Vol. 215, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 4, 1995 Pages 405-411

Identification of Cellular Protein That Can Interact Specifically with the Basic Helix-Loop-Helix Domain of the Aromatic Hydrocarbon Receptor*

Anwar Hossain, Hideaki Kikuchi¶, Shuntaro Ikawa, Ikuko Sagami†, and Minro Watanabe

Department of Molecular Genetics, Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan

Received August 30, 1995

Summary: The Ah receptor is a basic helix-loop-helix (bHLH)-PAS protein and functions as a ligand-activated DNA binding protein directly interacting with target genes by binding to xenobiotic responsive elements. We have sought to identify possible cellular proteins that can interact with the Ah receptor. The bHLH domain of the Ah receptor was fused to glutathione-S-transferase (GST), and the resulting fusion protein was used as a probe to help us to identify receptor associated protein(s). At least one such protein, 45kDa (p45), was detected in mouse liver extracts, but it does not bind to the bHLH domain of the Ah receptor nuclear translocator, nor to the transactivation domain of Ah receptor or GST alone.

Introduction:

The aromatic hydrocarbon (Ah) receptor is the prototype of a newly described family of transcription factors. Biochemical and toxic effects of halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls (PCB) are mediated by this receptor (see

* This work was supported in part by a Grant in Aid for Scientific Research on priority Areas from the Ministry of Education, Science, and Cultuere of Japan and by a grant from the Men-Earth System.

¶To whom correspondence should be addressed at Seiryo-machi 4-1, Aoba-ku, Sendai 980-77, Japan. Fax: 81-22-272-4927.

†Present address: Institute for Chemical Reaction Science, Katahira 2-1-1, Aoba-ku, Sendai 980-77, Japan.

The abbreviations used are: a.a., amino acids; Ah receptor, Aromatic hydrocarbon receptor; mAHR, mouse Ah receptor; ARNT, Ah receptor nuclear translocator; hARNT, human ARNT; bHLH, basic helix-loop-helix; CYP1A1, Cytochrome P450 1A1; GSH-Sepharose, Glutathione Sepharose 4B; GST, glutathione-S-transferase; HEDG, HEPES-EDTA-DTT-glycerol; HIF-1a, Hypoxia inducible factor-1α; PAS, per-Ah receptor/ ARNT-Sim; HSP90, 90 kDa heat shock protein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, Xenobiotic responsive elements.

0006-291X/95 $12.00

reviews, 1, 2). As a ligand activated transcription factor, the Ah receptor activates the transcription of xenobiotic metabolizing enzymes cytochromeP4501A1 (CYP1A1) and cytochrome P4501A2 (CYP1A2) as well as other xenobiotic and drug metabolizing enzymes.

Much has been learned from cDNA cloning and sequencing of the Ah receptor in different species including the mouse (3,4) , the rat (5), and human (6,7). From the cDNA cloning of the Ah receptor, this receptor has been shown to harbor a bHLH domain (3,4). The Ah receptor also belongs to a family of proteins as shown by a region containing 200 aminoacids with high sequence similarities, termed the PAS domain (3,4). The PAS protein family is so named for the three originally identified members, i.e., the *Drosophila* circadian rhythm protein Per (8), the *Drosophila* neurogenic protein Sim (9), the Ah receptor nuclear translocator protein (ARNT) (10) and the newly identified Hypoxia inducible factor 1α (HIF-1α) (11). Of the five PAS proteins thus far identified, four contain a bHLH domain next to the N-terminal of the PAS domain. For the Ah receptor, the PAS domain appears to play a role with ARNT in heterodimerization, interactions with heat shock protein 90 (HSP90) and ligand binding (12,13). In the absence of a ligand, the Ah receptor is located in the cytosol in association with HSP90 (14,15,16). Treatment with ligands initiates the conversion of the receptor to a nuclear form and results in its translocation into the nucleus and its association with ARNT (17). The resultant AHR-ARNT-ligand heterocomplex binds to xenobiotic responsive elements (XRE) upstream of dioxin responsive genes, resulting in increased initiation rates of target gene transcription (18,19).

