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CPEB1 Regulates β -Catenin mRNA Translation and Cell Migration in Astrocytes

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Abstract

A crucial step in directed cell migration is the recruitment of cytoskeletal regulatory and signaling proteins to the leading edge of the cell. One protein localized to the leading edge of a migrating astrocyte is β -catenin. Using an *in vitro* wound healing assay, we show the localization of β -catenin to the leading edge is dependent upon new protein synthesis at the time of wounding. We examined the mRNA encoding β -catenin for potential regulatory elements and identified a conserved cytoplasmic polyadenylation element in the 3'-untranslated region (UTR). We now show that the CPE-binding protein (CPEB1) is expressed in astrocytes and that translation of β -catenin mRNA is regulated by CPEB1. Further, expression of a mutant CPEB1 protein in astrocytes not only blocks β -catenin protein localization, it inhibits cell migration. These findings demonstrate a role for CPEB1-mediated protein synthesis in the localization of β -catenin protein to the leading edge of migrating astrocytes and in regulating directed cell motility.

Keywords

glia; cell culture; glioblastoma

Introduction

Astrocytes are motile cells during development and in the mature CNS. For example, astrocytes respond to brain trauma by migrating to the site of injury where they form a glial scar. The scar helps repair the damage caused by trauma, including mending the blood brain barrier, limiting excitotoxic neural degeneration, and containing inflammation (Bush et al. 1999; Faulkner et al. 2004). In transformed astrocytes, increased cell migration leads to a rapid spreading of glioblastoma tumors and a high recurrence rate following treatment (Stupp et al. 2005). Therefore, understanding the mechanisms regulating astrocyte guidance and motility may have wide ranging implications.

Using a cell culture assay to study astrocyte motility, Etienne-Manneville and Hall (2001) found that astrocytes entering a wound reorient microtubules perpendicular to the wound site prior to migration into the wound. This reorientation of the axis involves integrin mediated activation of Cdc-42 leading to the downstream activation of par6/PKC ζ (Etienne-Manneville and Hall 2001). They hypothesized that Par6/PKC ζ in turn inactivates GSK3 β leading to a sparing of β -catenin protein from degradation and a localization of β -catenin, adenomatous polyposis coli (APC) and EB1 to the leading edge of the migrating astrocyte

(Etienne-Manneville and Hall 2003). APC is required for the cell polarity changes, while the localization of β -catenin protein may play an as yet unspecified role in cell migration (Etienne-Manneville and Hall 2003).

This rapid localization in β -catenin protein to the leading edge suggested to us a potential role for local protein synthesis. Therefore, we examined β -catenin mRNA for known regulatory *cis*-elements. We determined that the 3'-untranslated region (UTR) of β -catenin contains multiple conserved cytoplasmic polyadenylation elements (CPEs). The translation of CPE-containing mRNA in oocytes and neurons is regulated by the CPE-binding protein CPEB1 (Mendez and Richter 2001). Here, CPEB1 bound mRNAs are held in a translationally dormant state until CPEB1 is phosphorylated at Thr¹⁷¹ and Ser¹⁷⁷, this phosphorylation leads to mRNA polyadenylation and translation (Mendez et al. 2000; Wu et al. 1998). CPEB1 is critical for oocyte maturation and cell division (Stebbins-Boaz et al. 1996; Tay et al. 2000), and in post-mitotic neurons it regulates some forms of synaptic plasticity (Alarcon et al. 2004; McEvoy et al. 2007; Shin et al. 2004; Wu et al. 1998). The current study represents the first evidence that CPEB1 is present in astrocytes. Further, we show that β -catenin mRNA is polyadenylated in activated astrocyte and that β -catenin protein localization to the leading edge of migrating cells is dependent upon CPEB1. Finally, disruption of CPEB1-mediated protein synthesis inhibits cell migration in glioblastoma cells.

Materials and Methods

Cell Culture Preparation

Astrocytes were isolated from cortices of postnatal day 1 rat pups as previously described (Banker 1991). Briefly, cortices were removed, treated with trypsin, physically dissociated, and plated at ~100,000 cells/ml on 60mm tissue culture dishes, 6 well plates or 18mm round glass coverslips (Carolina Biologicals, Burlington, NC., coated with 1 μ g/ml polyD lysine). Astrocytes were maintained in culture for two weeks in growth medium containing MEM with glutamine (Invitrogen, Carlsbad, CA.), 10% horse serum (Atlanta Biologicals, Lawrenceville GA.), glucose (1 mg/ml), pyruvate (1mM), and pen/strep (100 mg/ml). For cultures free of neurons, cells were plated in 75mm flasks and grown for 1–2 weeks prior to trypsinization and replating on tissue culture dishes. CNS-1 cells, a gift from Rick Matthews (SUNY Upstate Medical University), were cultured in RPMI (Invitrogen) supplemented with glucose (1 mg/ml) pyruvate (1mM), and pen/strep (100 mg/ml). Wounds were introduced by scratching the dish or coverslip (1–20 times) with a 0.1–10 μ l plastic pipet tip.

Western blot analysis

Cultured cortical astrocytes grown to confluence in 60 mm dishes were scratched numerous (~ 20) times. Cultures were then lysed at various times post-scratch in a 1% SDS solution containing 2mM EDTA, 2mM EGTA, 10mM Tris pH 7.5, and 1mM sodium orthovanadate (Sigma, St Louis MO). In some cases cultures were treated just prior to scratching with either cycloheximide (100 μ M), actinomycin D (5 μ M; Sigma) or the Aurora kinase inhibitor ZM447439 (200 nM; Tocris Bioscience; Ellisville, MO). One hour after the scratch all drugs were washed out and the media replaced with fresh media for the duration of the experiment. Cordycepin (20 μ M; Sigma) was added 15 minutes prior to scratching and remained for one hour post-scratch. Following harvesting of the cell lysates, proteins were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed with antibodies against β -catenin (1:5000; Chemicon/Millipore, Billerica, MA) and α -tubulin (1:10,000; Sigma). Phospho-CPEB1 (1:5000; Atkins et al. 2004) and total CPEB1 (1:2500; Shin et al. 2004) were also used to probe western blots. Blots were developed using femto ECL (Pierce, Rockford IL) and analyzed for densitometry using EpiChem II

Darkroom and Labworks software (UVP, Upland, CA). The ratios of β -catenin to tubulin or phosphorylated CPEB1 to total CPEB1 were calculated.

