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KRIT1 association with the integrin-binding protein ICAP-1: a new direction in the elucidation of cerebral cavernous malformations (*CCM1*) pathogenesis

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Mutations in KRIT1, a protein initially identified based on a yeast two-hybrid interaction with the RAS-family GTPase RAP1A, are responsible for the development of the inherited vascular disorder cerebral cavernous malformations (*CCM1*). As the function of the KRIT1 protein and its role in CCM pathogenesis remain unknown, we performed yeast two-hybrid screens to identify additional protein binding partners. A fragment containing the N-terminal 272 amino acid residues of KRIT1, a region lacking similarity to any known protein upon database searches, was used as bait. From parallel screens of human fetal brain and HeLa cDNA libraries, we obtained multiple independent isolates of human integrin cytoplasmic domain-associated protein-1 (ICAP-1) as interacting clones. The interaction of KRIT1 and ICAP-1 was confirmed by GST-KRIT1 trapping of endogenous ICAP-1 from 293T cells. The α isoform of ICAP-1 is a 200 amino acid serine/threonine-rich phosphoprotein which binds the cytoplasmic tail of β_1 integrins. We show that mutagenesis of the N-terminal KRIT1 NPXY amino acid sequence, a motif critical for ICAP-1 binding to β_1 integrin molecules, completely abrogates the KRIT1/ICAP-1 interaction. The interaction between ICAP-1 and KRIT1, and the presence of a FERM domain in the latter, suggest that KRIT1 might be involved in the bidirectional signaling between integrin molecules and the cytoskeleton. Furthermore, these data suggest that KRIT1 might affect cell adhesion processes via integrin signaling in *CCM1* pathogenesis.

INTRODUCTION

Cerebral cavernous malformations (CCMs) are inherited or sporadic vascular anomalies of the central nervous system characterized by grossly enlarged capillary-like blood vessels with no intervening neural tissue and lack of mature blood vessel elements. Minimal blood flow occurs within the lesions (1), which may be single or multiple and range in size from a few millimeters to a few centimeters. Cavernous malformations usually present clinically during the third to fifth decades of life, when patients manifest with seizures, intracerebral hemorrhage leading to stroke, or recurrent headaches and focal neurological deficits (2–5). Intracerebral hemorrhage of the vascular lesion can be fatal and is often the first and only clinical presentation of the disorder (6).

KRIT1 has been identified as the disease gene for an autosomal dominant form of *CCM1* (7,8). Two other loci for which the disease gene has not been identified, *CCM2* and *CCM3*, are linked to markers mapping to chromosome bands 7p15–p13 and 3q25.2–q27 (9). *KRIT1* mutations identified in these (7,8) and subsequent studies (10–14) were predominantly found to be frame-shift, nonsense and invariant splice site sequence mutations, all predicted to result in a truncated protein and loss of function.

The cellular function of KRIT1 and its role in *CCM1* pathogenesis remain unknown. The primary insight into KRIT1 function came from its original identification as a yeast two-hybrid binding partner for the RAS-family GTPase RAP1A (15), a protein originally identified by its ability to revert the transformed phenotype of cells expressing an oncogenic *RAS* gene (16). This suggested KRIT1 involvement in RAP1A/RAS signal transduction pathways as well as possible involvement in tumor suppression processes. As additional evidence for KRIT1 to be involved in such processes, the putative Chinese hamster ovary (CHO) cell *KRIT1* ortholog was found to be transcriptionally upregulated upon chemically induced reversion of transformed CHO cells (17).

The presence of two characterized structural motifs provides additional clues to the function of the KRIT1 protein: a series of four putative ankyrin repeats, a motif present in a multitude of proteins of diverse function and known to mediate protein–protein interactions (18); and a C-terminal FERM domain, found in a number of proteins including ezrin, radixin, moesin and merlin, which mediates the interaction of the actin cytoskeleton

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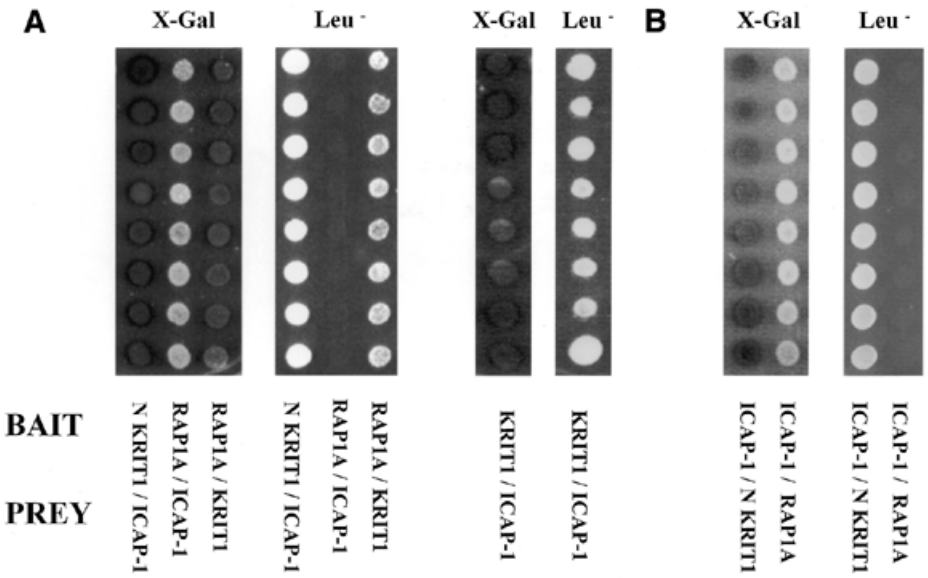