The best though still poorly understood role of the Ah receptor in vivo is induction of CYP1A1 (19,20). Recent evidence suggests that besides ARNT and HSP90, other cellular factor(s) may play important role(s) in Ah receptor function (21). In this study, we sought to identify, through an affinity purification procedure using GST-AHR.bHLH, bHLH domain of Ah receptor, a cellular protein that interacts with the Ah receptor. We herein report, the identification of one such protein of approximately 45 kDa which we have designated as p45.

Materials and Methods

Materials: Anti-mAHR (mouse Ah receptor) rabbit antiserum was prepared against amino acids 9-418 of the mAh receptor expressed in *E. coli* as a $(His)_6$-tag fusion protein (22). Goat anti-rabbit IgGs was obtained from Promega (Madison, WI, U.S.A.). Glutathione Sepharose 4B was purchased from Pharmacia, Biotech AB (Uppsala, Sweden). The Hepa-1 cell line was obtained from Dr. Sogawa of Tohoku University and the HepG2 cell line from Dr. Hori of Saga School of Medicine.

Methods

Preparation of mouse extracts: C57BL/6J mice, 6-8 weeks of age were sacrificed. Different tissues from the mice were collected and homogenized in 2 volumes of HEDG (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 400 mM of KCl. Homogenization was performed using 4 bursts of 5 seconds each with

an Ultra Turrax tissue homogenizer. The preparation was initially centrifuged at 10,000 x g for 20 minutes at 4°C. The supernatant was recentrifuged at 100,000 x g for 1 hour. The resulting clear supernatant was collected and kept at -80° C.

Construction of GST expression vector: To generate the vector pGEX2T.mAHR.bHLH, the nucleotide region of 79-961 of mAHR cDNA from C57BL/6J was amplified usingthe polymerase chain reaction with the following primers: 5'-GTGGATCCGCTGAAGGAATT-3' and 3'-TAGGTACGACGTCTGT-5' . The PCR product was digested with *Bam*H I and *Eco*R I and ligated into pUC19, which had been digested with*Pst* I and *Hin*P I to yield a fragment of nucleotide region 79-354 that encodes amino acid residues Ala_{27} to Gln_{118} of mAh receptor. The resulting fragment was blunt-ended with Klenow enzyme and digested with *Bam*H I. Digested fragments were ligated into pGEX2T which hadbeen digested with *Bam*H I and *Sma* I. To generate the vector pGEX2T. mAHR.Q, the nucleotide region 1449-2422 of mAh receptor cDNA from C57BL/6J was amplified using the following primers:5'-AGTGCGGGAGTTGGCAAGAC-3' and 3'-TCGTTCCACGTCTCAACTCCAC - 5'. The PCR product was digested with*Hae* III. Digested fragments were ligated into pGEX2T which had been digested with *Sma* I. These constructs were mapped by restriction digestion and the junction at the 5' and 3' ends were sequenced. Construction of the plasmid encoding GST.hARNT.bHLH is described elsewhere (22).

Expression and purification of fusion protein: The expression and purification GST.mAHR.bHLH and pGEX2T.mAHR.Q were carried out as previously described (23) with the following exception to minimize degradation and increase the solubility. *E. coli* JM 109 cells harboring the expression plasmids were induced with 0.5 mM isopropyl-ß-thiogalactoside at 22°C for 1 hour. Cells were collected by cetrifugation and lysed in buffer A (20 mM Tris. Cl, pH 8.0, 200 mM NaCl, 10% glycerol, 0.5% NP 40, 0.1% ß-mercaptoethanol, 5 µg/ml each of pepstatin, leupeptin, chymostatin, antipain, and elastinal) containing 5 mg lysozyme andbriefly sonicated. The lysates were cleared by centrifugation at 15000 rpm. in microcentrifuge and the supernatant was passed through a 1 ml column of50% (v/v) Glutathione Sepharose 4B. The column was subsequently washed with15 ml of buffer A, 10 ml of buffer A containing 1M NaCl and 15 ml of buffer A and stored at 4°C as 50% slurry in buffer A for subsequent studies. The amount of fusion protein was estimated by 10% SDS-PAGE and staining the gel with Coomassie Blue.

