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LYRIC/AEG-1 overexpression modulates BCCIPα protein levels in prostate tumor cells

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ABSTRACT

LYRIC/AEG-1 is a unique protein that has been shown to promote tumor cell migration and invasion through activation of the transcription factor NF-κB. We performed yeast two-hybrid screening to detect LYRIC/AEG-1 associated proteins, and identified BCCIP, a CDKN1A and BRCA2-associated protein involved in cell cycle regulation and DNA repair. Here, we demonstrate association between LYRIC/AEG-1 and BCCIP in mammalian cells, and define the region of interaction. Co-expression of the two proteins resulted in decreased levels of BCCIPα, an effect partially abrogated by proteasome inhibition. A truncated LYRIC/AEG-1 construct lacking the interaction region did not alter BCCIPα protein levels. Coincidentally, it was observed that overexpression of BCCIPα in DU145 prostate tumor cells induced an apparent neuroendocrine differentiation. In summary, our data suggest LYRIC/AEG-1 is a negative regulator of BCCIPα, promoting proteasomal degradation either through direct interaction, or potentially through an indirect mechanism involving downstream effects of the NF-κB signaling pathway.

LYRIC, also known as AEG-1 [1] and metadherin [2], is a highly conserved protein of unknown function. Elevated expression has been reported in breast and prostate tumors, melanoma and glioblastoma [1–4], and evidence suggests that LYRIC/AEG-1 actively contributes to malignant progression. LYRIC/AEG-1 overexpression activates both NF-κB and Akt signaling pathways [5,6], and the protein acts synergistically with Ha-Ras to promote anchorage-independent growth [1,7]. In prostate tumor cell lines, LYRIC/AEG-1 knockdown resulted in reduced viability and invasiveness [3]. Overexpression of LYRIC/AEG-1in non-tumorigenic cells is not sufficient to induce transformation [1,7], but studies to date suggest that LYRIC/AEG-1 is an important factor promoting progression or metastasis of a variety of tumors.

In our ongoing characterization of LYRIC/AEG-1, we performed a yeast two-hybrid screen to identify associated proteins. We here present evidence that LYRIC/AEG-1 interacts with BCCIP (TOK-1), a BRCA2- and CDKN1A ($p21^{Cip1/Waf-1}$)-associated protein with two major isoforms, BCCIPα and BCCIPβ. Co-expression of LYRIC/AEG-1 with BCCIPα in prostate tumor cells resulted in decreased BCCIPα protein levels, relative to control, suggesting LYRIC/AEG-1 as a possible negative regulator of BCCIPα activity. BCCIPα binds to the cell cycle regulator p21, and enhances p21-mediated inhibition of Cdk2 kinase [8]. Loss of BCCIP impairs cell cycle G1/S checkpoint activation following DNA damage [9], and in conjunction with BRCA2, BCCIP plays a role in homologous recombination repair of DNA damage [10], and contributes to maintenance of chromosome stability [11].

Reduced BCCIPα expression has been observed in breast cancer and glioma cell lines, and exogenous expression of the protein causes growth delay [12], thus BCCIPα has properties of a tumor suppressor. Roversi et al. [13] identified BCCIP as a positional candidate gene lost during glioma progression. In contrast, LYRIC/AEG-1 expression increases in malignant glioma, and contributes to migration and invasiveness [14]. Our data suggest that the inverse relationship between these two proteins is not merely coincidental, and we postulate that one mechanism for down regulation of BCCIPα may be increased expression of LYRIC/AEG-1, within the context of other changes contributing to tumor progression.

Materials and methods

Cell culture and transfection. Tissue culture materials were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Human embryonic kidney 293T cells were maintained in Dulbecco's Modified Eagle Medium with 10% FBS (Hyclone, Logan, UT) and 50 μg/ml gentamycin. DU145 human prostate tumor cells were grown in RPMI with 10% FBS and 50 μg/ml gentamycin. Transfections

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were carried out using Lipofectamine LTX (Invitrogen) as per the manufacturer's protocol.

