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Development and characterization of monoclonal antibodies against human aryl hydrocarbon receptor

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ABSTRACT

Aryl hydrocarbon receptor (AhR), a ligand-dependent nuclear receptor, is involved in a diverse spectrum of biological and toxicological effects. Due to the lack of three dimensional (3D) crystal or nuclear magnetic resonance structure, the mechanisms of these complex effects of AhR remain to be unclear. Also, commercial monoclonal antibodies (mAbs) against human AhR protein (hAhR), as alternative immunological tools, are very limited. Thus, in order to provide more tools for further studies on hAhR, we prepared two mAbs (1D6 and 4A6) against hAhR. The two newly generated mAbs specifically bound to amino acids 484–508 (located in transcription activation domain) and amino acids 201–215 (located in Per-ARNT-Sim domain) of hAhR, respectively. These epitopes were new as compared with those of commercial mAbs. The mAbs were also characterized by enzyme-linked immunosorbent assay, western blot, immunoprecipitation and indirect immunofluorescence assay in different cell lines. The results showed that the two mAbs could recognize the linearized AhRs in six different human cell lines and a rat hepatoma cell line, as well as the hAhR with native conformations. We concluded that the newly generated mAbs could be employed in AhR-based bioassays for analysis of environmental contaminants, and held great potential for further revealing the spatial structure of AhR and its biological functions in future studies.

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor which belongs to the basic helix-loop-helix (bHLH)-Per-ARNT-Sim (PAS) family (Burbach et al., 1992). Functional domains of AhR are classified to a bHLH region, two PAS domains (A and B) and a transcription activation domain (TAD). The aryl hydrocarbon receptor can be activated by exogenous chemicals, such as halogenated aromatic hydrocarbons, non-halogenated polycyclic aromatic hydrocarbons, and other dioxin-like chemicals (DLCs), as well as endogenous compounds,

such as 6-formylindolo-[3,2-b]-carbazole, bilirubin and lipoxinA4 (Denison and Nagy, 2003; Nguyen and Bradfield, 2007). The activation of AhR by these compounds leads to regulation of the expression of a battery of genes resulting in diverse biological and toxicological effects, including dermal, hepatic, cardiac and immunotoxic response, wasting syndrome, reproductive and developmental toxicities (Beischlag et al., 2008; Flaveny et al., 2010; Birnbaum and Tuomisto, 2000; Bock, 1994).

Apart from the presence of endogenous ligands, studies using AhR knockout (KO) mice further indicated that AhR also played roles in normal physiology and pathology. It has been reported

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that almost half of the AhR-deficient mice died shortly after birth. Although the survivor reached maturity and were able to be fertile, they showed decreased accumulation of lymphocytes in the spleen and lymph nodes (Fernandez-Salguero et al., 1995). In the immune system, AhR was involved in regulating the antigen responses in Th17 cells, dendritic cells, and other adaptive immune cells (Esser, 2009; Esser et al., 2009; Tian et al., 2015). In addition, smaller liver size and hepatic deformation were observed in adult AhR KO mice (Harstad et al., 2006), and the animals were more likely to develop liver tumors after exposure to diethylnitrosamine (Fan et al., 2010). Besides, a recent investigation demonstrated that AhR KO mice had a higher risk of developing bladder stones (Butler et al., 2012). However, the mechanisms for AhR being involved in these physiological and pathological responses were not well-understood.

Given the biological and toxicological importance of AhR exhibited via interactions with various exogenous and endogenous compounds of diverse chemical structures, the mysterious molecular events upon its ligand bindings have been attracting increasing attentions. Due to the lack of 3D crystal or nuclear magnetic resonance structure of AhR, monoclonal antibodies (mAbs), binding specifically to various functional domains of AhR, could serve as effective tools for sophisticated studies on the molecular events and mechanisms. Apart from that, mAbs can also be used to detect AhR ligands in the environment, such as in Ah-Immunoassay (Ah-I) (Tian et al., 2012).

