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The cholangiocyte marker, BD. 1, forms a stable complex with CLIP170 and shares an identity with eIF3a, a multifunctional subunit of the eIF3 initiation complex

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Abstract

We have previously described the generation of a monoclonal antibody recognizing a novel cholangiocyte marker, designated BD.1, that is expressed by fetal and adult rat cholangiocytes but not hepatocytes or the hepatic progenitor cells known as oval cells. In the present report, we have undertaken a comprehensive examination of BD.1 expressed by long-term cultures of bile duct epithelial cells (BDEC) and prostate epithelial cells (PEC). We show that with continued passage, the levels of BD.1 expressed by BDEC and PEC drop significantly, a decrease that is temporally associated with transition from a diploid to an aneuploid karyotype. Cell cycle analysis revealed cell cycle dependent expression of BD.1 characterized by decreased BD.1 levels within the first 10 h after release from serum starvation followed by reacquisition as cells entered S phase. MAb BD.1 recognized a 170 kDa protein in Western blots and showed strong reactivity with a 170 kDa band in blots prepared from phosphoproteins isolated by metal affinity chromatography. Analysis by mass spectrometry of tryptic peptides generated from BD.1 purified by continuous elution electrophoresis identified the plus end microtubule-binding protein, CLIP170, in the fraction reactive with MAb BD.1. Double immunofluorescence with MAb BD.1 and a MAb specific for CLIP170 showed that both were reactive with intrahepatic bile ducts. However, overexpression or siRNA knockdown of CLIP170 in 293T cells did not significantly alter BD.1 levels, indicating that CLIP170 and BD.1 were distinct, co-migrating proteins. Immunoprecipitation analysis with MAb BD.1 and anti-CLIP170 antibodies showed that under microtubule depolymerizing conditions the two proteins could be co-precipitated with both antibodies, leading us to conclude they were capable of forming stable complexes. Two different protocols were devised to enrich for the CLIP170 binding protein recognized by MAb BD.1. Analysis of tryptic peptides by LC-ESI-MS/MS identified BD.1 as eIF3a, the largest subunit of the elongation initiation factor 3 (eIF3) complex. This identity was confirmed by the simultaneous knockdown of both BD.1 and eIF3a by eIF3a-specific siRNAs and by the strong reactivity of MAb BD.1 with the 170 kDa protein immunoprecipitated with the anti-eIF3a antibody, 5H10. Based on these findings, we concluded

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

that the BD.1 antigen was identical to eIF3a, a multifunctional subunit of the eIF3 complex shown here to associate with microtubules through its interactions with CLIP170.

Keywords

eIF3a; p170; CLIP170; Cholangiocytes; Prostate epithelial cells; Neoplastic conversion

Introduction

Under normal circumstances mature hepatocytes and cholangiocytes are capable of mediating complete liver regeneration. However, physical insult or exposure to hepatocarcinogenic or hepatotoxic agents, such as D-galactosamine or retrosine, that compromise the proliferative ability of hepatocytes and cholangiocytes, induces the rapid expansion of immature ductular cells (Dabeva and Shafritz, 2003; Hixson and Fowler, 1997; Lowes et al., 2003). These immature cells, known as oval cells because of their oval nuclei, are believed to constitute a reserve progenitor cell compartment that is activated in situations of severe liver injury in which hepatocytes or cholangiocytes are incapable of mounting an appropriate proliferative response (Fausto et al., 1993; Lowes et al., 2003; Yang et al., 1993b). Oval cells are a heterogeneous population composed of cholangiocytes, transitional cells with characteristics of both hepatocytes and cholangiocytes, and bipotent stem cells capable of differentiation along a biliary or hepatocyte lineage (Agelli et al., 1997; Fausto and Campbell, 2003; Fausto et al., 1993; Lowes et al., 2003; Yang et al., 1993b). Although oval cells display phenotypic features that distinguish them from cholangiocytes composing the biliary tree, they share a number of cholangiocyte markers, suggesting a possible origination from terminal biliary ductules (Strick-Marchand and Weiss, 2003; Yang et al., 1993a). Further evidence for a biliary origin comes from the observation that many of the antibodies recognizing oval cells also react with fetal or adult cholangiocytes (Yang et al., 1993a). One of the exceptions is monoclonal antibody (MAb) BD.1, an antibody raised against fetal cholangiocytes that reacts uniformly with fetal ductal cells, heterogeneously with both normal and carcinogen-treated adult rat cholangiocytes, but weakly or not at all with oval cells (Yang et al., 1993a). This differential reactivity of MAb BD.1 did not appear to be related to the immaturity of oval cells because both BD.1 positive and negative bile ducts in the adult rat liver displayed several mature cholangiocyte markers (Yang et al., 1993a).

In a previous report, analysis of BD.1 expression by continuous lines of bile duct epithelial cells (BDEC) demonstrated that BD.1 levels rapidly elevated following methotrexate induced G1/S arrest and then decreased during G2/M (Yang et al., 1993b). We reported that BDEC maintained in culture for more than 30 passages expressed lower levels of BD.1 and no longer showed the characteristic increase exhibited by low passage cells arrested in G1/S by methotrexate (Yang et al., 1993b). In the present report, we have extended our previous investigations and show that expression of BD.1 changes in a cell cycle dependent manner. Arrest in G1 by serum starvation resulted in increased expression of BD.1. Return to serum-supplemented medium produced a rapid drop in BD.1 levels followed by a rise in expression as cells entered S phase. Examination of BD.1 localization during the cell cycle revealed

high levels on the spindle apparatus in early anaphase raising the possibility that BD.1 was a microtubule-associated protein, a possibility supported by the discovery that BD.1 could form a stable complex with the microtubule-plus end binding protein CLIP170. Based on results from LC-ESI-MS/MS analysis of BD.1 purified from rat prostate epithelial cells (PEC) by two different protocols, the protein recognized by MAb BD.1 was identified as elongation initiation factor 3a (eIF3a), a microtubule-binding subunit of the eIF3 translation initiation complex. We discuss the significance of the cell type specific expression of BD.1/eIF3a and possible functional roles.

Materials and methods

Antibodies

Monoclonal antibodies against the following markers were used for phenotypic analysis: Hsp70, Affinity Bioreagents Inc., Golden, CO; Tubulin, Zymed; and Myc, Invitrogen, Carlsbad, CA. The construction of the hybridoma secreting MAb against the cholangiocyte antigen BD.1 has been previously described (Yang et al., 1993a). With the exception of MAb BD.1 (IgM isotype), all of the MABs were IgGs. MAb BD.1 used for affinity purification of BD.1 was purified from ascites by euglobulin precipitation (Garcia-Gonzalez et al., 1988). Ascites was dialyzed overnight at 4 °C against distilled H₂O. The precipitate containing primarily IgM was collected by centrifugation at 30 K × g for 30 min at 4 °C. The pellet was resuspended in PBS and the protein concentration determined. Polyclonal antibodies against CLIP170 and GFP were both purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Alexa fluor 594-conjugated goat anti-mouse IgM and Alexa fluor 488-conjugated goat anti-mouse IgG secondary antibodies were obtained from Molecular Probes, Eugene, OR; the HRP-conjugated IgM and IgG were from Biosource International, Camarillo, CA. A rabbit anti-elf3a polyclonal antibody was obtained from Cell Signaling Technologies (Danvers, MA). 5H10, a monoclonal antibody raised against synthetic peptides corresponding to sequences in p167, a centrosomin A-like protein identical to elf3a (Scholler and Kanner, 1997) was a gift from Kanner et al. (1990).

