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Polyclonal Antibodies to LIM Proteins CRP2 and CRIP2 Reveal Their Subcellular Localizations in Olfactory Precursor Cells

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Abstract—In this study, we describe the presence of CRP2 (cysteine- and glycine-rich protein 2) and CRIP2 (cysteine-rich intestinal protein 2), which are members of group 2 LIM proteins, in rat olfactory precursor cells by reverse transcription polymerase chain reaction. We have developed polyclonal antibodies against CRP2 and CRIP2 individually. Specificity of the antibodies was demonstrated by Western blot analysis, using CRP2 and CRIP2 transfected cells. No cross-reactivity was observed between the antibodies. Furthermore, we used the antibodies to determine the expression and localization of CRP2 and CRIP2 in olfactory precursor cells by Western blot analysis and immunofluorescence staining. Our results demonstrated that in undifferentiated olfactory precursor cells CRP2 was distributed both in the nucleus and the cytoplasm, whereas CRIP2 was predominantly localized in the cytoplasm. While the olfactory precursor cells differentiated into end cells, only the expression of CRIP2 would be detected. The function of these LIM proteins in olfactory precursor cells warrants further study.

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Key words: CRP2, CRIP2, LIM protein, polyclonal antibody, olfactory precursor cells

In the adult mammalian brain, new neurons and glia are continuously generated but molecular factors regulating their differentiation, proliferation, and lineage relationships are largely unknown. Neuroepithelial precursor cells have the potential to differentiate into neurons, astrocytes, and oligodendrocytes and the ability to self-renew, indicating that they have the neural stem cells feature [1, 2]. Progenitor cells in the subventricular zone (SVZ) proliferate throughout life in rodents. These proliferating progenitor cells migrate into the olfactory bulb (OB) and differentiated into the interneurons. Multipotent precursors with stem cell features can be isolated from the SVZ but also from the entire rostral extension, including the distal portion within the OB [3]. SVZ/OB progenitor cells could serve as a useful source for investigating cell proliferation and differentiation.

LIM proteins mediate specific protein–protein interactions and are of fundamental importance for cell differentiation, cytoskeletal remodeling, and transcriptional regulation. The LIM domain is one distinct sequence motif that contains two independent zinc fingers that function as a protein interaction module, mediating specific contacts with other proteins [4]. The LIM domain has been found in a variety of transcriptional regulators, proto-oncogene products, and proteins associated with sites of cell–substratum contact, some of which also contain homeodomains and are clearly DNA-binding transcription factors [5, 6]. The LIM domain is a crucial protein structure for the biological functions of many LIM proteins [4, 7]. Due to the plethora of proteins containing the LIM domain, schemes have been proposed to classify them into three groups. Group 2 LIM proteins lack classical DNA-binding homeodomains and could act as adapters to facilitate protein–protein interaction [8]. Members of this group are evolutionarily conserved proteins that have been implicated in the process of cell proliferation and differentiation [7, 9-11]. This group of LIM domain proteins includes cysteine-rich intestinal proteins (CRIPs) and the cysteine- and glycine-rich proteins (CRPs).

Abbreviations: CRIPs, cysteine-rich intestinal proteins; CRPs, cysteine- and glycine-rich proteins; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IPTG, isopropyl-β-D-thiogalactopyranoside; OB, olfactory bulb; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; SVZ, subventricular zone.

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CRP2 and CRIP2 are encoded by *csrp2* and *crip2*, respectively. CRP2 contains two tandemly arranged LIM domains and is implicated in diverse processes linked to cellular differentiation and growth control. CRP2 is expressed mainly in vascular smooth muscle cells and is essential to the maintenance of normal cell function [12, 13]. CRIP2 is a novel LIM protein and was first characterized by van Ham et al. [14]. It consists of two LIM domains spaced by a short amino acid stretch. It serves as a novel LIM-only protein that is highly homologous to rat ESP1 and CRP2 sequences. CRIP2 displays a wide, overlapping tissue distribution with protein tyrosine phosphatase, which has a potential role in the dynamics of the actin cytoskeleton. CRIP2 displays amino sequence identities of 50% compared with the sequence of CRP2. On the protein level, the two proteins share 42% similarity. It has been reported that these two proteins have distinct tissue distribution in various tissues, suggesting that each might serve related but specific roles in tissue organization or function [15-17]. Although LIM proteins have been found to play essential roles in cell proliferation and differentiation, there has been no report showing the expression of group 2 LIM proteins in the stem cells or precursor cells.