Currently, the commercial anti-hAhR mAbs are specifically raised against the synthetic peptide RKRRKPVKPIAEGIK (amino acid (a.a.) 12–17 & a.a. 22–31) or recombinant proteins corresponding to a.a. 721–821, 637–848 or 1–848 of hAhR. Given the homology of hAhR with murine AhR, functional domains of hAhR could be predicted based on those of mouse AhR (mAhR, Fukunaga et al., 1995; Whitelaw et al., 1994), including the PAS B domain (a.a. 230–397) of mAHR for exogenous ligand binding, the bHLH region (a.a. 27–79) and the region encompassing the PAS B repeat (a.a. 182–374) for Hsp 90 binding, a part of bHLH region (a.a. 27–39) for DNA binding, regions encompassing a.a. 40–79 and a.a. 121–289 for dimerization and the Q-rich region (a.a. 490 to 805) in the TAD domain for transcription activation. Thus, the epitopes of the commercial mAbs might specifically bind to the peptides located in only two function regions of hAhR, bHLH and Q-rich domains, which is insufficient to study the complex molecular events upon activation of hAhR involving different functional domains. Therefore, in the present study, we employed a recombinant protein of full-length hAhR expressed in *Escherichia coli* BL21 cells aiming to generate new mAbs with novel epitopes belonging to different functional domains of hAhR. Two new mAbs were selected and subjected to series characterizations to reveal the recognition ability, the epitopes and potential applications.

1. Materials and methods

1.1. Animals and cell culture

All mice care and experimentation were approved by the Committee of Animal Care at the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences

(RCEES). Female BALB/c mice, six weeks of age, were purchased from Vital River Laboratories (Beijing, China). The mice were fed on a standard laboratory chow diet, housed four or three per cage, maintained at $(20 \pm 1)^{\circ}\text{C}$, relative humidity of $50\% \pm 10\%$, with a 12 hr/12 hr light/dark cycle and sacrificed via CO_2 asphyxiation.

Two hepatoma cell lines HepG2 (human) and H4IIE (rat) were kind gifts from Dr. Michael S. Denison (University of California, Davis, CA, USA). Mouse skeletal muscle cell line (C2C12) was from Dr. Karl WK Tsim (The Hong Kong University of Science and Technology, Hong Kong, China). Human renal tubular epithelial cell line (HKC) was from Dr. Wei Liu (Peking University Hospital, Beijing, China). Human cervical epithelial cell line (HeLa) was from Dr. Benzhan Zhu (RCEES, Beijing, China). Human embryonic kidney 293 T (HEK 293 T) cell line, human breast cancer cell line (MCF-7), human neuroblastoma cell line (SK-N-SH) and mouse myeloma cell line (SP2/0) were obtained from the cell resource center of Chinese Academy of Medical Sciences (Beijing, China). The hepatoma cell lines were cultured in α -MEM (alpha Eagle's minimal essential medium). MCF-7 cells, HeLa cells, HEK293T cells, C2C12 cells and SK-N-SH cells were grown in DMEM (Dulbecco's Modified Eagle Medium). HKC cells were cultured in DMEM/F12 medium. SP2/0 cells and hybridoma cells were cultured in RPMI 1640 medium. All cell culture medium were supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin–Streptomycin Solution (P/S). The cell lines were seeded in 100 mm dishes or 96-well plates and incubated at 37°C under a 5% CO_2 , 95% humidity environment. All reagents for cell culture were purchased from Invitrogen (CA, USA).

1.2. Preparation and characterization of mAbs against hAhR

The prokaryotic expressing plasmid pGEX4T-1/GST-hAhR was constructed using primers of 5'-AAC AGT CGA CTC ATG AAC AGC AGC AGC GCC AA-3' and 5'-AAA AGC GGC CGC TAC AGG AAT CCA CTG GAT GTC A-3' with Sal I and Not I sites (underlined), respectively. The hAhR sequence (Accession: NM_001621.4) was inserted. Glutathione S-transferase (GST)-tagged hAhR fusion proteins (GST-hAhRs), and GST proteins were overexpressed in *E. coli* BL21 by transformation of plasmids, pGEX4T-1/GST-hAhR and pGEX4T-1/GST, respectively. The GST-hAhRs were present in the inclusion bodies. Mixed proteins at around 110 kDa were obtained from the inclusion bodies which were separated on 6% SDS-PAGE. The GST proteins, used as control, were purified on glutathione-agarose beads. Seven adult female BALB/c mice were immunized GST-hAhRs (20 μg per mouse) for 4 times by subcutaneous injection. Subsequently, according to the standard methodology (Köhler and Milstein, 1976), spleen cells from three selected immunized mice were fused with SP2/0 cells in polyethylene glycol (PEG1500, Merck, Germany), and the hybridoma cells were cultivated.