Figure 1. Confirmation of specificity of KRIT1/ICAP-1 two-hybrid interaction. (**A**) Reintroduction of ICAP-1 prey construct into yeast harboring N-terminal KRIT1 bait or full-length KRIT1 bait yielded a positive interaction phenotype, whereas concurrent introduction of ICAP-1 into yeast harboring an unrelated bait, RAP1A, did not activate the two-hybrid reporter genes. Positive interactions were assessed by growth on galactose media lacking leucine and blue color upon X-gal treatment on galactose media. The two-hybrid interaction of RAP1A and KRIT1 in the context of the original screen identifying KRIT1 (15) was utilized as a positive control for activation of the two reporter genes. (**B**) Swapping the bait–prey orientation of the N-terminal KRIT1/ICAP-1 two-hybrid constructs satisfies the phenotypic requirements for an authentic interaction. Concurrent introduction of an unrelated prey construct, RAP1A, into yeast harboring ICAP-1 bait failed to activate the two-hybrid reporter genes.

and the plasma membrane (19). The presence of these domains strongly suggests that KRIT1 might interact with multiple protein binding partners, and that the identification of these partners will be a crucial initial step in elucidating KRIT1 function.

The recent identification of additional 5′ *KRIT1* sequence revealing the presence of four additional exons encoding 207 additional amino acids beyond those initially reported provided fundamental information for future studies aimed at elucidating KRIT1 function (12,20,21). This newly identified N-terminal region of the KRIT1 protein is lacking similarity to any known protein in the databases and is devoid of any known functional motifs, suggesting a KRIT1-specific function for this region. Since the identification of this new N-terminal region, a truncating *CCM1* patient mutation has been identified in this region (12).

In order to gain insight into KRIT1 function and *CCM1* pathogenesis we utilized this new N-terminal region of KRIT1 as bait in yeast two-hybrid screens to identify additional protein binding partners. In this report we describe the identification of a KRIT1 binding partner, ICAP-1, the confirmation of the two-hybrid interaction, as well as the identification of a KRIT1 amino acid motif critical for its association with ICAP-1.

RESULTS

Identification of ICAP-1 as a specific KRIT1 binding partner

The N-terminal region of KRIT1 was used as bait in yeast two-hybrid screening to identify binding partners specific for this unique region of the protein. Utilizing the dual bait two-hybrid system (22), we created a LexA fusion construct corresponding to the N-terminal 272 amino acid residues of KRIT1 to be used as bait in the library screens. This region includes the entire N-terminus just prior to the ankyrin repeat domain.

A yeast strain harboring the bait construct was mated in parallel to two galactose-inducible cDNA library yeast strains: (i) derived from human fetal brain, in the interest of identifying partners potentially specific to *CCM1* pathology and (ii) derived from HeLa cells. Approximately 1×10^6 transformants were screened for each library. Initial colonies were obtained by selecting for growth on galactose media lacking leucine. From an initial 48 putative positive colonies in the screen from both libraries, nine colonies satisfied the phenotypic requirements of an authentic interactor. All nine were identified by sequence analysis as either full-length or nearly full-length clones of the α isoform of human integrin cytoplasmic domain-associated protein-1 (ICAP-1).

We next sought to confirm the specificity of the N-terminal KRIT1/ICAP-1 interaction within the context of the yeast two-hybrid system. Reintroduction of a full-length ICAP-1 isolate into yeast harboring the N-terminal KRIT1 bait construct reproduced the positive interaction phenotype, whereas parallel introduction of the clone into yeast containing a RAP1A bait construct was negative for the interaction phenotype (Fig. 1A). Reciprocal bait-prey swapping with the N-terminal KRIT1 and ICAP-1 fragments reproduced the phenotype requirements for an authentic interactor (Fig. 1B). These experiments confirmed the specificity of the interaction and verified that the interaction phenotype was not due to intrinsic basal transcription activity of ICAP-1.

ICAP-1 interaction with KRIT1 structural domains

To test the possibility that ICAP-1 can also interact with regions other than the N-terminus of KRIT1, we tested the