Cell culture

BDEC were grown in vitro as previously described in medium supplemented with Normocin (0.1 mg/ml, InvivoGen, San Diego, CA) (Rozich et al., 2010). PEC were maintained in a 1:1 mixture of RPMI 1640 (Gibco, Carlsbad, CA) and MCDB 153 (Sigma-Aldrich, St. Louis, MO) supplemented with sodium bicarbonate (1.9 g/L), sodium pyruvate (0.5%), fetal bovine serum (FBS) (5%, Hyclone, Logan, UT), epidermal growth factor (0.02 µg/ml, BD Biosciences, San Jose, CA), bovine pituitary extract (5 µg/ml, BD Biosciences), dexamethasone (2 mM in 95% EtOH), glutamine (1%), gentamycin (0.1 mg/ml, Gibco), ITS (0.25%, BD Biosciences), forskolin (2.5 µg/ml, Calbiochem, San Diego, CA) and Normocin. PC3 and DU145 human prostatic carcinoma cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA). These cell lines were maintained in RPMI 1640 supplemented with 5% FBS and gentamycin (0.1 mg/ml). 293T human kidney cells obtained from ATCC were grown in DMEM medium supplemented with FBS (10%), glutamine (1%) and gentamycin (0.1 mg/ml). Maintenance of AS30D, a rat hepatocellular carcinoma cell line, as an ascitic tumor has been previously described (Faris et al., 1990).

Rat prostate epithelial cell (PEC) cultures

An abbreviated description for the development of the rat PEC line was first described by (Britt et al., 2004). Here, we provide a more detailed description of the PEC isolation. Dorsal and lateral lobes from male Fischer rats (200–250 g) were removed, freed of connective tissues, rinsed in Hanks Balanced Salt Solution, HBSS (Sigma-Aldrich), containing gentamycin (0.25 mg/ml) and 2 mM EGTA and minced. Resuspended tissue fragments collected by centrifugation in HBSS supplemented with 0.025 mM CaCl_2 , 0.1% BSA, 0.1% collagenase type I (158 units/mg, Gibco BRL), 0.1% protease type XIV (5.3 units/mg, Sigma) were incubated in a shaking water bath for 1 h at 37 °C. Cells and tissue fragments pelleted at 150 $\times g$ were resuspended in RPMI 1640 medium (5% FBS). After settling at 1 $\times g$ for 5 min, the supernatant containing dissociated cells was collected and the undigested fragments were incubated in 10 ml Dispase digestion medium (BD Biosciences) for 30 min at 37 °C. Cells and tissues collected at 150 $\times g$ were resuspended in RPMI (5% FBS), combined with those from the first digestion, pelleted at 150 $\times g$, resuspended in RPMI medium, passed through an 18 gauge needle, and resuspended in complete PEC medium supplemented with 12.5 $\mu\text{g/ml}$ fungizone (InvivoGen), and cholera toxin (20 ng/ml). One ml suspensions were seeded in 12-well culture dishes. After 1 h at 37 °C, unattached cells were transferred into fresh wells and maintained at 37 °C for 3–4 days. Plating medium was then replaced with PEC complete medium. After 2 weeks, primary cultures were incubated for 30 min at 37 °C in PBS containing 2 mM EDTA. Cultures were subsequently subcultured every 7–14 days using trypsin/EDTA. The epithelial origin of the PEC culture has been previously described (Britt et al., 2004; Halpert et al., 1996).

Cell cycle analysis by FACS

BDEC or PEC were plated in 24-well plates in triplicate for each time point at a starting concentration of 5×10^4 cells/ml. Cells were allowed to reach 50–60% confluence before arrest in G1/S by serum starvation. Cultures were placed in serum-free stock medium supplemented with Normocin and gentamycin but lacking all growth factors. After 48 h, serum-free medium was removed and replaced with complete medium. For each time point following release into serum containing medium, cells were harvested from 3 plates by washing with HBSS, incubating with trypsin/EGTA (0.5%) and resuspending cells in 5 ml of complete medium with fetal calf serum to neutralize trypsin activity. Cell suspensions were gently disrupted by pipetting until a single-cell suspension was obtained. Following two washes at 4 °C with Ca^{2+} and Mg^{2+} free PBS containing glucose at 1 g/L cells were resuspended at a concentration of 1×10^7 cells/ml and pelleted at 400 $\times g$ at 4 °C. The supernatant was decanted and the cell pellet vigorously vortexed in the remaining fluid for 10 s, after which 1 ml of ice-cold ethanol (70%) was slowly added dropwise while continuing to vortex. Samples were fixed in ethanol overnight at 4 °C and stored in ethanol at 4 °C. For propidium iodide (PI) staining, cell pellets were resuspended and centrifuged for 5 min at 1200 $\times g$ and the ethanol decanted. Pellets were vortexed in residual ethanol before adding 500 μl of PI staining solution consisting of propidium iodide (0.05 mg/ml) (Roche, Indianapolis, IN), and RNase A (100U/ml) (Sigma) in glucose–PBS sample buffer. Cells were vortexed, incubated for 30 min at room temperature with gentle rocking and analyzed within 24 h.

Microtiter immunocytochemical ELISA (MICE)

Levels of BD.1 expression were measured using a modified version of the microtiter immunocytochemical ELISA (MICE) (de la Monte et al., 1999). In the modified ELISA, viable, metabolically active cells were quantified by the MTS Assay (Promega, Madison, WI) instead of Coomassie blue binding (Bernas and Dobrucki, 1999; de la Monte et al., 1999). BDEC or PEC were seeded in 24-well plates at a concentration of 5×10^4 cells/well. At 96 h after seeding when cultures had reached 50–60% confluence, complete medium was removed and replaced with serum-free medium without growth factors for synchronization. Following a 48-h incubation, medium was replaced with complete medium and at each time point MTS values were obtained prior to fixation. Plates were then fixed in acetone (100%) at 4 °C. Cells were permeabilized (0.2% TX-100), treated with 0.03% H₂O₂, blocked with 1% NGS/TBS, and incubated sequentially with MAb BD.1 and HRP-conjugated mu chain specific Goat anti-mouse IgM. Following incubation with ABTS (Pierce), a normalized MICE index was calculated by dividing the level of BD.1 expression (OD at 650 nm) by the total number of cells/well (MTS value at 490 nm).

