ACHERON, AN NOVEL LA ANTIGEN FAMILY MEMBER, BINDS TO CASK AND FORMS A COMPLEX WITH ID TRANSCRIPTION FACTORS

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Abstract: Acheron, a Lupus antigen ortholog, was identified as a novel death-associated transcript from the intersegmental muscles of the moth Manduca sexta. Acheron is phylogenetically-conserved and represents a new sub-family of Lupus antigen proteins. Acheron is expressed predominantly in neurons and muscle in vertebrates, and regulates several developmental events including myogenesis, neurogenesis and possibly metastasis. Using Acheron as bait, we performed a yeast two-hybrid screen with a mouse embryo cDNA library and identified CASK-C, a novel CASK/Lin-2 isoform, as an Acheron binding partner. Acheron and CASK-C bind via the C-terminus of Acheron and the CaMKII-like domain of CASK-C. Co-immunoprecipitation assays verify this interaction and demonstrate that Acheron also forms a complex with all members of the Id (inhibitor of differentiation) proteins. Taken together, these
data suggest a mechanism by which Acheron may regulate development and pathology.

**Key words**: Programmed cell death, *Manduca sexta*, Muscle, Apoptosis, Lupus antigen protein

**INTRODUCTION**

Acheron (Achn) is a newly described protein that is structurally related to the LA/SSB (Lupus antigen/Sjögren Syndrome) protein but defines a distinct subfamily that is highly conserved across phylogeny [1]. This 64 kDa protein contains a highly conserved N-terminal three imperfect Lupus antigen (LA) repeats consisting of 71 amino acids (amino acids 99-171), RNA recognition motif (amino acids 184–296), an RNA binding motif, and functional nuclear localization and export signals. As well, it has several putative phosphorylation and amidation sites.

Achn was identified as a novel death-associated transcript from the intersegmental muscles (ISMs) of the moth *Manduca sexta* [1]. Moth and human Achn display 31% identity and 40% similarity overall, and 42% identity and 54% similarity in a conserved 227 amino acid region [1]. Western blot analysis of mouse tissue has shown that Achn is most widely expressed in neurons and skeletal muscle [1]. Developmental studies in zebrafish embryos and mouse myoblasts have demonstrated that Achn acts upstream of the muscle specific transcription factor MyoD and plays an essential role in myogenesis (Wang, Z., Glenn, H., Liu, J.X., Seth, A., Karlstrom, R.O. and Schwartz, L.M., submitted). In addition, Achn expression is elevated in some basal-like human breast cancers and ectopic expression of Achn is sufficient to drive angiogenesis and tumor growth in human breast cancer cells in xenograph animal models (Shao, Scully, Yan, Bentley, Mueller, Brown and Schwartz, submitted).

To help determine how Achn might function as a signal transduction protein, we performed a yeast two-hybrid screen. In the present study we demonstrate that human Achn binds to CASK/Lin-2 (Ca2+/calmodulin-dependent serine protein kinase). CASK was originally identified as a neurexin-interacting partner in rat brain [2]. CASK is a membrane-associated guanylate kinase protein (MAGUK) [3] and regulates lineage-specific decisions in a variety of taxa, including nematodes and mammals [4,5]. CASK is considered to be a pseudokinase because the functionally important residues within CaMKII domain are mutated [6]. CASK functions as a scaffold protein that is targeted to neuronal synapses and cell-cell junctions. It has been shown to participate in the regulation of signaling by ion channels, receptors, cell adhesion molecules, and heparan sulfate in several cell types such as epithelial cells, motor neurons, and muscles [7]. CASK can also function as a transcriptional co-activator although it lacks a nuclear localization signal and is dependent on binding partners for translocation into the nucleus [8].
CASK binds to members of the inhibitor of differentiation (Id) family of helix-loop-helix (HLH) transcription factors, which play key roles in proliferation, differentiation and oncogenesis [9]. In this study, we have used yeast two-hybrid assays to identify a new isoform of CASK as a novel Achn-interacting protein and defined the interaction domains between these two proteins. We also demonstrate that Achn binds with all known Id protein isoforms. Our data suggest that Achn/CASK/Id form a novel complex and that may regulate developmental processes and/or cancer.

MATERIALS AND METHODS

Yeast strains and manipulations

Saccharomyces cervesiae strains Y190 and CG1945 (Clontech, Matchmaker GAL4 Two-Hybrid User Manual) were maintained in YPD medium: YP (yeast extract plus peptone) medium plus 2% glucose; SD medium contains 2% glucose; or Drop Out medium (DO) lacking the appropriate selection medium (e.g. SD/-ura/-trp/-leu media lacking uracil, tryptophan or leucine). Plasmid DNAs were introduced into yeast by standard LiOAc-mediated transformation.