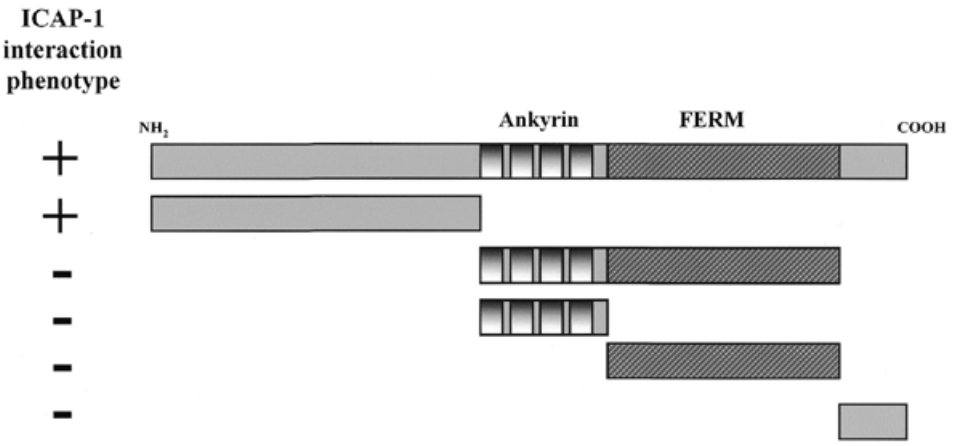

Figure 2. Summary of ICAP-1 two-hybrid interaction with KRIT1 structural domains. Shown are the functional motifs of the KRIT1 protein—a C-terminal FERM domain preceded by a series of four ankyrin repeats. Schematics of KRIT1 bait constructs used to assay two-hybrid interaction with ICAP-1 prey are shown. In addition to the N-terminus of KRIT1, only full-length KRIT1 produces a two-hybrid interaction phenotype with ICAP-1.

interaction of various KRIT1 bait constructs in the yeast two-hybrid assay. LexA–bait fusions were constructed with the following regions of KRIT1: (i) the four putative ankyrin repeats alone; (ii) the FERM domain alone; (iii) the extreme C-terminus (excluding the FERM domain) alone; (iv) the ankyrin repeats and FERM domain together; and (v) full-length KRIT1. Each of these bait constructs was analyzed for its ability to interact with the full-length ICAP-1 prey construct (Fig. 2). Only the full-length KRIT1 bait construct reproduced the strong interaction phenotype observed with the N-terminal KRIT1 bait (Fig. 1A). This suggests that the site of ICAP-1 binding is limited to the unique N-terminal region of KRIT1.

Biochemical confirmation of KRIT1/ICAP-1 interaction

To confirm the KRIT1/ICAP-1 association, we performed an *in vitro* binding assay to provide independent biochemical confirmation of the interaction. The N-terminal KRIT1 region used in the two-hybrid screening and full-length KRIT1 were expressed as GST fusions in *Escherichia coli*. GST alone was expressed in parallel as a negative control. A lysate of 293T cells (a human embryonic kidney cell line) was incubated with immobilized N-terminal KRIT1–GST and full-length KRIT1–GST fusion protein or GST control protein. Proteins which bound specifically to the GST fusion proteins were subjected to SDS–PAGE followed by western blotting with an anti-ICAP-1 antibody. GST–N-terminal KRIT1 was able to trap endogenous ICAP-1 from 293T cells, whereas no ICAP-1 was detected in the GST alone lane (Fig. 3A). Significantly, full-length KRIT1–GST also resulted in the trapping of endogenous ICAP-1 from 293T cells (Fig. 3A) despite lower levels of fusion protein production in *E.coli* relative to N-terminal KRIT1–GST production (Fig. 3B).

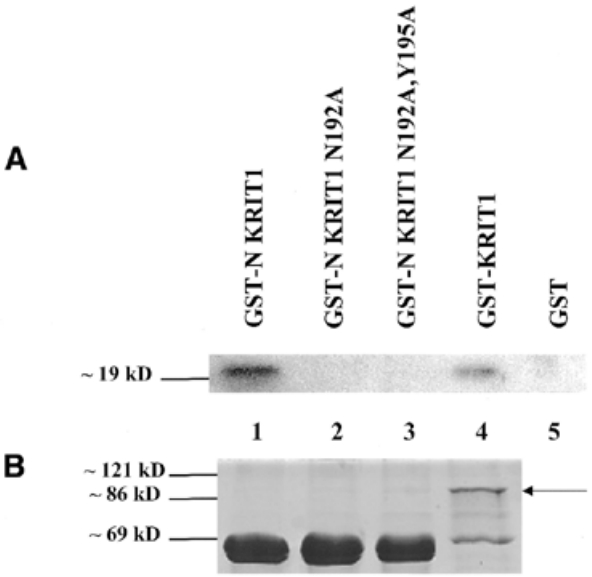

Figure 3. GST fusion affinity precipitation confirming KRIT1/ICAP-1 interaction. (**A**) Western analysis with anti-ICAP-1 antibody detects an ICAP-1 band after subjecting GST–N-terminal KRIT1 (lane 1) or GST–full-length KRIT1 (lane 4) to 293T cell lysate, but not in the GST alone control lane (lane 5). NPXY motif mutations N192A (lane 2) and N192A/Y195A (lane 3) in N-terminal KRIT1–GST completely disrupt trapping of ICAP-1 from 293T cell lysate. (**B**) Coomassie-stained gel of GST fusion protein used for western analysis in (A) corresponding to lanes 1–4. The band corresponding to full-length KRIT1–GST, produced less efficiently in *E.coli* relative to N-terminal KRIT1–GST, is indicated by the arrow. The amount of GST alone control protein (data not shown) utilized in the binding assay was normalized to the level of the N-terminal KRIT1–GST fusion proteins.