Indirect immuno fluorescence microscopy

Cells were plated on chamber slides under sterile conditions and grown in serum-supplemented medium until 60–70% confluent. Slides were washed three times in PBS, fixed in ice-cold acetone for 5 min at 4 °C and stored at –80 °C. For indirect immunofluorescence (IIF), slides were blocked with normal goat serum in PBS (1% NGS/PBS), incubated with primary antibody and treated with either Alexa fluor 594-conjugated goat anti-mouse IgM or Alexa fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) (Hixson et al., 1997). For double-labeling experiments, slides were incubated sequentially with anti-tubulin antibodies and MAb BD.1, secondary antibodies were also added sequentially following the appropriate primary. For double labeling with MAb BD.1 and anti-CLIP170, cells or tissues were incubated sequentially with mixtures of the two primary antibodies and the two secondary antibodies. Labeled slides were observed under a Nikon Eclipse E800 fluorescence microscope (Nikon Instruments, Inc., Melville, NY, USA) and digital images acquired using a SPOT camera (SPOT Imaging Solutions, Sterling Heights, MI, USA).

Cell lysates and immunoblotting

Cells were harvested with trypsin/EGTA (0.5%), and washed with HBSS. Cell pellets containing 2×10^7 cells were resuspended in 1 ml lysis buffer containing 1% TX-100, 10 mM tris (pH 7.4) and 20 µl pro-tease inhibitor cocktail (Calbiochem, San Diego, CA), vortexed and incubated on ice. After 15 min, extracts were centrifuged at 4000 ×g, 4 °C for 10 min. The supernatant was removed and the resulting pellet was resuspended in 0.5 ml of lysis buffer and 10 µl protease inhibitor cocktail, and incubated on ice for an additional 15 min. Insoluble material was pelleted at 4000 ×g, 4 °C for 10 min, and the first and second supernatants were combined to make the final detergent extract. For SDS-PAGE, 1 volume of 5× reducing SDS sample buffer was mixed with four volumes of detergent extract (Laemmli, 1970). For comparative analysis, each sample was prepared with an equal volume of detergent extract. After heating at 70 °C samples were separated by SDS-PAGE on 7.5%

gels. The gels were Western blotted onto nitrocellulose membranes, blocked with 5% milk, incubated with primary antibody, and treated with HRP-conjugated anti-IgM or anti-IgG. Bound HRP was visualized by chemiluminescence using SuperSignal West Peroxide Solution (Pierce, Rockford, IL). Samples were immunoblotted onto PVDF membranes (Bio-Rad) previously soaked in ethanol for 30 s and rinsed with water. Transfer was performed using Bio-Rad's semi-dry transfer apparatus for 30 min at 25 V. After transfer, membranes were incubated in 8% acetic acid for 15 min, rinsed with water, and air-dried. PVDF membranes were rehydrated with methanol, rinsed with water and blocked in 5% milk overnight at 4 °C. Blots were labeled with antibodies using the immunoblot protocol described below.

Microtubule depolymerization and immunoprecipitations

Cell pellets were resuspended in 1–2 ml of lysis buffer pH 7.4 containing 30 mM Hepes, 100 mM KCl, 1% TX-100, 20 µl/ml protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem), and 20 µl/ml phosphatase inhibitor cocktail (Halt Phosphatase Inhibitor Cocktail, Pierce), vortexed, and incubated while rocking at 4 °C to depolymerize microtubules (Hoogenraad et al., 2000). After 40 min, insoluble material was removed by pelleting extracts at 13,000 rpm, 4 °C for 12 min. Supernatants were analyzed by SDS-PAGE as describe above. Immunoprecipitations were carried out as previously described (Hoogenraad et al., 2000). IgM coupled sepharose (BD.1) or Protein G (CLIP170) was washed three times with PBS-T. All subsequent steps were performed at 4 °C. Supernatants were pre-cleared for 45 min with washed IgM- or Protein G-Sepharose. After removal of Sepharose beads by centrifugation, MAb BD.1 or MAb CLIP170 was added to the pre-cleared supernatant together with fresh aliquots of anti-IgM-(MAb BD.1) or Protein G (MAb CLIP170) Sepharose. Following a 2-h incubation at 4 °C, Sepharose beads with bound immune complexes were collected by centrifugation, resuspended in PBS-T, and washed by pelleting through a 5% sucrose cushion. Immune complexes were eluted by incubating the Sepharose beads in 70 µl 1× reducing SDS sample buffer, at 70 °C for 15 min. The supernatant removed from the beads prior to the sucrose wash (unbound) and bound fractions were analyzed by SDS-PAGE as described above.

Purification of BD.1

Since PEC grew more rapidly than BDEC, did not require collagen gels and expressed significantly higher levels of BD.1, they provided a valuable source of BD.1 for LC-ESI-MS/MS analysis. Two different approaches were used to purify BD.1. Both took advantage of the increase in solubility following disruption of microtubules.

Protocol 1—PEC cells growing in 100 mm culture dishes were washed twice at room temperature with Pipes/Hepes buffer (PHEM, 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM MgCl₂, pH 6.9) and lysed for 5 min at room temperature with PHEM buffer containing 0.15 M Brij 58. Detergent resistant cytoskeletons that remained attached to the plate were washed 2 times for 1 min at room temperature in low ionic strength microtubule stabilizing buffer (20 mM Pipes, 2 mM MgSO₄, 1 mM EGTA, 1 mM GTP, pH 6.9). Microtubules were depolymerized by incubating cytoskeletons for 30 min on ice in Pipes/Hepes buffer containing 1 mM MgCl₂, 1 mM CaCl₂, pH 6.9. Cells were removed with a

rubber scraper and the pooled buffer was centrifuged at $30 \text{ K} \times \text{g}$ for 15 min at 4°C . The pellet was resuspended in 1% TX100 in 10 mM Tris, pH 8.0 with 10 mM EGTA and protease inhibitors. After centrifugation at $15 \text{ K} \times \text{g}$ for 15 min at 4°C , the supernatant and the pellet (25 μg of each) were mixed with non-reducing SDS sample buffer, heated at 70°C for 10 min and resolved by SDS-PAGE on a 7.5% gel. One lane was immunoblotted with MAb BD.1 and the remainder of the gel was stained with Silver Quest. The silver stained band with the same mobility as the 170 kDa band recognized with MAb BD.1 was cut from three lanes, gel slices digested with trypsin and submitted to the Proteomics Core Facility for the Rhode Island Hospital Center of Biomedical Research Excellence (COBRE) for Cancer Research Development (CCRD) for analysis by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) on a QSTAR XL mass spectrometer (Applied Biosystems, Framingham, MA, USA).