Immunocytochemistry

Astrocytes were grown to confluence on 18mm coverslips, scratched (3–5 times) and fixed 6 hours later in 4% paraformaldehyde in PBS and 4% sucrose. To determine β -catenin and CPEB1 localization, the cells were blocked with 10% horse serum for 15 minutes prior to incubation in mouse anti- β -catenin (1:1000) and/or rabbit anti-CPEB1 (1:1000) overnight at 4°C. Following a rigorous wash, species specific secondary antibodies conjugated to either FITC or Cy3 (Jackson ImmunoResearch, West Grove, PA) were added at room temperature for 1 hour. Coverslips were then washed and mounted onto slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Images were acquired with a Nikon Eclipse E800 fluorescent microscope equipped with a Spot camera. Images were processed using Adobe Photoshop 7.0 and labeled in Adobe Illustrator 10.

Reverse transcription-PCR

To determine if CPEB1 mRNA was expressed in astrocytes, total RNA was isolated from astrocytes cultures free of neurons using trizol reagent (Invitrogen) and treated with Dnase (10 μ g/ml). 1 μ g RNA was used as template in a random primed reverse transcription reaction and cDNA was amplified using primers P1 and P2 (Wu et al. 1998). To verify that the astrocyte cultures were devoid of neurons, we performed Western blot analysis using an antibody against NeuN, a neuronal marker (data not shown; Chemicon/Millipore). In addition, by visual inspection the cultures were comprised of roughly 95% cells maintaining characteristic type I astrocytic morphologies and 5% astrocytic type II morphologies.

In situ hybridizations

In situ hybridizations were performed as described (Shin et al. 2004). Briefly, oligonucleotide probes were end-labeled with digoxigenin (DIG) according to manufacturer specifications (DIG oligonucleotide tailing kit second generation; Roche Applied Science, Indianapolis, IN). Here, 100 pmol of oligo was incubated in reaction buffer with .05 nmol DIG ddUTP and 20 U terminal transferase for 15 minutes at 37°C. Cells were fixed for 15 minutes at room temperature with 4% paraformaldehyde in 1x PBS and 5 mM MgCl₂. Cells were permeabilized for 5 minutes at room temperature with 0.1% tritonX-100 in 1x PBS with 5 mM MgCl₂ and blocked for 30 minutes in 1x PBS with 5mM MgCl₂ and 1% acetylated BSA. CPEB1 protein was localized prior to mRNA detection. Anti-CPEB1 was diluted 1:1000 in blocking buffer and incubated overnight at 4°C. Localization of β -catenin mRNA was determined using 50 ng of each of two probes:

ctccctaccaagtctttctgagttctgcaggcagagtaaagtattcacc and
gcgcaggtgaccacatttatcatcagaacccagaagctgcactagagt. Cells were washed 3x in 1x PBS with 5 mM MgCl₂ before 20 minute equilibration at room temperature in 1x SSC, 50% formamide, and 10 mM sodium phosphate (pH 7.0). Coverslips were hybridized face down for 5 hours at 37°C on a 40 μ l drop of probe/hybridization buffer mixture placed on parafilm. Hybridization buffer contained 50ng of each probe in 50% formamide and 2x SSC, 10% dextran sulfate, 40 μ g tRNA, 0.2% BSA, 10 μ g salmon sperm DNA, 20 μ M VRC, and 10 mM sodium phosphate. After hybridization, cells were washed for 20 minutes at 37°C in 50% formamide with 1x SSC. Cells were then washed 3 by 10 minutes at room temperature in 1x SSC on a rocker. Cells were blocked for 1 hour at room temperature in 0.1 M tris (pH 7.5), 0.15 M NaCl and 1% acteylated BSA, and incubated overnight at 4°C with a 1:100 dilution of goat anti-rabbit FITC secondary and a 1:300 dilution of mouse anti-DIG-cy3 in block solution. Coverslips were then washed in PBS and mounted on slides with Vectashield mounting medium.

Immunoprecipitation

Immunoprecipitation of CPEB1 was performed as described in (Shin et al. 2004). Briefly, astrocytes grown in flasks were trypsinized and replated into 100 mm tissue culture dishes and grown to approximately 70% confluence. Cells were lysed in lysis buffer containing 10 mM HEPES pH 7.4, 200mM NaCl, 30 mM EDTA, and 0.5% Triton X-100 with 200 U/ml RNase inhibitor (Roche) and harvested by scraping. Anti-CPEB1 or rabbit IgG was added and incubated for 2 hours at room temperature. Protein A- agarose beads (Sigma) equilibrated in lysis buffer were then added and incubated with rotation for 30 minutes. Beads were isolated by centrifugation and washed 6 times with wash buffer containing 10 mM HEPES pH 7.4, 500mM NaCl, 30 mM EDTA, and 0.5% Triton X-100. RNA was isolated from the precipitation using a Qiagen RNeasy micro-column and used as template for an RT-PCR reaction. cDNA template was amplified using primers against β -catenin and histone H1. (PRIMER SEQUENCES: β -catenin AAATGGTCCGATTAGTTTCCT and TGAATGAATTAAAAGTTTAATTCTG; histone H1 GGTGGCTTTCAAGAAGACCAA and TGAGGTCTGTTTGCTGTCCTT). PCR conditions were 1.5 mM $MgCl_2$, at 52°C annealing temperature and 40 cycles.

