Research Article

Generation and Characterization of Novel Human IRAS Monoclonal Antibodies

Bo Wang,1 Ying Liu,1 Yajun Shan,2 Zhenyu Yao,2 Xiaolan Liu,2 Ruibin Su,1 Qihong Sun,2 Yuwen Cong,2 and Jin Li1

1 Department of Pharmacology, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China
2 Department of Pathophysiology, Beijing Institute of Radiation Medicine, Beijing 100850, China

Correspondence should be addressed to Jin Li, jinli9802@yahoo.com

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Imidazoline receptors were first proposed by Bousquet et al., when they studied antihypertensive effect of clonidine [1]. Based on their physiologic and pharmacological properties, imidazoline receptors are classified into three main types: I1R, I2R, and I3R [2–4]. I1R and I2R have been implicated in hypertension and psychiatric disorder regulation, respectively, while I3R may be involved in insulin secretion [5–9]. Compared with mitochondrial I2R, which resides within the monoamine oxidase protein [10], the clonidine-preferring imidazoline binding sites (known as I1R) are localized to plasma membrane fractions [11, 12] and specifically to synaptic plasma membranes [13].

A strong candidate for I1R, known as imidazoline receptor antisera-selected protein (IRAS), has been cloned from human hippocampus [14]. hIRAS is a larger protein of 1504 amino acids consisting of an NH2-terminal phox (PX) domain, 5 putative leucine-rich repeats, a predicted coiled-coil domain, and a long COOH-terminal region. Several evidence supported the identity of native I1R and IRAS protein in tissue distributions, ligand binding properties, some cellular functions and downstream signal pathways [14–18]. The murine form of IRAS, Nischarin, truncated at the N-terminal 244 amino acids including the PX domain compared with the hIRAS, was a soluble cytosolic protein involved in cytoskeletal organization [19]. It has been shown that decreasing the expression of rat IRAS or Nischarin in PC12 rat pheochromocytoma cells could attenuate the activation of extracellular signal-regulated kinase (ERK) or reduce the radioligand binding to I1R, which further supported that hIRAS or Nischarin might serve as I1R itself, or at least a functional subunit of I1R [20]. Recently, Molderings et al. have reported that the “I1-imidazoline receptors” mediating effects of clonidine and moxonidine in PC12 and the transfected HEK293 cells belonged to the S1P-receptor family, in particular, representing a mixture of sphingosine-1-phosphate (S1P)1- and S1P3-receptors and/or heterodimers of both. It was then proposed that an increased expression of IRAS or Nischarin may improve...
the receptor-trafficking from cytosolic S1P-receptors to the cell membrane and thereby increase the number of binding sites in the plasma membrane for radioligand binding [21]. In our previous study, we also reported that IRAS mediated agmatine-induced inhibition of opioid dependence in morphine-dependent cells [22]. Despite intensive efforts, the molecular base of the I1R had not yet been elucidated.

To elucidate the functional and structure properties of I1R, several different epitope-specific antisera against IRAS have been raised in rabbits [23]. Because of IRAS splice variants or nonspecificity of these antisera, more sizes of IRAS (≈33, ≈85, ≈170 KDa) have been visualized in various tissue and cells, which limited their uses on western blot. IRAS was reported to target to the endosomes by a combined action of a PX domain and a coiled-coil region. The PX domain, consisting of 10–130 amino acids, was first identified from the sequence analysis of two SH3 domain-containing cytosolic components of NADPH oxidase, p47phox and p40phox [24]. Therefore, in the present study, we developed the newly monoclonal antibody against the N-terminal hIRAS region including the PX domain (10–120aa). This development has great utility for immunoblotting, indirect immunofluorescent staining, immunoprecipitation, and flow cytometry. These monoclonal antibodies will provide powerful reagents for the further investigation of hIRAS protein functions.

2. Materials and Methods

2.1. Generation of the NusA-IRAS(10–120aa) Fusion Protein. E. coli BL21(DE3) (F-ompT gal dcm hsdSB (rB-mB-)) λ (DE3) (Novagen) cells were transformed with recombinant plasmid, pET43.1-IRAS(10–120aa). Transformants were selected from ampicillin-containing Luria Bertani (LB) lates. Selected colonies were cultured in ampicillin-containing LB media. Isopropyl-β-D-thio galactopyranoside (IPTG) was added to a final concentration of 1 mM. The incubation was continued for 3 hours at 30°C. Cells were harvested, mixed with lysis buffer (phosphate buffered saline [pH 7.3], 1.0 mM EDTA, 1.0 mM phenylmethylsulfonyl fluoride, 0.5 mg/mL lysozyme (Roche)), and sonicated. The high-speed supernatant which contained the pET43.1-fluoride, 0.5 mg/mL lysozyme (Roche)), and sonicated. 