In this study, we for the first time identified the presence of CRP2 and CRIP2 in rat olfactory precursor cells by reverse transcription polymerase chain reaction (RT-PCR). Then we expressed the full-length CRP2 and CRIP2 in *Escherichia coli* and generated antibodies, which are able to specifically recognize not only recombinant His-CRP2 and His-CRIP2 proteins, but the endogenously expressed CRP2 and CRIP2 proteins individually. More importantly, with these antisera we analyzed the expression and subcellular localization of CRP2 and CRIP2 in the rat olfactory precursor cells by Western blot and immunofluorescence staining. We believed these findings will provide a novel approach to elucidate CRP2 and CRIP2 biofunctions and regulation mechanisms in olfactory precursor cells.

MATERIALS AND METHODS

Bacteria strains, plasmids, and cell culture. *Escherichia coli* strains BL21(DE3) and DH5α were purchased from Novagen (USA). The plasmid vectors pGEM-T-Easy, pRSET A, and pcDNA3.1(–) were purchased from Invitrogen (USA). Cell culture and identification for olfactory precursor cells was performed as described previously [18]. For precursor cell differentiation, 10% fetal calf serum (FCS) was added into the medium. Cos-7 cells were grown in RPMI 1640 (Gibco, USA) supplemented with 10% FCS, 20 U/ml penicillin, and 20 mg/ml streptomycin.

RT-PCR and construction of expression plasmids. The coding sequence of *csrp2* (GenBank Ac. No. NM_177425) and *crip2* (GenBank Ac. No. NM_022501) were obtained from undifferentiated olfactory precursor cell cDNA by RT-PCR using the primers listed in the table (CSRP2a, c and CRIP2a, c). The sequenced full-length *csrp2* and *crip2* were ligated into the expression vector pRSET A with N-terminal His tag. Resulting plasmids were checked by DNA sequencing.

Primers for PCR amplification of *crip2* and *csrp2*

Primer	Site	Sequence (5′-3′)
CRIP2a	*Bam*HI	CGGGATCCGCCTCAAAGTGTCCCAGGTG
CRIP2b	*Bam*HI	CGGGATCCGCCACCGCCTCAAAGTGTCCCAGGTG
CRIP2c	*Hind*III	CCAAGCTTGGACTAGGCAGCAGATCACT
CSRP2a	*Bam*HI	CGGGATCCATGCCTGTCTGGGGCGGTGG
CSRP2b	*Bam*HI	CGGGATCCGCCACCATGCCTGTCTGGGGCGGTGG
CSRP2c	*Hind*III	CCAAGCTTCAAGTGCTGGCTGTTTCA

Another construct was made to transfect Cos-7 cells and analyze the specificity of the antibodies. The forward primers CSRP2b and CRIP2b (see table) contain a Kozak translation initiation sequence for proper initiation of translation. The vector was made by cloning the coding sequence of *csrp2* and *crip2* in pcDNA3.1(–) plasmid individually.

Expression and purification of recombinant protein. *Escherichia coli* BL21(DE3) was used to express CRP2 and CRIP2. Expression was induced by 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Cells were harvested and resuspended in 1 ml buffer A (50 mM Tris-HCl and 200 mM NaCl, pH 7.5). The suspension was sonicated on ice 20 times for 10 sec with 10 sec intervals. Fractions were then analyzed by 12% SDS-PAGE. For purification of each His-tagged protein, the bacterial pellet was resuspended in 20 ml of lysis buffer. The sample was centrifuged at 12,000*g* at 4°C for 15 min. The recombinant protein was purified using a column with Ni-NTA Agarose (Qiagen, USA) following the manufacturer's manual, and this was verified by Western blotting using anti-His monoclonal antibody (Sigma, USA). The concentration of each purified protein was measured using BCA kits (Bios, China).