Yeast two-hybrid screen

Human Acheron cDNA (Achn) [1] lacking the coding region for the first 33 amino acids, was amplified by PCR with primers containing the SalI restriction sites and subcloned in frame to SalI site of pAS2-1 to form pAS2-1-Achn as the bait. pAS2-1-Achn was transformed into yeast CG1945 and the expression of the fusion protein verified by Western blotting using an anti-Achn antiserum [1]. No autonomous activation was observed in yeast carrying the bait pAS2-1-Achn vector.

Mouse 17-day embryo cDNA library cloned into the pACT2 vector was obtained from Clontech, transformed into E. coli and then amplified on LB/amp plates at high density. All the colonies were collected and the plasmid DNA was isolated and purified using a Qiagen plasmid DNA purification (Promega) Kit. The pACT2 plasmid library was transformed into the CG1945 yeast containing the pAS2-1-Achn plasmid and a total of $4.8 \times 10^6$ independent colonies were plated on SD/-ura/-trp/-leu/-his + 3 mM 3-AT (3-amino-1,2,4-triazole). Large colonies were streaked and subjected to β-galactosidase colony-lift filter assay. Yeast colonies displaying both HIS3 and LacZ reporter gene activation were selected for subsequent analysis. HIS3$^+$ and LacZ$^+$ colonies were selected and the plasmids isolated and transfected into E. coli (KC8) followed by selection on M9 minimal medium lacking leucine. Library plasmids encoding proteins that appeared to interact with the Achn bait were sequenced and analyzed via BLAST searches.

To confirm the interaction between Achn and the candidate partners, the library and the pAS2-1-Achn bait plasmids were co-transformed into yeast strain Y190.
and grown on SD/-ura/-trp/-leu/-his + 50 mM 3-AT and a β-galactosidase colony-lift filter assay was performed.

**GST pull-down assays**
The CaM kinase II domain of mouse CASK-C (mCASK-C) cDNA was amplified by PCR, cloned in-frame into the SmaI site of pGEX-2T, and transformed into *E. coli* BL21 cells. Cultures were induced with 0.4 mM IPTG and recombinant proteins were affinity-purified from bacterial lysates using glutathione-Sepharose 4B beads (Pharmacia Biotech). For pull-down assays, both $^{35}$S-labeled Achn and $^{35}$S-labeled luciferase were synthesized from pET-25b(+)Achn and pET-25b(+)luciferase plasmids using the In Vitro TNT Quick Coupled Transcription/Translation System (Promega) with $^{35}$S-methionine as the sole source of methionine, following the manufacturer’s instructions. 5 μl of $^{35}$S-methionine-Achn or $^{35}$S-methionine-luciferase were incubated with equal amounts of GST or GST-mCASK-C (CaM kinase II domain) bound to glutathione-Sepharose 4B beads respectively, under constant rocking for 45 min in 2 ml of NETN binding buffer at room temperature. $^{35}$S-methionine-luciferase and GST served as the controls in the binding assays. The Sepharose beads were pelleted, washed extensively with the binding buffer and fractionated by size via SDS 10% PAGE and analyzed via film autoradiography.

**Constructs for deletion analysis**
Achn deletion mutants were generated by PCR amplification with primers complementary to the pAS2-1-Achn template. The PCR products were digested with SalI and subcloned in-frame into the SalI site of pAS2-1. The resulting constructs were named after the amino acids they encoded: pAS2-1-Achn (47-380); pAS2-1-Achn (47-405); pAS2-1-Achn (47-432); pAS2-1-Achn (47-472); and pAS2-1-Achn (373-480). The BamHI fragment from pAS2-1-Achn was cloned in frame to BamHI site of pAS2-1 to generate pAS2-1-Achn (47-283). The BamHI digested pAS2-1-Achn vector was self-ligated to form pAS2-1-Achn (238-480).

mCASK-C deletion mutants were generated by PCR amplification with complementary primers to pACT2-mCASK-C template. The PCR products were digested with EcoRI and XhoI and then cloned in frame to pACT2. The generated constructs were: pACT2-mCASK-C (1-105); pACT2-mCASK-C (1-280); pACT2-mCASK-C (1-304); pACT2-mCASK-C (1-315); pACT2-mCASK-C (1-339); and pACT2-mCASK-C (350-897).