Mapping the site(s) of interaction between KRIT1 and ICAP-1

ICAP-1 was originally identified as a binding partner for the cytoplasmic tail of β_1 integrin (23,24). This interaction is dependent on the amino acid motif NPXY of the β_1 integrin cytoplasmic tail (23,24). As N-terminal KRIT1 contains an NPXY motif at amino acid positions 192–195 (Fig. 4A), we reasoned that this motif might also be critical for ICAP-1 binding to KRIT1. We created two mutant N-terminal KRIT1 bait constructs and assessed their effects on ICAP-1 binding using quantitative yeast two-hybrid liquid β-galactosidase assays. The first mutation substitutes an Ala for the first Asn in the putative NPXY binding motif. The second construct was a compound mutation including the first mutation, and a second, substituting Ala for Tyr in the last position of the motif. The

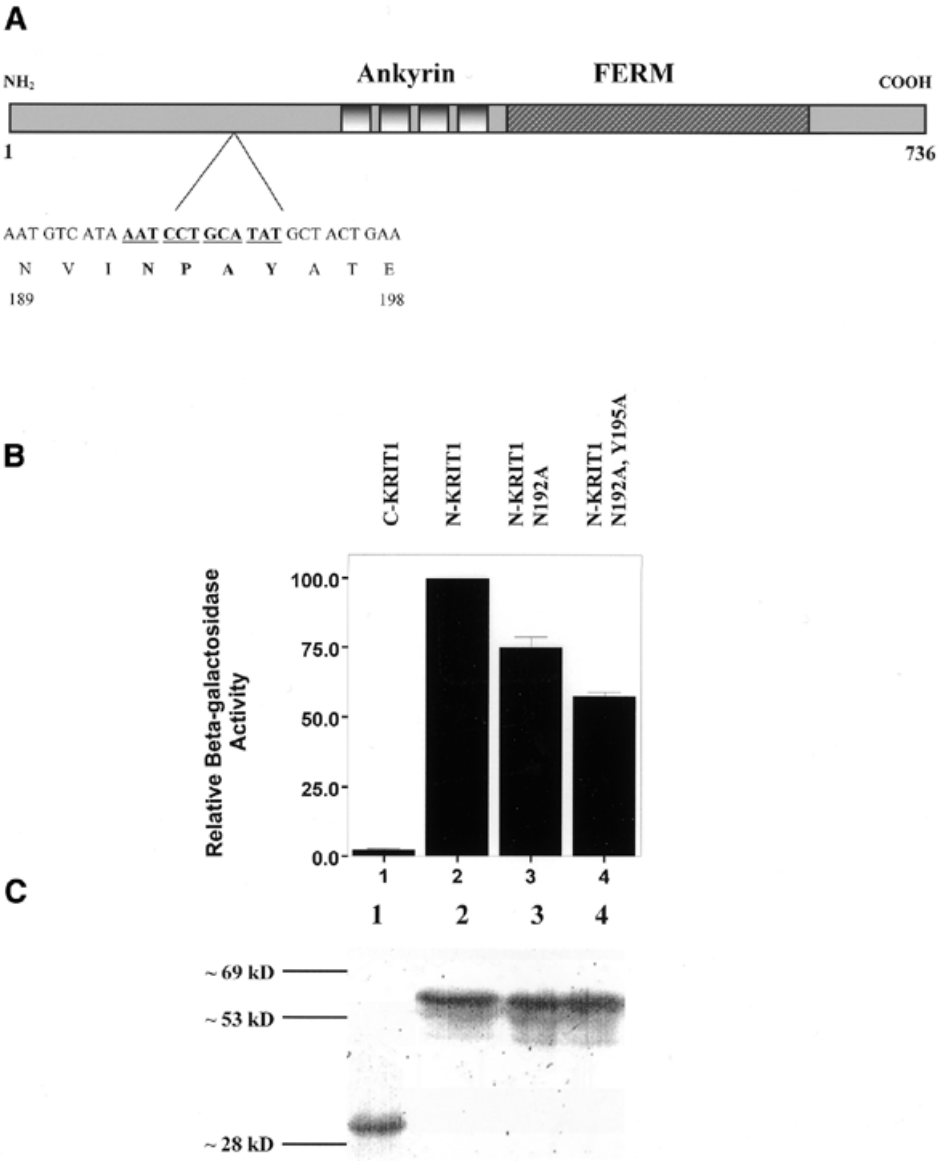

Figure 4. Effect of targeted NPXY mutations on ICAP-1 interaction. (**A**) The position of the KRIT1 NPXY motif—a motif in the β_1 integrin cytoplasmic tail critical for ICAP-1 binding—is shown relative to the functional domains of KRIT1. (**B**) Quantitative liquid β-galactosidase assays assessing the relative strength of two-hybrid interactions were performed in triplicate using independent colonies and averaged. Shown are the averaged relative β-galactosidase activity values for targeted NPXY motif missense mutations N192A (lane 3) and N192A/Y195A (lane 4) shown as a percentage of the wild-type N-terminal KRIT1/ICAP-1 (lane 2) strength of interaction (defined as 100% activity). The C-terminal KRIT1/ICAP-1 interaction (lane 1) was utilized as a negative control. Bars represent means; error bars represent mean ± 0.5 SE. (**C**) Anti-LexA western blot showing approximately equal expression levels of corresponding bait fusion proteins (lanes 1–4) used for the liquid β-galactosidase assay in (B).