Preparation of polyclonal antibodies. Two New Zealand white rabbits for each protein were immunized subcutaneously with 1 mg of the purified protein emulsified in Freund's complete adjuvant. Three booster injections were given with 0.5 mg of the protein emulsified in

Freund's incomplete adjuvant at 2-week intervals. A week after the last injection, sera were collected to test the titers. The rabbit with the best affinity to the target protein was sacrificed, and the serum was purified by protein G affinity chromatography. At the same time, eight BALB/c mice were subcutaneously immunized similarly except that 50 mg of protein was injected at each time point. The titers of the antisera were examined using an indirect enzyme-linked immunosorbent assay (ELISA). We also tested the cross-reactivity of two prepared antibodies by ELISA. The specificity of sera was evaluated by Western blot analysis.

Transfection of Cos-7 cells. Cos-7 cells were passaged and subcultured on coverslips up to 50-70% confluence. Cells were washed with phosphate-buffered saline, and then 1.5 ml serum-free medium was added to each well. Cos-7 cells were transfected transiently with plasmids pcDNA3.1(–), pcDNA3.1(–)-*csrp2*, and pcDNA3.1(–)-*crip2*, respectively, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation for 6 h, the solution in each well was replaced with 2 ml complete medium and incubated for 48 h. For Western blot analysis, the cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4) containing proteinase inhibitors.

Fluorescence microscopy. Olfactory precursor cells were grown on coverslips in a six-well plate. After washing with cold PBS, cells were fixed with 2 ml of 4% paraformaldehyde for 20 min. Cells were then washed three times with cold PBS and permeabilized with 2 ml of PBS with 0.1% Triton X-100 for 15 min. After washing four times with cold PBS, cells were incubated with the mouse anti-CRP2 antiserum and rabbit anti-CRIP2 polyclonal antiserum (diluted 1 : 100 with 1% BSA) overnight at 4°C. Cells were washed three times with PBS and then incubated with FITC-conjugated goat anti-mouse IgG (Takara, Japan) and Cy3-conjugated sheep anti-rabbit IgG (Sigma) for 40 min at 37°C. To detect the nuclei, specimens were further incubated with 10 μg/ml of Hoechst 33258 (Sigma) for 5 min. Background staining was removed by washing with PBS three times. The coverslips were mounted on slides and examined using a laser microscope (Nikon, Japan).

Western blot analysis. Olfactory precursor cells were solubilized in Laemmli buffer; proteins were run on a 12% SDS-PAGE gel and then transferred onto nitrocellulose membrane. The membrane was blocked with 5% (w/v) skim milk powder in PBS for 1 h at room temperature followed by incubation with the antisera diluted in blocking solution overnight at 4°C. After being washed 3 times (10 min each) with 0.05% Tween 20 in PBS, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG at room temperature. The membrane was washed and then analyzed using the enhanced chemiluminescence detection system (Genmed, USA).

RESULTS

Cloning of *csrp2* and *crip2* and construction of expression vectors. Complete sequences of *csrp2* and *crip2* were amplified using cDNA synthesized from undifferentiated olfactory precursor cells (Fig. 1). *Bam*HI and *Hind*III sites were designed in the primers to facilitate cloning into pRSET A expression vector. The PCR products were first cloned into pGEM-T-Easy vector which was supplied linearized with single 3′-T overhangs for TA cloning to improve the efficiency of ligation with PCR products. The *Bam*HI-*Hind*III fragments were then subcloned in the pRSET A expression vector, respectively (Fig. 2). The insert sizes were confirmed by digestion and further verified by sequencing.