**Co-immunoprecipitation assays**
COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum plus 1% penicillin/streptomycin in 5% CO₂. Equal amounts of HA-CASK-C or RFP-CASK-C were co-transfected into COS-1 with either pBABE empty vector or FLAG-Achn (3.0-3.5 mg) using Lipofectamine 2000 (Invitrogen). Forty eight hours later, cells were washed
3 times with PBS and lysed in 500 ml of modified lysis buffer (1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride in isopropanol, 5mM aprotinin, 0.2 trypsin inhibitor units/ml) prepared in TSA solution (0.1M Tris-HCl buffer, pH 8.0, 0.14M NaCl, 0.025% NaN3). Cell lysates were incubated for 1 hour at 4°C with gentle rotation, followed by centrifugation for 5 min. at 4°C at maximum rpm in a microcentrifuge. The supernatants were transferred into new tubes and pre-cleared with Sepherose-G beads (Amersham Pharmacia Biotech) for 2 hours at 4°C. After one minute-centrifugation at 4°C, the supernatants were transferred to new tubes and 10% of each sample’s volume (60 ml) was removed. The rest of the sample was incubated with monoclonal anti-FLAG antibody, M2, (1:150) (Sigma) with Sepherose-G beads at 4°C overnight. After one-minute centrifugation at 4°C, the supernatants were discarded and precipitates were washed 3X with PBST (PBS+ 5% Tween-20). After final washing, an equal volume (60 ml) of PBST and 2X sample buffer was added to bead-containing precipitates. 40 μl of each sample was loaded into 4-15% PAGE gel (BioRad) followed by Western blotting using polyclonal anti-CASK antibody (1:1000) (Zymed). Signals were enhanced by ECL (PerkinElmer) and visualized on Kodak film. As a positive control, we performed co-immunoprecipitation assays with myc-human parkin and HA-CASK-C using polyclonal anti-CASK antibody for immunoprecipitation and monoclonal anti-myc antibody (9E10: Covance). In order to determine if FLAG-Achn interacts with Id proteins, an equal amount of plasmids encoding FLAG-Achn and one of GFP-Ids (GFP-Id1-4 (generously provided by Dr. Kurooka, University of Fukui) or pEGFP-N1 (Invitrogen) were transfected into COS-1 cells. The same procedures were conducted except using polyclonal anti-GFP antibody (1:1000) (Molecular probe) for Western blots.

RESULTS

Yeast two-hybrid screen for Achn-interacting proteins
The human Achn cDNA (lacking the first 33 amino acids) was cloned in-frame with the C-terminus of the DNA-binding domain of the yeast GAL4 transcription factor to create the bait in the plasmid pAS2-1. pAS2-1-Achn was transformed into the yeast strain CG1945 carrying two reporter genes, HIS3 and LacZ. Western blotting with an anti-human Achn antiserum was used to confirm the expression of the Achn-Gal4 fusion protein (data not shown). Expression of the bait protein alone was insufficient to activate the expression of either of the two reporter genes used in this study. To identify the potential Achn binding partners, a day 17 mouse embryo cDNA library was amplified and transformed into yeast strain CG1945 containing bait pAS2-1-Achn. Approximately 4.8 × 10^6 transformants were plated and two separate clones were confirmed positive for both HIS3 and LacZ expression. The two prey plasmids were isolated and subjected to DNA sequence analysis. One of the plasmids encoded mouse ariadne, a ubiquitin E3 ligase that is the subject of a separate report (Wang, Z., Parelkar, S., Kim, C. and Schwartz, L.M.
unpublished). The second plasmid encoded a full-length member of the CAMGUK family that we have named mCASK-C (described below). To further confirm the interaction between Achn and mCASK-C, we conducted a second two-hybrid assay utilizing yeast strain Y190 (Fig. 1). Isolated prey plasmid pACT2-mCASK-C, bait plasmid pAS2-1-Achn, vector plasmid pAS2-1 and pACT2 were co-transformed into yeast strain Y190 in various combinations.

Fig. 1. Achn physically interacts with mCASK-C in yeast two-hybrid assays. The yeast strain Y190 was co-transfected with bait (pAS2-1) and prey (pACT2) plasmids with different combinations of Achn and mCASK-C. The cells were then streaked on plates containing drop out medium lacking: tryptophan, histidine, uracil, and leucine. The plates also contained 50 mM 3-amino-1,2,4-triazole (AT) to inhibit HIS3 protein. Only the combination of pAS2-1-Achn and pACT2-mCASK-C was viable. The transformants were subjected to the growth on SD/-ura/-trp/-leu/-his + 50 mM 3-AT and β-galactosidase colony filter lift assays were performed. Only yeast strain Y190 carrying both pAS2-1-Achn and pACT2-mCASK-C displayed both survival in the absence of histidine and was positive LacZ expression. Transformants carrying any other combinations of plasmids failed to either survive (Fig. 1) or express LacZ (data not shown), suggesting that the GAL4-BD-Achn fusion protein did not interact with GAL4-AD and the GAL4-AD-mCASK-C fusion protein did not interact with the GAL4-BD protein. Thus co-expression of the LacZ and HIS3 reporter genes required the interaction of GAL4-BD-Achn and GAL4-AD-mCASK-C fusion proteins.

