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AP-2α AND AP-2β REGULATE DORSAL INTERNEURON SPECIFICATION IN THE SPINAL CORD

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Abstract—To date, five AP-2 genes that encode AP-2α, β, γ, δ and ε have been identified in vertebrates and they have been reported to be key regulators of embryonic development. However, the role of AP-2 family members in the development of central nervous system (CNS) has not been characterized. In the present study, we systematically examined the spatiotemporal expression pattern of AP-2 genes in the developing spinal cord of mouse and chick embryos and found that AP-2α and AP-2β are specifically expressed in post-mitotic dorsal interneurons. Loss-of-function analysis using *in ovo* electroporation in embryonic chick spinal cord preliminarily demonstrated that cAP-2α and cAP-2β regulates dorsal Class A and Class B interneuron specification, respectively. Gain-of-function experiments further revealed that misexpression of cAP-2α, but not cAP-2β, was able to induce the ectopic generation of Class A interneurons. Together, our studies indicated that AP-2 family members, AP-2α and AP-2β, have distinct functions in the regulation of dorsal interneuron development.

Key words: AP-2, dorsal interneuron specification, chick embryo, *in ovo* electroporation.

INTRODUCTION

AP-2 genes are an important family of transcription factors that regulate various aspects of tissue development during embryogenesis. To date, five members of this family have been identified in vertebrates, named AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε (Williams et al., 1988; Snape et al., 1991; Moser et al., 1995; Oulad-Abdelghani et al., 1996; Shen et al., 1997; Zhao et al., 2001a, 2003; Feng and Williams, 2003; Knight et al., 2005). All AP-2 proteins share a highly conserved helix-span-helix dimerization motif at the C-terminus, followed by a basic region and a less conserved transactivation domain rich in proline and glutamine at the N-terminus. AP-2 factors form hetero- or homodimers to bind to GC-rich sequences in the promoter regions to mediate transcriptional activation or repression (Williams and Tjian, 1991a,b; McPherson and Weigel, 1999).

The critical roles of AP-2 genes during embryonic development have been demonstrated by their unique spatiotemporal expression patterns and knockout animal models (Eckert et al., 2005). In mice, AP-2α, AP-2β and AP-2γ are predominantly expressed in the neural crest, kidney, eye, facial mesenchyme, and limbs (Oulad-Abdelghani et al., 1996; Moser et al., 1997b). AP-2α-deficient mice exhibit mild abnormalities, including anencephaly, body-wall defects and malformation of the outflow tract of the developing tract (Schorle et al., 1996; Zhang et al., 1996; Brewer et al., 2002). Further analysis revealed that AP-2α regulates the switch between neuroepithelial cell proliferation and differentiation through the EGFR-PI3K-Akt pathway (Wang et al., 2006). Loss of AP-2β has firstly been reported to cause congenital polycystic kidney disease due to excessive apoptosis and renal epithelial cells, ultimately resulting in terminal renal failure (Moser et al., 1997a). More recent research has uncovered that several point mutations in AP-2β coding region are associated with Char Syndrome, a human autosomal dominant disorder (Satoda et al., 2000; Zhao et al., 2001b). Further studies performed in chick embryos indicated that AP-2β indeed functions as a transcriptional regulator for digit morphogenesis (Seki et al., 2015). The majority of AP-2γ knockout mice are not able to survive beyond 8.5 days post coitum (dpc), as they fail to establish a normal maternal-embryonic interface due to malformed extra-embryonic tissues (Werling and Schorle, 2002). Unlike these three AP-2 genes, AP-2δ and AP-2ε are expressed in more restricted patterns. The signal of AP-2δ is detected in the retina and the developing heart (Zhao et al., 2001a, 2003). AP-2ε is prominently expressed in the developing olfactory bulb, and its null mutation leads to disorganized olfactory bulb lamination (Feng and Williams, 2003; Feng et al., 2009). Taken together, comparison of mutant phenotypes revealed that loss of AP-2 transcription factor activity generally impairs cell proliferation and induces premature differentiation or apoptosis in tissue development. Thus, AP-2 proteins

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Abbreviations: APC, Adenomatous Polyposis Coli; CNS, central nervous system; dI, dorsal interneuron; dpc, days post coitum; ISH, *In situ* hybridization; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

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are viewed as gatekeepers controlling the balance between proliferation and differentiation during embryogenesis. Although much is known about the expression and function of AP-2 genes in various tissues, little is known about their roles in the development of central nervous system (CNS).

In the present study, we examined the expression pattern of AP-2 factors in the developing mouse and chick spinal cord, and demonstrated that only AP-2α and AP-2β are specifically expressed in the dorsal interneurons during early neurogenesis. Loss-of-function analysis with dominant-negative constructs in embryonic chick spinal cord demonstrated that they are required for the proper differentiation of distinct dorsal interneuron subpopulations. Gain-of-function study revealed that overexpression of cAP-2α, but not cAP-2β, was able to induce ectopic formation of Class A neurons. Collectively, these studies indicated that AP-2α and AP-2β have distinct functions in the regulation of dorsal interneuron specification.

