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EPIGENETIC REGULATION OF KAPPA OPIOID RECEPTOR GENE IN NEURONAL DIFFERENTIATION

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Abstract—The gene of mouse kappa opioid receptor (KOR) utilizes two promoters, P1 and P2. P1 is active in various brain areas and constitutively in P19 mouse embryonal carcinoma cells. P2 is active in limited brain stem areas of adult animals and only in late differentiated cells of P19 induced for neuronal differentiation in the presence of nerve growth factor (NGF). NGF response of P2 was found to be mediated by a specific binding site for transcription factor activation protein 2 (AP2) located in P2. Electrophoretic gel shift assay showed specific binding of this AP2 site by AP2β, but not AP2α. Knockdown of endogenous AP2β with siRNA abolished the stimulating effect of NGF on the expression of transcripts driven by P2. Binding of endogenous AP2β on the endogenous KOR P2 chromatin region was also confirmed by chromatin immunoprecipitation. The effect of NGF was inhibited by LY2942002 (phosphatidylinositol 3-kinase, PI3K inhibitor), suggesting that PI3K was involved in signaling pathway mediating the effect of NGF stimulation on KOR P2. The chromatin of P2 in P19 was found to be specifically modified following NGF stimulation, which included demethylation at Lys9 and dimethylation at Lys4 of histone H3 and was consistent with the increased recruitment of RNA polymerase II to this promoter. This study presents the first evidence for epigenetic changes occurred on a specific KOR promoter triggered by NGF in cells undergoing neuronal differentiation. This epigenetic change is mediated by recruited AP2β to this promoter and involves the PI3K system.

Key words: kappa opioid receptor, epigenetic, NGF, retinoic acid, P19 neuronal differentiation, AP2.

It is known that the effects of opioid drugs are mediated through, primarily, three opioid receptors μ-, δ- and κ- (MOR, DOR, and KOR, respectively) opioid receptor. Opioid receptors belong to the superfamily of G protein-coupled seven transmembrane receptors. Activated opioid receptors convey opioid signals via inhibition of adenylyl cyclase activity (Prather et al., 1993; Smart et al., 1997; Ozawa et al., 1999), increased phospholipase C activity and transient surge in intracellular Ca^{2+} levels (Johnson et al., 1994; Spencer et al., 1997), activation of inward-rectifying K^+ channels (Henry et al., 1995), inhibition of Ca^{2+} channels (Tallent et al., 1994; Piros et al., 1995), and activation of the mitogen-activated protein kinases Erk1/two (Fukuda et al., 1996; Li and Chang, 1996). The three opioid receptors are encoded by three different genes. Each gene produces multiple mRNA variants through alternative promoter usage, splicing and/or polyadenylation (Zimprich et al., 1995; Pan et al., 2001; Pan, 2003; Wei et al., 2004).

The activity of these receptors is regulated at different levels, including the protein level such as receptor phosphorylation and desensitization (Pei et al., 1995; Arden et al., 1995; Appleyard et al., 1997), the transcriptional level (Wei and Loh, 2002; Law et al., 2004), and the post-transcriptional level like alternative splicing and polyadenylation (Lu et al., 1997; Wei et al., 2000; Pan et al., 2001; Hu et al., 2002), mRNA stability (Wei et al., 2000), mRNA transport (Bi et al., 2003, 2006, 2007) and translation (Tsai et al., 2006). Distribution of opioid receptors and their mRNAs has been extensively examined (Fowler and Fraser, 1994; Hu et al., 2002). These studies demonstrate that these receptors are expressed in different, but slightly overlapping, patterns, and that each gene contains various specific regulatory elements upstream of the somewhat similar promoter region. However, it remains unclear as to how these genes can be specifically activated in neurons where the activities and functions of these receptors are mostly expected.