To determine if Achn binds to itself, we subcloned Achn in-frame into the pACT2 vector. pACT2-Achn and pAS2-1-Achn were co-transformed into yeast strain Y190 and the transformants tested for LacZ expression. No signal was detected suggesting that Achn did not associate with itself to form dimmer (data not shown).

**Coding region of mCASK-C**

The coding region of mCASK-C spans 2694 nucleotides and encodes a protein of 897 amino acids. It shares 95% identity at the DNA level and 99.6% identity at protein level with human CASK, with only three amino acid differences
Like human and rat CASK and mouse CASK-B, the putative mCASK-C is composed of a series of well characterized domains: the N-terminal CaM kinase II domain (aa 1-339) that contains both a protein kinase (aa 12-276) and calmodulin binding subdomains (aa 305-315); the C-terminal PDZ domain (aa 483-558); an SH3 domain (aa 587-652); and a guanylate kinase-like domain (GUK) domain (aa 710-831). This combination of N-terminal CaM kinase II domain and C-terminal MAGUK domains places mCASK-C in the in the CAMGUK family of kinases [10]. The CaM kinase II and PDZ domains of mCASK-B and C, human CASK, and rat CASK are all identical except one amino acid difference between mouse CASK-B and others (L<sup>298</sup> against F<sup>298</sup>). The SH3 domain and GUK domains of these four proteins are highly conserved. However, mCASK-C lacks 6 amino acids (aa 340-345) that are present just downstream CaMKII in CASK-B and another 23 amino acids (aa 580-602) downstream PDZ domain. Based on intron-exon analysis, it appears that the loss of these two regions results from alternative splicing of the CASK transcript (data not shown).

**SH3 Domains**

|        | mCASK-C | hCASK | mCASK-B | rCASK |
|--------|---------|-------|---------|-------|
|        | TVRAQFEYDF AKDDLIPCKE AGIRFRVGD1IQIISKDDKN WWQKLENSK |
|        | NGAAGQEPF SLQWR |

**GUK Domains**

|        | mCASK-C | hCASK | mCASK-B | rCASK |
|--------|---------|-------|---------|-------|
|        | RKLTVLGAH GVGRHRKNT LITKHEDRFAY PITPHTRRP KDDEBARGNY |
|        | YFVSHDQQQ DISNNEYSEY GSHEDAGKGT KLETIRKHE QGLIAIDVE |
|        | PQALKVLRTAEAPFVVF1AAP |

Fig. 2. Protein sequence comparisons of the conserved domains SH3 and GUK among mCASK-C, human CASK, mouse CASK-B and rat CASK. Identical residues are blocked in black, conserved residues are shaded.
In vitro binding assays
To further verify the physical interaction between Achn and mCASK-C, we performed two different in vitro binding assays: GST pull-down and co-immunoprecipitation. For the former, $^{35}$S-labeled Achn was synthesized by coupled in vitro transcription/translation and then incubated with either GST or GST-mCASK-C (CaMKII domain from amino acid 1 to 339) immobilized on glutathione-Sepharose 4B beads. Following washing, the bound proteins were extracted, fractionated by PAGE and detected via film autoradiography (Fig. 3). Achn was found to bind with GST-mCASK-C but not with GST alone. As an additional control, we incubated $^{35}$S-labeled luciferase with GST-mCASK-C and observed that there was no binding, suggesting that the interaction between Achn and mCASK-C is specific.

![Fig. 3. Interaction of Achn and mCASK-C in vitro. Achn physically interacted with CaM kinase II domain of mCASK-C (amino acid 1-339) but not with GST. CaM kinase II domain of mCASK-C did not associate with control protein luciferase. The Achn band is located at 64 kDa.](image)

To perform co-immunoprecipitation assays, COS-1 cells were co-transfected with HA-tagged mCASK-C and either the empty pBabe vector, or one encoding FLAG-tagged Achn. After 48 hours, immunoprecipitation was performed with an anti-FLAG antibody bound to Sephasrose-G beads. The proteins were then fractionated via PAGE and used to generate a Western blot that was probed with a polyclonal anti-CASK antiserum. No specific bands were detected in the negative control assays using pBabe and either HA-CASK or RFP-CASK (Fig. 4A). As a positive control, we demonstrated the interaction between human CASK and parkin [11]. Both endogenous CASK-B/C and exogenous HA-CASK-C and RFP-CASK-C forms were co-precipitated with FLAG-Achn (arrows). The similar sizes of the CASK B and C preclude us from determining which endogenous isoforms were co-precipitated. Another protein that may be CASK-A, based on size, also precipitated in this assay (arrow head).
Fig. 4. Co-Immunoprecipitation assays with Achn, CASK and Id proteins. A – Achn interacted with both endogenous CASK and exogenous CASK. COS-1 cells were transfected with FLAG-Achn and HA-CASK or RFP-CASK. After lysis, immunoprecipitation was conducted with mouse monoclonal anti-FLAG. The precipitants were then fractionated in 4-15% PAGE gels followed by Western blotting with a rabbit anti-CASK polyclonal antibody. The samples from cells co-transfected with empty pBABE vector and either HA-CASK or RFP-CASK did not display positive signals. Co-transfection with plasmids encoding CASK and its binding partner parkin served as a positive control. MWt = protein sizes in kDa. B – COS-1 cells were transfected with FLAG-Achn plus one of the mammalian expression constructs encoding eGFP fusions to each of the four known Id proteins. After 48 hours, cells were lysed and immunoprecipitated with a mouse monoclonal anti-FLAG antibody. The precipitants were then fractionated in 4-15% PAGE gels followed by Western blotting with a polyclonal anti-GFP antibody. Achn interacted with all isoforms of eGFP-Ids. The samples from Achn and EGFP transfected cells served as a negative control and did not display signals.