Protocol 2—PEC (6×10^7 cells) harvested with trypsin/EGTA solution (Sigma) were divided into 3 aliquots containing 2×10^7 cells. Each aliquot of cells was pelleted and resuspended in 1 ml of lysis buffer (1% TX100, 30 mM Hepes, 100 mM KCl, pH 7.4) with 20 μl each of phosphatase and protease inhibitor cocktails (Calbiochem, San Diego, CA). After a 40 min incubation on ice to depolymerize microtubules, extracts were clarified at $30 \text{ K} \times \text{g}$ for 12 min at 4°C and pre-cleared for 45 min with anti-mouse IgM-Sepharose which had been washed with PBS containing 0.1% Tween (PBS-T). Sepharose beads were removed by centrifugation and 85 μg of MAb BD.1 (IgM isotype), together with a fresh aliquot of anti-mouse IgM-Sepharose, was added to each lysate. After a 2-h incubation at 4°C , Sepharose beads with bound immune complexes were collected by centrifugation, resuspended in PBS-T and washed by pelleting through a 5% Sucrose cushion. Immune complexes were eluted by incubating the Sepharose beads in 70 μl of $1 \times$ reducing SDS-PAGE sample buffer at 70°C for 15 min. After pelleting the Sepharose beads by centrifugation, the supernatants were combined and analyzed by reducing SDS-PAGE. A gel Code Blue stained band at 170 kDa was cut from the gel, digested with trypsin and submitted to the COBRE Proteomics Core for LC-ESI-MS/MS analysis.

Phosphoprotein enrichment column

Phosphorylated proteins were extracted from PEC using the phosphoprotein enrichment kit from BD Biosciences Clontech (Mountain View, CA) following protocol PT3731-1. Briefly, PEC were treated with extraction buffer, mixed gently and incubated at 4°C for 10 min. Following the incubation, the cell extract was centrifuged at $10,000 \times \text{g}$ for 20 min at 4°C . After centrifugation, the clarified super-natant was removed and added to an equilibrated column. Column and sample were gently agitated at 4°C for 20 min on a rocker to allow the phosphorylated proteins to bind to the column. Following incubation, the extract that did not bind to the column (unbound) was collected, the column washed and the bound material eluted with the provided buffer. Unbound and eluted fractions were analyzed for BD.1 expression by immunoblot as described above.

Plasmids and transfection

A GFP-CLIP170 expression plasmid for rat CLIP170 was a generous gift from Niels Galjart; Erasmus University, The Netherlands (Hoogenraad et al., 2000). A pGEM-Myc-

CLIP170 expression plasmid containing human CLIP170 was generously provided by Franck Perez; University of Geneva, Switzerland (Perez et al., 1999; Pierre et al., 1994). A pCMV6-AC-GFP expression plasmid containing a GFP-tagged ORF clone of human eIF3a was obtained from Origene (Rockville, MD). 293T cells were transfected using Lipofectamine 2000 according to the manufacturer's recommended protocol (Invitrogen, Carlsbad, CA). Cells were harvested 48 h post-transfection by washing with HBSS and incubating with trypsin/EGTA (0.5%). Cells were pelleted, washed once with HBSS and frozen at -70°C until analyzed by immunoblot as described above.

siRNA-mediated knockdown of CLIP170 and eIF3a proteins

293T cells were transfected using Lipofectamine 2000 according to the manufacturer's recommended protocol for siRNA (Invitrogen, Carlsbad, CA). CLIP170 and eIF3a siRNAs were obtained from Ambion (Austin, TX) and Origene (Rockville, MD), respectively. Following transfection with CLIP170 siRNA, cells were harvested at 48 and 72 h. Cells transfected with eIF3a siRNA were re-transfected after 72 h and cells were collected at 48 h. Cell pellets were lysed as described above under microtubule depolymerizing conditions and the samples analyzed by Western blot for BD.1, CLIP170 or eIF3a. To establish percent knockdown, Quantum One (Bio-Rad) was used to measure the density of each band. Protein expression was normalized using the negative control siRNA. Percent reduction equaled the difference in expression between untreated and treated cells after normalization.

Results

MAB BD.1 recognizes a 170 kDa protein expressed by both rat and human prostate cells

In previous work we have demonstrated that monoclonal antibody BD.1 reacts with a 170 kDa protein expressed by rat fetal and adult cholangiocytes, cultured rat BDEC, the rat hepatocellular carcinoma-derived line H5D, and the human hepatoma cell line HTB 52 (Yang et al., 1993a, 1993b). Here, we extend these findings and demonstrate that MAB BD.1 also reacts strongly with rat and human PEC lines. When non-tumorigenic (RWPE) and malignant (PC3 and DU145) human prostate cells were labeled by IIF with MAB BD.1, each cell line showed strong cytoplasmic labeling (Fig. 1A). Western blot analysis of RWPE, PC3 and DU145 protein extracts confirmed that MAB BD.1 reacted with a 170 kDa protein (Fig. 1B). We have shown previously by indirect immunofluorescence that rat BDEC express high levels of BD.1 antigen at low passage ($p < 35$ passages) but showed a marked decrease in expression levels at mid ($p 36-84$) and high ($p > 85$) passage (Rozich et al., 2010). As shown by immuno-blot analysis, BD.1 expression in rat PEC also varied with passage number as low ($p 30$ passages) and high ($p > 70$ passages) passage cells showed the strongest and mid-passage ($p 31-69$) the weakest reactivity with MAB BD.1 (Fig. 1C).

BD.1 expression by BDEC and PEC is cell cycle dependent

To define the relationship between BD.1 expression and cell-cycle status, BD.1 levels in low passage BDEC and PEC were determined following arrest in G0/G1 by serum starvation and after release in serum-supplemented medium. As shown in Fig. 2A, MTS values for BDEC did not change but BD.1 levels steadily increased during a 48-h serum starvation. However, when cells were switched to serum-supplemented medium, there was an immediate and

sustained decrease in BD.1 levels that mirrored the increase in MTS values between 96 and 148 h as cells transitioned into M phase. As shown in Fig. 2B, arrest of PEC cells in G0/G1 by serum starvation resulted in a much more pronounced increase in BD.1 levels than that observed with BDEC. Release into serum containing medium resulted in a precipitous drop in BD.1 expression that was followed by a gradual rise as cells transitioned into S phase. FACS analysis of PI stained BDEC and PEC showed that the majority of cells were in G0/G1 at the end of starvation and began to enter S phase between 10 and 24 h after release (data not shown). At approximately 100 h after the start of starvation, MTS values for both BDEC and PEC started to increase and BD.1 levels began to fall as cells presumably progressed through G2/M (Yang et al., 1993b).