Eukaryotic expression constructs. A PCR cloning strategy was used to amplify the desired region of cDNA using primers incorporating appropriate restriction sites (Table 1). PCR reactions were routinely 35 cycles annealing at 55 °C, using Platinum Taq DNA Polymerase HiFi (Invitrogen). Products were purified by agarose gel electrophoresis and GeneClean (QBiogene, Inc., Carlsbad, CA), restriction digestion and ligation were conducted using standard protocols [15] and plasmids were transformed into NovaBlue competent cells (EMD biosciences, Gibbstown, NJ). Plasmid DNA was purified using Qiagen (Valencia, CA) kits, and constructs were verified by sequencing at the Yale University WM Keck Facility. MacVector software (MacVector, Inc., Cary, NC) was used for primer design and sequence analysis.

FLAG-tagged full-length rat LYRIC (RNF) was described previously [16]. FLAG-tagged truncation constructs were generated by PCR from rat liver cDNA, and cloned into pFLAG-CMV-5a or pFLAG-CMV-2, for C-terminal or NH_2-terminal FLAG tag, respectively (Sigma–Aldrich, St. Louis, MO). The rat FLAG constructs were on hand at the time of the yeast two-hybrid screen, and used for initial co-precipitation experiments. Transfections in DU145 cells used human LYRIC/AEG-1 constructs. Full-length human cDNA encoding LYRIC/AEG-1 was cloned into the pcDNA4/TO expression vector (Invitrogen), with no epitope tag. A control pcDNA4-lacZ construct was provided by Invitrogen. The truncated construct, hsNΔ169, was amplified by RT-PCR from DU145 RNA, and cloned into pFLAG-CMV2. RNA was isolated using an RNeasy kit (Qiagen) and cDNA was synthesized using random hexamers and Superscript III (Invitrogen).

Full-length BCCIPα and splice variant c were amplified by RT-PCR from commercially available human prostate RNA (Ambion, Austin, TX), and cloned into the pCMV-HA vector (Clontech, Mountain View, CA), incorporating an HA tag at the NH_2-terminus of the expressed protein. The HA-tagged control for immunofluorescence, HA-CLASP1, is a partial cDNA encoding the C-terminal 583 amino acids of the protein cloned into pCMV-HA.

Yeast two-hybrid screening. Protein interactions were identified using the CytoTrap yeast two-hybrid system (Stratagene, La Jolla, CA) as per the manufacturer's protocol. Human LYRIC/AEG-1 cDNA encoding amino acids 71–582 was amplified by PCR and cloned into the pSos vector. A commercially available (Stratagene) human prostate cDNA library cloned in the pMyr target vector was co-transformed with pSos-LYRIC into yeast strain cdc25H using an *S.c.* EasyComp Transformation kit (Invitrogen). Control transformations, media and growth conditions were performed as per the CytoTrap kit protocol. Colonies containing putative interacting proteins were cultured in YPD broth, plasmid DNA was isolated using a Y-DER Yeast DNA Extraction Reagent Kit (Pierce, Rockford, IL) and the insert was partially sequenced and identified by BLAST search. Interactions of interest were confirmed by co-transforming pSos-LYRIC individually with candidate pMYR target plasmids.

Table 1
Primers used for cloning

Construct	Primer sequences (forward and reverse)
pSOS-LYRIC	5′-GGCGGATCCGCTGGGCCGCGGCTTGC-3′ 5′-GGCGTCGACTCACGTTTCTCGTCTGGC-3′
HA-BCCIPα	5′-GCGGAATTCACATGGCGTCCAGGTCTAAGC-3′ 5′-GCGCTCGAGATTATGACAGAGCAATTCCAAC-3′
HA-BCCIPc	5′-GCGGAATTCACATGGCGTCCAGGTCTAAGC-3′ 5′-GCGCTCGAGTGGCTTCATTCATCCTGGCTTG-3′
Δ463	5′-CGCGAATTCGACGGGAGGGAAGATGGCTG-3′ 5′-CGCGGATCCTTCTGTGTCCTGGGTGATAGAG-3′
Δ238	5′-CGCGAATTCGACGGGAGGGAAGATGGCTG-3′ 5′-CACGGATCCTGCAGTTGTAAGTTGCTCGGTG-3′
NΔ280	5′-CACGAATTCAGTCAATGGAGGAGGCTGGAGTG-3′ 5′-GTGGGATCCTTCACGTTTCCCGTCTGGC-3′
NΔ169	5′-CACGAATTCAAAGTCAGATGCTAAAGCAGTG-3′ 5′-GTGGGATCCTTCACGTTTCCCGTCTGGC-3′
hsNΔ169	5′- CACGAATTCAAAGTCAGATGCTAAAGCAGTG-3′ 5′-GTGGGATCCTTCACGTTTCTCGTCTGGC-3′