Defining the Achn and mCASK-C interaction domains

To define the domain(s) within Achn that physically interact with mCASK-C, a series of Achn deletion mutants were generated and fused in-frame with the DNA-binding domain of Gal4 in pAS2-1. Each of these constructs was co-transformed with pACT2-mCASK-C into the yeast Y190 strain and the transformants subjected to the colony-lift filter assay to test for LacZ expression. A region in the carboxy-terminus of Achn (aa 373-472) was found to be necessary and sufficient for physical interaction with mCASK-C (Fig. 5A). Longer regions of Achn that contained this N-terminal region were also able to both bind to mCASK-C and activate LacZ expression, but loss of this region abrogated interaction.

In a complementary set of experiments, we created a series of deletion mutations in mCASK-C and tested them to determine which domain(s) were required for interaction with Achn (Fig. 5B). A domain within the mid-region of mCASK-C (between amino acids 280 and 304) was found to be required for interaction with Achn. A portion of the N-terminus containing amino acids 1-280 failed to induce LacZ expression, while a slightly larger fragment (amino acids 1-304) was effective. Structural analysis suggests that the region between mCASK-C 280 and 304 maps to the C-terminal region of the protein kinase domain of CASK.
Fig. 5. Determine the interaction regions for Achn and mCASK-C. A – Yeast strain Y190 was co-transformed with pACT2-mCASK-C and each of indicated pAS2-1- Achn deletant. The transformants were subjected to β-galactosidase assay. The La is indicated as dark gray box. Nucleus localization sequence (NLS) is indicated as light gray box. The number above each construct represents amino acid positions of Achn protein.

| Construct               | β-Galactosidase Assay |
|-------------------------|-----------------------|
| pAS2-1-Achn             | +                     |
| pAS2-1-Achn (47-238)    | -                     |
| pAS2-1-Achn (238-480)   | +                     |
| pAS2-1-Achn (47-380)    | -                     |
| pAS2-1-Achn (373-480)   | +                     |
| pAS2-1-Achn (47-472)    | +                     |
| pAS2-1-Achn (47-432)    | -                     |
| pAS2-1-Achn (47-405)    | -                     |

B

| Construct               | β-Galactosidase Assay |
|-------------------------|-----------------------|
| pACT2-mCASK-C           | +                     |
| pACT2-mCASK-C (1-339)   | +                     |
| pACT2-mCASK-C (350-897) | -                     |
| pACT2-mCASK-C (1-105)   | -                     |
| pACT2-mCASK-C (1-280)   | -                     |
| pACT2-mCASK-C (1-304)   | +                     |
| pACT2-mCASK-C (1-315)   | +                     |

CaM kinase II Domain
protein kinase subdomain
binding subdomain

Y190 was co-transformed with pAS2-1- Achn and each of indicated pACT2-mCASK-C deletant. The transformants were subjected to β-galactosidase assay. The CaM kinase II domain is indicated as light gray box. Inside there are two subdomains, protein kinase subdomain and calmodulin binding subdomain, indicated as dark gray and black box, respectively. The number above each construct represents amino acid positions of mCASK-C protein.
**Achn forms a complex with CASK and all known ID proteins**

CASK binds to Id1, a transcription factor that has been implicated in both differentiation and oncogenesis [12]. Since we have demonstrated that CASK binds to Achn, we wanted to test the hypothesis that Achn forms a complex that includes CASK and Id1. COS-1 cells were transfected with a FLAG-Achn construct and expression constructs encoding eGFP-fusions with Id1, Id2, Id3 or Id4. Expression of eGFP alone was used as a negative control. Co-immunoprecipitations were performed with anti-FLAG mouse monoclonal antibody and the resulting Western blots probed with a rabbit anti-GFP polyclonal antiserum (Fig. 4B). Precipitation of Achn also pulled down each of the Ids. Since COS-1 cells express high levels of endogenous CASK (Kim and Schwartz, unpublished), it is unclear if the interaction between Achn and the Id proteins is direct or via CASK. Nevertheless, these data support the hypothesis that CASK, Achn and the Id proteins interact within cells.