The individual cultures were subjected to a screening test for their content of specific antibodies against GST-hAhRs but not GST proteins by standard indirect enzyme-linked immunosorbent assay (ELISA) with slight modifications. The 96-well ELISA plates were firstly incubated with either purified GST-hAhRs or GST in 0.1 mol/L Na_2CO_3 (pH = 9.2) overnight at 4°C at a concentration of 0.1 $\mu\text{g}/\text{well}$. After washing, 150 μL of 5% BSA was added to each well for 1 hr at 37°C . The coated plates were

Table 1 – Sequences and location in the hAhR of the customized peptides being used to reveal the recognition sites of the two new mAbs.

Name of peptides	Sequences of peptides	Amino acid position
B1	SQCTESGQGIEATG	181–195
B2	EEATGLPQTVVCYNP	191–205
B3	VCYNPDQIPPENSPL	201–215
B4	ENSPLMERCFCRLR	210–225
E1	MDPLPLRTKNGTSGK	424–438
E2	GTSGKDSATTSTLSK	434–448
E3	STLSKDSLNPSSLLA	444–458
E4	DESIYLYPASSTSST	464–478
E5	STSSTAPFENNFFNE	474–488
E6	NFFNESMNECRNWQD	484–498
E7	RNWQDNTAPMGNDTI	494–508

hAhR: human aryl hydrocarbon receptor; mAbs: monoclonal antibodies.

subsequently incubated with 50 μ L of hybridoma supernatant for 1 hr at 37°C, followed by washing and incubation with horseradish peroxidase solution (HRP)-conjugated rabbit anti-mouse IgG antibody (1:10,000 (V/V), Sigma-Aldrich, MO, USA) for 1 hr at 37°C. Then substrate solution (100 μ L/well of 3,3',5,5'-tetramethylbenzidine liquid-1 component, AMRESCO, Ohio, USA) was added to the plates and incubated at 37°C for 30 min. Finally, 100 μ L/well of 1 mol/L H₂SO₄ was used to end the reaction. The light absorbance of each well was tested at 450 nm (infinite F200 PRO, TECAN, Switzerland). Colonies derived from single cells of suitable hybridomas were isolated by the limiting dilution cloning method (Puck and Marcus, 1955).

The mAbs were purified by protein A/G (Yuekebio, Shanghai, China) column chromatography from ascites of BALB/c female mice which were injected monoclonal cell lines. The concentrations of mAbs were detected by NanoDrop (NanoDrop 2000 spectrophotometer, Thermo Scientific, USA), and their titers were also detected by indirect ELISA. According to the manufacturer's protocol, the isotype of the new mAbs was determined using a mouse antibody isotyping kit (Sigma-Aldrich, MO, USA). The specific recognition of the new

mAbs against synthetic peptides was also tested by indirect ELISA. The plates were pre-coated with 10 μ g/well of the synthetic peptides (customized from Life Tein LLC, Beijing, China, Table 1), and then detected by 0.1 μ g/well of the mAbs.

1.3. Plasmid construction and transfection for epitope study

The hAhR was divided into eight different segments, the encoding sequences of which were amplified from the pGEX4T-1/GST-hAhR (constructed in our lab). These eight amplified segments were constructed into pGEX4T-1/GST vector (Promega, Madison, WI, USA), separately. Two restriction sites, BamHI (5'-GGATCC-3') and XhoI (5'-CTCGAG-3'), were used for cloning and included in primer sequences (Table 2). The constructed plasmids were transformed into *E. coli* BL-21 cells to overexpress the target protein segments.

1.4. Western blot

GST and GST-hAhRs and eight different hAhR segments, which were overexpressed in *E. coli* BL-21, were migrated separately in a 10% SDS/PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. After the transfer, the PVDF membranes were incubated with newly prepared antibodies at 4°C for 18 hr, and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (HRP, Goat anti-mouse IgG(H + L) (Sigma-Aldrich, MO, USA) at room temperature for 50 min. Enhanced chemiluminescence (ECL; Millipore, Billerica, MA) method was used to develop luminescence to detect these bound antibodies.