In vitro binding of GST-mAHR.bHLH fusion protein to tissue or cell extracts: Appropiate amounts of liver extracts (1 mg) were diluted in assay buffer (25 mM Tris.Cl, pH 8.0, 150 mM NaCl, 1mM ß-mercaptoethanol, 1 mM EDTA, 1 % NP 40, 10 µg/ ml each of leupeptin, pepstatin, antipain, chymostatin, and elastinal) to a volume of 1.5 ml. To preclear the extracts , 50 µl of 50% slurry of Glutathione Sepharose 4B was added to the mixture and incubated overnight on ice. GST.mAHR.bHLH bound 10 µl of 50% slurry of Glutathione Sepharose was addedand incubated on ice for 2-3 hours with shaking on a rocker platform. Beads were collected by brief centrifugation and non-specifically bound protein was removed by extensive washing with the assay buffer without protease inhibitors five times. The pellet was collected, suspended in 20 µl of 2x SDS-PAGE sample buffer and boiled for 5 minutes. Bound protein was separated by electrophoresis and visualized by Coomassie Blue staining.

Immunobloting: Proteins were separated by SDS-PAGE and transferred onto the nitrocellulose membrane. After blocking overnight with 20% non-fat dry milk powder in Tris buffered saline (TBS), the blot was incubated for 4 hours at room temperature with anti-mAHR sera. Antigen bound antibodies were visualized with a second anti-body conjugated with alkaline phophatase. The blots were visualized by nitro

blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate disodium at room temperature.

Results and Discussion: To identify cellular proteins that bind to the bHLH domain of the Ah receptor, different constructs, pGEX2T. mAHR (9-166 a.a.), pGEX2T. mAHR (27-167 a.a.) and, pGEX2T. mAHR.bHLH were used to express the bHLH domain of the Ah receptor as a GST fusion protein in *E. coli*. Constructs pGEX2T. mAHR (9-166a.a.) and pGEX2T. mAHR (27-167 a.a.) yielded fusion proteins as bacterial inclusion bodies. Construct pGEX2T. mAHR.bHLH encoding the bHLH domain of the Ah receptor maximized the solubility of the fusion protein, and therefore was used for the entire study. The size of the fusion protein was in good agreement with the predicted structure: GST, 28 kDa; GST. mAHR.bHLH, 38 kDa. As a second check on structure, the fusion protein was probed with a rabbit polyclonal antisera raised against 9-418 a.a. of the mAh receptor, expressed as a His tag fusion protein in *E. coli* (22). As expected, the antibody recognized both the endogenous mAh receptor as well as the GST.mAHR.bHLH fusion protein (Fig 1b). Fusion proteins which contained intact the bHLH and PAS domain retained the ability to bind to ARNT. Conversely, fusion proteins that contain only the bHLH domain are known to abrogate the Ah receptor binding to ARNT (18). We intentionally avoided