**DISCUSSION**

Achn was initially discovered in a molecular screen to identify genes that are induced when the intersegmental muscles of moth initiate programmed cell death at the end of metamorphosis [1]. Transcripts for Achn are barely detectable in the ISMs of *Manduca* until late on day 17 of pupal-adult development, when the cells become committed to die, at which point it becomes a very abundant mRNA. Treatment on day 17 with the steroid hormone 20-hydroxyecdysone delay both ISM death and the expression of Achn mRNA [1]. Thus, expression of Achn mRNA is regulated by the same endocrine factor that controls ISM death. These data suggest that Achn is a signal transduction molecule that can regulate developmental decisions, one of which may be death. This hypothesis was further supported by studies with C2C12 mouse myoblasts, where several lines of evidence demonstrate that Achn plays an essential permissive role for both myotube formation and apoptosis following the loss of growth factors (Wang, Z., Glenn, H., Liu, J.X., Seth, A., Karlstrom, R.O., and Schwartz, L.M. submitted). We obtained similar results in zebrafish embryos injected with an Achn antisense morpholino.

Database analysis revealed that Achn represents a new subfamily of Lupus antigen/Sjögren’s syndrome B (LA/SSB) proteins [1]. Human La was identified as a major target of autoimmune response in patients with Sjögren’s syndrome or systemic lupus erythematosus. Its functions are elusive, but several lines of evidence suggest that La proteins contain an RNA recognition motif (RRM) and bind nascent RNA polymerase III transcripts. The La proteins function as chaperone for both RNA maturation and retention in nucleus [13-15]. Achn homologs are found in the genomes of chordates and arthropods, but unlike La protein, it does not appear to be present in nematodes or yeast [1]. La is not essential for viability in yeast but loss-of-function mutations in La in mammals
results in death prior to the formation of the inner cell mass [16, 17]. Beyond that, little is known about the role of Lα protein in developmental decisions. To gain better insight into the molecular mechanisms that might mediate Achn function, we performed a yeast two-hybrid analysis with human Achn as the bait and an embryonic mouse cDNA library as the prey. This genetic screen produced two positive binding partners. The first was ariadne, a ubiquitin E3 ligase that shares substantial structural and functional identity with the parkin E3 ligase [Wang, H, Wang, Z., Valavanis, C. and Schwartz, L.M. unpublished, 18]. The second cDNA isolated in this screen encoded CASK-C, a novel member of the CASK family of MUGUK proteins. The primary difference between CASK-C and the previously described CASK-A and CASK-B isoforms is the absence of six amino acids downstream of the CaMK-like domain in CASK-B, and another 23 amino acids downstream of the PDZ domain. It is not know how the loss of these 29 amino acids affects the function and/or binding of CASK, but it may provide a tool for differential regulation of CASK-dependent signaling events.

CASK is a unique member of membrane-associated guanylate kinase (MAGUK) family proteins since it is the only one that contains a CaMKII-like domain. Like other in MAGUKs, CASK is a scaffold protein in many different cell types including: epithelial cells, neurons, and muscles [19-23]. CASK has been shown to bind a number of signaling proteins, including: potassium channels (eg. Ether-á-go-go [24] and the inward rectifier Kir2 potassium channels [25]; calcium channels [26]; cytoplasmic adaptor proteins (eg. Mint1, Veli/mLin-7/Mals [25, 27], SAP97 [25], caskin [28], CIP98 [29]; adhesion proteins (eg. neurexin [2] and syndecans [30]. CASK can form complexes with a variety of proteins at cell adhesion regions and is involved in cell polarization, channel-mediated signaling pathways, and exocytosis of synaptic vesicles.

CASK can shuttle between the membrane and the nucleus where it facilitates target gene transcription. For example, CASK binds to the T-box transcription factor Tbr-1 via its GUK domain and translocates to the nucleus where it binds the Id1 inhibitor of differentiation and the CINAP, a nucleosome assembly protein [8, 9, 31]. In cerebellar neurons, CASK can drive the expression of the secreted extracellular glycoprotein reelin [8]. Interestingly, Achn is highly expressed in the axons of cerebellar neurons in both mouse and human (Schwartz, L.M., Moral, L., Zoeller, T., Bentley, B., Brown, C. and Mueller, J. unpublished).