N192A mutation in KRIT1 resulted in a reduction of the relative strength of the ICAP-1 interaction to ~75% of the wild-type KRIT1/ICAP-1 interaction, and the compound mutation in the putative binding motif further reduced the relative interaction strength to ~58% of the wild-type interaction (Fig. 4B). The reduction of the relative strength of the two-hybrid interaction was not due to reduced expression of the mutant constructs, as the LexA–bait fusions used in the quantitative two-hybrid liquid β-galactosidase assays were expressed at approximately equal levels upon western blotting (Fig. 4C).

We next sought to independently verify the results obtained by the quantitative two-hybrid assay indicating the importance of the NPXY motif on the KRIT1/ICAP-1 interaction by employing the GST fusion affinity precipitation experiment used above. The N192A and N192A/Y195A N-terminal KRIT1 mutations were expressed in tandem in *E.coli* as GST fusions and incubated with 293T cell lysates followed by immunoblotting with anti-ICAP-1 antibody. Both NPXY mutations completely abrogated the N-terminal KRIT1/ICAP-1 interaction as no detectable ICAP-1 was present following immunoblotting (Fig. 3A, lanes 2 and 3). The NPXY mutant GST fusions were produced equally as well as wild-type N-terminal KRIT1–GST in bacteria and equal amounts of mutant and wild-type N-terminal KRIT1–GST protein were utilized in the affinity assay (Fig. 3B). These biochemical data

confirm the importance of the N-terminal KRIT1 NPXY motif as a crucial binding site for ICAP-1.

DISCUSSION

The identification of *KRIT1* as the *CCM1* gene has raised the question of its cellular function, and specifically its role in the angiogenesis and/or remodeling of the cerebrovasculature. Models of KRIT1 function and *CCM1* pathogenesis have hitherto focused on its association with the RAS-family small GTPase RAP1A, suggesting that the loss of KRIT1 function contributes to loss of RAP1A-regulated cell proliferation, resulting in the development of cavernous malformations. In the present study we sought to identify additional protein binding partners that would aid in the characterization of KRIT1 cellular function and provide insight into associated signal transduction pathways. The identification of ICAP-1 as a KRIT1 binding protein represents a new direction in understanding KRIT1 function and *CCM1* pathogenesis—that of cell adhesion processes and extracellular matrix (ECM) interactions mediated by integrin signaling.

Integrin molecules are heterodimeric transmembrane receptors that mediate cell–cell and cell–ECM interactions. Integrin signaling is bidirectional; binding activity to ECM ligands such as fibronectin, laminin, vitronectin and collagen, is influenced by intracellular signaling events ('inside-out' signaling) while binding a particular ECM ligand generates a signal that is transmitted into the cell ('outside-in' signaling) (25). Consistent with the role of integrin and ECM influences in *CCM1* pathogenesis, recent ultrastructural analysis of CCM patient lesions revealed abnormal basal lamina underlying the endothelial cells (1). Abnormalities in the basal lamina might reflect a perturbation of 'inside-out' integrin signaling, potentially due to reduction of the KRIT1/ICAP-1 interaction by *CCM1* mutations.

As the cytoplasmic tails of integrin molecules lack intrinsic enzymatic activity (25), regulation of this 'inside-out' and 'outside-in' integrin signaling is dependent on adaptor proteins that possess enzymatic activity or serve as linkers connecting the integrin tail and the actin cytoskeleton. ICAP-1 is one such integrin-adaptor protein. ICAP-1 is a 200 amino acid phosphoprotein identified as a yeast two-hybrid binding partner for the β_1 integrin cytoplasmic tail (23,24). Northern analyses (23,24) have, similar to KRIT1 (7,15), suggested a relatively broad tissue distribution for *ICAP-1* transcripts. However, the putative murine ortholog of *ICAP-1*, *bodenin*, exhibits a more restricted expression of a gene trap LacZ insertion, which includes the visual system, the heart, and particularly relevant to *CCM1* pathogenesis, specific regions of the forebrain and cerebellum of newborn and adult mice (26).

The ICAP-1 association with the β_1 integrin cytoplasmic tail is highly specific, as demonstrated by lack of interaction with a panel of other β integrin and α cytoplasmic tails (23,24). Other proteins that are capable of binding the β_1 integrin cytoplasmic tail include the protein kinases integrin-linked kinase (27) and focal adhesion kinase (28), and the cytoskeletal proteins filamin (29), α-actinin (30), paxillin (31) and talin (32). These molecules participate in either integrin-mediated signaling as signal transducers, or as cytoskeletal linkers. ICAP-1 lacks significant similarity with any of these β_1 integrin cytoplasmic tail binding proteins (24), and thus does not fall neatly into either the kinase or cytoskeletal protein categories.

ICAP-1 is phosphorylated upon β_1 integrin-mediated cell adhesion (23,24) and may additionally participate in cell migration mediated by β_1 integrins (24). Our biochemical confirmation of the KRIT1/ICAP-1 interaction resulted in the trapping of a single species of ICAP-1. The intensity or migration of this trapped ICAP-1 species upon western analysis with anti-ICAP-1 antibody did not change upon phosphatase inhibitor treatment during 293T lysis (data not shown). Additional experiments will be required to define the exact phosphorylation status of this species of ICAP-1. There is, however, some debate in the literature concerning the number and phosphorylation status of ICAP-1 isoforms (24). Nonetheless, these results suggest that phosphorylation of ICAP-1, and subsequent interaction with KRIT1, might be a crucial regulatory event for integrin-mediated adhesion or cell migration processes.

