the possibility that CAS also interacts with  $\alpha$ -catenin. However, as

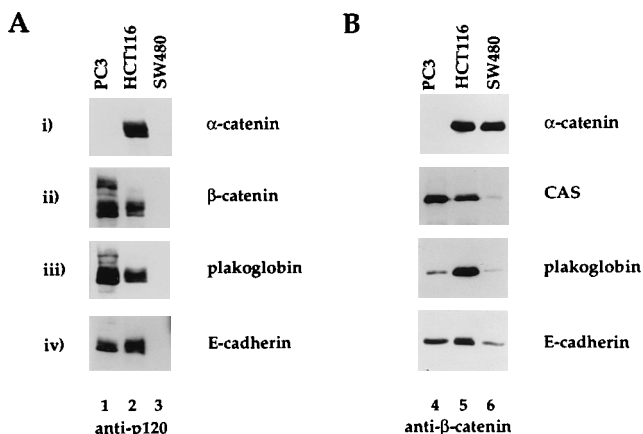


FIG. 2. Immunoblot detection of CAS (A) and  $\beta$ -catenin (B), which coprecipitated with the various components of the cadherin-catenin complex. Whole-cell lysates from PC3, HCT116, and SW480 cells were immunoprecipitated with antibodies to  $\alpha$ -catenin, CAS,  $\beta$ -catenin, plakoglobin, or E-cadherin (as indicated to the right of each blot). Precipitated proteins were detected by Western blotting with CAS MAb pp120 (A) or polyclonal sera to  $\beta$ -catenin (B). No CAS was coprecipitated from SW480 cell lysates by any component of the complex (panel A, lane 3, i to iv). The molecular masses of the proteins detected are listed in the legend to Fig. 1.

shown in Fig. 2A, CAS was not present in  $\alpha$ -catenin immunoprecipitates from E-cadherin-deficient SW480 cell lysates (lane 2 versus lane 3). Furthermore, when immunoprecipitates from PC3 cell lysates lacking  $\alpha$ -catenin were examined, we found CAS in immunoprecipitates by using antibodies to  $\beta$ -catenin, plakoglobin, and E-cadherin but not by using antibodies to  $\alpha$ -catenin (Fig. 2A, lane 1). These data suggested that  $\alpha$ -catenin was not required for CAS association with the complex and that CAS did not directly associate with  $\alpha$ -catenin.

To extend these observations, we performed the reciprocal experiment and obtained similar results. In HCT116 cell lysates, we found that  $\alpha$ -catenin coprecipitated with CAS,  $\beta$ -catenin, and E-cadherin (Fig. 3A). However, in SW480 cell lysates,  $\alpha$ -catenin coprecipitated only with  $\beta$ -catenin (Fig. 3B). These observations indicate that CAS does not have detect-

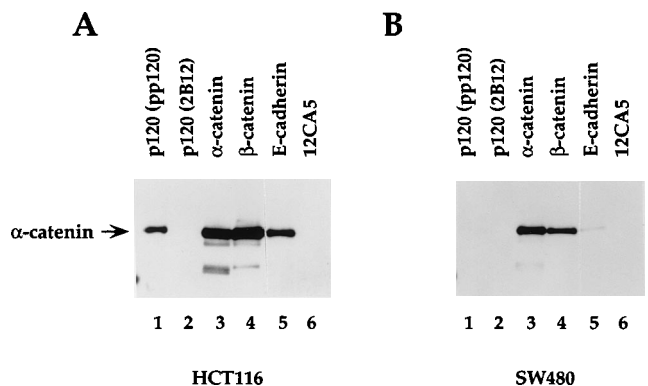


FIG. 3. Western blot analysis of  $\alpha$ -catenin coprecipitated with APC and various components of the cadherin-catenin complex in HCT116 (A) and SW480 (B) cells. Lysates from HCT116 and SW480 cells were immunoprecipitated with antibodies to CAS (MAb 2B12 or pp120),  $\alpha$ -catenin (MAb 1G5),  $\beta$ -catenin (polyclonal antisera), plakoglobin (MAb 5F11), and E-cadherin (MAb E9) as indicated above the lanes and Western blotted with an  $\alpha$ -catenin polyclonal antibody which detected the 102-kDa protein. Anti-Flu MAb 12CA5 was used as a control.