The pull-down of CPEB1-interacting mRNA was performed by transfecting dishes of astrocytes with a 6x-His-tagged CPEB1 RNA-binding domain (CPEB1-RBD). This construct was made by cloning nucleotides 930–1715 of mouse CPEB1 mRNA (NM_007755) into the pcDNA 4 HISMAX TOPO vector (Invitrogen). Cells were then lysed in the same manner as the IP (above) except buffers did not contain EDTA. His-tagged protein was purified with Talon beads (BD biosciences) equilibrated in lysis buffer. RNA was extracted from the precipitate using the Qiagen RNeasy micro kit and reverse transcribed. The cDNA was PCR amplified using primers for β -catenin and glial fibrillary acidic protein (GFAP). GFAP primers: GCTAATGACTATCGCCGCCAACT and ATCCCGCATCTCCACCGTCTTTAC

mRNA polyadenylation assays

Polyadenylation test (PAT) assays were used to determine the size distribution of the poly(A) tail of specific mRNAs. RNA was extracted from confluent monolayers of astrocytes before and after scratching using trizol reagent (Invitrogen). PAT assays were performed as described (Wu et al. 1998). Briefly, astrocytes were taken as unscratched controls or scratched approximately 20 times and lysed using Trizol reagent at time points following scratching. RNA was extracted according to manufacturer's protocol. RNA was DNase treated and reverse transcribed using the PAT primer GCGAGCTCCGCGGCCGCGTTTTTTTTTTTTT. cDNA was PCR amplified (54°C annealing temperature, 1.5 mM $MgCl_2$ and 35 cycles) using the PAT primer and gene specific primers for β -catenin and GAPDH. β -catenin primer: TGGGGATACGTGCGGTGGGGTAAAT; GAPDH primer: CAGCAACTCCCACTCTTCCACCTT.

CPEB1-RBD Expression

Astrocytes were cultured on coverslips to confluence and transfected using lipofectamine 2000. Briefly, 0.5 μ g of plasmid DNA was mixed with 3 μ l of transfection reagent in 200 μ l of OptiMem (Invitrogen). DNA and reagent were incubated for 30 minutes at room temperature before addition to the astrocytes in 1 ml of culture medium. Cells were transfected for 6 hours before the transfection mixture was replaced with fresh medium. 3–5 scratches/coverslip were introduced 12 hours later. Constructs used for transfection encoded either EGFP or an EGFP tagged CPEB1-RBD. The GFP-CPEB1-RBD construct consisted of the CPEB1-RBD sequence described above cloned in-frame into the XhoI site of pEGFP-C1 (BD Biosciences). Cells were fixed 6 hours after scratching and immunostained for β -

catenin. Localization was assessed in expressing cells on the edge of the scratch. Cells were scored as positive for β -catenin localization if increased β -catenin immunoreactivity was apparent at > 50% of the surface of the leading edge. Cells were examined with phase microscopy to ensure the increase in β -catenin protein at the leading edge was specifically due to increased protein localization and not due to detritus on top of the cell resulting from the scratch injury or folding of the cell membrane as an artifact of processing.

Migration Assays

CNS-1 glioma cells were used to study the effects of CPEB1 inhibition on long term migration due to their higher transfection efficiency. CNS-1 cells were cultured as described (Kruse et al. 1994). Cells were plated on coverslips coated with 1 μ g/ml poly-D-lysine. Cells were transfected with Lipofectamine 2000 with 0.5 μ g of either pEGFP-C1 and monomeric dsRED (BD Biosciences) or GFP-CPEB1-RBD and monomeric dsRED plasmids. dsRED fills the entire cell relatively evenly, thus, co-transfection with the experimental construct was used to visualize cell shape. Cultures were scratched 6 hours post-transfection and cell migration was assessed 24 hours later. Cultures were fixed, mounted onto slides and imaged. Quantification of transfection efficiency was determined by counting the number of transfected cells in 3 confluent (non-scratched) fields per coverslip. Fields containing scratches were visualized and selected at 20x magnification under brightfield phase optics to ensure a non-biased sample. The number of transfected cells inside and outside the scratch was then determined under fluorescent optics. For each field, the number of transfected cells inside the wound borders was also compared to the total number of cells inside the wound borders as measured by DAPI staining.

Tumor Implantation Assays

CNS-1 cells stably expressing RFP were plated overnight in soft agar (1% agarose in OptiMEM; Invitrogen), to form cell spheres. Cell spheres were transfected overnight with either GFP or GFP-CPEB1-RBD using Lipofectamine 2000 as described above. Brain slices (300 μ m thick) were prepared from P1 rat pups and placed on tissue culture filters (Milipore) and fed from below using RPMI with 20% horse serum. Transfected CNS-1 cell spheres (1–3 per slice) were placed on top of the brain slices and incubated at 37° for 96 hours. Tumor size was determined using RFP fluorescence at 24, 48, 72 and 96 hours post-implantation by analyzing the images with Photoshop 7.0 software (Adobe Systems Inc, San Jose, CA). Specifically, the outer boarder of the RFP fluorescence was obtained using the magic wand tool set at a tolerance of 32 (similar results were obtained with a tolerance of 16) and selecting for the fluorescence of a cell on the leading edge of the tumor mass. This outlines the tumor with pixels that match the selected intensity. This method was used to allow for uniform, consistent and unbiased determination of tumor size. The diameter of each tumor outlined in this fashion was measured using Metamorph software (Molecular Devices, Sunnyvale, CA) and quantified by taking the average of 4 measurements that each intersect the middle of the tumor at 45° angles from each other.

