Brain cytoplasmic 200 RNA (BC200 RNA) is a neuron-specific non-coding RNA, implicated in the inhibition of local synaptodendritic protein synthesis, and is highly expressed in some cancer cells. Although BC200 RNA has been shown to inhibit translation in vitro, the cellular location of this inhibition is unknown. In this study, we used a BC200 RNA-recognition antibody to identify the cellular locations of BC200 RNA in HeLa cervical carcinoma cells. We observed punctate signals in both the cytoplasm and nucleus, and further discovered that BC200 RNA co-localized with the p-body decapping enzyme, DCP1A, and the heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2). The latter is a known BC200 RNA-binding partner protein and a constituent of p-bodies. This suggests that BC200 RNA is localized to p-bodies via hnRNP E2. [BMB Reports 2017; 50(6): 318-322]

INTRODUCTION

In 1993, brain cytoplasmic 200 RNA (BC200 RNA) was first identified by Tiedge’s group as a non-coding RNA expressed in human neuronal cells (1). A possible correlation between BC200 RNA and cancer was reported by the same group in 1997 (2). The authors detected BC200 RNA in human germ cells and several immortalized cell lines that did not originate from neurons. Moreover, in situ hybridization experiments showed that BC200 RNA was abnormally overexpressed in various cancers, including breast, lung, tongue, esophagus, ovary, and cervix (2). A later study found that particularly high levels of BC200 RNA were expressed in invasive breast cancer cells and several immortalized cell lines that did not originate from neurons. Furthermore, in non-small-cell lung cancers, BC200 RNA was confirmed that MabBC200-A3 can be used to immunostain BC200 RNA in HeLa cells, and thereafter used it to study the cellular localization of BC200 RNA in these cells. We found that the antibody yielded concentration-dependent immunostaining signals for BC200 RNA in the tested cell line, and the BC200 RNA was localized as punctuates in both the cytoplasm and the nucleus of HeLa cells.

The binding of proteins to BC200 RNA could play an important role in its subcellular localization. Recently, we identified heterologous nuclear ribonucleoprotein E2 (hnRNP E2) as a binding partner of BC200 RNA, as assessed using a yeast three-hybrid assay (13). hnRNP E2 is a multifunctional protein that participates in a variety of cellular processes, including RNA metabolism (14, 15) and translational enhancement (16). Although it is mostly located in the nucleus, a considerable portion of hnRNP E2 is found in the cytoplasm,
enriched in the p-bodies and stress granules, where RNA-processing factors function to control the RNA metabolism (17). Since hnRNP E2 is a constituent of p-bodies, we suspect that BC200 RNA might be localized to p-bodies through its binding to hnRNP E2. Indeed, our immunostaining analysis with MabBC200-A3 shows that BC200 RNA and hnRNP E2 co-localize along with the p-body decapping enzyme, DCP1A.

RESULTS AND DISCUSSION

To investigate the localization of BC200 RNA, we first examined whether the MabBC200-A3 antibody (12) could immunostain the BC200 RNA in HeLa cells. When total cell lysates were treated with the antibody, about half of the cellular BC200 RNA molecules were immunoprecipitated by the antibody (Fig. 1B), suggesting that the antibody effectively recognizes the BC200 RNA in the cell. However, about 50% of the BC200 RNA molecules were not recovered by immunoprecipitation. This reflects that some proteins capable of interacting with the MabBC200-A3 binding motif of BC200 RNA (nts 63-107) compete with the antibody for RNA binding (12), enabling some BC200 RNA molecules to avoid interacting with the antibody.

Next, we immunostained the cellular BC200 RNA and subjected the cells to confocal fluorescence microscopy. When permeabilized cells were treated with increasing amounts of MabBC200-A3, we found that the fluorescent signal increased dose-dependently, up to 1 μg (Fig. 1C). To examine whether this saturation point reflected a limited amount of cellular BC200 RNA available for antibody binding, we transfected HeLa cells with increasing amounts of a BC200 RNA-expressing plasmid (pSUPER-BC200), and examined whether the fluorescent signal increased with the amount of cellular BC200 RNA. Indeed, we found that the transfected cells showed dose-dependent increase in the fluorescent signal (Fig. 2A and B), proportional to the cellular content of BC200 RNA (Fig. 2C). Finally, we used the validated antibody to further investigate the subcellular localization of BC200 RNA. We observed a dispersed fluorescence throughout the cells, including both punctate staining in the nuclei and fine punctates throughout the cytoplasm (Fig. 3A).