EXPERIMENTAL PROCEDURES

Animal

C57BL/6N mice were obtained from the Jackson Laboratory, provided free access to food and water and housed in a 12-h light/dark cycle. Fertilized chicken eggs (White Leghorn, SPAFAS) were incubated at 39 °C in a humidified incubator for 48 hours and windowed to reveal the embryo. All research procedures using animals were approved by the Institutional Animal Care and Use Committee at Hangzhou Normal University, Zhejiang, China. All efforts were made to minimize the number of animals used and their suffering.

In situ hybridization

In situ hybridization (ISH) was performed according to Scheer et al. (2001) with minor modifications. Animals were deeply anesthetized and perfused with 4% paraformaldehyde (PFA), and tissues were isolated and postfixed in 4% PFA at 4 °C overnight. Following fixation, tissues were saturated in 30% sucrose in phosphate-buffered saline (PBS) overnight. Tissues were then embedded in OCT medium and sectioned on a cryostat. Frozen sections (16-μm-thick) were subjected to ISH with digoxigenin-labeled riboprobes according to Schaeren-Wiemers and Gerfin-Moser (1993) with minor modifications.

Immunofluorescence

Single or double-immunofluorescent procedures were described previously (Qi et al., 2001). The dilution ratio of primary antibodies is as follows: mouse anti-AP-2α (DSHB, 1:50), mouse anti-Pax7 (DSHB, 1:50), mouse anti-Brn3a (Chemicon, 1:200), mouse anti-Islet1 (DSHB, 1:50), mouse anti-Pax2 (Sigma, 1:200); rabbit anti-Lbx1 (provided by Dr. Carmen Birchmeier, 1:10,000), mouse anti-Flag (Sigma, 1:1000), mouse anti-HA (Sigma, 1:1000) and mouse anti-Lhx1/5 (DSHB, 1:50). Slides were incubated with primary antibodies overnight at 4 °C and then washed three times with PBS, incubated with Alexa-488- or Alexa-594-conjugated secondary antibodies (Invitrogen, 1:3000). Fluorescent images were collected by Nikon epifluorescence microscope.

In ovo electroporation

The full-length or truncated fragments encoding the cAP-2α and cAP-2β fused with different tags (myc, HA and Flag) were cloned into a pRCASBP retrovirus vector to construct the RCASBP-myc-AP-2α, RCASBP-HA-AP-2β, RCASBP-dnAP-2α and RCASBP-dnAP-2β expressing vectors. GFP fragment was subcloned into pCAGGS expression vector to construct pCAGGS-GFP. The AP-2β^{A264D} and AP-2β^{R289C} point-mutation dominant negative forms were amplified using primers with single nucleotide substitution as previously reported (Seki et al., 2015) and cloned into pCAGGS expression vector. All recombinants were reconfirmed by sequencing. Fertilized chick embryos were incubated at 39 °C. Neural tubes of chick embryos at cE2 were co-injected unilaterally with the recombinant vectors (4 mg/ml with 0.2% fast green) and pCAGGS-GFP at the thoracic levels. Square-wave current (five pulses, 20V, 5 ms) was generated using BTX Electro Square Porator T820 (Itasaki et al., 1999). Embryos were then incubated at 39 °C in a humidified incubator and harvested 48 hours after electroporation. Embryos with high level of GFP fluorescence in the neural tube were fixed in 4% PFA and further processed for cryosectioning.

Statistical analysis

Quantitative data are indicated as number of cells in the electroporated site that express a marker relative to the cells expressing the same marker in an equivalent area in the control side. Quantitative data were expressed as mean ± s.d.; $n \geqslant 3$ embryos per experimental point, at least four sections per embryo were counted. Significance was evaluated by Student's *t*-test. Differences were considered statistically significant at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Restrictive expression of AP-2 genes in the developing spinal cord

To determine the spatiotemporal expression of AP-2 genes in the developing spinal cord, we performed RNA *in situ* hybridization in wild-type mouse spinal cords at various embryonic stages. Expression of all five AP-2 members was not detected in the spinal cord at embryonic day 10.5 (E10.5). Starting at E12.5, AP-2α and AP-2β signals were observed with a similar pattern in the mantle zone of dorsal spinal cord immediately adjacent to the ventricular zone (Fig. 1A, D). At E14.5, the AP-2α+ and AP-2β+ cells were dispersed laterally in the lower dorsal region (Fig. 1B, E). As development proceeded, their signals were detected more ventrally by E18.5 (Fig. 1C, F). In contrast, AP-2γ, AP-2δ and AP-2ε did not have detectable expression in the developing spinal cord (Fig. 1G–O). Thus, among the