Expression and purification of recombinant protein. Under the control of IPTG-inducible phage T7 promoter, *csrp2* cDNA in pRSET A is predicted to encode a recombinant protein of 193 a.a. with a molecular weight of ~24 kDa. The *crip2* cDNA in pRSET A is predicted to encode a recombinant protein of 208 a.a. with a molecular weight of ~27 kDa. Small-scale cultures of the positive clones were subjected to IPTG induction to identify clones capable of expressing the predicted recombinant proteins. Recombinant proteins migrated at apparent molecular weight that matched the prediction (Fig. 3). The results confirmed that CRP2 and CRIP2 could be efficiently expressed in *E. coli* host cells. Expression was not detected in uninduced recombinant strains. Western blot results confirmed that the expressed proteins were the target proteins (Fig. 3).

To examine the relative distribution of the expressed recombinant protein in the soluble and insoluble fractions, both the supernatant and the pellet of the cell lysate were examined to detect the recombinant proteins. The

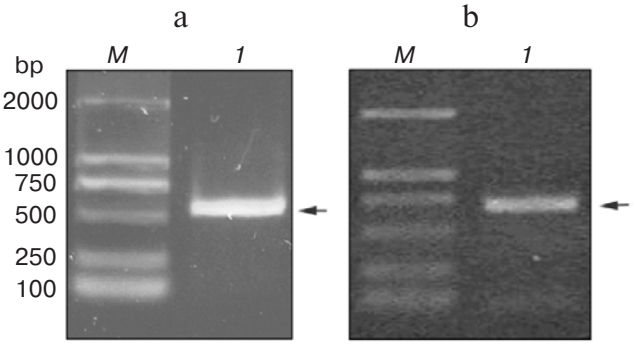

Fig. 1. PCR amplification of *csrp2* (a) and *crip2* (b). Lanes: M, DNA marker DL2000 (Takara); *1*) PCR product. The specific bands are indicated with arrows.