2.2. Antiserum Titer Determination by Indirect Enzymelinked Immunosorbent Analysis (ELISA). The purified NusA-IRAS(10–120aa) protein (50 μg in a volume of 80 μL) was mixed with an equal volume of Freund’s complete adjuvant. The antigen-adjuvant mixture was injected into 6 female BALB/c mice and was followed by three booster injections at 2-week interval in incomplete Freund’s adjuvant. The mouse with the highest antibody titer tested by ELISA was boosted intraperitoneally with 100 μg NusA-IRAS(10–120aa) protein without adjuvant 3 days before the cell fusion. Feeder layer cells were prepared 1 day prior to fusion. Splenocytes from mice with the highest ELISA antibody titers were fused with murine myeloma cells SP2/0 following standard procedures [25]. Culture supernatants were collected after fusion and initially screened by ELISA with purified NusA-IRAS(10–120aa) fusion proteins as antigens. Positive hybridoma clones were selected with the limiting dilution method, and stable hybridoma clones were obtained after 3 cloning cycles. Isotypes of antibodies were identified with a mouse subsisotype panel (Bio-Rad). The pristine-primed BALB/c mice were injected intraperitoneally with 1 × 10⁶ hybridoma cells per mouse in order to acquire abundant mAbs. The ascitic fluid was collected and mAbs were purified with a protein A/G affinity column (Amersham Pharmacia Biotech).

2.3. Production of Antihuman IRAS Monoclonal Antibodies. The purified NusA-IRAS(10–120aa) protein (50 μg in a volume of 80 μL) was mixed with an equal volume of Freund’s complete adjuvant. The antigen-adjuvant mixture was injected into 6 female BALB/c mice and was followed by three booster injections at 2-week interval in incomplete Freund’s adjuvant. The mouse with the highest antibody titer tested by ELISA was boosted intraperitoneally with 100 μg NusA-IRAS(10–120aa) protein without adjuvant 3 days before the cell fusion. Feeder layer cells were prepared 1 day prior to fusion. Splenocytes from mice with the highest ELISA antibody titers were fused with murine myeloma cells SP2/0 following standard procedures [25]. Culture supernatants were collected after fusion and initially screened by ELISA with purified NusA-IRAS(10–120aa) fusion proteins as antigens. Positive hybridoma clones were selected with the limiting dilution method, and stable hybridoma clones were obtained after 3 cloning cycles. Isotypes of antibodies were identified with a mouse subsisotype panel (Bio-Rad). The pristine-primed BALB/c mice were injected intraperitoneally with 1 × 10⁶ hybridoma cells per mouse in order to acquire abundant mAbs. The ascitic fluid was collected and mAbs were purified with a protein A/G affinity column (Amersham Pharmacia Biotech).

2.4. Western Blotting Analysis. Transfected cells were harvested 48 hours after transfection. Total cell lysate preparation and western blot analysis were performed according to previously described procedures [26]. In brief, cell lysates were prepared, electrophoresed on SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% [v/v] Tween 20) and incubated with IRAS or GFP mAbs (Cell Signaling Technology Inc.). Blots were incubated with horseradish peroxidase (HRP) conjugated goat antimouse antibodies (Santa Cruz) after primary antibody incubation, and blots were developed with enhanced electrochemiluminescence (ECL, Cell Signaling Technology Inc).

2.5. Confocal Microscopy Analysis. The cellular localization of the IRAS protein was identified according to previously described procedures [27]. Cells on glass cover slips were rinsed with PBS, fixed with 3% paraformaldehyde for 30 minutes, and permeabilized with 0.2% [v/v] Triton X-100/PBS. Permeabilized cells were incubated with IRAS mAbs and TRITC-conjugated affinipure goat antimouse
2.6. Immunoprecipitation Analysis. The HEK293 cells were transfected with PCMV-myc-IRAS or PCMV-myc plasmid to analyze IRAS mAbs immunoprecipitated with IRAS. Cell lysates were prepared in a modified RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [v/v] Nonidet P40, 0.5% [w/v] sodium deoxycholate) containing Complete Protease Inhibitor Cocktail Tablets (Roche). Cell lysates were centrifuged to remove debris. Recovered lysates were incubated with IRAS or c-myc mAbs (Santa Cruz) overnight at 4°C followed by protein A/G agarose beads (50 μL, Santa Cruz) for another 3 hours to capture the immunoprecipitates. Recovered immune complexes were extensively washed in lysis buffer. Samples were boiled to release the bound proteins and appropriate supernatant aliquots were run on SDS-PAGE gels for western blot analysis as described above.