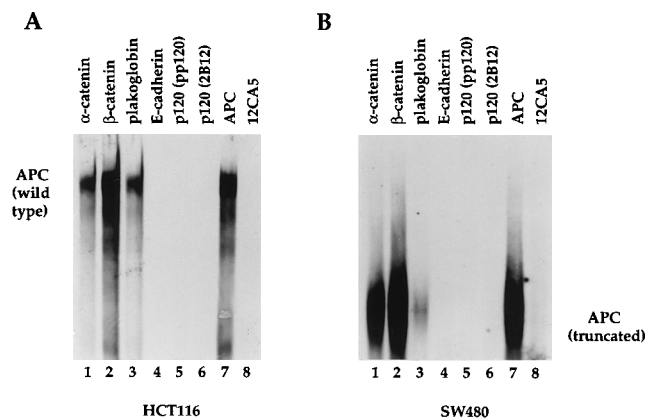


FIG. 4. Coprecipitation of APC with E-cadherin–catenin complex components. Lysates from HCT116 (A) and SW480 (B) cells were immunoprecipitated with the antibodies indicated above the lanes, and immunoprecipitates were electrophoretically separated as described in Materials and Methods. The membrane-blotted full-length (300-kDa) and truncated (147-kDa) APC proteins were then detected with an antibody to APC (Fe9).

able affinity for free  $\alpha$ -catenin or  $\alpha$ -catenin– $\beta$ -catenin heterodimers.

**CAS does not bind APC.** Previous reports have indicated that the interaction between  $\beta$ -catenin and APC is mediated by the  $\beta$ -catenin Arm domain (13, 42), raising the possibility that other Arm-containing proteins, such as CAS, also interact with APC. To determine if CAS associates with APC, HCT116 and SW480 cell lysates were immunoprecipitated with antibodies to the individual components of the cadherin–catenin complex and the precipitated proteins were examined by Western blot analysis with an anti-APC antibody. In accordance with previous reports, APC coprecipitated with  $\alpha$ -catenin,  $\beta$ -catenin, and plakoglobin but not with E-cadherin (Fig. 4A) (13, 41, 42, 48). However, no APC was coprecipitated by CAS in either cell line (Fig. 4A and B, lanes 5 and 6) or in the prostate tumor cell line PC3 (data not shown), indicating that in contrast to  $\beta$ -catenin and plakoglobin, CAS lacks detectable affinity for APC. Interestingly, plakoglobin coprecipitated relatively small amounts of the truncated APC protein found in SW480 cells (Fig. 4B, lane 3), suggesting that the truncated APC protein has lost part of its binding site for plakoglobin or that the truncated APC has an altered conformation that decreases its binding to plakoglobin. This result also indicates that the interaction between  $\beta$ -catenin and APC differs from that between plakoglobin and APC.

**Protein–protein interactions in the yeast two-hybrid system.** To confirm that CAS does indeed directly interact with E-cadherin, we used the yeast two-hybrid system to further examine the protein–protein interactions between CAS and E-cadherin. Figure 5 is a schematic representation of the full-length and mutant forms of CAS used in this analysis. A summary of their interactions with E-cadherin, as assessed by growth on media lacking histidine and by  $\beta$ -galactosidase activity, is also presented in Fig. 5. Only full-length CAS and deletion mutants  $\Delta$ N-term and  $\Delta$ R11 $\Delta$ C, lacking either the amino or carboxyl terminus, respectively, were able to interact with E-cadherin. CAS deletion mutants lacking any of the remaining 10 Arm repeats failed to interact with E-cadherin. In addition, CAS did not homodimerize (data not shown) or directly interact with  $\alpha$ -catenin,  $\beta$ -catenin, or plakoglobin (Table 1), thus corroborating the results of our immunoprecipitation analyses. One mutant,  $\Delta$ R3-5, independently activated the reporter genes when cotransformed with the pGAD424 vector

