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Research paper

Phosphorylation of poly(rC) binding protein 1 (PCBP1) contributes to stabilization of mu opioid receptor (MOR) mRNA via interaction with AU-rich element RNA-binding protein 1 (AUF1) and poly A binding protein (PABP)

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ABSTRACT

Gene regulation at the post-transcriptional level is frequently based on cis- and trans-acting factors on target mRNAs. We found a C-rich element (CRE) in mu-opioid receptor (MOR) 3′-untranslated region (UTR) to which poly (rC) binding protein 1 (PCBP1) binds, resulting in MOR mRNA stabilization. RNA immunoprecipitation and RNA EMSA revealed the formation of PCBP1-RNA complexes at the element. Knockdown of PCBP1 decreased MOR mRNA half-life and protein expression. Stimulation by forskolin increased cytoplasmic localization of PCBP1 and PCBP1/MOR 3′-UTR interactions via increased serine phosphorylation that was blocked by protein kinase A (PKA) or (phosphatidyl inositol-3) PI3-kinase inhibitors. The forskolin treatment also enhanced serine- and tyrosine-phosphorylation of AU-rich element binding protein (AUF1), concurrent with its increased binding to the CRE, and led to an increased interaction of poly A binding protein (PABP) with the CRE and poly(A) sites. AUF1 phosphorylation also led to an increased interaction with PCBP1. These findings suggest that a single co-regulator, PCBP1, plays a crucial role in stabilizing MOR mRNA, and is induced by PKA signaling by conforming to AUF1 and PABP.

1. Introduction

In eukaryotes, the 3′-untranslated region (3′-UTR) of mRNA regulates many post-transcriptional regulatory pathways, such as mRNA localization, stability, and translation efficiency, via either its length or specific sequence elements for 3′-UTR-binding proteins and microRNAs (Pesole et al., 2001). The average length of the 3′-UTR increases strikingly from 200 nucleotides (nt) in plants and fungi (Tanaka et al., 2011) to >1000 nt in humans and other vertebrates (Pesole et al., 2001), suggesting that long 3′-UTRs play a role in the regulation of the more complicated gene expression in higher vertebrates. Several target genes known to be involved in human diseases are depended on cis- and/or trans-factors acting at the 3′-UTR of mRNA (Conne et al., 2000; Chatterjee and Pal, 2009; Han et al., 2010; Chaudhury et al., 2011). A major class of cis-acting elements that regulates mRNA stability includes AU-rich elements (AREs) and is often found in the 3′-UTR of short-lived mRNA (Caput et al., 1986; Shaw and Kamen, 1986; Zou et al., 2010). AREs mainly recruit mRNA degradation proteins (i.e., AUF1, also called hnRNPD) (Pan et al., 2005; Barker et al., 2012); however, mRNA stabilizing proteins, such as HuR and pp32, have also been reported to bind to and be recruited to AREs (Peng et al., 1998; Brennan et al., 2000). Reports have shown the importance of 3′-UTR length and ARE location within the 3′-UTR as key determinants of RNA/protein interaction and translational control of beta2-adrenergic receptor mRNA (Subramaniam et al., 2004; Subramaniam et al., 2011).

C-rich element (CRE) has also been reported to be involved in the regulation of mRNA stability. In erythroid cells, α-globin mRNA stability is regulated by a sequence-specific RNA-protein complex at a CRE region in the 3′-UTR of α-globin mRNA (Weiss and Liebhaber, 1994). This α-complex is a multisubunit structure and may be a general

Abbreviations: PCBP1, poly(rC) binding protein 1; MOR, mu opioid receptor; AUF1, AU-rich element RNA-binding protein 1; PABP, poly (A)-binding protein; 3′-UTR, 3′-untranslated region; PKA, protein kinase A; PKC, protein kinase C; CRE, C-rich element; RNA-EMSA, RNA electrophoretic mobility shift assay; ARE, AU-rich elements; hnRNPs, heterogeneous nuclear ribonucleoproteins; HuR, human antigen R; RNPs, ribonucleoproteins; ILEI, interleukin-like EMT inducer; Dab2, disabled-2; STR, short tandem repeats; RIP, RNA immunoprecipitation; nt, nucleotide; rpL32, ribosomal protein L32; IRES, internal ribosome entry segment; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DICE, differentiation-control element; LOX, lipoxygenase; LMB, leptomycin B; LUC, luciferase; PMA, phorbol 12-myristate 13-acetate.