GSK3- β inhibition

The presence of Aurora A kinase was detected by RT-PCR. Total RNA was reverse transcribed using random primers and the resulting cDNA was PCR amplified using primers specific to Aurora A (CTGTTCTTCGGTCCGAAACGAGTC and CGCAGGCTGTGGCTGCTACTCTTCT). Aurora A protein was determined by Western blot of pure astrocyte cultures probed with an Aurora A antibody at 1:1000 (Abcam, Cambridge UK). In order to study the effects of GSK3 β inhibition, astrocytes were grown to ~70% confluence and treated with 20 mM LiCl (Sigma) for up to one hour to inhibit GSK3- β . Cells were lysed and processed for Western blot as described above. In some cases, 20 μ M cordycepin or 20 μ M adenosine was applied 30 minutes prior to LiCl addition, other

drugs were applied simultaneously with LiCl. The proteasome inhibitor MG-132 (10 μ M; Sigma) was applied to unscratched cultures for 1 hour prior to lysis.

Results

Wound invasion is dependent on new protein synthesis

To determine if new protein synthesis is required for successful astrocyte migration, we used an *in vitro* assay for cell migration. Here, a monolayer of astrocytes is scratched and the cells migrate to fill the wound. In control experiments, 71.8% of the wound area had been filled by 24 hours post-scratch. However, when protein synthesis was inhibited with cycloheximide (100 μ M) or anisomycin (20 μ M) for just the first hour following wounding, only 16.6% or 26.6% (respectively) of the wound area was filled by 24 hours (Figure 1). The inhibition of migration was striking considering that the effects of cycloheximide and anisomycin on protein synthesis *in vitro* wash out within 15 minutes (Colombo et al. 1965; Grollman 1967). By 48 hours following wounding, control cells had completely filled the wound (92.4% of wound area covered), while cells treated with protein synthesis inhibitors for 1 hour had filled only 24.1% (cycloheximide) or 39.8% (anisomycin) of the wound (Figure 1).

β Catenin Localization to the Leading Edge is Protein Synthesis Dependent

During the early stages of migration, astrocytes located along the wound rapidly localize β -catenin protein to the leading edge (Etienne-Manneville and Hall 2003). To determine if β -catenin was one of the proteins synthesized following wounding, astrocyte cultures were scratched in the presence of cycloheximide and β -catenin localization was examined. Cycloheximide applied at the time of the scratch and washed out one hour post-scratch, completely eliminated the localization of β -catenin at the leading edge 6 hours post-scratch (Figure 2). However, β -catenin was still localized in the presence of the transcriptional inhibitor actinomycin D, indicating that β -catenin at the leading edge was newly synthesized from mRNA present at the time of the scratch and not recruited from existing cellular pools of the protein.

To confirm that β -catenin protein is newly synthesized in this assay, total levels of β -catenin protein were assessed by Western blot following multiple scratches. Consistent with the localization data, β -catenin levels increased by 1.9 fold in the control condition after 6 hours and this increase was unchanged in the presence of actinomycin D (Figure 2b, Supplemental Figure 1). However, the increase was blocked when cycloheximide was present for the first hour post-scratch. Anisomycin, another mRNA translation inhibitor, also eliminated the increase in total β -catenin protein levels and the localization to the leading edge following a scratch (data not shown). This again suggests that β -catenin is being synthesized from mRNA present at the time of injury.

A Role for CPEB1 in β -Catenin Translation and Localization

To determine if there were known regulatory elements in the β -catenin mRNA, we analyzed the 3'-UTR of β -catenin mRNA. This analysis revealed the presence of potential cytoplasmic polyadenylation elements (CPE; Figure 3a). A CPE was defined as a nucleotide sequence consisting of U₄₋₅A₁₋₂U in close proximity to the cleavage and polyadenylation hexanucleotide (HEX) sequence that denotes the end of the 3'-UTR (Mendez and Richter 2001; Pique et al. 2008). CPE1 and CPE3 were conserved in human, mouse, rat and other mammals where sequence was available (data not shown). CPE2 was conserved in all mammalian species examined except for rat. Therefore, we examined pure astrocytes cultures for the presence of the CPE-binding protein CPEB1. Reverse transcription-polymerase chain reactions (RT-PCR) and Western blot analysis demonstrated that CPEB1

mRNA and protein are expressed in astrocytes (Figure 3b). In addition, immunolocalization of CPEB1 protein in a confluent layer of astrocytes shows a predominantly cytoplasmic/peri-nuclear staining. However, in migrating astrocytes, CPEB1 protein is found at the leading edge where it co-localizes with β -catenin protein (Figure 3c). Since CPEB1 activation of mRNA translation is accompanied by an increase in the poly(A)-tail length of responsive mRNA, the polyadenylation inhibitor cordycepin was used in the migration assay and had a similar, though less dramatic effect, on migration as that of the mRNA translation inhibitors (Figure 1). In addition, cordycepin effectively inhibited both the localization of β -catenin to the leading edge and the increase in β -catenin protein following wounding (Figure 2).

To determine the distribution of β -catenin mRNA, we performed fluorescent *in situ* hybridization (FISH) using probes specific for β -catenin mRNA. Figure 3 shows β -catenin mRNA localized to the leading edge of a migrating cell, where it co-localizes with CPEB1 protein. In order to confirm an interaction between β -catenin mRNA and CPEB1 protein, two approaches were used. First, CPEB1 was immunoprecipitated from astrocyte cultures under native conditions. RNA was then isolated from the precipitates and used as template in a reverse transcription reaction and PCR amplified using primers specific for either β -catenin or a control, non-CPE containing mRNA GFAP. β -catenin mRNA co-precipitated with anti-CPEB1 antibodies, but did not co-precipitate with rabbit IgG antibodies and GFAP mRNA was not detectable under either condition (Figure 4c).