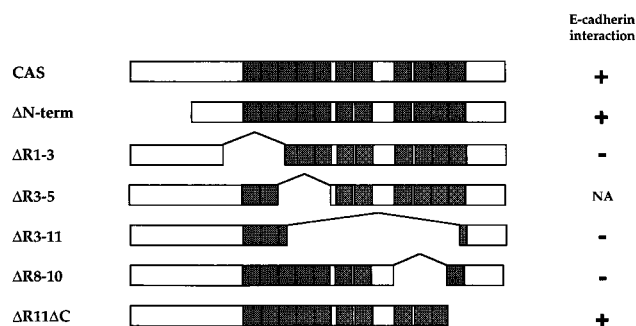


FIG. 5. Schematic representation of full-length and mutant CAS constructs used in the yeast two-hybrid system. The 11 internal 42-amino-acid repeats that make up the Arm domain are indicated by the hatched boxes. The following amino acid residues were deleted in the mutant CAS proteins:  $\Delta$ N-term, 1 to 158;  $\Delta$ R1-3, 233 to 387;  $\Delta$ R3-5, 365 to 495;  $\Delta$ R3-11, 390 to 820;  $\Delta$ R8-10, 657 to 789;  $\Delta$ R11 $\Delta$ C-term, 789 to 911. A summary of the binding interactions with E-cadherin in the two-hybrid analysis is presented on the right. NA, not addressed; the binding of  $\Delta$ R3-5 to E-cadherin could not be addressed because this mutant transactivated the reporter genes when cotransformed with the GAL4 activation domain vector alone.

alone. Hence, data from this mutant could not be interpreted and were omitted from the analysis.

## DISCUSSION

The association of CAS with the cadherin–catenin complex (36, 43) implies a role for CAS in cadherin-mediated activities. In this study, we have shown that within the cadherin–catenin complex, CAS associates directly with E-cadherin. Our *in vivo* experiments suggest that E-cadherin is necessary and sufficient for the association of CAS with cadherin–catenin complexes and that no other catenin ( $\alpha$ ,  $\beta$ , or  $\gamma$ ) is required. Furthermore, CAS and E-cadherin associated directly in the yeast two-hybrid assay, and by using a panel of deletion mutants, we mapped the CAS site of interaction to Arm repeats 1 to 10. This region is similar to the region of  $\beta$ -catenin which binds E-cadherin and is consistent with the notion that CAS is a member of the group of cadherin-associated proteins called catenins. Unlike  $\beta$ -catenin and plakoglobin, however, CAS did not associate with the actin-binding protein  $\alpha$ -catenin or with the tumor suppressor protein APC. These data are the first to substan-

TABLE 1. Yeast two-hybrid analysis of protein–protein interactions assayed by  $\beta$ -galactosidase activity<sup>a</sup>

Fusion construct	Interaction with:				
	pGAD424	E-Cadherin	$\alpha$ -Catenin	$\beta$ -Catenin	Plakoglobin
CAS	–	+	–	–	–
$\Delta$ N-term	–	+	–	–	–
$\Delta$ R1-3	–	–	–	–	–
$\Delta$ R3-5 <sup>b</sup>	+	NA <sup>c</sup>	NA	NA	NA
$\Delta$ R3-11	–	–	–	–	–
$\Delta$ R8-10	–	–	–	–	–
$\Delta$ R11 $\Delta$ C	–	+	–	–	–

<sup>a</sup> Yeast cells cotransformed with the constructs of interest were grown in medium that lacked leucine, tryptophan, and histidine. After 3 to 4 days, the cotransformants were assayed for  $\beta$ -galactosidase activity. The left column represents DNA-binding domain fusion constructs, and activation domain constructs are indicated across the top.

<sup>b</sup> This DNA-binding domain fusion construct could not be assayed because it activated the reporter genes when cotransformed with the pGAD424 vector that lacked an insert.

<sup>c</sup> NA, not applicable.

tially differentiate CAS from the other Arm motif-containing catenins and suggest that the role of CAS in the cadherin-catenin complex is fundamentally different from that of  $\beta$ -catenin and plakoglobin; i.e., CAS does not serve as a linker between E-cadherin and  $\alpha$ -catenin.

Accumulating evidence indicates that  $\beta$ -catenin and plakoglobin form mutually exclusive complexes in which they bind to identical or closely overlapping sites on E-cadherin (10, 13, 36, 42). Since we and others were able to coprecipitate either  $\beta$ -catenin or plakoglobin with CAS (36, 43), we conclude that (i) CAS does not form a mutually exclusive complex with E-cadherin but rather forms CAS-E-cadherin- $\beta$ -catenin or CAS-E-cadherin-plakoglobin complexes and (ii) CAS does not compete with  $\beta$ -catenin or plakoglobin for a single site on E-cadherin but instead interacts with E-cadherin at a distinct site. Interestingly, a recent study showed that CAS does not associate with a truncated E-cadherin molecule which lacks the carboxy-terminal 37 amino acids necessary for catenin binding (43). This indicates that although the CAS and  $\beta$ -catenin binding sites on E-cadherin are distinct, they are contiguous and in close proximity. Consequently, CAS and  $\beta$ -catenin are closely juxtaposed in the complex, and even though we and others have not detected a direct interaction between these two proteins, they are in a position to sterically affect each other, and the cadherin complex, by conformational changes produced by, for example, tyrosine phosphorylation.