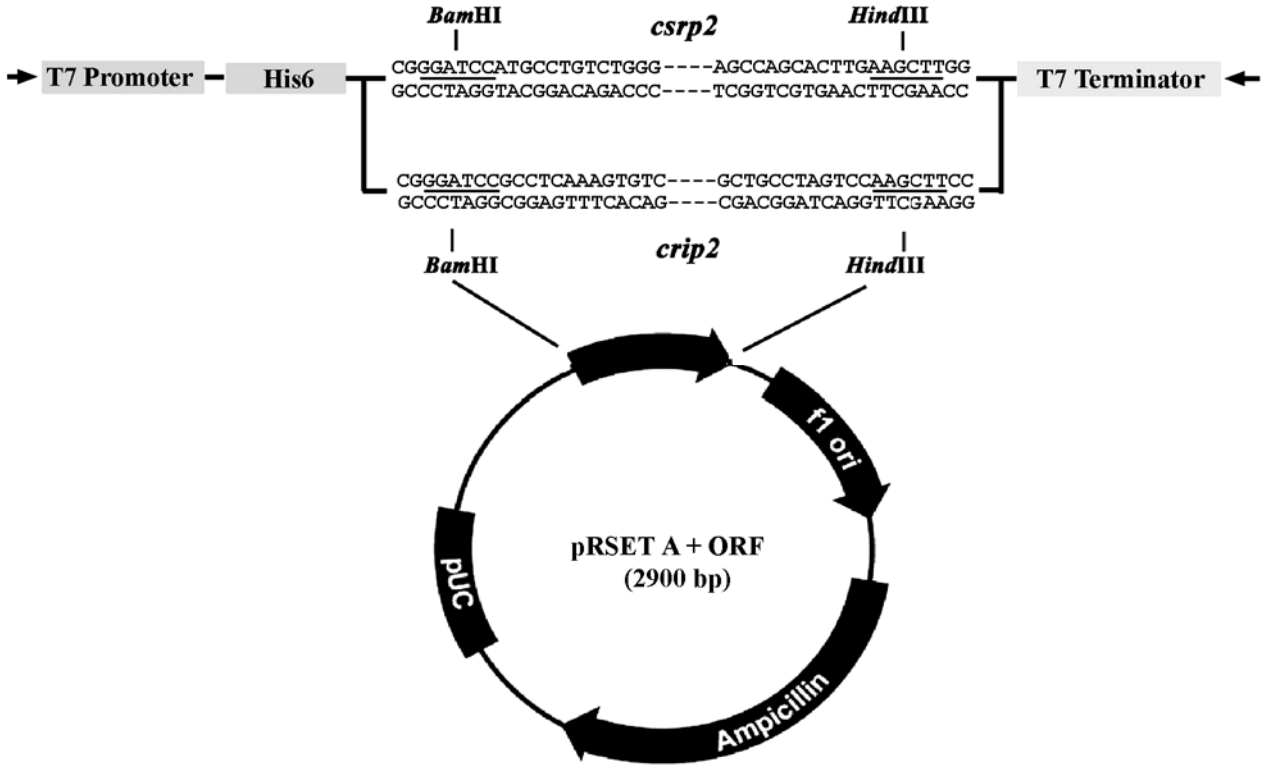

Fig. 2. Construction of pRSET A-*csrp2* and pRSET A-*crip2*. Schematic of open reading frames of *csrp2* and *crip2* inserted in pRSET A expression vector.

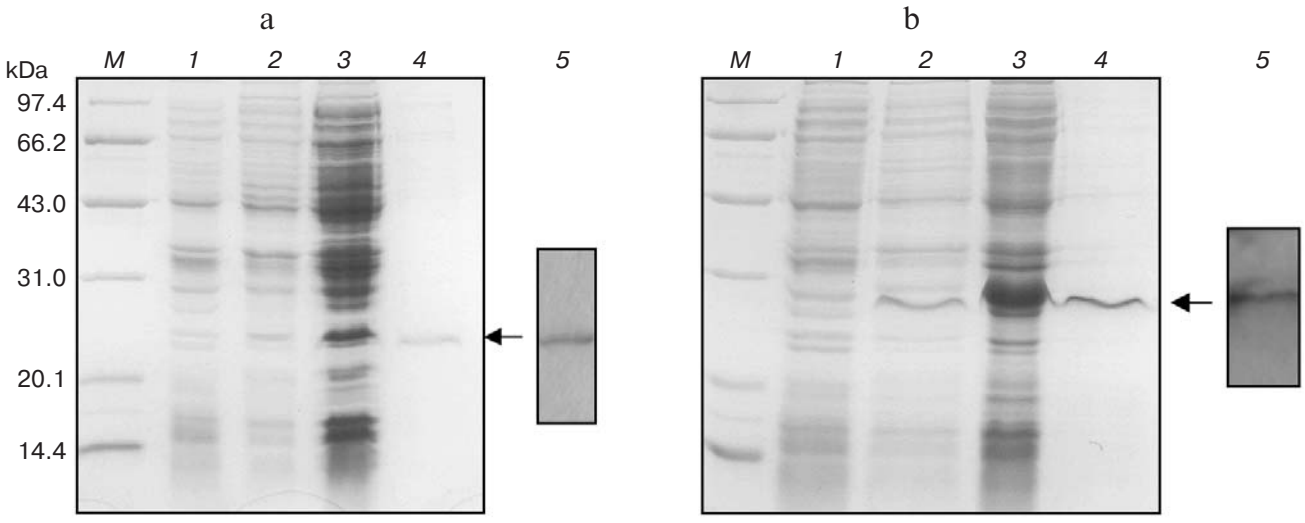

Fig. 3. Purification and Western blot analysis of CRP2 (a) and CRIP2 (b). Lanes: M, protein molecular weight markers (Takara); *1*) whole cell lysate from uninduced recombinant strain; *2*) soluble fractions of total bacterial lysate from IPTG-induced recombinant strain; *3*) insoluble fraction of the bacterial lysate from IPTG-induced recombinant strain; *4*) purified recombinant protein; *5*) Western blot analysis using anti His-tag monoclonal antibody.

expression of both recombinant proteins was detected predominantly in the soluble fraction (Fig. 3).