Second, astrocyte cultures were transfected with a hexa-histidine tagged RNA binding domain of CPEB1 (CPEB1-RBD). Complexes containing the CPEB1-RBD and associated mRNAs were then isolated on a cobalt column. β -catenin mRNA co-precipitated on the column from transfected cells, but not untransfected cells, while the highly expressed but non-CPE containing mRNA for glial fibrillary acidic protein (GFAP) did not co-precipitate under either transfection condition (Figure 4c). This suggests a direct interaction between CPEB1 and β -catenin mRNA, and demonstrates that CPEB1-RBD can compete with endogenous CPEB1 for binding to CPE-containing mRNAs *in vivo*. Further, since CPEB1-RBD lacks the critical activation site (T¹⁷¹, S¹⁷⁷) it should serve as a dominant negative CPEB1 in astrocytes as it does in oocytes (Mendez et al. 2000).

Since CPEB1-mediated mRNA translation is activated following phosphorylation of T¹⁷¹, S¹⁷⁷, we used a phospho-specific antibody that recognizes this site to determine the activation status of CPEB1 (Atkins et al. 2004). CPEB1 phosphorylation (pCPEB1) was significantly increased ($p < 0.05$) within 1 hour of the scratch and continued for at least 6 hours (Figure 4d). To determine if CPEB1 phosphorylation resulted in β -catenin mRNA polyadenylation, the length of the poly(A)-tail of β -catenin was measured using a modified RT-PCR reaction, known as a polyadenylation test (PAT assay; (Wu et al. 1998). The poly(A)-tail of β -catenin begins to increase at 1 hour after the scratch and remains long over the 6 hour experiment. However, the poly(A)-tail of a non-CPE containing mRNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), remains unchanged over the same time period.

CPEB1 inhibition interferes with cell migration

To determine the effect of inhibiting CPEB1-mediated protein synthesis on astrocyte migration, we expressed a GFP-tagged version of CPEB1-RBD in astrocytes and glioblastoma cells and determined their ability to migrate in the scratch assay. We used the expression of β -catenin at the leading edge as an indication that we had indeed blocked CPEB1-mediated protein synthesis. In astrocytes located along the edge of the scratch expressing GFP alone, 67% localized β -catenin protein. However, β -catenin localized in only 17% of GFP-CPEB1-RBD expressing cells along the scratch ($p < 0.01$, $n = 4$).

experiments, 3–7 fields per experiment; Figure 4). Thus, inhibiting CPEB1 function interferes with β -catenin localization to the leading edge. In astrocytes, expression of CPEB1-RBD did not block the initial protrusion into the wound (4–6 hours post-scratch), but by 6 hours the cells expressing CPEB1-RBD appeared to be falling behind the neighboring untransfected cells (Figure 5).

To examine cell migration further we used a glioblastoma cell line (CNS-1) to take advantage of a higher transfection efficiency and a rapid migration rate (Kruse et al. 1994). CNS-1 cells were transfected with either GFP or GFP-CPEB1-RBD and the ability of the transfected cells to invade the scratch was quantified 16–24 hours post-scratch. Untransfected CNS1 cells had completely “healed” the wound at this time (Figure 6a). In these experiments CNS-1 cells were co-transfected with EGFP-CPEB1-RBD and red fluorescent protein (RFP) to visualize cell shape, as RFP will distribute evenly throughout the cell. Cells expressing either GFP or GFP-CPEB1-RBD had similar morphologies when the soluble RFP was used to visualize cell shape. To ascertain transfection efficiency, transfected cells in confluent (unscratched) fields were counted for each condition. Transfection efficiencies did not vary between GFP and GFP-CPEB1-RBD (data not shown). We next quantified the number of transfected cells in the wound from regions of interest selected under brightfield optics to avoid bias (the edges of the wound are still detectable). While the transfection efficiency was the same for GFP and GFP-CPEB1-RBD cultures the percentage of transfected cells within the wound was reduced from 47% in GFP expressing cells to 30% in GFP-CPEB1-RBD expressing cultures. When quantified as a ratio of the total number of cells in the wound, there was a 40% reduction in GFP-CPEB1-RBD transfected cells in the wound ($p \leq .01$; Figure 6). Although this experimental design does not identify the starting position of transfected cells relative to the scratch, it does suggest that CPEB1-RBD expressing cells are less able to migrate into the wound.

To determine if CPEB1 inhibition could affect migration in a system more akin to *in vivo* migration, we expressed the CPEB1-RBD in CNS1 glioblastoma tumors implanted onto rat brain slices grown in culture (Nakada et al. 2004; Yoshida et al. 2003). CNS-1 cells stably expressing RFP were grown in non-adherent soft agar allowing the formation of cell spheres or “tumors”. Cell spheres were transfected with either EGFP or EGFP-CPEB1-RBD and 24 hours after transfection they were placed on rat brain slices previously harvested from p1 rat pups and grown in culture for 1 day. The same tumors were imaged using RFP fluorescence 24, 48, 72, and 96 hours post implantation and tumor size determined at each time point (Figure 6c,d). When the CNS-1 cells were expressing GFP-CPEB1-RBD the tumors grew approximately 65% less than GFP expressing tumors over the course of the experiment. This inhibition of tumor size could be due to an alteration of cell proliferation, cell migration or both. Further examination of this process will be required to distinguish between these possibilities, but the significant inhibition of expansion of the leading edge of the tumor is consistent with an inhibition of cell migration following blockade of CPEB1-mediated protein synthesis.

CPEB1 is Activated following GSK3 β Inhibition

In astrocytes a cdc-42 dependent pathway is activated upon scratching. This pathway leads to the Par 6/PKC ζ dependent inhibition of GSK3 β (Etienne-Manneville and Hall 2003). Lithium, an inhibitor of GSK3 β (Klein and Melton 1996), was used to test if inhibition of GSK3 β resulted in the synthesis of β -catenin protein and the phosphorylation of CPEB1. LiCl (20mM) treatment alone (no scratch) for 1 hour was enough to induce a phosphorylation of CPEB1 that was accompanied by an increase in β -catenin (Figure 7). Further, the LiCl-induced increase in β -catenin was dependent upon new protein synthesis and mRNA polyadenylation (Figure 7c). GSK3 β inhibition is known to prevent the phosphorylation of β -catenin and subsequent proteasome mediated degradation (Aberle et al.