We also demonstrated that CAS does not bind  $\alpha$ -catenin. This result was, perhaps, expected since the binding site for  $\alpha$ -catenin maps to the first 130 amino acids of  $\beta$ -catenin (13, 17, 29, 42) and there is very little amino acid identity between CAS and  $\beta$ -catenin in this region. Nevertheless, because of the close juxtaposition of CAS to  $\beta$ -catenin on the E-cadherin molecule, we wanted to formally exclude the possibility that CAS might associate with  $\alpha$ -catenin via a domain of  $\alpha$ -catenin distinct from the one that associates with  $\beta$ -catenin and plakoglobin. However, using two independent methods, we were unable to detect an interaction between CAS and  $\alpha$ -catenin under conditions favorable for the association of  $\alpha$ -catenin with either  $\beta$ -catenin or plakoglobin. Hence, it is appears that CAS differs from the classical  $\beta$ - and  $\gamma$ -catenins and is likely to recruit into the complex other distinct factors that contribute uniquely to cadherin function or mitogenic signaling.

Another difference between CAS on one hand and  $\beta$ -catenin and plakoglobin on the other was the failure of CAS to associate directly with APC. Although the Arm domains of  $\beta$ -catenin and plakoglobin mediate binding to both E-cadherin and APC (13, 42), in contrast to  $\beta$ -catenin and plakoglobin, the CAS Arm domain appears to mediate binding only to E-cadherin. Interestingly, the E-cadherin and APC protein binding sites in  $\beta$ -catenin overlap at the core of the Arm domain, i.e., Arm repeats 4 to 10 (13). Therefore, the differences in amino acid identity between the Arm domains of CAS and  $\beta$ -catenin (20% identity), compared with  $\beta$ -catenin and plakoglobin (70% identity) (34), not only generate a distinct CAS-E-cadherin binding interaction but prohibit a potential CAS-APC interaction. We cannot exclude the possibility that there is a low-affinity CAS-APC interaction which was not detected in our immunoprecipitation analysis. However, since we were unable to detect a CAS-APC interaction under mild detergent conditions which favored easily detectable  $\beta$ -catenin-APC and plakoglobin-APC interactions, it appears that a CAS-APC interaction does not exist.

The heterogeneous expression of CAS in the different cell lines and the complete absence of the CAS1 isoforms in the HCT116 colon carcinoma cell line are intriguing observations whose functional significance is unknown. Differential expres-

sion of the isoforms could represent a novel mechanism of regulating or modulating cadherin function in different cell types. The association of CAS with E-cadherin cell adhesion complexes and the unusual heterogeneity of expression of CAS isoforms in HCT116 cells and other human colon tumor cell lines (unpublished observations) raise the possibility that defects in CAS expression or splicing contribute to the metastatic phenotype or other observed defects of these cell lines.

The association of CAS with E-cadherin at a site distinct from  $\beta$ -catenin and plakoglobin, coupled with its inability to interact with  $\alpha$ -catenin, highlights the uniqueness of CAS as a component of the cadherin-catenin cell adhesion complex. The cadherin-catenin complex proteins are major constituents of the adherens junction, a dynamic structure which appears capable of rapid assembly and disassembly in response to mitogenic signals. Since we and others have demonstrated that CAS,  $\beta$ -catenin, and plakoglobin are tyrosine phosphorylated in response to growth factor treatment (3, 12, 44), it is possible that the catenins regulate the equilibrium of the complex, and thus the adherens junction, to mediate cell-cell adhesion or proliferation. The differences between CAS and  $\beta$ -catenin, as reported in this study, imply that they mediate different aspects of this process. It is possible that they respond to different incoming signals, or alternatively, CAS and  $\beta$ -catenin could respond to the same signals but produce different effects via interactions with their own distinct effector molecules, e.g.,  $\beta$ -catenin via APC. It is therefore important to identify CAS-specific binding partners to fully understand the role of CAS and tyrosine phosphorylation in cadherin-mediated cell-cell adhesion and mitogenic signaling.

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