After purifying the recombinant proteins by affinity chromatography, predominantly one band of the recombinant protein could be seen in the SDS-PAGE and Western blot, indicating that the recombinant proteins were highly purified (Fig. 3). The protein yields were 0.7 and 0.6 mg/ml for CRP2 and CRIP2, respectively.

Characterization of antisera. After immunizing rabbits and mice with His-tagged CRP2 and CRIP2, respectively, anti-CRP2 and anti-CRIP2 sera were purified by protein G affinity chromatography. Titers of both antibodies were analyzed by ELISA using purified CRP2 or CRIP2 as antigen and were showed to be approximately 1 : 1600. There was no cross-reactivity between the two antibodies.

It is known that antibodies that recognize proteins in their denatured form (SDS-PAGE gel) are not always able to detect the same proteins in a native form, and vice versa [19]. To determine if the prepared antibodies detect endogenic CRP2 and CRIP2 in eukaryotic cells, the antibodies were used in lysate of Cos-7 cells transfected with pcDNA3.1(–)-*csrp2* and Cos-7 cells transfected with pcDNA3.1(–)-*crip2*. The results presented a protein of ~21 kDa detected by Western blot analysis using rabbit anti-CRP2 antibody (Fig. 4a). Similarly, a protein of ~24 kDa was blotted with the rabbit anti-CRIP2 antibody (Fig. 4b). In contrast, no band was visualized in the Cos-7 cells transfected with pcDNA3.1(–) using either antibody (Fig. 4). These results indicate that these two antibodies are specific to CRP2 and CRIP2, respectively. The same results were achieved using mouse antibodies that we prepared (data not shown).

Subcellular localization and expression analysis of CRP2 and CRIP2 proteins in rat olfactory precursor cells. With the prepared CRP2 and CRIP2 antibodies, we detected the subcellular localization of CRIP2 and CRP2 in undifferentiated olfactory precursor cells and in differentiated end cells by immunofluorescence staining. Hoechst 33258 was used as a marker for nuclei. The results presented in Fig. 5b (see color insert) show that in the undifferentiated olfactory precursor cells, CRP2 was localized both in the nucleus and cytoplasm, which is consistent with previous reports that CRP2 is localized in the cytoplasm along the cytoskeleton and in the nucleus [20-22]. In contrast, CRIP2 was localized predominantly in the cytoplasm, which is consistent with a previous report that CRIP2 is present in the cytoplasm of epithelial cells [14]. In the differentiated end cells, only the expression of CRIP2 in the cytoplasm can be detected and anti-CRP2 does not detect any protein (Fig. 5). Western blot analysis showed similar results (Fig. 5). The colocalization of CRIP2 and CRP2 is evident in the merge image.

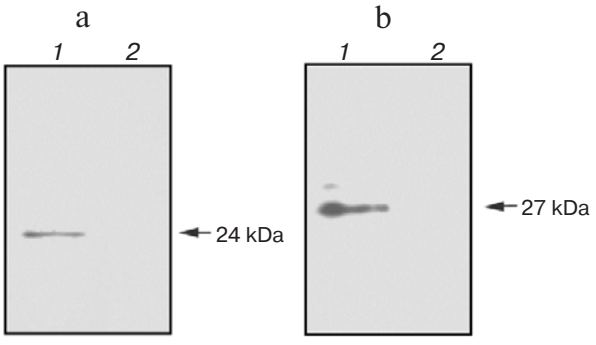

Fig. 4. Specificity analysis of the antisera by Western blotting with cell lysate of Cos-7 cells transfected with pcDNA3.1(–)-*csrp2* (a) or pcDNA3.1(–)-*crip2* (b). Lanes: *1*) cell lysate of Cos-7 cells transfected with *csrp2* or *crip2* expression vector; *2*) cell lysate of Cos-7 cells transfected with pcDNA3.1(–) as a control.