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determinant of mRNA stability (Chkheidze et al., 1999). Sustained stability of α-globin mRNA is important during all stages of erythropoiesis in order to have steady expression of α-globin mRNA and its protein (Waggoner and Liebhaber, 2003). PCBP1 as well as several other RNA binding proteins, such as PCBP2, AUF1, hnRNPK, and poly(A)-binding protein (PABP), are constituents of the α-complex that binds to the CRE motif to prevent mRNA degradation (Kiledjian et al., 1997; Chkheidze et al., 1999; Wang et al., 1999; Chaudhury et al., 2010). PCBP1 has been shown to regulate stability of many other mRNAs, such as androgen receptor (Yeap et al., 2002), β-globin (Yu and Russell, 2001), collagens I and III (Thiele et al., 2004), disabled-2 (Dab2) and interleukin-like EMT inducer (ILEI) (Chaudhury et al., 2011), endothelial nitric oxide synthase (Ho et al., 2013), erythropoietin (Czyzyk-Krzeska and Bendixen, 1999), folate receptor (Tang et al., 2011), neurofilament-M (Thyagarajan and Szaro, 2008), renin (Skalweit et al., 2003), and tyrosine hydroxylase (Czyzyk-Krzeska and Beresh, 1996).

Recent reports indicate that microRNAs regulate the MOR gene by binding to target sites in the 3′-UTR of MOR (Hwang et al., 2012). Opioids increased the expression of three microRNAs, miR-23b (Wu et al., 2009), let-7 (He et al., 2010), and miR-339-3p (Wu et al., 2013), resulting in downregulation of the MOR gene at the post-transcriptional level. Inhibition of the expression of the microRNAs in vivo by microRNA inhibitors resulted in upregulation of the MOR gene (Wu et al., 2009, 2013) and partially attenuated opioid tolerance in the case of let-7 (He et al., 2010). These studies suggest that microRNAs may play a causal role, at least partially, during development of opioid tolerance by increasing MOR expression. Besides microRNAs, several other factors also regulate gene expression by binding to the non-coding regions of the RNA. For example, a report suggested that short tandem repeats (STRs) in MOR 3′-UTR, especially T and TA repeats, may contribute to mRNA stability by affecting the binding of mRNA degradation or stabilizing proteins, which consequently leads to the high morphine sensitivity observed in mouse strains with long T and TA STRs (Shigeta et al., 2008). Several ARE motifs have been found at positions 4–5 and 8–9 kb (mouse) and 11–12 kb (human) downstream from the stop codon in the 3′-UTR (Wu et al., 2005; Kasai et al., 2006). The current search for conserved motifs in the MOR 3′-UTR reveals several other conserved motifs known to function as cis-acting elements for up- or downregulation of genes. However, until now the roles of these cis-acting elements have not been fully studied.

In this study, we found that PCBP1 binds to a novel target site (CRE) of MOR 3′-UTR and enhances MOR mRNA stability by binding to several other factors. We studied the effect and regulation of PCBP1 binding on the MOR gene and identified novel roles for PCBP1 interaction with different RNA-binding factors. We have attempted to demonstrate for the first time that a single regulator can function as a major factor in the formation of an RNA-binding protein complex that stabilizes the target gene MOR.

2. Materials and methods

2.1. Materials

Forskolin (344270), JNK inhibitor II, Ly294002, NF-κB inhibitor [6-amino-4-(4-phenoxyphenylethylamino)quinazoline], PD98059, PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine], and PP3 [4-amino-7-phenylpyrazolo(3,4-d)pyrimidine] were purchased from EMD Biosciences. SB203580 and U0126 were purchased from Cell Signaling. Actinomycin-D, cycloheximide, H89 (H-89 dihydrochloride hydrate), and leptomycin B were purchased from Sigma. Phorbol-12-myristate-13-acetate (PMA) was purchased from Fisher Scientific. siRNAs of hnRNPK (s67648), PCBP1 (s76740), and negative control (#1, 4390843) were purchased from Invitrogen. We obtained control shRNA and AUF1 shRNA (pSilencer-AUF1) (Chang et al., 2010) through the kindness of Dr. Wengong Wang (Peking University, China). PCBP1 shRNA (sc-38269-SH) was purchased from Santa Cruz.