Disruption of the cytoskeleton increases the detergent solubility of BD.1

The strong expression of BD.1 by AS30D hepatocellular carcinoma cells and the ability to generate ascites containing large numbers of tumor led us to evaluate AS30D as a source of purified BD.1. Immuno-blot analysis of material extracted in 1% Triton-X 100 (TX-100) from AS30D cells and the insoluble material collected by centrifugation and solubilized in SDS demonstrated that the 170 kDa band recognized by MAb BD.1 was present entirely in the TX-100 insoluble pellet (Fig. 3A, panel a), raising the possibility that BD.1 was associated with the cyto-skeleton. Consistent with this possibility, confocal microscopic analysis of PEC double-labeled by IIF with MAb BD.1 and anti-tubulin antibodies showed co-localization of BD.1 with microtubules composing the spindle apparatus (Fig. 3B). To obtain additional evidence for the cytoskeletal association of BD.1, immunoblot analysis was performed to determine if disruption of the cytoskeleton would increase the solubility of BD.1 in TX-100. Results showed that treatment of AS30D ascites with cytochalasin D, used to depolymerize actin, followed by incubation at 4 °C to disrupt microtubules, shifted BD.1 into the TX-100 soluble fraction, suggesting a major portion of BD.1 was associated with the TX-100 insoluble cytoskeleton (Fig. 3A, panel b).

A high percentage of BD.1 from PEC cells could be solubilized in TX-100

Although the retention of BD.1 in the TX-100 insoluble pellet from AS30D cells offered an effective means of enrichment, subsequent solubilization of BD.1 from the detergent resistant pellet required SDS. The necessity of removing SDS prior to immunoaffinity purification added an additional step that greatly reduced the recovery of BD.1 protein. To avoid the need for SDS, we reoriented our purification strategy towards increasing the solubility of BD.1 in TX-100 by disrupting the cytoskeleton. A survey of several cell lines showed that low passage PEC ($p < 30$) showed strong reactivity with MAb BD.1 and contained a high percentage of TX-100 soluble BD.1, a percentage that could be further increased by disrupting microtubules (Fig. 3A, panel c). Immunoblot analysis of fractions collected during the separation of PEC TX-100 extracts by continuous elution electrophoresis (CEE) (Lim et al., 1993) identified three fractions containing a 170 kDa band reactive with MAb BD.1. When pooled and concentrated, the three fractions displayed a 170 kD band detectable by silver stain that showed an identical mobility to the MAb BD.1 reactive protein (data not shown). Analysis of 25 different mass spectra obtained by SELDI-ToF-MS analysis of tryptic peptides from this 170 kDa band demonstrated the presence of CLIP170 (cytoplasmic linker protein 170), one of a diverse group of plus-end tracking

proteins involved in regulating the dynamic instability of microtubules (Sawin, 2000). However, because CEE separates proteins based only on size, the presence of CLIP170 in the MAb BD.1 positive CEE fractions was not sufficient to establish an identity between the two proteins. To clarify the relationship between BD.1 and CLIP170, several additional experiments were carried out. Double-labeled IIF analysis showed that BD.1 and CLIP170 were co-localized in PEC cultures (Fig. 4A). In normal rat liver, intrahepatic bile ducts were labeled by both anti-CLIP170 and BD.1 antibodies (Fig. 4B). Immunoblot analysis of PEC lysates confirmed the expression of CLIP170 and showed that CLIP170 and BD.1 displayed identical mobilities when resolved by SDS-PAGE (Fig. 4A, inserts). Together, these results were consistent with either an identity or a functional interaction between BD.1 and CLIP170.

BD.1, like CLIP170, is a phosphorylated protein

Numerous microtubule-associated proteins are regulated by phosphorylation. Additionally, previous studies have shown that phosphorylation regulates the binding of CLIP170 to microtubules (Perez et al., 1999; Pierre et al., 1994). To determine the phosphorylation status of BD.1, phosphorylated proteins present in PEC lysates prepared under microtubule depolymerizing conditions were isolated on a phosphoprotein affinity column. Immunoblot analysis of the bound and unbound material showed that BD.1 was found only in the bound fraction, suggesting that most of the BD.1 present in PEC cells is phosphorylated (Fig. 5).

Overexpression or suppression of CLIP170 expression did not produce a corresponding change in BD.1 levels

IIF analysis showed that 293T human kidney cells expressed both BD.1 and CLIP170 (data not shown). Transfection of 293T cells with a rat GFP-CLIP170 expression plasmid (Hoogenraad et al., 2000) induced high levels of the GFP-CLIP170 fusion protein as demonstrated by immunoblot analysis with anti-GFP antibodies. However, a corresponding increase in BD.1 levels was not observed in transfected cells (Fig. 6A) suggesting that BD.1 and CLIP170 were distinct proteins. Transfection experiments were repeated with a human myc-CLIP170 fusion protein (Perez et al., 1999). Immunoblot analysis with anti-CLIP170 detected two reactive bands representing endogenous CLIP170 and the larger myc-CLIP170 fusion protein labeled with anti-myc antibodies (Fig. 6B). In contrast, MAb BD.1 detected only one reactive band with a mobility identical in size to endogenous CLIP170 (Fig. 6B). Taken together, these findings indicated that BD.1 and CLIP170 were distinct proteins. Further evidence supporting this conclusion was obtained by separately transfecting 293T cells with three different siRNAs specific for CLIP170. Immunoblot analysis of cell lysates isolated under microtubule depolymerizing conditions at 48 and 72 h following transfection showed a significant reduction in CLIP170 expression without a noticeable decrease in BD.1 levels (data not shown).

Co-immunoprecipitation confirmed an association between BD.1 and CLIP170

To examine the possibility that BD.1 was present in a stable complex with CLIP170, MAb BD.1 immunoprecipitates from 293T cells transfected with the GFP-CLIP170 fusion protein were isolated under microtubule depolymerizing conditions, a requirement for the immunoprecipitation of CLIP170 in previous studies (Hoogenraad et al., 2000). Immunoblot

analysis showed that GFP-CLIP170 was present in MAb BD.1 immunoprecipitates (Fig. 7A). In addition, anti-GFP immunoprecipitates contained a distinct 170 kDa band reactive with MAb BD.1. Immunoblot analysis of immunoprecipitates from PEC lysates produced similar results. Fig. 7B shows that endogenous CLIP170 was co-precipitated with MAb BD.1 and a faint but clear band reactive with MAb BD.1 was co-precipitated with anti-CLIP170. Taken together, these results demonstrated that under microtubule depolymerizing conditions, BD.1 forms a detergent soluble complex with both exogenous and endogenous CLIP170.

LC-ESI-MS/MS analysis identified BD.1 as eIF3a

Two purification protocols were devised, both of which resulted in BD.1 preparations depleted of CLIP170. The first took advantage of the association of BD.1 with the cytoskeleton. PEC cultures were extracted in Brij 58 under conditions that solubilized cellular membranes and removed organelles, leaving a stabilized cytoskeletal network attached to the culture dish (Schliwa et al., 1981). Following depolymerization of microtubules at 4 °C in Ca^{2+} containing buffer, cytoskeletal components released from culture dishes were centrifuged and the resulting supernatant and a pellet were solubilized in SDS-PAGE sample buffer. In the second protocol, proteins reactive with MAb BD.1 were isolated by immunoprecipitation from TX-100 extracts of PEC cells prepared at 4 °C to depolymerize microtubules. Coomassie or silver stained bands with an apparent MW of 170 kDa were cut from the gel, digested with trypsin and analyzed by LCESI-MS/MS analysis. As shown in Table 1, tryptic digests of purified BD.1 contained a large number of peptides derived from elongation initiation factor 3a (eIF3a), the largest subunit (170 kDa) of the eIF3 initiation complex (Benne and Hershey, 1976).