We recently identified a heterogeneous ribonucleoprotein E2 (hnRNP E2, also known as PCBP2) as a BC200 RNA-interacting protein (13). hnRNP E2 binds to BC200 RNA mainly through the unique 3’ C-rich domain (nts 162-200) of BC200 RNA (Fig. 1A), which does not overlap with the antibody-binding motif. To evaluate whether the interaction of BC200 RNA and hnRNP E2 is detected by immunostaining with MabBC200-A3, we transfected cells with an hnRNP E2-eCFP-expressing plasmid, immunostained BC00 RNA with MabBC200-A3, and examined the distribution of BC200 RNA and the hnRNP E2-eCFP fusion protein by confocal microscopic analysis. As shown in Fig. 3B, the hnRNP E2-eCFP fusion protein exhibited both dispersed and punctate staining patterns. The punctate staining pattern overlapped with that of BC200 RNA, suggesting that BC200 RNA and hnRNP E2 co-localize in HeLa cells.

Since hnRNP E2 is known to be a constituent of p-bodies (17), we used DCP1A as a p-body marker protein (18), and examined whether BC200 RNA localizes to p-bodies. When
HeLa cells were transfected with a DCP1A-mCherry fusion construct, the DCP1A fluorescence staining pattern overlapped with that of BC200 RNA (Fig. 4A). Additional transfection experiments with both hnRNP E2-eCFP and DCP1A-mCherry fusion constructs confirmed that hnRNP E2 co-localized with DCP1A (Fig. 4B).

BC200 RNA acts as a general translation inhibitor in vitro, by interacting with components of the translational machinery (7-9). However, recent in vivo studies indicate that only a limited number of genes were altered by BC200 RNA (11), suggesting that one or more mechanisms govern the specificity of BC200 RNA-mediated inhibition of translation in vivo. In neuronal cells, BC200 RNA is normally restricted to dendrites, and thus acts on the dendritic mRNAs (19). In cancer cells, such as HeLa cells, BC200 RNA may also affect the specifically localized mRNAs. Thus, our present observation that BC200 RNA is localized in p-bodies suggests that BC200 RNA inhibits the translation of mRNAs localized in p-bodies. BC200 RNA is known to be targeted to dendrites by hnRNP A2/B1 in neuronal cells (19). Likewise, it is likely that BC200 RNA could be targeted to p-bodies by hnRNP E2 in HeLa cells because the p-body constituent (hnRNP E2) is a binding partner of BC200 RNA (13).

Although the cellular localization of BC200 RNA can be visualized by RNA fluorescence in situ hybridization (FISH) using antisense nucleic acids (20), the use of MabBC200-A3 has three major advantages over FISH. 1) Since both the sequence and structure of RNA are crucial for the antibody-RNA interaction, the recognition of RNA by an antibody is
more specific than a complementarity-based RNA interaction, which is generally associated with nonspecific signals. 2) Whereas antisense nucleic acids are used under conditions that prevent the retention of structure, an antibody can be used to monitor complex and dynamic behaviors of RNAs that have various conformations and are involved in the formation of different ribonucleoproteins. 3) FISH is a multistep procedure that generally involves cell fixation and denaturation of target RNA (20), making it difficult (if not impossible) to detect RNA dynamics over time in living cells.

MATERIALS AND METHODS

Cell culture and transfection

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic (Gibco), at 37°C with 5% CO₂. For transfections, cells were seeded and incubated for 24 h, and plasmid DNA transfections were performed using Lipofectamine 3000 (Invitrogen), according to the manufacturer’s protocol.