Since CASK does not have its own nuclear localization or export signals, it is dependent upon other proteins like Tbr-1 to facilitate its translocation [8]. Achn, like Tbr-1, has functional nuclear localization and export signals (Shao et al., submitted) and binds to CASK. However unlike other nuclear proteins that interact with CASK, yeast two-hybrid assays demonstrated that Achn binds to its CaMKII-like domain rather than the more typical GUK domain [9]. It should be noted that indirect interactions between Achn and CASK may also exist. Co-immunoprecipitation data has shown that both ectopic (Fig. 4A) and endogenous CASK (data not shown) can bind to Achn in vivo. However, since
endogenous CASK-A may also bind, despite the lack of a CaMKII domain, it is possible that some of these interactions are indirect and may be mediated by a common partner(s), such as Id proteins. Nevertheless, the yeast two-hybrid data demonstrate a specific physical interaction between CASK and Achn.

Several questions remain to be addressed. First, does Achn facilitate nuclear translocation in some cells to mediate CASK-dependent developmentally regulated gene expression? Second, does the binding of Achn to CASK prevent other kinases from phosphorylating Achn?

In summary, we have demonstrated that Achn physically binds to a novel member of the CASK family of MUGUK proteins and that this interaction requires a C-terminal region of Achn and the CaMKII-like domain of mCASK-C. We have further demonstrated that Achn can bind, directly or indirectly, to all known members of the Id family of transcriptional repressors. These data provide new insights into CASK signaling and help define possible mechanisms that mediate Achn function.

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REFERENCES

1. Valavanis, C., Wang, Z., Sun, D., Vaine, M. and Schwartz, L.M. Acheron, a novel member of the Lupus antigen family, is induced during the programmed cell death of skeletal muscles in the moth *Manduca sexta*. Gene 393 (2007) 101-109.

2. Hata, Y., Butz, S., and Sudhof, T.C. CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. *J. Neurosci.* 16 (1996) 2488-2494.

3. Dimitratos, S.D., Woods, D.F. and Bryant, P.J. Camguk, Lin-2, and CASK: novel membrane-associated guanylate kinase homologs that also contain CaM kinase domains. *Mech. Dev.* 63 (1997) 127-130.

4. Caruana, G. Genetic studies define MAGUK proteins as regulators of epithelial cell polarity. *Int. J. Dev. Biol.* 46 (2002) 511-518.

5. Atasoy, D., Schoch, S., Ho, A., Nadasy, K.A., Liu, X., Zhang, W., Mukherjee, K., Nosyreva, E.D., Fernandez-Chacon, R., Missler, N., Kavalali, E.T. and Sudhof, T.C. Deletion of CASK in mice is lethal and impairs synaptic function. *Proc. Natl. Acad. Sci. USA* 104 (2007) 2525-2530.

6. Boudeau, J., Miranda-Saavedra, D., Barton, G.J. and Alessi, D.R. Emerging roles of pseudokinases. *Trends Cell Biol.* 16 (2006) 443-452.
7. Hsueh, Y.P. The role of the MAGUK protein CASK in neural development and synaptic function. *Curr. Med. Chem.* 13 (2006) 1915-1927.
8. Hsueh, Y.P., Wang, T.F., Yang, F.C. and Sheng, M. Nuclear translocation and transcription regulation by the membrane-associated guanylate kinase CASK/LIN-2. *Nature* 404 (2000) 298-302.
9. Qi, J., Su, Y., Sun, R., Zhang, F., Luo, X., Yang, Z. and Luo, X. CASK inhibits ECV304 cell growth and interacts with Id1. *Biochem. Biophys. Res. Commun.* 328 (2005) 517-521.
10. Laverty, H.G. and Wilson, J.B. Murine CASK is disrupted in a sex-linked cleft palate mouse mutant. *Genomics* 53 (1998) 29-41.
11. Fallon, L., Moreau, F., Croft, B.G., Labib, N., Gu, W.J. and Fon, E.A. parkin and CASK/LIN-2 associate via a PDZ-mediated interaction and are co-localized in lipid rafts and postsynaptic densities in brain. *J. Biol. Chem.* 277 (2002) 486-491.
12. Fong, S., Debs, R.J. and Desprez, P.Y. Id genes and proteins as promising targets in cancer therapy. *Trends Mol. Med.* 10 (2004) 387-392.
13. Alfano, C., Sanfelice, D., Babon, J., Kelly, G., Jacks, A., Curry, S. and Conte, M.R. Structural analysis of cooperative RNA binding by the La motif and central RRM domain of human La protein. *Nat. Struct. Mol. Biol.* 11 (2004) 323-329.
14. Maraia, R.J. and Intine, R.V. La protein and its associated small nuclear and nucleolar precursor RNAs. *Gene Expr.* 10 (2002) 41-57.
15. Ohndorf, U.M., Steegborn, C., Knijff, R. and Sondermann, P. Contributions of the individual domains in human La protein to its RNA 3' end binding activity. *J. Biol. Chem.* 276 (2001) 27188-27196.
16. Wolin, S.L. and Cedervall, T. The La protein. *Annu. Rev. Biochem.* 71 (2002) 375-403.
17. Park, J.M., Kohn, M.J., Bruinsma, M.W., Vech, C., Intine, R.V., Fuhrmann, S., Grinberg, A., Mukherjee, I., Love, P.E., Ko, M.S., DePamphilis, M.L. and Maraia, R.J. The multifunctional RNA-binding protein La is required for mouse development and for the establishment of embryonic stem cells. *Mol. Cell Biol.* 26 (2006) 1445-1451.
18. Ardley, H.C., Tan, N.G., Rose, S.A., Markham, A.F. and Robinson, P.A. Features of the parkin/ariadne-like ubiquitin ligase, HHARI, that regulates its interaction with the ubiquitin-conjugating enzyme, Ubch7. *J. Biol. Chem.* 276 (2001) 19640-19647.
19. Lehtonen, S., Lehtonen, E., Kudlicka, K., Holthofer, H. and Farquhar, M.G. Nephrin forms a complex with adherens junction proteins and CASK in podocytes and in Madin-Darby canine kidney cells expressing nephrin. *Am. J. Pathol.* 165 (2004) 923-936.
20. Sanford, J.L., Mays, T.A. and Rafael-Fortney, J.A. CASK and Dlg form a PDZ protein complex at the mammalian neuromuscular junction. *Muscle Nerve* 30 (2004) 164-171.
21. Schuh, K., Uldrijan, S., Gambaryan, S., Roethlein, S. and Neyses, L. Interaction of the plasma membrane Ca2+ pump 4b/Cl with the Ca2+/calmodulin-dependent membrane-associated kinase CASK. *J. Biol. Chem.* **278** (2003) 9778-9783.