Immunoprecipitation analysis and siRNA knockdown confirmed the identity of BD.1 as eIF3a

Two approaches were used to confirm the identity between the MAb BD.1 reactive protein and eIF3a. In the first, proteins immuno-precipitated by MAb 5H10 were resolved by SDS-PAGE and incubated with MAb BD.1. As shown in Fig. 8A, MAb BD.1 recognized a 170 kDa band in MAb 5H10 immunoprecipitate thereby demonstrating that the 170 kDa band carried epitopes recognized by MAb BD.1 and MAb 5H10 specific for eIF3a. To further confirm that MAb BD.1 reacted with eIF3a protein, 293T cells were transfected with an eIF3a-specific or a scrambled control siRNA. A second transfection with siRNA was performed after 72 h. Three days following the second transfection, cells were harvested and tested for eIF3a expression by immunoblot analysis with MAb 5H10 and MAb BD.1 antibodies. As shown in Fig. 8B, no eIF3a or the 170 kDa BD.1 reactive protein was detected in immunoblots of siRNA transfected 293T cells labeled with MAb 5H10 or MAb BD.1. Taken together, the results from LCESI-MS/MS analysis, the immunological identity and the simultaneous knockdown of BD.1 and eIF3a lead us to conclude that the 170 kDa protein recognized by MAb BD.1 is eIF3a.

Discussion

In two previous reports, we described the generation and characterization of a monoclonal antibody, MAb BD.1, that showed strong reactivity with mature cholangiocytes but was negative with hepatocytes and oval cells, the latter, a population of bipotent progenitors resident in the biliary tree (Yang et al., 1993a, 1993b). To the best of our knowledge, MAb BD.1 is still one of the only antibodies capable of distinguishing between oval cells and cholangiocytes. Using LC-ESI/MS/MS to determine the primary sequence of tryptic fragments from BD.1 purified by two different schemes, we established a tentative identity between BD.1 and eIF3a, the 170 kDa subunit of the 800 kDa eIF3 initiation complex. eIF3a is a heavily charged protein containing a central region with an 88% identity to centrosomin A, a N-terminal protein-protein interaction domain, a C-terminal alpha helical domain involved in complex formation and an actin binding region composed of spectrin repeats (Scholler and Kanner, 1997). eIF3a is highly conserved among species (Saletta et al., 2010), a characteristic consistent with the strong reactivity of MAb BD.1 with both human and rat eIF3a/BD.1. In addition to size, BD.1 and eIF3a share a number of additional similarities. Like many other proteins involved in the initiation of translation, BD.1 and eIF3a are phosphorylated (Pincheira et al., 2001a). Both are located primarily in the cytoplasm (Pincheira et al., 2001a), both interact with microtubules (Hasek et al., 2000; Shanina et al., 2001), both show cell cycle dependent patterns of expression, both are expressed at high levels in fetal tissues and both are overexpressed by tumor cells (Bachmann et al., 1997; Chen and Burger, 2004; Dellas et al., 1998; Pincheira et al., 2001b; Yang et al., 1993b). That eIF3a is the antigen recognized by MAb BD.1 was confirmed by the ability of eIF3a specific siRNAs to downregulate the expression of BD.1 and by the reactivity of anti-eIF3a antibodies with Western blots prepared with a MAb BD.1 immunoprecipitate.

Intuitively, it would seem that the absence of eIF3a/BD.1 in oval cells and hepatocytes would in some way compromise their translational machinery. However, results from a number of studies indicate that eIF3a is nonessential for the eIF3-mediated formation of preinitiation complexes. eIF3a is not part of the wheat germ eIF3 complex and mammalian eIF3, with or without eIF3a, did not differ significantly in their initiation activity (Saletta et al., 2010). Based on these and other findings, it is now thought that eIF3a is not required for global translation but is needed for translation of a specific subset of mRNAs involved in differentiation and cell cycle regulation (Saletta et al., 2010). eIF3a/BD.1 has also been linked to the translational regulation of the cell cycle inhibitor, p27, alpha tubulin and ribonucleotide reductase (RRM2), the key enzyme in converting ribo to deoxyribonucleotides (Dong and Zhang, 2003; Dong et al., 2004). Down or up regulation of eIF3a increases or decreases, respectively, p27 expression but has the opposite effect on alpha tubulin and RRM2. A role in differentiation is suggested by the tissue specific differences in eIF3a/BD.1 levels during murine fetal development (Liu et al., 2007). In the kidney, for example, highest levels were present at embryonic day (ED) 12 but by postnatal day 90 expression could no longer be detected. In the small intestine and stomach, in contrast, expression levels were high at ED 15 but had fallen to undetectable levels at ED 16 and remained negative thereafter, suggesting that differentiation was preceded by the loss of eIF3a/BD.1. Liu et al. arrived at a similar conclusion based on experiments showing that

siRNA knockdown or overexpression of eIF3a, respectively, resulted in the enhancement or suppression of differentiation of colon cancer cells suggesting that for these cells, eIF3a is a negative regulator of differentiation (Liu et al., 2007). This does not appear to be the case for BDEC (Yang et al., 1993b) and PEC (data not shown), both of which retain expression of eIF3a/BD.1 as they undergo differentiation/maturation.

In a previous report, we noted a rapid decrease in BD.1 positive bile ducts following partial hepatectomy from a high of 70% in resting liver to a low of 30% at 72 h post-surgery (Yang et al., 1993b). This decrease was closely paralleled by an increase in the percentage of BD.1 negative cells labeled with ³H-thymidine, suggesting a loss of BD.1 expression as bile ductal cells entered the cell cycle. This conclusion was reinforced by results from in vitro studies showing a rapid decrease in BD.1 expression by PEC and BDEC cells during the first 10 h after release from serum starvation followed by increasing levels as cells moved into S phase. A rise in BD.1 expression was also observed when cells were blocked in G0/G1 by serum starvation (Fig. 2) or in G1/S by methotrexate treatment (Yang et al., 1993b), a finding at odds with a report by Dong et al. where significant levels of eIF3a were not observed until 16 h after release from serum starvation, a time when the majority of cells (87%) were in S phase (Dong et al., 2009). Although the basis for these discrepancies remains to be determined, they may reflect differences in the role played by eIF3a/BD.1 in the NIH 3T3 cells used by Dong et al. (2009) and the epithelial cell lines used in the present study, a possibility suggested by the differences in BD.1 expression displayed by high passage PEC and BDEC cells.