Plasmids

For expression of hnRNP E2-eCFP, the hnRNP E2-encoding sequence was PCR amplified from a human cDNA library and cloned into the HindIII/BamH I sites of pECFP(n1) (Clontech). For expression of the DCP1A-eGFP and DCP1A-mCherry fusion proteins, the DCP1A-encoding sequence was PCR amplified and cloned into the XhoI/SacII sites of pEGFP(c1) (Clontech) or the HindIII/BamH I sites of pmCherry(c1) (Clontech). For ectopic expression of BC200 RNA, the BC200 RNA-encoding sequence was PCR amplified and cloned into pSUPER (Clontech) to generate pSUPER-BC200.

RNA pull-down assay

Cells were washed, suspended in 10% FBS/PBS, permeabilized by 0.1% PBST, and incubated with MabBC200-A3 (5 μg) for 30 min at room temperature. MabBC200-A3 was prepared as previously described (12). The cells were washed twice with cold PBS, and lysed in cold RIPA buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Na-deoxycholate (m/v), 0.1% SDS (w/v), 1% Triton X-100 (v/v), 2 mM EDTA]. The cell lysates were incubated with protein G agarose beads (Invitrogen) for 1 h at room temperature. The beads were collected and washed three times with cold RIPA buffer, and the immunoprecipitated RNAs were purified by phenol extraction and examined by Northern blotting. As an input control, whole-cell lysates were prepared in parallel without any antibody.

Northern blot analysis

Northern blot analysis was performed as described previously (21). Total RNA was fractionated on a 6% polyacrylamide gel containing 7 M urea, and transferred to a Hybond-XL membrane (GE Healthcare). Antisense oligonucleotides were 5’ end labeled with [γ-32P] ATP (PerkinElmer Life Sciences) by T4 polynucleotide kinase (Enzynomics) and used as probes for hybridization. Hybridization was carried out in Rapid-Hyb buffer (GE Healthcare), and samples were incubated at 40°C overnight (for BC200 RNA) or at 42°C for 1 h (for the 5S RNA). The membrane was washed twice (20 min each time) at 25°C in 2× SSC buffer (20 mM sodium phosphate, pH 7.4, 0.3 M NaCl, 2 mM EDTA) containing 0.1% SDS, and twice (20 min each time) in 0.2× SSC buffer containing 0.1% SDS. The membrane was exposed to an imaging plate (Fuji BAS-IP), and the results were analyzed on a phospho-image analyzer (Fuji FLA-7000).

Confocal laser scanning microscopy

Confocal laser scanning was performed as described previously (22). Briefly, HeLa cells were seeded on sterile cover slips (Marienfeld) in a 24-well plate (100,000 cells/well), and transfected with or without fluorescent fusion protein-encoding plasmids. For immunostaining, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, followed by incubation with 1% BSA to block non-specific binding. The MabBC200-A3 antibody (500 ng/100 μl) was used to stain BC200 RNA, following which it was visualized using Cy3-labeled donkey anti-human IgG (1:200, Jackson Immunoresearch). Imaging was performed using a confocal Zeiss microscope (Zeiss LSM780).

ACKNOWLEDGEMENTS

This study was supported by National Research Foundation of Korea (NRF) funded by the Korea government (MSIP) (2011-0020322 and 2017R1A6A1A1031308) and the Intelligent Synthetic Biology Center of Global Frontier Project funded by MSIP (2013M3A6A8073557).

CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

REFERENCES

1. Tiedge H, Chen W and Brosius J (1993) Primary structure, neural-specific expression, and dendritic location of human BC200 RNA. J Neurosci 13, 2382-2390
2. Chen W, Bocker W, Brosius J and Tiedge H (1997) Expression of neural BC200 RNA in human tumours. J Pathol 183, 345-351
3. Iacoangeli A, Lin Y, Morley EJ et al (2004) BC200 RNA in invasive and preinvasive breast cancer. Carcinogenesis 25, 2125-2133
4. Wu DI, Wang T, Ren C et al (2016) Downregulation of BC200 in ovarian cancer contributes to cancer cell proliferation and chemoresistance to carboplatin. Oncol Lett 11, 1189-1194