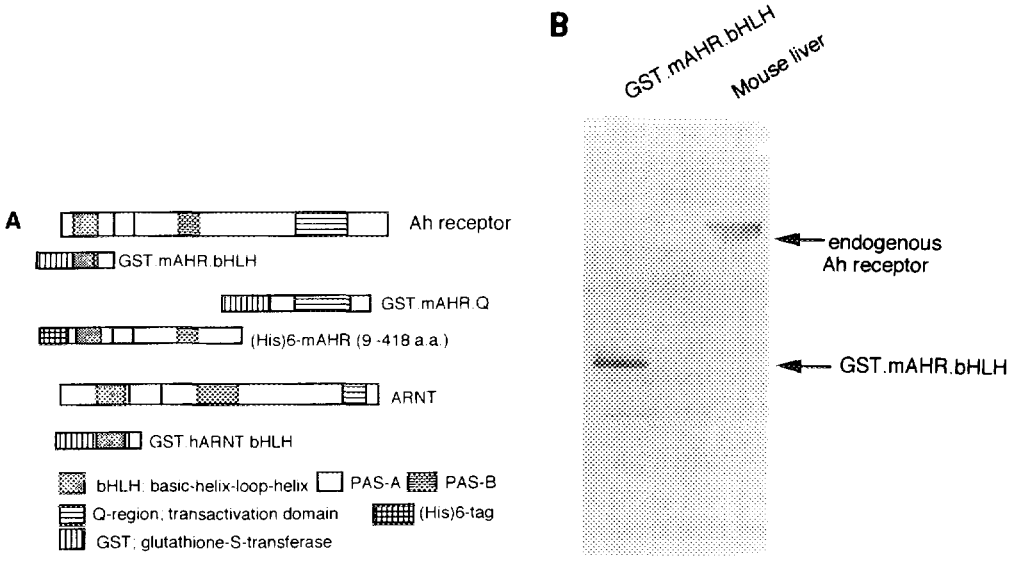

Fig 1A. Schematic diagram of fusion proteins: The full-length Ah receptor and ARNT are shown with the bHLH domain(bHLH), the PAS region (PAS-A and PAS-B) and transactivation domain (Q-region). Fusion proteins used for this study are also shown in diagram.

Fig 1B. Immunoblot analysis with anti-Ah receptor antibody: Mouse liver extracts (500 µg) and purified GST.mAHR.bHLH (0.5 µg) were run on SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with anti-Ah receptor antibody as described under experimental procedures. Arrowheads indicate the relative position of the endogenous Ah receptor and GST.mAHR.bHLH fusion protein.

those constructs, because ARNT might interfere with the binding of Ah receptor fusion protein to one or more of these potential cellular factors in vitro. As expected, this GST. mAHR.bHLH failed to recognize endogenous ARNT (data not shown).

We tested whether the bHLH domain of the Ah receptor contained enough structural information to interact with protein from mouse liver. Mouse liver was chosen for this experiment because this tissue is highly responsive to TCDD and related compounds. Thus, we reasoned that possible proteins might also be present in this tissue. One protein in mouse liver extracts was found to associate specifically with GST.mAHR.bHLH. The molecular mass of this protein was approximately 45 kDa (Fig. 2A). This protein, here after referred to as p45, did not bind to the fusion protein GST.mAHR.Q which contained the transactivation domain of the Ah receptor, or to GST alone. In an attempt to show that p45 interacts specifically with the bHLH domain of the Ah receptor, i.e., that it was not the general bHLH domain binding protein, mouse liver extracts were incubated with a fusion protein carrying the bHLH domain of hARNT. The results displayed in Fig. 2B demonstrate that p45 did not bind to the GST.hARNT.bHLH containing the bHLH domain of ARNT. To further document the specificity of the interactions between the GST.mAHR.bHLH and p45, binding competition was carried out. A thrombin-cleaved mAHR (27-118 a.a.) fragment was incubated with the protein-protein interaction assay before the addition of the GST fusion protein. The thrombin-cleaved fragment blocked virtually almost all specific binding sites of GST. mAHR.bHLH fusion protein (Fig. 2B, Lane 3). Therefore, this fusion protein exhibited a major Ah receptor functional property in a manner indistinguishable from that of the intact Ah receptor. This technique has proven useful in identifying proteins associated with a number of receptors (24,25). Recently, Semenza *et. al* cloned the hypoxia inducible factor, composed of HIF-1α and ARNT complex, ARNT-HIF-1α dimer bind to the promoter regions of the different genes (11). Our data suggest that bHLH domain of the Ah receptor could interact not only with ARNT but with other proteins in a putative transducing complex. This interaction could be important *in vivo* since we could not explain the diverse action of TCDD with Ah receptor-ARNT complex. We can speculate that like ARNT-HIF-1α complex, the Ah receptor and p45 heterocomplex could also binds to the different responsive elements in different cellular environment.