1997). Therefore, we examined if sparing β -catenin from degradation could contribute to the early increase in β -catenin protein levels. We applied the proteasome inhibitor MG-132 to astrocytes in culture and examined β -catenin levels 1 hour later. No increase in β -catenin could be detected, indicating that sparing β -catenin from degradation can not account for the increase seen following LiCl treatment.

Inhibition of GSK3 β in oocytes has been linked to the activation of Aurora A kinase, that in turn can phosphorylate and activate CPEB1 (Sarkissian et al. 2004). To determine if GSK3 β inhibition activated CPEB1 through Aurora kinase, the Aurora kinase inhibitor ZM447439 was applied to cells along with LiCl. ZM447439 is a specific Aurora kinase inhibitor with an IC50 of 110nM (Ditchfield et al. 2003). The phosphorylation of CPEB1 and the increase in β -catenin protein seen with LiCl stimulation were abolished in the presence of ZM447439 (Figure 7).

Discussion

Here we demonstrate a role for new protein synthesis in the migration of astrocytes into a wound. Using an *in vitro* migration assay we show that new protein synthesis at the time of the scratch is essential for wound invasion and one of the new proteins synthesized is β -catenin. Further, the newly synthesized β -catenin protein is localized to the leading edge of migrating astrocytes, and this process is regulated by the mRNA-binding protein CPEB1. Our results suggest that following a scratch GSK3 β inhibition leads to an activation of Aurora kinase and subsequent phosphorylation of CPEB1. pCPEB1 in turn activates the synthesis of β -catenin protein through a mechanism dependent upon polyadenylation of the mRNA.

A role for mRNA translation localized to a specific cellular compartment has been demonstrated previously for fibroblast migration *in vitro* (Condeelis and Singer 2005). Here, β -actin mRNA localizes to the protruding edge of a motile fibroblast, and a 54 nucleotide segment located in the 3'UTR of β -actin mRNA called the "zip code" was responsible for this localization (Kislauskis et al. 1994; Kislauskis et al. 1997). An mRNA binding protein, zip code binding protein 1 (ZBP1), was later identified and shown to be responsible for β -actin mRNA localization and translation. Localization of the β -actin mRNA toward the leading edge correlated with fibroblast movement in that direction and an increased motility compared to fibroblasts without localization of β -actin mRNA (Gu et al. 2002; Ross et al. 1997). In addition, ZBP1 was recently found to repress translation of the β -actin mRNA during transport until src kinase phosphorylates ZBP1 and relieves translational repression (Huttelmaier et al. 2005). In astrocytes, we show that CPEB1 is regulating β -catenin mRNA translation, and that this translation is necessary for β -catenin localization of the leading edge.

Astrocytes along the edge of a scratch will reorient microtubules perpendicular to the wound and migrate into the wound (Etienne-Manneville and Hall 2001). Independent of the reorientation of the microtubule organizing center (MTOC), there is an increase in β -catenin protein and localization to the leading edge of the astrocyte (Etienne-Manneville and Hall 2003). To demonstrate that this increase was due to new synthesis and not redistribution of existing β -catenin, we show this localization is inhibited by blocking either protein synthesis or mRNA polyadenylation. By Western blot analysis β -catenin protein levels are detectably higher within two hours following wounding; however, protein synthesis inhibition in the first hour eliminates this increase even 6 hours post-scratch. This indicates that there is a temporal window within the first hour where protein synthesis is activated and required for β -catenin localization.

How is β -catenin mRNA regulated? We present several lines of evidence suggesting that β -catenin mRNA is regulated by CPEB1. First, β -catenin mRNA contains conserved CPE sequences in its 3'-UTR. Conservation of these sequences in the 3'-UTR suggests a functional domain. Second, β -catenin mRNA binds to CPEB1 *in vivo* and co-localizes with CPEB1 at the leading edge of a migrating astrocyte. Next, β -catenin mRNA becomes polyadenylated following a scratch. Polyadenylation is detectable approximately one hour after the scratch and is considerably increased by two hours. This slightly precedes the detectable increase in β -catenin protein consistent with a model where mRNA polyadenylation leads to mRNA translation. Polyadenylation is a characteristic of CPEB1-mediated regulation and precedes translational enhancement of the message (Mendez and Richter 2001). Finally, to test for a direct connection between CPEB1 function and β -catenin localization a GFP-CPEB1-RBD fusion protein was expressed in astrocytes and CNS1 glioblastoma cells. The RNA binding domain of CPEB1 is capable of interacting specifically with CPE-containing constructs and competing with native CPEB1. However, CPEB1-RBD lacks the critical phosphorylation domain and thus bound mRNA will not be translationally activated under conditions where endogenous CPEB1 is activated (Mendez et al. 2000). Importantly, expression of the CPEB1-RBD disrupts both β -catenin localization and cell migration in the *in vitro* scratch assay. To determine if inhibition might alter glioblastoma tumor advancement, we utilized an *in vivo* model for tumor growth and showed that expression of the CPEB1-RBD also inhibits tumor expansion. The inhibition of tumor growth may not be solely due to a block of cell migration, as CPEB1 has been shown to influence cell proliferation in oocytes (Tay and Richter 2001); however, it is consistent with decrease in the spread of cells away from the main tumor mass.