2.7. Flow Cytometry. Cells (2 × 10^6) were transfected with PCMV-myc-IRAS for each sample and fixed with 70% [v/v] cold ethanol at 4°C overnight. Cells were permeabilized in 0.1% [v/v] Triton X-100/PBS and were incubated with IRAS or c-myc mAbs (Santa Cruz). Cells were washed and resuspended in an FITC-conjugated affinity pure goat antimouse IgG (Santa Cruz). Finally, cells were washed twice and samples were analyzed on a flow immunocytometry machine (Becton-Dickinson) [28].

2.8. Cell Culture and Transfection. HEK293 cells were grown in Dulbecco’s modified Eagle medium (DMEM) ( Gibco BRL) supplemented with 10% fetal bovine serum. Cells were transfected with different plasmid DNAs using a Lipofectamine 2000 (Invitrogen) when cell densities reached 70% confluence per the manufacturer’s instructions. Hybridoma cells were grown in DMEM with 10% fetal bovine serum.

2.9. Construction of Expression Vector pET43.1a-IRAS, pCMV-myc-IRAS, and pEGFPc1-IRAS. The cDNA fragment of the human IRAS N-terminal (10–120aa) was inserted into the pET-43.1a (+) expression vector (Novagen) by PCR amplification of the human IRAS cDNA (AF082516) using the following oligomers: sense, 5'-CGGGATCCTAGCGGCAGCCGCGCACCTTCG-3', and antisense, 5'-CGGAATTCATAGAAGTGAAAATGCAAGAAGTG-3'. The full-length human IRAS was obtained by PCR amplification of the entire coding region, and the resulting 4512-bp PCR product was ligated into the pEGPC1 and PCMVM-myc vectors in a similar fashion using the following oligomers, respectively: sense, 5'-CGCGAATTCTATGGCGACCGGC-GGCACACCTG-3', and antisense, 5'-CGGGATCTTACGC-CGGTGAGCTGCAGACGCG-3', sense, 5'-CGCGAATTCCGGATTGCACCGGCACCTTCG-3', and antisense, 5'-CGCGTCCGAGCTAGCCGGTGAAGCTGACAGCC-3'. All plasmid sequences were confirmed by sequencing analysis.

3. Results and Discussion

3.1. Generation of Murine Monoclonal Antibodies (mAbs) against a Recombinant NusA-IRAS (10–120aa) Fusion Protein. IRAS is a large protein comprising of 1504 amino acids. Its NH2-terminal phox domain is important for membrane association and cellular localization. The N-terminal of IRAS (10–120aa) covering the PX domain was cloned into the pET43.1a (+) vector (Figure 1(a)) [29]. The supernatant fusion protein was purified by His-tag affinity purification and was subsequently used to generate the monoclonal antibody. The dissolved protein yielded one major band of 78 kDa expected molecular weight (Figure 1(b)) with high purity and integrity. The NusA protein used as a control generated the 66 kDa expected molecular weight. The recombinant protein was also confirmed with western blot using anti-His mAbs (right column, Figure 1(b)). BALB/c mice (n = 3) were immunized with the NusA-IRAS (10–120aa) fusion protein, and blood was collected from the mice after multiple injections. Antibody titers were tested by ELISA on plates coated with the NusA-IRAS (10–120aa) Protein (data not shown). The 2 mice
with the highest titers were sacrificed and spleens from both mice were fused to myeloma cells following standard procedures. Individual hybridomas was grown and 125 hybridomas were further characterized. Supernatant from the growing hybridoma clones was screened with ELISA. Screening was performed on plates coated with NusA-IRAS\textsubscript{(10–120aa)}, NusA protein, and GST-IRAS\textsubscript{(10–120aa)} fusion protein to determine antibody specificity. A total of 5 hybridomas (DA041, DD015, BE073, BA022, and AH021) reacted selectively with the NusA-IRAS\textsubscript{(10–120aa)} protein in all 3 assays and were further evaluated. Isotype analysis revealed that all mAbs were of the IgG1 subtype.