In accordance with the manufacturer's protocol of total protein extraction kit (BSP003, Sangon Biotech, Shanghai, China), the total protein containing AhR from eight different cell lines (HepG2, MCF-7, HeLa, HEK293T, HKC, SK-N-SH, H4IIE and C2C12) was extracted. Equal amount of total protein was electrophoresed in 8% SDS-PAGE and blotted on nitrocellulose membrane (NC membrane). After blotting, the NC membrane was incubated with commercial antibodies, the new mAbs alone or together with relevant synthetic peptides, at 4°C for 18 hr with subsequent incubation with secondary antibody (dilution 1:10,000 (V/V); IRDye 680RD goat anti-mouse IgG (H + L), Odyssey,

Table 2 – Primers used for amplification of the DNA fragments for prokaryotic expression of hAhR segments.

Name of genes	Sequences of primers	Amino acid position in hAhR (segment size)
hAhR-1	5'-CGGGATCCAACAGCAGCAGCGC-3' 5'-CCGCTCGAGTCTTGTAAAGTTCAGGC-3'	a.a. 1–114 (38.6 kDa)
hAhR-2	5'-CGGGATCCGCAAGAAGGAGAATTCTTA-3' 5'-CCGCTCGAGTAGACGACATATGAA-3'	a.a. 113–224 (38.4 kDa)
hAhR-3	5'-CGGGATCCTGCTTCATATGTCGTCTAA-3' 5'-CCGCTCGAGTTCAGTATATCTCTAAAACA-3'	a.a. 219–312 (36.4 kDa)
hAhR-4	5'-CGGGATCCGGATATACTGAAGCAGAG-3' 5'-CCGCTCGAGAGTGGTAAACATAAAAGGC-3'	a.a. 309–408 (37.1 kDa)
hAhR-5	5'-CGGGATCCCCTTTTATGTTTACCACTGG-3' 5'-CCGCTCGAGAAATGAGTTCACATCCTG-3'	a.a. 403–523 (37.2 kDa)
hAhR-6	5'-CGGGATCCGATGTGAACACTCATTGCTG-3' 5'-CCGCTCGAGCTTTTGGTGATGTTGCT-3'	a.a. 519–628 (38.2 kDa)
hAhR-7	5'-CGGGATCCCAACAGCAACATCACCAA-3' 5'-CCGCTCGAGCTAGAAGTAGTGGGTAT-3'	a.a. 622–734 (38.5 kDa)
hAhR-8	5'-CGGGATCCCCCACTACTTCTAGTTTAGA-3' 5'-CCGCTCGAGGGTAGTCTGAGTGTATT-3'	a.a. 729–823 (36.5 kDa)

Table 3 – Concentration, titer and isotype of the two new mAbs against hAhR.

Name of mAb	Concentration (mg/mL)	Titer	Isotype
1D6	0.24	204,800	IgG1
4A6	0.79	409,600	IgG1

mAbs: monoclonal antibodies; hAhR: human Ah receptor.

Nebraska, USA) at room temperature (RT) for 50 min. Finally, the immune-complex was detected using double color infrared laser imaging system (Odyssey-9140, LI-COR, USA).

1.5. Immunoprecipitation (IP)

HepG2 cells (5×10^6 cells/dish) were seeded in 100 mm dishes and incubated in fresh medium for 24 hr. The cells were rinsed with PBS and lysed in 1 mL TBS-1% Triton X-100 with protease inhibitor cocktail (complete EDTA-free, protease inhibitor cocktail tablets, Roche, Germany) for 1 hr on ice, and the cell extract was centrifuged at 13,300 r/min for 15 min at 4°C. The supernatant was firstly incubated with protein A/G, and then pre-absorbed with primary Abs at 4°C for 2 hr. After that, the complexes were washed 4 times in TBS-1%Tween-20 (centrifugation at 2000 r/min for 2 min at 4°C for each washing). Subsequently, the protein A/G beads were boiled in 20 μ L 2 $\times$ SDS loading buffer at 100°C for 7 min to collect precipitated protein, which was subjected to 8% SDS-PAGE and western blotting. Finally, hAhR was detected by ECL.