The signal transduction pathway activated following a scratch has been partially defined. The scratch initially induces recruitment of scribbled (scrib) protein to the leading edge of the migrating astrocyte and scrib binds and recruits the cdc-42 guanine exchange factor β Pix to the leading edge, resulting in local activation of cdc-42 (Osmani et al. 2006). The localized activation of cdc-42 is essential for cell migration and activates several pathways. In addition to inducing an increase in β -catenin, it leads to a localization of APC to the plus ends of microtubules at the leading edge. This localization is essential for reorientation of the centrosome and MTOC perpendicular to the scratch (Etienne-Manneville and Hall 2003). Disks large 1/SAP97 (Dlg1) is also recruited to the leading edge in a cdc-42 dependent manner. The Dlg1 localization is independent of APC, but once localized it interacts with APC and potentially tethers the APC associated microtubules to the basal plasma membrane (Etienne-Manneville et al. 2005). Localized active cdc-42 at the leading edge initiates signaling through potentially parallel pathways leading eventually to the formation of polarity and cell migration into the wound.

How might CPEB1 contribute to this signaling cascade? Inhibition of GSK3 β stabilizes β -catenin by preventing its proteasome mediated degradation (Aberle et al. 1997). However, we have demonstrated that stabilization of β -catenin protein cannot account for the initial increase and localization of β -catenin following GSK3 β inhibition. New synthesis of β -catenin is essential. While the function of β -catenin at the leading edge is unknown, it seems possible that regulating the local concentration of β -catenin, and β -catenin interacting proteins such as APC, could regulate microtubule dynamics at the leading edge of the cell (Kroboth et al. 2006; Nathke et al. 1996; Zumbunn et al. 2001). For example, in NIH 3T3 cells β -catenin localizes APC to membrane clusters, stabilizing the plus ends in cellular protrusions (Sharma et al. 2006).

While CPEB1 function is required for localization of β -catenin to the leading edge at an early stage of migration, at later time points CPEB1-mediated translation is required for successful invasion into the scratch. The effect of inhibiting CPEB1's function on cell

migration is almost certainly not due solely to its affect on β -catenin translation. Instead, a number of CPE-containing transcripts are likely regulated in a similar manner, and the cumulative dysregulation of translation of CPE-containing mRNA in cells expressing CPEB1-RBD results in a loss of cell migration. For example, IRSp53, an adaptor protein implicated in actin regulation, was recently shown to be regulated by CPEB1 in neurons (McEvoy et al. 2007). In neurons, it appears that CPEB1 function is not required for constitutive synthesis of IRSp53, but rather the activity-induced increase in IRSp53 at synapses (McEvoy et al. 2007). In astrocytes, CPEB1 function is not required for the establishment of cell polarity or the initial protrusion into the scratch; instead, CPEB1 function may be responsible for the persistence of cell migration and the continued ability of an astrocyte to fully migrate to the site of a wound.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

New protein synthesis is required for astrocyte migration. **(A)** Phase contrast images of astrocytes migrating into a wound 24 and 48 hours after wounding (control) or sister cultures treated with cycloheximide (100 μ M), anisomycin (20 μ M) or cordycepin (20 μ M). Dotted lines indicate the borders of the wound (scale = 200 μ m). **(B)** Quantification of astrocyte invasion. Fields containing a scratch were selected randomly with phase microscopy. Total wound area and area devoid of cells within the wound were measured using Image J software. The percentage of the wound containing cells was calculated and expressed as % wound closure. Data represent 4 fields per coverslip and at least 3 coverslips per condition (*= p <0.01).

Figure 2.

β -catenin protein localization to the leading edge of migrating astrocytes is protein synthesis dependent. **(A)** β -Catenin immunocytochemistry in astrocytes 6 hours after wounding (control) or sister cultures treated for 1 hour post-wounding with Actinomycin D (5 μ M), cycloheximide (100 μ M) or cordycepin (20 μ M). β -Catenin protein localizes to the leading edge in control and actinomycin D. treated (large arrows) but is not present at the leading edge of cycloheximide or cordycepin treated cells (small arrows). Scale Bar=20 μ m. **(B)** Total β -catenin levels analyzed by Western blot on tissue harvested from astrocytes grown in culture following multiple scratches. Blots were quantified by densitometry, and normalized to tubulin levels. Data represents the fold change in β -catenin protein at times indicated post-scratch compared to unwounded cultures. Cycloheximide (CX), actinomycin D (AD), and cordycepin (Cdy) were applied for 1 hour post-scratch (n=6; *= p <0.05 compared to unscratched cultures).

Figure 3. CPEB1 expression in astrocytes. (A) Schematic of the mouse β -catenin mRNA showing 3 potential CPE sequences and the hexanucleotide sequence (HEX) in the 3'-UTR. (B) Astrocyte cultures devoid of neurons were analyzed by RT-PCR for the expression of CPEB1 mRNA and Western blot analysis for CPEB1 protein expression (α -CPEB1). (C) Immunolocalization of β -catenin and CPEB1 protein (arrows) in astrocytes 6 hours after wounding (left panels). Overlay of the boxed region of the two images is shown on the right, demonstrating the co-localization of the two proteins toward the leading edge of the cell (scale bars=10 μ m).

Figure 4.