From our mutational analyses of the putative NPXY binding motif we conclude that, as with β_1 integrins, the NPXY motif in KRIT1 is crucial for ICAP-1 interaction. Sharing of the ICAP-1 binding motif by KRIT1 and β_1 integrins suggests the possibility of functional competition between the two molecules, perhaps utilizing a phosphorylation-dependent or ECM-dependent mechanism. Our GST affinity precipitation data clearly show that the KRIT1 NPXY motif is necessary for ICAP-1 binding. However, residual strength of the KRIT1/ICAP-1 interaction following NPXY mutagenesis in our quantitative two-hybrid assay suggests that it is possible that additional residues in KRIT1 contribute to the NPXY-mediated interaction. Consistent with this hypothesis, a valine residue upstream of the NPXY sequence in the β_1 integrin tail was found to significantly contribute to the integrin/ICAP-1 interaction (23).

In addition to their role as molecular bridges connecting integrin cytoplasmic tails to the actin cytoskeleton, integrin cytoplasmic tails sit at the crossroads for multiple signal transduction pathways. These include signal transduction pathways that drive cell cycle progression, as well as pro-apoptotic pathways (25). Specific integrins are also associated with growth factor receptor pathways and are necessary for optimal activation of growth factor receptors (25). In particular, vascular endothelial growth factor receptor requires appropriate cell adhesion conditions mediated by integrins for proper ligand activation (33). Thus, the KRIT1/ICAP-1 interaction might influence signaling cascades involved in cell cycle progression, apoptotic pathways, or growth factor receptor crosstalk events, any one of which might be affected by mutation of KRIT1.

These diverse signaling pathways may ultimately lead back to the RAS-family member, RAP1A. Although KRIT1 was originally isolated as a binding partner for RAP1A (15), the role of KRIT1 in RAP1A signaling still remains to be determined. The KRIT1/RAP1A interaction has yet to be verified outside the context of the yeast two-hybrid system by more direct biochemical binding assays, and we have observed that KRIT1 and RAP1A in the opposite bait–prey orientation from the original screen (15) fails to yield a two-hybrid interaction.

Nonetheless, our identification of ICAP-1 as a KRIT1 binding partner provides a potential connection between integrin and RAP1A signaling. In this context, KRIT1 may serve as a RAP1A effector, responsible for linking integrin signaling and RAP1A signaling—thus placing the KRIT1/RAP1A interaction

in a new paradigm. Consistent with this hypothesis, RAP1A is known to function in the activation of integrin molecules and has been shown to be a crucial player in integrin-mediated cell adhesion (34–36). More specifically, RAP1A has been implicated in 'inside-out' integrin signaling, as RAP1A mediates the activation of integrins by stimulation of cell surface receptors. As an example, CD31 [platelet endothelial cell adhesion molecule (PECAM)]-induced adhesion is blocked by inactive RAP1A (Rap1N17) (34). RAP1A may also play a role in 'outside-in' signaling (37), where cell adhesion events affect RAP1A activity. Additionally, RAP1A-associated molecules have been shown to regulate integrin adhesive events. There is evidence that a GTPase activating protein for RAP1, SPA-1, affects the regulation of integrin-mediated cell adhesion (38). Thus, *CCM1* mutations in KRIT1 may disrupt its function as a RAP1A effector molecule—thereby disrupting a link between integrin-mediated cell adhesion and downstream RAP1A signaling.

In summary, we have identified a new protein-binding partner for the *CCM1* gene product, the integrin-binding protein ICAP-1. Reduction of the KRIT1/ICAP-1 interaction by *CCM1* protein-truncating mutations may contribute to integrin-mediated processes influential in *CCM1* pathogenesis. This finding represents a new direction for studies aimed at elucidating the molecular mechanisms of *CCM1* pathogenesis—that of cell–ECM adhesive interactions mediated by integrins.

MATERIALS AND METHODS

All plasmids constructed for this study were sequenced to verify the absence of PCR-induced errors.

Cloning of *KRIT1* cDNA

Total HUVEC RNA was used as template for first strand cDNA synthesis with M-MuLV Reverse Transcriptase (Roche, Indianapolis, IN) using oligo dT_{15} (Roche) and random hexamer (Roche) primers according to the manufacturer's instructions. One microliter of the first strand cDNA reaction was used as template for subsequent Pfu Turbo™ (Stratagene, La Jolla, CA) PCR reactions amplifying *KRIT1* in two segments with the following primers: *KRIT1* fragment 1, 5′-CCGGAATTCGGAAATCCAGAAAAC-3′ (sense) and 5′-GCTGAGTTTCTTGAGAGAGACGC-3′ (antisense). KRIT1 fragment 2, 5′-TGTCACAGAAGACAAGGAACGAC-3′ (sense) and 5′-GATCTCGAGTCATGAATTTCTTTCA-3′ (antisense).