The immunoreactivities of the 5 representative mAbs with NusA-IRAS\textsubscript{(10–120aa)} were shown in Figure 2, all of which specifically recognized a 78 kDa protein band which corresponded to the purified NusA-IRAS\textsubscript{(10–120aa)} protein, but not to the 66 kDa NusA protein. Anti-His mAbs reacted with both proteins in the same experiment (Figure 2(a)) as controls. We evaluated the specificity of mAbs in mammalian cells by inserting IRAS cDNA into expression vectors to allow the production of GFP fusion proteins under the control of a CMV promoter. The pEGFPC1 and pEGFPC1-IRAS plasmids were separately transfected into HEK293 cells. Western blot analysis with the anti-GFP antibody demonstrated that chimeric proteins were expressed and migrated separately at expected molecular masses of approximately 19 or 27 kDa (Figure 2(b)). However, the expected 190 kDa band whose molecular weight corresponded to the full-length IRAS protein was only detected with the mAbs DA041, DD015, and BE073 in GFP-IRAS expressed cells. The remaining BA022 and AH021 mAbs were negative (Figure 2(b)). The same samples were also incubated with preimmune serum with no reactivity (data not shown). Results revealed that all 5 selected mAbs specifically recognized bacterially expressed NusA-IRAS\textsubscript{(10–120aa)} proteins, but only 3 mAbs recognized recombinant IRAS in mammalian cells.

3.2. Cellular Localization and Flow Cytometry Analysis for IRAS Expression. IRAS mAbs were used to detect the cellular localization of IRAS protein based on specificity characterized by western blot analysis. The pEGFPC1-IRAS plasmid was transfected into HEK293 cells. IRAS protein localization was tested by fluorescence confocal microscopy after 48 hours transfection. GFP-IRAS was primarily located in the cytoplasm in a punctate pattern (Figure 3(a)) with concentrations on discrete loci and spot fluorescence (panel A, E, I,
Figure 3: Binding of IRAS mAbs to IRAS-expressing cells by immunofluorescence and flow cytometry analysis. (a) HEK293 cells were transfected with the plasmid pEGFPC1-IRAS. Cellular localization of IRAS was observed by scanning fluorescence confocal microscopy. Green represented EGFP fluorescence (A, E, I, M, and Q) from the GFP-IRAS fusion protein, blue (C, G, K, O, and S) fluorescence represented Hoechst stained cell nuclei, red (B, F, J, N, and R) fluorescence represented cells expressing GFP-IRAS labeled with TRITC-conjugated goat antimouse antibody using IRAS mAbs as the primary antibody, and yellow (D, H, L, P, and T) represented overlapping green and red fluorescence. All 3 panels had the same field of view. Scale bar, 100 μm. (b) Samples were collected and separately analyzed by flow cytometry for the ability to bind the preimmune serum (control), the c-myc mAb, and the mAb DA041 after 48 hours transfection of PCMV-myc-IRAS. Results were expressed as histograms with the DNA content on x-axis and the number of fluorescent cells on y-axis. Cells were distributed in 2 populations, and the second population (R2) with high fluorescence reflected the population of transfected cells recognized by the mAb DA041.
Myc-IRAS
WB:anti-myc
IP:c-myc
Lysate
IP:DA041
Lysate
IP:DD015
Lysate
IP:BE073
Lysate
IP:BA022
Lysate
IP:AH021
Lysate
Myc-IRAS
WB:DA041
175 KDa
(a)
WB:DA041
175 KDa
(b)

Figure 4: Analysis of immunoprecipitates revealed that the IRAS mAbs specifically recognized the native state of the IRAS protein. The HEK293 cells were transiently transfected with the empty vector PCMV-myc (negative control) and PCMV-myc-IRAS. Whole cell lysates were prepared and immunoprecipitated with the c-myc mAb or the mAbs DA041, DD015, BE073, BA022, and AH021. Immunoprecipitation of the same lysates using mouse normal IgG did not result in the detection of any protein species. Immunocomplexes were analyzed by western blot with indicated antibodies.

Our results suggested that the mAbs DA041, DD015, and BE073 were reactive for both immunofluorescence and immunoblotting. Therefore, these mAbs likely recognized linear epitopes in the IRAS protein. In addition, the 3 mAbs were capable of recognition of native full-length IRAS proteins by immunoprecipitation. However, the mAbs BA022 and AH021 specifically recognized bacterially expressed IRAS immunogens and did not detect recombinant IRAS in mammalian cells. This could be related to the different IRAS folding patterns, since misfolded IRAS proteins could result in the exposure of unique immunogenic epitopes different from native proteins. The mAbs BA022 and AH021 could be used as backup reagents to safeguard against antibody-specific artifacts. In addition, analyzing the overall homology of the amino acid sequence of the PX domain, Sorting Nexins 13 (SNX13) is the most relative protein to IRAS [31]. We
found monoclonal antibody DA041 against hIRAS could not react with SNX13 by immunofluorescence assay (data not shown). It was proposed that these antibodies developed here were specific to the PX domain of IRAS.

In summary, we generated specific mAbs directed against the human IRAS N-terminal. The mAb DA041 exhibited the best performance in a variety of assays including immunoblotting, immunoprecipitation, indirect immunofluorescence staining, and flow cytometry. Specific mAbs may provide an ideal reagent for further investigation of the function of IRAS proteins.

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