1.6. Indirect immunofluorescence assay (IFA)

HeLa cells were cultured in 4-chamber glass-bottomed dishes. After 24-hour-plating, the cells were fixed with 4%

Fig. 1 – Specific detection of human aryl hydrocarbon (Ah) receptor (hAhR) by the two newly prepared mAbs in western blot analysis. Glutathione S-transferase-tagged hAhR fusion proteins (GST-hAhR) and Glutathione S-transferase (GST) protein were expressed in a prokaryotic system. In the western blots, 0.05 μ g of the purified proteins was loaded per lane, and the testing mAbs, 1D6 (left panel) and 4A6 (right panel), at 1 μ g/mL were used in the primary Ab incubations separately. A representative blotting is shown.

Fig. 2 – Detection of hAhR protein segments by the newly generated mAbs. Eight hAhR protein segments expressing *E. coli* were obtained as mentioned in Section 1.3. The *E. coli* with overexpressed protein segments were directly lysed in direct lysis buffer with 2% SDS, 10% glycerol and 200 mmol/L 2-mercaptoethanol and loaded to the SDS-PAGE. In western blots, 1D6 at 0.1 μ g/mL (A) and 4A6 at 0.1 μ g/mL (B) were used to detect the segmental hAhR proteins. Lane 1: segment-hAhR-1; lane 2: segment-hAhR-2; lane 3: segment-hAhR-3; lane 4: segment-hAhR-4; lane 5: segment-hAhR-5; lane 6: segment-hAhR-6; Lane 7: segment-hAhR-7; lane 8: segment-hAhR-8. A representative blotting is shown.

paraformaldehyde in PBS for 15 min at RT. Following fixation, the cells were incubated with 50 mmol/L NH_4Cl /PBS for 1 hr at 4°C. Then 0.1% Triton X-100 was added for 10 min at RT for membrane permeabilization. After 1-hour-blocking (using 5% BSA/PBS at RT), the cells were firstly incubated with primary Abs (L-15, 10 μ g/mL, Santa Cruz, CA, USA; 1D6, 10 μ g/mL; 4A6, 10 μ g/mL) and then with secondary antibody donkey anti-mouse conjugated to AlexaFluor 488 (dilution 1:1000 (V/V); Invitrogen, CA, USA) containing NuBlue® live cell stain readyprobes TM reagent (Life Technologies, OR, USA) for 1 hr at room temperature in dark. Finally, specimens were examined by confocal microscopy (TCS SP5, Leica, Germany) and the fluorescent images of specimens which were incubated with L-15 and the newly prepared mAbs, respectively, were recorded under 40 $\times$ objective.

2. Results

2.1. Characterization of two newly generated mAbs against hAhR

Hybridoma fusion cell lines were subcloned and those excreting monoclonal antibodies against GST-hAhRs but not GST were

Fig. 3 – Functional domains predicted in hAhR and peptidic recognition of the newly generated mAbs. A schematic diagram illustrating the detection sites of the two selected mAbs is shown. 1D6 recognizes the a.a. 484–508 located in transcription activation domain (TAD), and 4A6 recognizes a.a. 201–215 located in PAS-A domain. bHLH: a member of the basic helix-loop-helix; PAS: Per-ARNT-Sim; Q-rich, glutamine-rich; TAD, transcription activation domain.

screened by indirect ELISA assay. Finally two monoclonal cell lines, named HCL-1D6 and HCL-4A6, were isolated. The survival rate of HCL-1D6 and HCL-4A6 examined by trypan blue exceeded 95%, and their recovery rates were about 60%–70%. In addition, the growth-speed of HCL-1D6 and HCL-4A6 was slow. Single cell was seeded in one well of a 96-well plate and grew into a cell cluster in one week. The mAbs secreted by HCL-1D6 and HCL-4A6 were named 1D6 and 4A6, respectively. The mAbs were enriched and purified from mouse ascites using protein A/G column. Characterization of the mAbs was carried out. Their isotype and concentration were detected by indirect ELISA; and western blot was used to test if mAbs could specifically recognize the hAhR expressed in *E. coli* BL21. As shown in Table 3, 1D6 and 4A6 were both IgG1 isotype, while the titers of 1D6 and 4A6 were 204,800× (at the concentration of 0.24 mg/mL) and 409,600× (at the concentration of 0.79 mg/mL), respectively. Additionally, both 1D6 and 4A6 specifically recognized purified GST-hAhRs (~130 kDa) but not purified GST proteins (~26 kDa) produced in *E. coli* BL21 cells by western blot (Fig. 1).