The resulting PCR product for fragment 1 was digested with *Eco*RI and *Hin*cII (New England Biolabs, Beverly, MA) and fragment 2 was digested with *Hin*cII and *Xho*I (New England Biolabs). The two *KRIT1* pieces were ligated together and cloned unidirectionally into pBS-SKII (Stratagene) as an *Eco*RI/*Xho*I insert. The clone was used as template for all subsequent PCR reactions creating the *KRIT1* constructs used in this report.

Yeast two-hybrid screening

The following PCR primer pair was used to amplify the N-terminal region of KRIT1; 5′-CCGGAATTCGGAAATC-CAGAAAAC-3′ (sense) and 5′-CCCTCGAGTCACTGCCA-TTTTTCCTGTTTA-3′ (antisense)

The resulting Pfu Turbo™ PCR product was cloned unidirectionally in-frame into the LexA fusion bait vector pMW103 as an *Eco*RI/*Xho*I insert. All two-hybrid plasmids and yeast strains used in this study are part of the LexA-based dual bait two-hybrid system (22; http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html). All liquid and solid yeast media were prepared using Complete Supplement Mixture™ (Qbiogene, Carlsbad, CA) dropout mixes and dropout base supplemented either with dextrose or 2% galactose/1% raffinose for induction of prey plasmid (pJG4-5) expression. pMW103-N-KRIT1 was introduced along with the LacZ reporter plasmid pDR8 (Invitrogen, Carlsbad, CA) into the *Saccharomyces cerevisiae* two-hybrid bait strain SKY 191 (MATα, *trp1*, *his3*, *ura3*, *2lexAop-LEU2*, *3cIop-Lys2*) by standard lithium acetate transformation. Absence of transcriptional activation capability of the LEU2 and LacZ reporters by the bait was verified prior to library screening. Expression of the LexA–N-terminal KRIT1 fusion was verified by western analysis with anti-LexA polyclonal antibody (Invitrogen). The N-terminal KRIT1 bait strain containing the reporter plasmid pDR8 was mated in parallel to ~10^8 c.f.u. of pretransformed HeLa (independent clones, 9.6×10^6; average insert size, 1.5 kb) and human fetal brain (independent clones, 3.5×10^6; average insert size 1.5 kb) pJG4-5-based, oligo-dT primed cDNA libraries (Invitrogen) in the *S.cerevisiae* strain SKY 473 (MATa, *his3*, *trp1*, *ura3*, *4lexAop-LEU2 3cIop-Lys2*). Mating efficiency was determined by plating serial dilutions on media lacking uracil, histidine and tryptophan. Library protein expression of the mated yeast was induced by incubation in galactose/raffinose media for 5 h at 30°C. Approximately 10^7 cells were plated on each of 20 Ura^-, His^-, Trp^-, Leu^- galactose/raffinose plates (10 cm) and allowed to grow for 5 days at 30°C. In subsequent replica platings, positive clones were defined as those which were able to grow on galactose/raffinose media lacking leucine and turned blue upon X-gal (Gold Biotechnology, St Louis, MO)/agarose overlays, while failing to grow and turn blue in parallel assays on media containing dextrose. Library plasmids (pJG4-5) from positive yeast clones were isolated by electroporation of *E.coli* with a yeast lysate followed by plating on media containing 50 μg/ml ampicillin.

Confirmation of specificity of the KRIT1/ICAP-1 two-hybrid interaction

Following isolation of pJG4-5-ICAP-1 (full-length), this plasmid was reintroduced into yeast containing pMW103-N-KRIT1 bait construct and pDR8 reporter plasmid by standard lithium acetate transformation. In parallel, pJG4-5-ICAP-1 was introduced into yeast containing an unrelated bait construct, pMW103-RAP1A. Assessing activation of the LEU2 and LacZ reporters was performed as described above. pMW103-RAP1A/pJG4-5-KRIT1 (full-length KRIT1 prior to the identification of the additional N-terminal exons) interaction was used as a positive control. To perform the bait–prey swapping experiment, the N-KRIT1 and ICAP-1 fragments were excised as *Eco*RI/*Xho*I fragments and cloned into pJG4-5 and pMW103, respectively. A SKY191 yeast strain was created that harbored these two clones and pDR8. A control strain was created in parallel that harbored pMW103-ICAP-1, pJG4-5-RAP1A and pDR8. Interaction phenotypes were assessed as described above.

Yeast two-hybrid assays testing the ICAP-1 interaction with KRIT1 structural domains

The following PCR primer pairs were used to amplify KRIT1 domains used as bait in two-hybrid assays.

Full-length, 5′-CCGGAATTCGGAAATCCAGAAAAC-3′ (sense) and 5′-GATCTCGAGTCATGAATTTCTTTCA-3′ (antisense).

Ankyrin repeats, 5′-GGAATTCGTAGATGATTTTCCTCTC-3′ (sense) and 5′-CCGCTCGAGTCATTTTTCATATGGTTT-3′ (antisense).

FERM domain, 5′-GGAATTCATTAACAAACCATATGAA-3′ (sense) and 5′-CCGCTCGAGTCAGACTTTATGATTGCT-3′ (antisense).

Ankyrin repeats/FERM domain, 5′-GGAATTCGTAGATGATTTTCCTCTC-3′ (sense) and 5′-CCGCTCGAGTCAGACTTTATGATTGCT-3′ (antisense).