The complexity of the TCDD response in different tissues (26) and cells (27) implies that the functional Ah receptor controls a variety of biological programs leading to effects ranging from CYP1A1 induction to the differentiation of keratinocytes. The present findings raise the interesting possibility that a heterodimer between the Ah receptor and different cellular factors may display differential activation and biological responses. A different type of Ah receptor heterodimer may lead to functionally distinct Ah-receptor actions, some being ARNT dependent and others not. A complete elucidation of the functions of p45 awaits the molecular cloning of the gene encoding it.

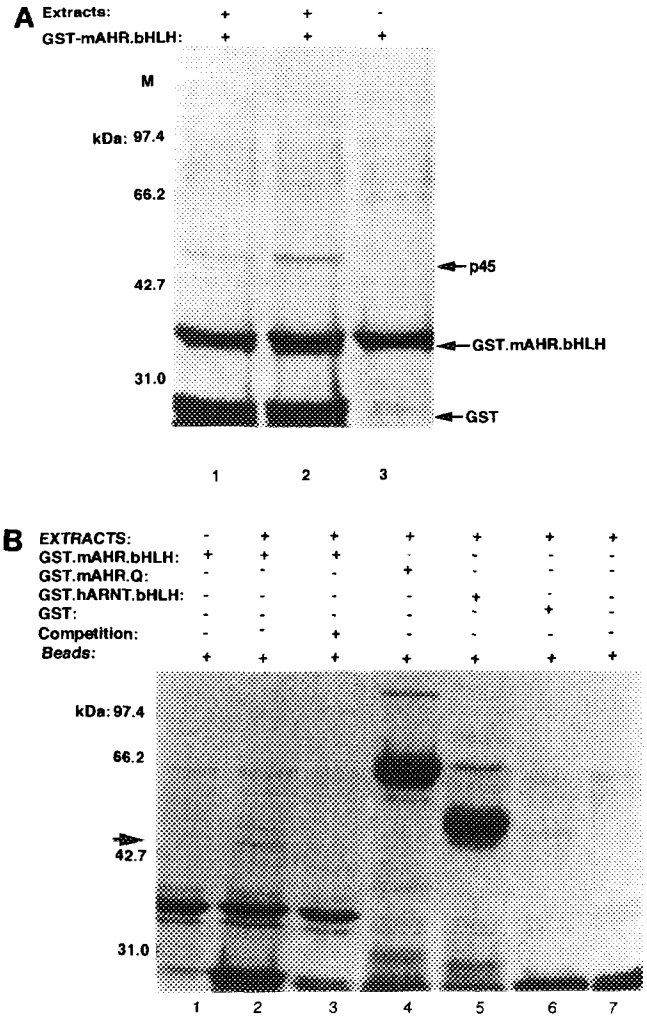

Fig2A. Specific interaction between the bHLH domain of the Ah receptor and cellular factor in mouse liver extracts: The coding sequence from nucleotide 79-354 b.p. of mouse Ah receptor was cloned into vector pGEX 2T and GST.mAHR.bHLH fusion protein encodes the amino acids 27-118 of mouse Ah receptor, prepared according to standard protocol. Mouse liver extracts were incubated with GST.mAHR.bHLH. After extensive washing the bound protein was analyzed by 10% SDS-PAGE and visualized by coomassie blue staining. Arrow indicates the position of 45 kDa protein (p45). Lane1; same as lane 2, except 500 mM NaCl was used in washing condition, lane 2; normal protein-protein interaction assay was performed as described under "materials and methods", and lane 3; no extracts (GST.mAHR.bHLH only). Molecular weight markers are indicated in left panel.

Fig2B. Specificity of interaction between the bHLH domain of the Ah receptor and cellular factor in mouse liver extracts: Mouse liver extracts were incubated with GST.mAHR.bHLH (lane 2), GST.mAHR.Q (lane 4), GST.hARNT.bHLH (lane 5), GST (lane 6), and only beads (lane 7). After extensive washing the bound protein was analyzed by 10% SDS-PAGE and visualized by coomassie blue staining. In lane 3, AHR.bHLH part of GST.mAHR.bHLH fusion protein was preincubated with mouse liver extracts and then used for assay. Lane 1, represents the GST.mAHR.bHLH only (no extracts). Molecular weight markers are indicated in left panel. Arrow indicates the p45.