2.2. Peptidic detection of the new mAbs

To determine the protein fragments detected by the selected mAbs, eight segments derived from hAhRs, namely hAhR-1, hAhR-2, hAhR-3, hAhR-4, hAhR-5, hAhR-6, hAhR-7 and hAhR-8 (Table 2), were obtained by a prokaryotic protein expression system. The full length of hAhR was divided into eight partitions unless encountering any border of the functional domain to get the segments, which were 94–121 a.a. in length, and 36.4–38.5 kDa in size (Table 2). The neighboring segments had overlapping regions of several amino acids in length. Western blot was used to identify which hAhR segment could be detected by the mAbs. The results showed that 1D6 and 4A6 could

recognize hAhR-5 (a.a. 403–523; Figs. 2A and 3) and hAhR-2 (a.a. 113–224; Figs. 2B and 3), respectively.

To further determine the epitopes, the antigenicity of the above hAhR segments was predicted by BepiPred (<http://www.cbs.dtu.dk/services/BepiPred/>) using antigenicity threshold of 0.35. According to the prediction results, 11 peptides were selected and synthesized (Table 1). By indirect ELISA, 1D6 was found to interact with two segments (a.a. 484–498 and a.a. 494–508, located in TAD domain), and 4A6 bind to a peptide sequence of a.a. 201–215 (located in PAS-A domain) (Table 4 and Fig. 3).

2.3. Detection of AhR by the mAbs in different mammalian cell lines

To test whether the newly generated mAbs could recognize endogenous AhR in different cell lines, AhR from eight cell lines was detected using the mAbs (as primary Abs) by western blot. As shown in Fig. 4A, where commercial mAbs, L-15 and B-11, served as positive controls, AhR was detected in a molecular weight between 100 and 130 kDa. L-15 and B-11 recognized one band in HepG2, HeLa, HKC, and H4IIE, two bands in MCF-7 and HEK293T, and three bands in SK-N-SH, but no band in C2C12. The performance of 1D6 and 4A6 binding to AhR was quite similar to L-15 and B-11. The results showed that the expression levels of AhR proteins varied remarkably in different cell lines, with the higher levels being found in MCF-7, HEK293T, SK-N-SH and the un-detectable levels in C2C12. Furthermore, the bands were detected in rat H4IIE cell lysate, which demonstrated that 1D6 and 4A6 could also recognize rat AhR.

Additionally, in order to distinguish the specific band recognized by the mAbs in the western blot for AhR of MCF-7, HEK293T and SK-N-SH cells, the synthetic peptides (a.a. 484–498 and a.a. 494–508 for 1D6 solution; a.a. 201–215 for 4A6) were co-incubated with the mAbs to compete with AhR protein in the primary Ab incubations. As shown in Fig. 4A, the multiple bands in MCF-7, HEK293T and SK-N-SH were detected by 1D6 and 4A6. The mAbs were able to specifically combine with the AhR with higher molecular weight in these three cell lines, the signals of which were obviously decreased after adding the competing peptides to the corresponding testing primary Abs (Fig. 4B, C, D, E). The signals of medium and light AhRs were barely changed upon the administration of competing peptides suggesting the non-specificity.

Table 4 – Peptide sequences recognized by the two new mAbs.

Name of mAb	Amino acid sequence	Amino acid position in hAhR
1D6	NFFNESMNECRNWQD	a.a. 484–498
	RNWQDNTAPMGNDTI	a.a. 494–508
4A6	VCYNPDQIPPENSPL	a.a. 201–215
mAbs: monoclonal antibodies; hAhR: human Ah receptor.

Fig. 4 – Recognition of AhR in different mammalian cell lines by the newly generative mAbs. Total proteins from eight mammalian cell lines were collected and subjected to SDS-PAGE, including HepG2, MCF7, HeLa, HKC, HEK293T and SK-N-SH cells from human, H4IIE from rat and C2C12 from mouse. The anti-hAhR primary Abs were 1D6 (0.8 μ g/mL), 4A6 (0.8 μ g/mL), L-15 (1:125 V/V, Santa Cruz, CA, positive control) and B-11 (1:250 V/V, Santa Cruz, positive control), separately (A, B, D). Antigenic peptide competitive inhibition experiment was carried out for the MCF-7, HEK293T and SK-N-SH as described in Section 2.3. Detected bands of heavy, medium and light molecules are indicated as AhR-Heavy (H), AhR-Medium (M) and AhR-Light (L) in B and D. Quantification of each target bands (as indicated) from the blots was performed by densitometry calibration which was normalized by corresponding β -actin bands on an image analyzer (C, E).