C-terminus, 5′-GGAATTCATCCCTGTGTATGTAGGAG-3′ (sense) and 5′-GATCTCGAGTCATGAATTTCTTTCA-3′ (antisense).

Resulting Pfu Turbo™ PCR products were cloned unidirectionally into pMW103 as *Eco*RI/*Xho*I fragments. Sequential lithium acetate transformations were performed to introduce each of the above pMW103 bait constructs along with pDR8 and pJG4-5-ICAP-1 into SKY191 yeast. Interaction phenotypes were assessed as described above.

Site-directed mutagenesis

QuikChange™ site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions for the introduction of NPXY missense mutations into pMW103-N-terminal KRIT1. The following primer pairs were used.

N192A, 5′-AAAAACTAATGTCATAGCTCCTGCATATGCTACTG-3′ (sense) and 5′-CAGTAGCATATGCAGGAGCTATGACATTAGTTTTT-3′ (antisense); N192A/Y195A, 5′-AAAAACTAATGTCATAGCTCCTGCAGCTGCTACTG-3′ (sense) and 5′-CAGTAGCAGCTGCAGGAGCTATGACATTAGTTTTT-3′ (antisense).

Cell culture and lysis

293T cells (a gift of Dr Bryan Cullen, Duke University Medical Center) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO) in a 5% CO_2 environment. DMEM was supplemented with penicillin/streptomycin (Invitrogen) and Amphotericin B (GensiaSicor™ Pharmaceuticals, Irvine, CA) at the manufacturers' recommended concentrations as antibacterial and antifungal agents, respectively. 293T cells were lysed at 4°C in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0) in the presence of a protease inhibitor cocktail (Product P 8340, Sigma) and phosphatase inhibitor cocktails (Products P 2850, P 5726; Sigma). Lysates were cleared of insoluble material by centrifugation at 10 000 *g* for 10 min.

GST fusion affinity precipitation

The N-KRIT1, N-KRIT1 N192A, N-KRIT1 N192A/Y195A and full-length KRIT1 inserts in pMW103 were subcloned as *Eco*RI/*Xho*I fragments into the bacterial expression vector pGEX-5X-1 (Amersham Pharmacia Biotech, Piscataway, NJ) to create GST fusion proteins in the *E.coli* strain BL21 (Amersham Pharmacia Biotech). Empty pGEX-5X-1 vector was transformed into BL21 in parallel as a negative control for the binding assay. Bacterial lysates were prepared and GST fusion proteins were bound to 25 µl of glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The yield of GST fusion proteins was determined by BCA Protein Assay Kit (Pierce, Rockford, IL) and purity was assessed by SDS–PAGE in 5% stacking 15% resolving polyacrylamide gels stained with Coomassie blue. Equal amounts of GST fusion protein immobilized on beads were washed three times with PBS and then incubated overnight at 4°C with 293T cell lysates (prepared as described above) that were precleared with glutathione–Sepharose beads for 1 h at 4°C. GST fusion protein bound beads were subsequently washed three times with PBS. SDS gel-loading buffer was added to the beads and the samples were boiled and subjected to SDS–PAGE on a 5% stacking 15% resolving polyacrylamide gel followed by transfer to Hybond™-P membrane (Amersham Pharmacia Biotech). The membrane was blocked in 1% dry milk/1× TBST overnight at 4°C, followed by incubation with polyclonal anti-ICAP-1 primary antibody (Dr David Chang, UCLA) and anti-rabbit-IgG-HRP (Amersham Pharmacia Biotech) secondary antibody. The membrane was washed four times, 5 min each with 1× TBST followed by ECL™ (Amersham Pharmacia Biotech) detection.

Quantitative liquid β-galactosidase assays

For each two-hybrid interaction to be tested, SKY 191 yeast harboring the pDR8 reporter plasmid, the LexA–bait construct and the prey construct were grown in 2% galactose/1% raffinose (to induce expression of the prey plasmid) dropout media selecting for each plasmid. Prior to the assay, all lithium acetate transformations were performed in parallel to avoid β-galactosidase activity differences due to varying transformation dates. Expression level of wild-type and mutant LexA–bait fusion proteins used in the liquid β-galactosidase assays was assayed by western blotting with anti-LexA polyclonal antibody (Invitrogen) of OD_{600}-normalized yeast culture lysates. Quantitative β-galactosidase assays using ONPG (Sigma) as the substrate were performed in triplicate using independent colonies for each assay. For each assay, β-galactosidase activity values in β-galactosidase units for each construct tested were expressed as a percentage of the wild-type N-KRIT1/ICAP-1 interaction (defined as 100% activity). The resulting percentages were averaged. The assays and β-galactosidase activity values were calculated as described in the Clontech (Palo Alto, CA) Yeast Protocols Handbook, protocol PT3024-1 (http://www.clontech.com/techinfo/manuals/index.shtml) with the following stipulations: (i) due to the strength of the interaction, the recommended 5-fold concentration of cells at step 7 was not performed in order to remain within the linear range of the assay and (ii) each ONPG reaction was allowed to proceed for 5 min before the addition of Na_2CO_3 (step 16).

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