DISCUSSION

CRPs have been shown to be involved in cell differentiation, transcriptional regulation, and the organization of the actin cytoskeleton. CRP2 was originally discovered on the basis of its strong suppression in avian fibroblasts transformed by retroviral oncogenes or chemical carcinogens [23]. The suppression of *csrp2* and its gene product, CRP2, was later directly linked to the transformed phenotype of fibroblasts in a conditional transformation system [12, 13]. Studies in both chick and rat revealed that CRP2 is present at highest levels in arterial sample [24, 25], and subsequent work has focused on a potential role for CRP2 in the vascular system [26]. CRP2 is localized in the cytoplasm along the cytoskeleton and in the nucleus [20, 22, 24]. The dual localization of CRP2 implies different roles of the proteins in the different cellular compartments. In the nucleus, CRP2 is implicated in differentiation processes by acting as cofactors of gene expression [27, 28]. In the cytoplasm, CRP2 is postulated to play a significant role in the regulation of the cellular function of cytoskeletal components. CRIP2 is a novel LIM-only protein that is highly homologous to human CRP2 sequence and was identified by Marco van Ham et al. [14] as a widely expressed and highly conserved CRIP. Mouse CRIP2 has a predicted molecular weight of 23 kDa and consists of two LIM domains spaced by 68 amino acids. To date, few functional studies on CRIP2 have been carried out, which is mainly due to the lack of availability of a specific antibody.

Multipotent precursor cells have neural stem cell features and can be isolated not only from the subventricular zone but also from the entire rostral extension, including the distal portion within the olfactory bulb. Undifferentiated precursor cells from the olfactory bulb can be differentiated into presumptive neurons and have the ability to proliferate throughout life [3]. Although LIM proteins have been found to play an essential role in cell proliferation and differentiation in many tissues and cells, no report has shown the expression of group 2 LIM proteins in the stem cells or precursor cells.

In this study, we have generated polyclonal antibodies against CRP2 and CRIP2. In order to obtain abundant target protein to generate polyclonal antibodies against CRP2 and CRIP2, a prokaryotic expression system was used. The recombinant proteins were highly and effi-

ciently expressed in *E. coli*. The purified recombinant proteins were used as immunogens to induce the production of polyclonal antibodies in rabbits and mice. The two proteins are highly conserved among different species of mammalian hosts. They are nearly identical between mouse and rat (≥99%). The identity of the two proteins is over 90% between mouse and human. Therefore, it is possible that we could not generate specific antisera. However, our Western blot analysis and ELISA results showed that the antisera we generated were efficient to recognize the target CRP2 or CRIP2.

Most importantly, we confirmed the expression of CRP2 and CRIP2 in undifferentiated olfactory precursor cells. CRP2 is localized in the cytoplasm and in the nucleus, whereas CRIP2 was predominantly localized in the cytoplasm. After the olfactory precursor cells differentiated into end cells, only the expression of CRIP2 could be detected. Due to its prominent expression and nuclear distribution in undifferentiated olfactory precursor cells, it is reasonable to conclude that CRP2 is required for proliferation and differentiation of olfactory precursor cells. Both proteins might regulate biofunctions of undifferentiated precursor cells such as cell cytoskeleton formation by LIM domain as shown in other tissues.

In summary, we have produced specific polyclonal antibodies against CRP2 and CRIP2 and detected CRP2 and CRIP2 expression in olfactory precursor cells. The group 2 LIM protein expression in precursor cells is of particular interest because these are the first data suggesting that CRP2 and CRIP2 might function in stem cells or precursor cells. As a result, further characterization of these proteins might offer insight into the proliferation and differentiation of olfactory precursor cells.

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