2.4. Detection of the native hAhR by the mAbs

In order to reveal the ability of these two new mAbs in recognizing native hAhR in HepG2 cells, IP assay was selected. As shown in Fig. 5, 1D6 and 4A6 were able to enrich the hAhR originated from HepG2 cells, with the same migration rate as those of the commercial mAb, B-11. Moreover, IFA assay was employed to test in situ expression of hAhR protein in HeLa cells by the two new mAbs. As a positive control, commercial mAb L-15 (Fig. 6B) could recognize hAhR in the cytoplasm of HeLa cells, while no signal could be detected in the negative control without adding any primary Ab (Fig. 6A). Compared with the commercial mAb, L-15, both of 1D6 and 4A6 could detect the hAhR in the HeLa cytoplasm more strongly (Fig. 6C, D).

3. Discussion

AhR is a molecular target of a variety of endogenous and exogenous ligands with structural diversity such as polyhalogenated aromatic hydrocarbons; coplanar polychlorinated biphenyls; polycyclic aromatic hydrocarbons, flavonoids and tryptamine (Denison et al., 2002). These ligands generated different toxic and biological effects in a diverse range of aspects, in different species, tissues and cell types resulting from differential alterations of gene expression (Hahn 2002; Denison and Heath-Pagliuso, 1998). It was reported that there were preferences for different ligands to bind AhR (Xing et al., 2012). Site-directed mutation and ligand binding analysis suggested that structurally diverse ligands, such as

Fig. 5 – Immunoprecipitation analysis using the new mAbs for enrichment of naturally folded hAhR in HepG2 cells. Proteins from the HepG2 cells were immunoprecipitated as described in Section 1.5 using 8 μ g 1D6, 4A6 and mIgG (negative control) or 1 μ g B-11 (positive control), separately. In the immunoblotting, B-11 was used at 1:1000 V/V as primary Ab to detect precipitated hAhR. A representative blotting is shown.

3-methylcholanthrene (MC), beta-naphthoflavone (BNF) and dibenzo-p-dioxins (TCDD), might interact with AhR through different sites, resulting in different transactivation activities and nuclear localizations (Goryo et al., 2007; Backlund and Ingelman-Sundberg, 2004; Whelan et al., 2010). In line with this notion, Zhao et al. suggested that the different consequences following the AhR activations triggered by different types of ligands, like TCDD and BNF, may be due to the distinct AhR transactivation process occurring before its translocation (Zhao et al., 2010). Thus, to closely investigate the differentially activated AhR, mAbs against various epitopes of AhR may be needed. However, the currently commercial available mAbs are limited for the N-terminal regions (a.a. 12–17&22–31), the LBD (a.a. 279–376) and the TAD (a.a. 721–821/820 and a.a. 637–848). The newly generated mAbs, 1D6 and 4A6, recognize two more sites, one on TAD (a.a. 484–508) and the other on PAS-A/dimerization region (a.a. 201–215), which may be helpful to distinguish different conformational changes upon binding with diverse AhR ligands. As shown in the characterization of the mAbs, they can be used in some commonly used biochemistry experiments, including ELISA, western blot, IP and IFA.

Aryl hydrocarbon receptor homologs were widely found in vertebrates and invertebrates, which were well discussed in

several reviews (Hahn, 1998, 2002). Moreover, the previous studies showed that the functional domains of AhR were conserved between human and murine (Dolwick et al., 1993; Ma et al., 1995; Jain et al., 1994). According to the alignments of the amino acid sequences of the epitopes for 4A6 and 1D6, the sequences of both rat and mouse had 60% of identity with that of human for 4A6, and 36% and 32% of identity for 1D6, respectively (hAhR, GenBank: AAH69390.1; mouse AhR, GenBank: AAK13443.1; rat AhR, GI: 3561054). Thus, it is possible for these mAbs to cross-react with rat and mouse AhR. As demonstrated in Fig. 4, the mAbs could recognize AhR in H4IIE cells derived from rat. In line with the commercial mAb, AhR was not detected in the mouse muscle cell line, C2C12 cells, by the mAbs, consistent with the relative low expression of AhR in muscles (Schrenk and Chopra, 2012). Moreover, the similar identical level compared to human were found in other species, including guinea pig (GenBank: AAT35817.1; 60% for 4A6 and 1A6), rabbit (Accession: NP_001075674.1; 60% for 4A6, 48% for 1A6), cow (Accession: NP_001192955.1; 80% for 4A6, 64% for 1A6), and dog (Accession: XP_532485.2; 6.6% for 4A6, 72% for 1A6). Thus both of the newly generated mAbs might be able to cross-react with these species due to the conservation of the recognition sequences between these mammals and humans.