References

1. Nebert, D. W., and Gonzalez, F. J. (1987) Ann. Rev. Biochem. 56, 945-993
2. Huff, J., Lucier, G., and Tritscher, A. (1994) Ann. Rev. Pharmacol.Toxicol. 34, 343-372
3. Ema, M., Sogawa, K., Watanabe, N., Chujoh, Y., Matsushita, N.,Gotoh, N., Funae, Y., and Fujii-Kuriyama, Y. (1992) Biochem. Biophys. Res. Commun. 184, 246-253
4. Burbach, K.M., Poland, A., and Bradfield, C.A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 8185-8189
5. Carver, L. A., Hogenesch, J. B., and Bradfield, C. A. (1994) Nuc. Acid. Res. 22, 3038-3044
6. Itoh, S., and Kamataki, T. (1993)Nucleic. Acids. Res. 21, 3578
7. Dolwick, K.M., Schimdt, J.V., Carver, L. A., Swanson, H. I., and Bradfield, C. A. (1993) Mol. Pharm. 44, 911-917
8. Jackson, F.R., bargiello, T.A., Yun, S.H., and Young, M.W. (1986) Nature 320, 185-188
9. Nambu, J. R., Lewis, J. O., Wharton, K. A., and Crews, S. T. (1991) Cell. 67, 1157-1167
10. Hoffman, E.C., Reyes, H., Chu, F.F., Sander, F., Conley, L.H., Brooks, B. A., and Hankinson, O. (1991) Science, 252, 954-958
11. Wang,L.G., Jiang, B-H., Rue, E.A., and Semenza,G.L. (1995) Proc. Natl. Acad. Sci. 92, 5510-5514
12. Dolwick, K. M., Swanson, H. I., and Bradfield, C. A. (1993) Proc. Natl. Acad. Sci. 90, 8566-8570
13. Whitelaw, M. L., G¨ø ttlicher, M., Gustafson, J. Å., and Poellinger, L. (1993) EMBO. J. 12, 4169-4179
14. Perdew, G. H. (1988) J. Biol. Chem. 263, 6708-6713
15. Wilhelmsson, A., Cuthill, S., Denis, M., Wikstorm, A.-C., Gustafson, J. Å., and Poellinger, L. (1990) EMBO J. 9, 69-76
16. Pongrtz, I., Mason, G. G. F., and Poellinger, L. (1992) J. Biol. Chem. 267, 13728-13734
17. Reyes, H., Reisz-Porszasz, S., and Hankinson, O. (1992) Science 256, 1193-1195
18. Swanson, H.I., and Bradfield, C. A. (1993) Pharmacogenetics 3, 213-230
19. Okey, A. B., Riddick, D. S., and Harper, P. A. (1994) Trends. Pharmacol. Sci. 15, 226-232
20. Nebert, D. W., Puga, A., and Vasiliou, V. (1993) Ann. NY. Acad. Sci. 624-640
21. Hankinson, O (1993) Arch. Biochem. Biophys. 300, 1-5
22. Hossain,A., Kikuchi,H., Ikawa,S., Sagami,I., and Watanabe,M. (1995) Biochem. Biophys.Res. Commun. 212, 144-150
23. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262-267
24. Halachimi, S., Marden, E., Martin, G. MacKay, H., Abbondanza, C., Brown, M. (1994) Science 264, 1455-1458
25. Sabatini, D. M., Erdjument-bromage, H., Lui, M., Tempst, P., and Synder, S. H. (1994) Cell. 78, 35-43
26. Gardin,K., Wihelmsson, A., and Berghard, A. (1993) J. Biol. Chem. 268, 4061-4068
27. Arellano, L. O., Wang, X., and Safe, S. (1993) Carcinogenesis. 14, 219-222