The gene of mouse KOR utilizes two promoters: P1 and P2 (Hu et al., 2002; Wei and Loh, 2002). Transcripts driven by P1 are widely detected in the brain of animals at different developmental stages whereas transcripts from P2 are detected only in very limited brain stem areas of adult animals. To study the regulation of these promoters, this laboratory has utilized a mouse embryonal carcinoma cell culture, P19, which can be induced for neuronal differentiation by retinoic acid (RA) (Bi et al., 2001; Li et al., 2002; Park et al., 2005). In the P19 neuronal differentiation model, P1 is constitutively active whereas P2 is activated only in later stages of RA-induced differentiation in the presence of nerve growth factor (NGF). P1 is constitutively active in P19 because its chromatin is in an open conformation (Park et al., 2005). P2, located within intron 1, is usually silenced in P19 stem cells (Bi et al., 2001) and in early differentiating cells because its chromatin is organized into a nucleosomal array, which is caused by histone deacetylation triggered by transcription factor Ikaros that recruits histone deacetylases to the Ikaros binding site in P2 (Hu et al., 2001). The search for a trigger that can activate P2 in neural tissues in adult animals has been a

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Abbreviations: AP2, activation protein 2; ChIP, chromatin immunoprecipitation; DOR, δ opioid receptor; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin; KOR, kappa opioid receptor; MOR, μ opioid receptor; NGF, nerve growth factor; PI3K, phosphatidylinositol 3-kinase; PLCγ, phospholipase Cγ; P1, promoter 1; P2, promoter 2; RA, retinoic acid; RT, reverse transcriptase.

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challenging task. But, detection of its transcripts in later stages of differentiated P19 cells in the presence of NGF suggests that P2 activation probably involves epigenetic changes triggered by neurotrophins.

NGF is one member of the neurotrophin family that also includes brain-derived neurotrophic factor, and neurotrophins 3 and 4. These neurotrophins regulate cell survival, proliferation, axon and dendrite growth and patterning, the determination of neural precursors, and regulation of gene expression and protein activity (Huang and Reichardt, 2003). NGF is known to activate TrkA receptor or the pan-neurotrophin receptor p75, resulting in activation of small G protein–mediated pathway and the pathways of phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), or mitogenic activation protein (MAP) kinase (Huang and Reichardt, 2003; Rong et al., 2003; Wu and Wong, 2005; Santos et al., 2007; Wehrman et al., 2007). This study examines the effects of NGF on P2, and uncovers NGF-triggered epigenetic changes on P2 that involves recruitment of a specific transcriptional activator, activation protein 2 (AP2) β, to its cognate binding site on P2 and is mediated by the pathway of PI3K, but not PLCγ.

EXPERIMENTAL PROCEDURES

Plasmid construction

Reporter constructs were made with luciferase coding sequence inserted following the initiation site of KOR P2 ([Hu et al., 2001] and [Park et al., 2002]) within the backbone of pGL3B (Promega, Madison, WI, USA). K18 contains the full length P2, including sequences of the whole intron one (P2) and exon 1. K18m was generated by site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) that converted the AP2 consensus sequence CCCGAGGG to TTTTAGGG, thus was mutated specifically on the AP2 site. Kd50 was constructed from K18 to retain exon 1 and only AP2 binding site (-404 to -210).

Cell culture, transfection and reporter assays

P19 cells were cultured in α-minimum essential medium supplemented with 7.5% defined calf serum and 2.5% defined fetal bovine serum and treated with 1.0×10^{-6} M all-*trans* RA. For neuronal differentiation initiated with aggregation, the procedure was performed as described previously (Bi et al., 2001; Park et al., 2005). Briefly, P19 cells were cultured on bacteriological Petri dishes in α-minimum essential medium containing 5% fetal bovine serum and 1.0×10^{-6} M RA. After 4 days, cell aggregates were subcultured onto gelatinized tissue culture dishes and treated with 5 μg/ml cytosine arabinoside in the absence of RA. NGF (1 ng/ml) was then added to differentiated cells without further RA treatment. PC12 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% donor horse serum and 5% fetal bovine serum. P19 or PC12 cells were transfected with reporter constructs using lipofectamine 2000® (Invitrogen, Carlsbad, CA, USA) followed by treatment of indicated reagents.

RNA interference

P19 cells were transfected with 10 nM siRNAs using Hiperfect reagent for 2–3 days (Qiagen). The siRNA was purchased from Qiagen (Valencia, CA, USA); AP2β (Mm_*Tcfap2b*_1 HP siRNA, SI00183883). Silencing effect was assessed by RT-PCR and Southern blot.