In cell lines derived from different human tissues, AhR expressions are different at mRNA level (Dolwick et al., 1993; FitzGerald et al., 1996; Frericks et al., 2007). The mRNA levels are higher in the placenta, lung, heart, pancreas and liver, and relatively lower in the brain, kidney and skeletal muscle (Dolwick et al., 1993). By using the newly generated mAbs, differential protein expression of AhR in various cell lines were detected as shown in Fig. 4, including HepG2, MCF-7, HeLa, HKC, HEK293T and SK-N-SH. Two different patterns of protein signals could be recognized by the mAbs in the western blot analysis. The AhR in HepG2, HeLa and HKC cells exhibited single-band-pattern migrated at 105 kDa, in line with the reported size in HeLa cells (Dolwick et al., 1993). A multiple-band-pattern was found in MCF-7, HEK293T and SK-N-SH cells, where non-specific signals at the relative light molecular weights were revealed by the antigen competition assay as shown in Fig. 4B–E. Thus, AhR from MCF-7, HEK293T and SK-N-SH cells are distinct to the others not only in the amount but also in the size. Previous work has shown that AhR could undergo several types of post-translational modifications. Post-translational modifications of protein, such as phosphorylation, acetylation, methylation, glycosylation and ubiquitylation, play pivotal roles in regulation of protein function (Beevers, 1982; Dennis et al., 1999). These post-modification played important roles in AhR activation (Backlund and Ingelman-Sundberg, 2004; Powis et al., 2011). It was also reported that phosphorylation of Ser-12 and Ser-36 inhibited nuclear import of hAhR (Ikuta et al., 2004a) and phosphorylation of Ser-68 in the nuclear inhibited the export of hAhR (Ikuta et al., 2004b). In addition, Lys-24 of hAhR was shown to be lysine acetylated (Lundby et al., 2012). Moreover, both N-glycosylation sites and O-glycosylation sites are predicted to be present in hAhR sequence (GenBank: AAH69390.1), by using NetOGlyc 4.0 server (<http://www.cbs.dtu.dk/services/NetOGlyc/>) (our unpublished work) (Khoury, et al., 2011; Chen et al., 2015). Thus, the relative high molecular weight of the AhR in HEK293T, MCF-7 or SK-N-SH at around 130 kDa may partly be explained by the possible posttranslational modifications.

Fig. 6 – Indirect immunofluorescence analysis using the new mAbs for revealing the in situ expression of AhR in HeLa cells. Permeabilized HeLa cells were stained with (B: L-15; C: 1D6; D: 4A6) or without (A) primary Abs. Images were acquired under the same detection condition. Representative images are shown.

4. Conclusions

In this study, we successfully generated and characterized two anti-hAhR mAbs (1D6 and 4A6) using full-length of the human AhR protein as antigen. We demonstrated that 1D6 could recognize a.a. 484–498 and a.a. 494–508, and 4A6 could detect a.a. 201–215. This is the first study to report anti-hAhR mAbs corresponding to these two amino acid positions. In addition, mAbs were verified in eight different tissue cell lines. It was implied that AhR was widely distributed in various cell lines, from human to rat, with different expression levels. We also found different molecular weight bands detected by mAbs in different cells, which was possibly because of the post-translational modifications of hAhR.

Moreover, the mAbs could recognize natural AhR protein conformation in different cell lines such as HepG2 and HeLa. Thus, we concluded that the newly generated mAbs, as helpful tools, not only could be used for quantitative bioassay for environmental contaminants based on the AhR signaling, but also held great potential for further investigating the molecular roles and spatial structure of AhR, as well as its potential biological functions in future studies.

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