2.2. Cell culture and plasmid construction

Cell culture and plasmid transfection in neuroblastoma NMB cells were performed as described previously (Hwang et al., 2004). For drug treatment of NMB cells, cells were plated to a density of 2×10^5 cells/well in 12-well culture plates 15 h before the drug treatment; 48 h after transfection, cells were harvested and used for isolation of total RNA or luciferase assay. Cultures of P19 cells and the procedures to differentiate P19 cells (AP4d) have been described previously (Hwang et al., 2007).

pMUTR and pSVUTR were described previously (Wu et al., 2008). PCBP1 expression plasmid (myc-tagged pcDNA4-PCBP1, also called pcDNA4-αCP1) was described previously (Choi et al., 2008). Mutations of PCBP1 for phosphorylation, NES, and NLS sites in pcDNA4-PCBP1 were generated by PCR site-directed mutagenesis using high-fidelity Pfu DNA polymerase according to the manufacturer's protocol (Quikchange, Stratagene) with the respective PCR primer sets (Table 1). To generate plasmid 5′-UTR, uAUG(+) plasmid (Song et al., 2007) was digested with *Hin*dIII and *Nco*I, and a 301-bp DNA fragment of the MOR 5′-UTR was isolated. This fragment was inserted into the *Hin*dIII/*Nco*I sites of pSVPA (Wu et al., 2008) and was designated 5′-UTR plasmid. DNA fragment A, containing +1 ~ +197-bp of the 3′-UTR fragment downstream of the MOR stop codon, was obtained by PCR amplification of the rpL32 region in pMUTR with primers Xba-S and RPfse-AS (Table 1). Fragment A was digested with *Xba*I and *Fse*I and inserted into the same sites of 5′-UTR plasmid to create 5′-UTR-Ag plasmid (Fig. 1B). Similarly, DNA fragment B (441-bp in size) was generated by PCR amplification of the AP1/Brd-containing region of pMUTR with primers XbaA-S and RPfseA-AS and inserted into the 5′-UTR plasmid, creating 5′-UTR-Bg plasmid. DNA fragment B contains two AP-1 sites (Activator Protein-1, 5′TGAGTCA-3′) (Halazonetis et al., 1988) in the region from +2582 to +2972 (downstream of the MOR stop codon) of MOR 3′-UTR and one Brd-box (Brd, 5′AGCTTTA-3′) at +2675 of the MOR 3′-UTR (Lai et al., 1998; Lai, 2002). DNA fragment C (172-bp in size) was generated by PCR amplification of the k-box-containing of pMUTR with primers XbaB-S and RPfseB-AS and inserted into the 5′-UTR plasmid to create 5′-UTR-Cg plasmid. DNA fragment C contains three conserved motifs, ADH-DRE (in reverse direction, 5′ AAGGCTGA-3′) (Parsch et al., 1999), k-box (k-box2 in the MOR 3′-UTR, 5′TGTGAT-3′) (Wu et al., 2008), and IGHA1 (immunoglobulin heavy constant alpha 1, 5′ATTTTCAT-3′) (Maeda et al., 1987). All vectors were confirmed by DNA sequencing and restriction enzyme analyses. RegRNA (Huang et al., 2006) was used to search for the regulatory RNA motifs in MOR 3′-UTR.

2.3. Transfection and luciferase reporter assay

Transfection of plasmid DNAs was performed with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Single (Luciferase Assay System, Promega) and dual luciferase assays (Promega) were performed as described (Wu et al., 2013). For transfection in neuronal differentiated P19 cells, we used cells grown in suspension for transfection with plasmids or shRNAs using Lipofectamine 2000 carried out as described (Jalali et al., 2011; Wu et al., 2013). 48 h after transfection, cells were plated at a density of 6×10^6 cells per 10 cm tissue culture dish; 4 days after plating, the cells were harvested and processed for flow cytometry.

To assay RNA stability in vitro, NMB cells were plated at a density of 7×10^5 cells per well in 6-well plates and transfected with the indicated DNAs (Fig. 3B), followed by treatment with Act-D (5 μg/ml) for 1 to 24 h. Analysis of mRNA decay profiles (with half-life) was performed as described previously (Wang et al., 2002; Wu et al., 2013). Relative mRNA levels of LUC and β-gal were measured by RT-PCR and real-time RT-

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