22. Lee, S., Fan, S., Makarova, O., Straight, S. and Margolis, B. A novel and conserved protein-protein interaction domain of mammalian Lin-2/CASK binds and recruits SAP97 to the lateral surface of epithelia. *Mol. Cell Biol.* **22** (2002) 1778-1791.

23. Martinez-Estrada, O.M., Villa, A., Breviario, F., Orsenigo, F., Dejana, E. and Bazzoni, G. Association of junctional adhesion molecule with calcium/calmodulin-dependent serine protein kinase (CASK/LIN-2) in human epithelial caco-2 cells. *J. Biol. Chem.* **276** (2001) 9291-9296.

24. Marble, D.D., Hegle, A.P., Snyder II, E.D., Dimitratos, S., Bryant, P.J. and Wilson, G.F. Camguk/CASK enhances Ether-á-go-go potassium current by a phosphorylation-dependent mechanism. *J. Neurosci.* **25** (2005) 4898-4907.

25. Leonoudakis, D., Conti, L.R., Radeke, C.M., McGuire, L.M. and Vandenberg, C.A. A multiprotein trafficking complex composed of SAP97, CASK, Veli, and Mint1 is associated with inward rectifier Kir2 potassium channels. *J. Biol. Chem.* **279** (2004) 19051-19063.

26. Maximov, A., Sudhof, T.C. and Bezprozvanny, I. Association of neuronal calcium channels with modular adaptor proteins. *J. Biol. Chem.* **274** (1999) 24453-24456.

27. Butz, S., Okamoto, M. and Sudhof, T.C. A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. *Cell* **94** (1998) 773-782.

28. Tabuchi, K., Biederer, T., Butz, S., and Sudhof, T.C. CASK participates in alternative tripartite complexes in which Mint 1 competes for binding with caskin 1, a novel CASK-binding protein. *J. Neurosci.* **22** (2002) 4264-4273.

29. Yap, C.C., Liang, F., Yamazaki, T., Muto, Y., Kishida, H., Hayashida, T., Hashikawa, T. and Yano, R. CIP98, a novel PDZ domain protein, is expressed in the central nervous system and interacts with calmodulin-dependent serine kinase. *J. Neurochem.* **85** (2003) 123-134.

30. Cohen, A.R., Woods, D.F., Marfatia, S.M., Walther, Z., Chishti, A.H. and Anderson, J.M. Human CASK/LIN-2 binds syndecan-2 and protein 4.1 and localizes to the basolateral membrane of epithelial cells. *J. Cell Biol.* **142** (1998) 129-138.

31. Wang, G.S., Hong, C.J., Yen, T.Y., Huang, H.Y., Ou, Y., Huang T.N., Jung W.G., Kuo T.Y., Sheng, M., Wang, T.F. and Hsueh, Y.P. Transcriptional modification by a CASK-interacting nucleosome assembly protein. *Neuron* **42** (2004) 113-128.