Reverse transcriptase-PCR (RT-PCR) and Southern blot hybridization

Total RNA was isolated from P19 cells using a Trizol® solution (Invitrogen) and endogenous KOR mRNA isoforms were detected with an established RT-PCR protocol (Li et al., 2002). PCR primers specific to each gene are: AP2β *Tcfap2b* (forward, 5′-CCC AAG CCA TAG CTC GAG ACT C-3′ and reverse, 5′-TGG CGG AGA CAG CAT TGC TGT TG-3′), p75 *Ngfr* (forward, 5′-ATG AGG AGG GCA GGT GCT GC-3′, and reverse, 5′-TCG TCT GCC TCC ACA CAG GG-3′), BM88 *Cend1* (forward, 5′-GAG GAA AGT CAG CCA GCA GC-3′, and reverse, 5′-TGT TGG ACT CGT CCT CCT CTG-3′), KOR *a* (forward, 5′-ATC AGC GAT CTG GAG CT-3′), KOR *b* (forward, 5′-TCA GCG ATC TGG AGC CCC-3′), KOR *c* (forward, 5′-ACA GGC AAA GTT TGT-3′), and a common reverse primer for KOR (5′-GCA AGG AGC ATT CAA TGA C-3′). Actin-specific primers were included for internal control in each RT-PCR. Amplified DNAs were transferred to nylon membranes and subjected to Southern blot using probes labeled with [α-^{32}P]dCTP, which was able to detect three isotypes of KOR transcripts (Park et al., 2005).

Electrophoretic mobility shift assay

AP2 binding site was found in KOR P2 by computer alignment. Electrophoretic mobility shift assays (EMSA) using three corresponding fragments from P2 were conducted as described previously (Park et al., 2002). Nuclear extracts (10 μg) prepared from P19 cells treated with RA and NGF were incubated in a 16 μl final reaction volume, which contains binding buffer (in mM: Hepes 10, pH 7.3; EDTA 1; dithiothreitol 1; KCl 25; 10% glycerol; 0.1 mg/ml poly[dI-dC] and 0.5% bovine serum albumin) and 2 ng of labeled DNA, at 4 °C for 30 min. For Supershift assay, antibodies against AP2α (sc-8975), AP2β (sc-8976) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and hemagglutinin (HA; H9653, Sigma-Aldrich) were incubated for 30 min at room temperature before addition of labeled DNA. Probe sequence used is 5′-CAACGCCCGAGGGTGAA-3′ (underlined is the putative AP2 binding site).

Western blot and chromatin immunoprecipitation (ChIP) assays

Nuclear proteins were extracted from P19 cells treated with 10^{-6} M RA and 1 ng/ml NGF and resolved on an SDS–acrylamide gel followed by Western blot using the indicated antibodies as described previously (Park et al., 2005). Cells treated with RA only or RA then NGF were cross-linked with 1% formaldehyde (Park et al., 2005). Sonicated cell extracts, which were adjusted to contain the same amount of proteins, were precipitated with 2 μg of the following antibodies at 4 °C overnight, followed by the addition of protein G beads for 1 h. The antibodies against acetylated histone H4 (AcH4; 06–866), Lys4 methylated histone H3 (H3-K4-me2; 07–030), Lys9 methylated histone H3 (H3-K9-me2; 07–422), heterochromatin protein 1α (HP1α, 07–346) and RNA polymerase II (RNA polII, 05–623) were purchased from Millipore (Lake Placid, NY, USA). Ten percent of cell extracts were used as input. Protein G beads were washed extensively, and the captured DNAs were eluted twice with 250 μl of elution buffer (1% SDS and 1 M $NaHCO_3$), which were subjected to reverse cross-link at 65 °C for at least 4 h. Precipitated DNA was amplified using P2-specific primers: forward, 5′-CAG CCA CAG GAG TGG ACA GCA CAA C-3′ and reverse, 5′-GAC TCC ATG GTG AGC GCT GCA GCT GG-3′.