http://bmbreports.org
5. Singh R, Gupta SC, Peng WX et al (2016) Regulation of alternative splicing of Bcl-x by BC200 contributes to breast cancer pathogenesis. Cell Death Dis 7, e2262
6. Hu T and Lu YR (2015) BCYRN1, a c-MYC-activated long non-coding RNA, regulates cell metastasis of non-small-cell lung cancer. Cancer Cell Int 15, 36
7. Kondrashov AV, Kiefmann M, Ebnet K, Khanam T, Muddashetty RS and Brosius J (2005) Inhibitory effect of naked neural BC1 RNA or BC200 RNA on eukaryotic in vitro translation systems is reversed by poly(A)-binding protein (PAPB). J Mol Biol 353, 88-103
8. Lin D, Pestova TV, Hellen CU and Tiedge H (2008) Translational control by a small RNA: dendritic BC1 RNA targets the eukaryotic initiation factor 4A helicase mechanism. Mol Cell Biol 28, 3008-3019
9. Wang H, Iacoangeli A, Pop S et al (2002) Dendritic BC1 RNA: functional role in regulation of translation initiation. J Neurosci 22, 10232-10241
10. Eom T, Berardi V, Zhong J, Risuleo G and Tiedge H (2011) Dual nature of translational control by regulatory BC RNAs. Mol Cell Biol 31, 4538-4549
11. Shin H, Lee J, Kim Y et al (2017) Knockdown of BC200 RNA expression reduces cell migration and invasion by destabilizing mRNA for calcium-binding protein S100A11. RNA Biol, DOI: 10.1080/15476286.2017.1297913
12. Jung E, Lee J, Hong HJ, Park I and Lee Y (2014) RNA recognition by a human antibody against brain cytoplasmic 200 RNA. RNA 20, 805-814
13. Jang S, Shin H, Lee J, Kim Y, Bak B and Lee Y (2017) Regulation of BC200 RNA-mediated translation inhibition by hnRNP E1 and E2. FEBS Lett 591, 393-405
14. Chkheidze AN, Lyakhov DL, Makeyev AV, Morales J, Kong J and Liebhaber SA (1999) Assembly of the alpha-globin mRNA stability complex reflects binary interaction between the pyrimidine-rich 3’ untranslated region determinant and poly(C) binding protein alphaCP. Mol Cell Biol 19, 4572-4581
15. Blyn LB, Swiderek KM, Richards O, Stahl DC, Semler BL and Ehrenfeld E (1996) Poly(rC) binding protein 2 binds to stem-loop IV of the poliovirus RNA 5’ noncoding region: identification by automated liquid chromatography-tandem mass spectrometry. Proc Nat Acad Sci U S A 93, 1115-1120
16. Blyn LB, Towner JS, Semler BL, Ehrenfeld E (1997) Requirement of poly (rC) binding protein 2 for translation of poliovirus RNA. J Virol 71, 6243-6246
17. Fujimura K, Katahira J, Kano F, Yoneda Y and Murata M (2009) Selective localization of PCBP2 to cytoplasmic processing bodies. Biochim Biophys Acta 1793, 878-887
18. Kedersha N, Tisdale S, Hickman T and Anderson P (2008) Real-Time and quantitative imaging of mammalian stress granules and processing bodies. Method Enzymol 448, 521-552
19. Muslimov IA, Patel MV, Rose A and Tiedge H (2011) Spatial code recognition in neuronal RNA targeting: role of RNA-hnRNP A2 interactions. J Cell Biol 194, 441-457
20. Werner M, Wilkers L, Aubele M, Nolte M, Zitzelsberger H and Komminoth P (1997) Interphase cytogenetics in pathology: principles, methods, and applications of fluorescence in situ hybridization (FISH). Histochem and Cell Biol 108, 381-390
21. Moon Y, Park B and Park H (2016) Hypoxic repression of CYP7A1 through a HIF-1alpha- and SHP-independent mechanism. BMB Rep 49, 173-178
22. Choi SH, Park BK, Lee KW, Chang J, Lee Y and Kwon HJ (2015) Effect of respiratory syncytial virus on the growth of hepatocellular carcinoma cell-lines. BMB Rep 48, 565-570