Western blotting and immunoprecipitation (IP). Protein extraction, IP, and Western blotting were performed as described previously [16]. Anti-FLAG agarose (Sigma) and the HA-Tag IP Application Set (Pierce) were used to IP FLAG-tagged and HA-tagged proteins, respectively. Anti-LYRIC PAb5393 was described previously [16]; anti-HA, anti-FLAG, anti-p21 and anti-β-actin were from Sigma–Aldrich; anti-BCCIP was from Novus Biologicals (Littleton, CO). Secondary antibodies were goat anti-rabbit HRP conjugate (Sigma–Aldrich) or goat anti-mouse HRP (Invitrogen). Densitometry analysis was performed using a Kodak 1D electrophoresis documentation and analysis system, with statistical comparisons by Student's *t*-test.

Indirect immunofluorescence (IIF). For IIF, cells were cultured on permanox plastic chamber slides (Nalge/Nunc-Fisher Scientific, Pittsburgh, PA) and stained as previously described [16]. IIF was visualized using a Nikon Microphot FX epifluorescence microscope equipped with a SPOT camera (Diagnostic Instruments, Inc., Sterling Heights, MI), and images were annotated for publication using Adobe Photoshop 7 for Apple Macintosh.

Results

BCCIP is identified as a LYRIC/AEG-1 associated protein

To gain insight as to a potential function for LYRIC/AEG-1, a yeast two-hybrid assay was performed to identify associated proteins. A cloned portion of LYRIC/AEG-1, lacking the first 70 NH_2-terminal amino acids encompassing the hydrophobic domain, was used as bait to screen a commercially available human prostate library. Potentially interesting candidates were re-tested individually and confirmed in the yeast system. One clone, chosen for further investigation, contained cDNA encoding amino acids 3–78 of a protein originally identified by two different groups as BCCIP, a BRCA2-interacting protein [12] and TOK-1, a p21(Cip1/Waf1) binding protein [8]. Three isoforms have been described, BCCIPα, BCCIPβ and BCCIP-c, which differ in their carboxy termini. To verify interaction with LYRIC/AEG-1, full-length cDNAs for BCCIPα and the BCCIP-c isoform were cloned into a vector providing an HA epitope tag. HA-BCCIP constructs were each co-transfected with FLAG-tagged LYRIC/AEG-1 into 293T cells, and LYRIC/AEG-1 was immunoprecipitated with anti-FLAG agarose. Western blot of cell lysates with anti-FLAG and anti-HA antibodies verified that LYRIC/AEG-1 and both BCCIP isoforms were expressed (Fig. 1A lanes 1–3), and that FLAG-LYRIC/AEG-1 was successfully immunoprecipitated (Fig. 1A lanes 4–6 upper panel). Both isoforms of BCCIP co-precipitated with LYRIC/AEG-1 and were detected by Western blot with anti-HA antibody (Fig 1A lanes 4–6 lower panel), demonstrating interaction between LYRIC/AEG-1 and BCCIP in mammalian cells.

Association between endogenous human LYRIC/AEG-1 and HA-BCCIPα was demonstrated by transfection of 293T cells with the HA-BCCIPα plasmid, and immunoprecipitation of native LYRIC/AEG-1 with polyclonal antibody (PAb) 5393. HA-tagged BCCIPα co-precipitated with endogenous LYRIC/AEG-1, while neither protein was precipitated by pre-immune rabbit serum used as a negative control (Fig. 1B). In the reverse of this experiment, HA-tagged BCCIPα transfected into 293T was precipitated with anti-HA antibody, and endogenous LYRIC/AEG-1 co-precipitated (Fig. 1C).

To define the region of LYRIC/AEG-1 necessary for association, a series of FLAG-tagged rat LYRIC/AEG-1 constructs with amino- or carboxy-terminal deletions were tested for interaction with HA-