Decrease or loss of eIF3a/BD.1 expression by BDEC at mid-passage was also closely paralleled by the onset of aneuploidy, further implicating eIF3a/BD.1 in cell division (Rozich et al., 2010). eIF3a/BD.1 contains the entire amino acid coding sequence for centrosomin A, a protein required for proper maturation of centrosomes into high capacity microtubule-organizing centers required for driving assemble of the bipolar spindle apparatus during mitosis. It has been suggested that centrosomins are derived from eIF3a by differential splicing, a possibility consistent with the presence of both eIF3a/BD.1 and the centrosomins on mouse chromosome 19, cytoband D. It has been suggested that loss or decreased levels of centrosomins compromises centrosome maturation, regulation of microtubule-organizing centers, and connection of centrioles to the apical cortex, the end result being errors in centriole segregation and an increased probability of improper segregation of chromosomes (Megraw et al., 1999).

In the course of our efforts to identify BD.1, LC-ESI-MS/MS analysis of a PEC extract enriched for BD.1 by continuous elution electrophoresis identified CLIP170 as a potential candidate. CLIP170 and BD.1 had identical mobilities by SDS-PAGE and were co-expressed by bile ducts. However, transfection of expression plasmids for myc- or GFP-tagged CLIP170 fusion proteins failed to increase BD.1 reactivity and transfection of CLIP170 siRNAs failed to significantly decrease BD.1 levels, indicating these were distinct proteins. Co-precipitation analysis further revealed that both exogenous and endogenous CLIP170 were capable of forming stable complexes with eIF3a/BD.1 leading to the conclusion that eIF3a/BD.1 and CLIP170 were distinct proteins capable of forming stable complexes. When combined with a report by Shanina et al. showing by Western blot that

eIF3a can be co-precipitated with microtubules through two polymerization/depolymerization cycles (Shanina et al., 2001), these findings suggest that the association of eIF3a/BD.1 with microtubules may occur indirectly by an interaction with CLIP170.

CLIP170 is the first reported member of a novel family of approximately 160–180 kDa proteins that bind to the plus-end of microtubules (Carvalho et al., 2003; Howard and Hyman, 2003). The plus-end localization of CLIP170 makes it a particularly attractive candidate as a regulatory factor for microtubule dynamics (Komarova et al., 2002; Perez et al., 1999). Recent studies have shown that CLIP170 binds to the kinetochore via its C-terminus and to microtubules via its N-terminus, allowing it to facilitate the capture of spindle MT by the kinetochore. Disruption of metaphase alignment following knockdown by RNA interference suggests an additional role for CLIP170 in chromosomal segregation (Tanenbaum et al., 2006; Wieland et al., 2004). Exactly where the interaction between eIF3a/BD.1 and CLIP170 fits into cell division and microtubule dynamics remains to be determined. It could be that association with eIF3a/BD.1 interferes with the binding of CLIP170 to the plus-end of microtubules, thereby hindering CLIP170 mediated growth of microtubules and/or their capture by the kinetochore, events that may become dominant during the disassembly of the spindle apparatus in telophase.

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Abbreviations

BDEC	bile duct epithelial cells
PEC	prostate epithelial cells
eIF3	elongation initiation factor 3
MAb	monoclonal antibody
FBS	fetal bovine serum
IIF	indirect immunofluorescence
p	tissue culture passage
CEE	continuous elution electrophoresis
PI	propidium iodide
MICE	microtiter immunocytochemical ELISA
ED	embryonic day

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Fig. 1. Monoclonal antibody BD.1 reacts with human and rat prostate epithelial cell lines. Panel A, Indirect immunofluorescence microscopy showed that the human prostate cell lines RWPE, PC3 and DU145 expressed high levels of the BD.1 antigen. Panel B, Immunoblot analysis of RWPE, PC3 and DU145 protein lysates confirmed that monoclonal antibody BD.1 reacted with a 170 kDa protein. Panel C, Immunoblot analysis of cell lysates low (p29), mid (p47) and high (p72, p104) passage rat prostate epithelial cells showed decreased expression of BD.1 in mid passage cells and increased expression in high passage cells. The anti-Hsc70 antibody was used to demonstrate equal loading of cell lysates in Panels B and C.

Fig. 2.

BD.1 expression is cell cycle dependent. Comparison of normalized BD.1 expression determined by MICE ELISA as described under “Materials and methods” with the number of viable cells as determined by MTS assay in low passage rat BDEC (p29, Panel A) and PEC (p26, Panel B). At each time point, MTS values were obtained and plates fixed for MICE. A normalized MICE index was determined by the ratio of BD.1 positive cells to total cell density per well. Arrows indicate the start and end of serum starvation.

Fig. 3.

BD.1 is associated with the cytoskeleton. (A) Cytoskeletal disruption is necessary for the release of BD.1 into the soluble fraction. *Panel a*, AS30D cells extracted with 1% Triton-X 100 were analyzed by immunoblot for BD.1 expression. Following extraction, BD.1 was found entirely in the insoluble pellet. *Panel b*, Disruption of the cytoskeleton with cytochalasin D for actin depolymerization, followed by incubation at 4 °C to disrupt microtubules, shifted the distribution of BD.1 entirely to the soluble fraction in AS30D cell lysates. *Panel c*, Treatment of PEC extracted with 1% Triton-X 100 at 4 °C for microtubule disruption released BD.1 entirely into the soluble fraction. (B) Confocal microscopy of rat prostate epithelial cells (p27) double-labeled with antibodies against tubulin and BD.1 demonstrated co-localization of the two proteins. Each image consists of nine compiled 12.5 micron sections.

Fig. 4. BD.1 protein co-localizes with the plus end microtubule binding protein CLIP170. (A) Double-labeled immunofluorescence microscopy for BD.1 and CLIP170 showed that the two proteins co-localized in rat prostate epithelial cells. Insets confirmed by immunoblot analysis that rat prostate epithelial cells express CLIP170 and showed that BD.1 and CLIP170 displayed identical mobilities of 170 kDa by SDS-PAGE. (B) Double-labeled immunofluorescence microscopy demonstrated that BD.1 and CLIP170 expression patterns were both restricted to intrahepatic bile ducts in normal rat liver. Original magnification 20 $\times$, scale bars represent 50 μ m.

Fig. 5.

BD.1 is a phosphorylated protein. Phosphorylated proteins from rat prostate epithelial cell lysate extracted under microtubule depolymerizing conditions were isolated on a phosphorylation affinity column as described under “Materials and methods.” Immunoblot analysis of the unbound material, representing unphosphorylated proteins, and the eluted fractions, containing phosphorylated proteins, was examined for BD.1 expression. BD.1 reactivity was specific to the eluted fractions.

Fig. 6.