CPEB1 interacts with β -catenin mRNA. **(A)** β -catenin mRNA and CPEB1 protein co-localize to the leading edge of migrating astrocytes. Fluorescent *in situ* hybridization using primers specific for β -catenin mRNA (top panel) in migrating astrocyte six hours post scratch. Images represent the overlay of the phase and fluorescent images. In the left panel an excess of un-labeled probe was added to the labeled probes to show specificity (control). β -catenin mRNA is located in the cytoplasm, but enriches at the leading edge (arrows top right panel). Immunohistochemistry for CPEB1 protein (bottom panel) shows its presence in the cytoplasm and localization to the leading edge (arrows lower left panel). When the two images are overlaid (lower right panel) you can clearly see co-localization (arrows; yellow) of β -catenin mRNA (red) and CPEB1 protein (green). Scale bar = 10 μ m. **(B)** Co-IP of CPEB1 and β -catenin mRNA. Astrocyte cultures were lysed and CPEB1 was IP. The mRNA that accompanies CPEB1 was isolated and an RT-PCR reaction was carried out using primers specific for GFAP or β -catenin. β -catenin mRNA is associated with endogenous CPEB1 but the non-CPE containing mRNA encoding GFAP is not (left panel). If a control IgG is used for the IP (control) neither β -catenin nor GFAP mRNAs are precipitated. β -catenin mRNA could also be pulled down by expressing a 6x-His tagged CPEB1 RNA-binding domain (CPEB1-RBD) in astrocyte cultures and isolating CPEB1-

RBD and associated RNA with Talon beads (right panel). RT-PCR analysis of this fraction reveals that β -catenin mRNA associates with the RBD but the non-CPE containing mRNA encoding GFAP does not. **(C)** CPEB1 is phosphorylated following a scratch. Astrocyte cultures were scratched and CPEB1 phosphorylation was analyzed by Western blot using a phospho-specific antibody (T¹⁷¹ and S¹⁷⁴; pCPEB). Quantification of the ratio of pCPEB1 to total CPEB1 is shown in the graph, with the ratio prior to wounding set at 1. A representative blot is shown above the graph (n=4, *p < 0.05). **(D)** β -catenin mRNA is polyadenylated following a scratch. The poly(A)-tail lengths of β -catenin and GAPDH mRNAs were analyzed using a PAT assay (see methods). An increase in β -catenin mRNA polyadenylation can be detected starting approximately 1 hour post scratch while the poly(A)-tail of GAPDH remains unchanged throughout the course of the experiment. Images represent a typical result from 3 separate experiments.

Figure 5. CPEB1-RBD expression inhibits β -catenin localization to the leading edge. Astrocyte cultures were transfected with either GFP (left) or GFP-CPEB1-RBD (right) and processed for β -catenin immunohistochemistry 6 hours post scratch. In cultures transfected with GFP, β -catenin protein (red) localization is readily detected in both transfected and untransfected cells (arrows). In cultures transfected with GFP-CPEB1-RBD no β -catenin protein was localized to the leading edge (arrowhead). However, there is abundant β -catenin in CPEB1-RBD expressing cells and in non-transfected cells β -catenin is still present at the leading edge (arrows). GFP-CPEB1-RBD cells also appear to lag behind neighboring untransfected cells. Quantification of cells expressing either GFP or GFP-CPEB1-RBD that have localized β -catenin protein to the leading edge is shown on the right (GFP $n = 14$ cells, GFP-CPEB1-RBD $n = 15$ cells, $* = p < 0.01$).

Figure 6.

Expression of CPEB1-RBD inhibits migration and glioblastoma tumor size. (A) CNS-1 glioblastoma cells were co-transfected with either GFP or GFP-CPEB1-RBD (GFP-RBD) prior to wounding. Cultures were fixed and visualized 24 hours post-scratch. Scratch sites were identified under brightfield phase optics (dotted lines) to avoid bias and cell migration into the wound was assessed by counting the number of transfected cells inside and outside the wound in 5 random fields per coverslip. In separate cultures GFP-RBD and red fluorescent protein (RFP) were co-expressed in CNS-1 cells and these cultures were co-stained with DAPI. The RFP allows for the visualization of the entire cell (right panel). (B) The number of cells entering the wound was used as a means of quantifying cell migration. Quantification of cell migration is expressed as the number of GFP expressing cells in the wound as either a percentage of the total cells in the wound (left) or the total transfected cells in the field (right). Migration was significantly decreased in the cells expressing GFP-RBD ($n = 4$ experiments, $*p < 0.05$). (C) CNS-1 cells stably transfected with RFP were grown in soft agar to form aggregates and transiently transfected with either GFP or GFP-RBD. Cell aggregates were then transferred onto a slice of rat brain (see methods) and monitored

for four days. Representative fluorescent images are shown of tumors expressing GFP or GFP-CPEB1-RBD taken at 24, 48, 72, and 96 hours post implantation onto a brain slice (scale bar = 500 μ m). **(D)** The size of each tumor was determined by outlining the tumor at each time point using Photoshop. An example of two representative tumors are illustrated by overlaying the outlines of the tumors at 24, 48, 72 and 96 hours (scale bar = 500 μ m). Quantification of tumor diameter is indicated by the graph demonstrating a significant decrease in tumor expansion following expression of GFP-CPEB1-RBD (* p =0.05, n =4 tumors/group).

Figure 7.

GSK3 β inhibition by LiCl results in an increase in pCPEB1 and β -catenin protein synthesis. (A) RT-PCR and Western blot analysis of tissue harvested from astrocytes cultures reveals the expression of Aurora A kinase mRNA and protein in astrocytes. (B) Astrocyte cultures treated with 20mM LiCl or LiCl in the presence of the Aurora kinase inhibitor ZM447439 (ZM) for 1 hour were analyzed by Western blot to determine the levels of pCPEB1. The blot was then stripped and re-probed with an antibody that recognizes total CPEB1. Graph represents the ratio of pCPEB1:total CPEB1 with the unstimulated ratio (time 0) set at 1 (n= 5, *p<0.05 compared to unstimulated). (C) Astrocyte cultures were treated with LiCl for 1 hour in the presence of either cycloheximide (CHX), actinomycin D (AD), cordycepin (Cdy) adenosine (Adn) or ZM447439 (ZM). Adenosine was used as a control for cordycepin, an adenosine analogue. β -catenin levels were assessed by Western blot, normalized to tubulin and reported as fold changes in β -catenin levels in relation to unstimulated (time 0). No change in β -catenin protein was detected in cultures treated with the proteasome inhibitor MG-132 (MG; n= 6, *p<0.05).