BD.1 and CLIP170 are distinct proteins. 293T cell lysates transfected with rat GFP-CLIP170 (A) or human myc-CLIP170 (B), and isolated under microtubule depolymerizing conditions as described under “Materials and methods”, were analyzed by Immunoblot analysis with antibodies to CLIP170 (top panels), BD.1 (bottom panels) and either GFP or Myc (A and B, respectively, middle panels). While transfections of CLIP170 induced high levels of exogenous CLIP170 protein, a corresponding increase in BD.1 protein levels was not observed.

Fig. 7.

Co-immunoprecipitation demonstrated that BD.1 forms a stable complex with CLIP170. (A) Immunoprecipitates of 293T cell lysates transfected with GFP-CLIP170 and isolated under microtubule polymerizing and depolymerizing conditions. MAb BD.1 immunoprecipitates were analyzed for GFP-CLIP170 (top panels) by probing with anti GFP antibody, and anti-GFP immunoprecipitates were analyzed for BD.1 (bottom panels) by probing with MAb BD.1. As shown, no complexes were observed under polymerizing conditions. (B) Immunoblot analysis of MAb BD.1 and anti-CLIP170 immunoprecipitates for BD.1 (bottom panels) and CLIP170 (top panels) expression from rat PEC lysates extracted under microtubule depolymerizing conditions.

Fig. 8.

Identity of BD.1 as eIF3a confirmed by immunoprecipitation analysis and siRNA knockdown of eIF3a. Panel A, Immunoprecipitated proteins using MAb 5H10 were resolved by SDS-PAGE. Western blot analysis showed that MAb BD.1 reacted with a 170 kDa band present in MAb 5H10 immunoprecipitate. MAb BD.1 did not show reactivity with Hsp70 IgG control immunoprecipitated proteins. Panel B, eIF3a siRNA decreases BD.1 antigen expression levels. 293T cells were transfected with eIF3a specific siRNA or negative control siRNA (scrambled). The effects of siRNA-mediated knockdown were examined by immunoblot analysis using MAb BD.1 and anti-eIF3a antibodies. The anti-Hsp70 antibody was used as a loading control for the cell lysates. Immunoblot analysis demonstrated that eIF3a siRNA provides highly efficient knockdown of both eIF3a and BD.1 expression levels as compared with the scrambled siRNA control (Scr). Results of 3 independent experiments are shown.

Table 1

LC-ESI-MS/MS analysis identified the 170 kDa BD.1 antigen as eIF3a. The 13 peptide fragments identified in the LC-ESI-MS/MS analysis are underlined and indicated by red letters. The grey region is the microtubule-binding domain. A second microtubule-binding domain is located at residues 25-157. The lack of C-terminal peptides is likely due to the high content of R and K that results in very small peptides.

1	MPAYFQRPEN	ALKR	<u>ANEFLE</u>	<u>VGKK</u>	QPALDV	LYDVMKSKKH	RTWQKIHEPI	ML	<u>KYLELCVD</u>
61	<u>LRK</u> SHLAKEG	LYQY	<u>KNICQQ</u>	<u>VNIKS</u>	LEDVV	RAYLKMAEEK	TEAAKEESQQ	MVL	DIEDLDN
121	IQTPESVLLS	AVSGEDTQDR	TD	<u>RLLLTPWV</u>	<u>KFLWESY</u>	<u>RQC</u>	<u>LDLLRN</u>	NSRV	<u>ERLYHDIAQQ</u>
181	<u>AFK</u> FCLQYTR	KAEFRKLCDN	LRMHLSQIQR	HHNQSTAINL	NNPESQSMHL	ETRLVQLDSA			
241	ISMELWQEAF	KAVEDIHGLF	SLSKKPPKPQ	LMANYYN	<u>KVS</u>	<u>TVFWK</u>	SGNAL	FHASTLHRLY	
301	HLSREMRKNL	TQDEMQRMS	RVLLATLSIP	ITPERTDIAR	<u>LLDMDGI</u>	<u>IIVE</u>	<u>KQRR</u>	LATLLG	
361	LQAPPT	<u>RIGL</u>	<u>INDMVR</u>	FNVL	QYVVPEVKDL	YNWLEVEFNP	LKLCERVT	<u>KV</u>	<u>LNWVRE</u>
421	EPQLQQYVPQ	LQNNITILRL	QQVSQIYQSI	EFSLRLTSLVP	FVDAFQLERA	IVDAARHCDL			
481	QVRIDHTS	<u>RT</u>	<u>LSFGSDLNYA</u>	<u>TREDAPIGPH</u>	<u>LQSMPS</u>	<u>EQIR</u>	<u>NQLTAMSSVL</u>	<u>AKALEVIKPA</u>	
541	<u>HILQEKEE</u>	QHLAVTAYLKN	SRKEHQRI	LA	RRQTIEERKE	<u>RLESLNIQRE</u>	KEELEQREAE		
601	LQKVRKAEEE	RLRQEAKERE	KERILQEHEQ	IKKKT	TVRERL	EQIKKTELGA	KAFK	DIDIED	
661	LEELDPDFIM	AKQVEQLEKE	KKELQERLKN	QEKKIDYFER	AK	<u>RLEEIPLI</u>	<u>KSAYEE</u>	QRIK	
721	DMDLWEQQEE	ERITTMQLER	EKALEHKNRM	SRML	EDRDLF	VMRLKAARQS	VYEEKL	KQFE	
781	ERLAEERHNR	LEERKRQRKE	ERRITYYREK	EEEEQ	RRAEE	QMLKEREERE	RAERAK	REEE	
841	LREYQERVKK	LEEVEKRRQ	RELEIEERER	RREEER	RLGD	SSLSRKDSRW	GDRD	SEGTWR	
901	KGPEADSEWR	RGPPEKEWRR	GEGRDEDRSH	RRDEER	PRRL	GDDEDREPSL	RPDDDR	VPRR	
961	GMDDDRGPRR	GPEEDRFSRR	GADDDRPSWR	NTDDDR	PPRR	IADEDRGNWR	HADDDR	PPRR	
1021	GLDEDRGSWR	TADEDRGPRR	GMDDDRGPRR	GGADDER	SSW	RNADDDRGP	RGLDDDR	GP	
1081	RGMDDDRGP	RGMDDDRGP	RGMDDDRGP	RGLDDDR	GPW	RNADDDRIP	RGAEDDR	GPW	
1141	RNMDDRLSR	RADDDRFP	RR	GDDSRPGPWR	PLVKPGGWRE	KEKAREESWG	PPRESRP	SEE	
1201	REWDREKERD	RDNQDREEND	KDPERERDRE	RDVDREDRFR	RPRDEGGWRR	GPAEES	SSWR		
1261	DSSRRDDRDR	DDRRERDDR	RDLRERRDLR	DDRDRRG	PPL	RSEREEVSSW	RRADDRK	DDR	
1321	VEERDPPRRV	PPPALS	RDRE	REGE	KEKASWRAEK	DRESLRRTKN	ETDEDG	WTTV	
1381	RR								