Development of an *In Vitro* Model for Investigating the Mechanism of Formation of Ligand Nuclear Ah Receptor Complexes

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ABSTRACT

Incubation of liganded cytosolic Ah receptor from wild-type mouse Hepa 1c1c7 cells with nuclei from the same cell line results in a time- and temperature-dependent formation of the nuclear Ah receptor complex. These results are similar to observations previously reported for both *in vivo* studies with rodents and with mammalian cells in culture. Moreover the molecular properties of the nuclear Ah receptor from the *in vitro* translocation were similar to those reported from transformed or nuclear Ah receptors in other studies. This model is ideally suited to probe for those cellular factors which are responsible for the formation of nuclear Ah receptor complexes.

INTRODUCTION

The cytosolic and nuclear Ah receptor from laboratory animals, humans and mammalian cells in culture have been extensively characterized[1]. The cytosolic receptor is a 270-300-kDa protein which reversibly binds 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds with high affinity. After initial binding, the cytosolic Ah receptor complex is transformed into a heterodimer which contains the Ah receptor ligand-binding protein and the Arnt protein which is involved in the cytosol to nuclear translocation of the ligand bound receptor complex[2]. The transformed cytosolic and nuclear Ah receptor complexes exhibit similar molecular properties which include high binding affinity for specific genomic sequences (dioxin responsive elements, DREs) which are target sites for the nuclear Ah receptor complex. The precise mechanism of the transformation and transport process has not been well defined for the Ah receptor complex. This paper describes a new *in vitro* model system which is ideally suited for probing the transport mechanism of cytosolic proteins into the nuclear compartment of cells. In this study, liganded cytosolic Ah receptor from Hepa 1c1c7 cells and nuclei from the same cell line are used. However, the system can be reconstituted with nuclei or cytosol from different cell lines and used to investigate the presence or absence of various factors which are required for intracellular transport processes.

METHODS

Cell growth and isolation of cytosol and nuclei Mouse Hepa 1c1c7 cells were grown in α-MEM and supplemented with 5% fetal bovine serum plus 10 ml antibiotic-antimycotic solution (Sigma). Cells were grown in 150-cm^2 culture flasks in an air:carbon dioxide (95:5) atmosphere at 37° C. After reaching confluence, the cultures were harvested by trypsinization. Cells were washed with used medium and centrifuged at 1000 g for 5 min at 4° C. Cell pellet was washed with HEGD buffer and centrifuged at 1000 g for 5 min at 4° C. Cell pellet was resuspended in 3 ml of HED buffer containing cytochalasin B (5 μg/ml) for 10

min and centrifuged at 1000 g for 10 min at 4° C. The cell pellet was resuspended in 1 ml of HEGDI buffer (HEGD plus aprotinin 1μg/ml; leupeptin 1μg/ml) and then homogenized. The homogenate was centrifuged at 2000 g for 10 min at 4° C. The nuclei were purified essentially according to the method of Blobel and Potter[3]. The crude nuclear pellet was resuspended in 1 ml HES (Hepes 5 mM, EDTA 5 mM, sucrose 250 mM, DTT 1 mM, NaCl 15 mM, KCl 80 mM) buffer and mixed with 2.5 ml of 2.3 M sucrose in HES in a centrifuge tube. After centrifugation for 30 min at 130,000 g, the purified nuclei were recovered and washed with HEGD buffer. The purified nuclei were resuspended in HEGD buffer and used immediately or frozen at -80° C for later use. The supernatant was centrifuged at 105,000 g for 30 min at 4° C, and the resulting supernatant was used as cytosol.

In vitro translocation assay Cytosol were incubated with [^{3}H]-TCDD (5 nM) for 1 hr on ice. After incubation, cytosol were incubated with dextran-coated charcoal at 4° C for 10 min and centrifuged at 2000 g for 10 min at 4° C to remove unbound TCDD. Purified nuclei from untreated cells were suspended in cytosol and incubated at various temperature for different time points. After incubation, nuclei were centrifuged and washed twice with HEGD buffer. The isolated nuclei were incubated with HEGDK (HEGD plus 0.5 M KCl) buffer for 1 hr at 4° C followed by centrifugation at 105,000 g for 1 hr at 4° C to give the nuclear extract.

Sucrose density gradient analysis Aliquots (300 μl) of the nuclear extracts were layered onto linear sucrose gradients (5-25%) prepared in HEG buffer containing 0.4 M KCl. Gradients were centrifuged at 4° C for 2.5 hr at 404,000 g. After centrifugation, 30 fractions were collected from each gradient, and radioactivity in each fraction was determined by liquid scintillation counting.

RESULTS

Cytosolic fractions obtained from wild-type Hepa 1c1c7 cells treated with 5 nM [^{3}H]-TCDD for 1 hr were isolated and velocity sedimentation analysis showed that the Ah receptor complex sedimented at 9-10 S (untransformed). Nuclei were isolated from untreated Hepa 1c1c7 cells. The liganded cytosolic fraction was incubated with nuclei for various time points and the results are summarized in Figure 1. After incubation at 37° C for 15 min the radiolabeled nuclear Ah receptor complex could be detected and the levels were maximized after 60 min. The effects of temperature on the formation of the nuclear Ah receptor complex were also investigated and the results are summarized in Figure 2. Only background levels of radiolabeled nuclear Ah receptor were detected after incubation at 4° C for 60 min. However, these levels significantly increased with increasing temperature and were maximized at 37° C. Extraction of the nuclear Ah receptor with high salt followed by velocity sedimentation analysis showed that the specifically-bound peak sedimented at 6-7 S (Figure 3). In addition, nuclear extracts incubated with a [^{32}P]-labeled consensus DRE followed by a gel shift assay showed a retarded band which corresponded to the nuclear Ah receptor-DRE complex (data not shown).

DISCUSSION

The intracellular transport of cytosolic proteins into the nucleus is a complex process which may involve several steps including protein modification (e.g. phosphorylation or dephosphorylation), specific protein-protein interactions (e.g. such as the formation of the heterodimeric Arnt-Ah receptor protein complex), interaction with transport proteins and ultimately transport through nuclear pores[4]. This multi-step process has been extensively investigated for many macromolecules using a number of different model systems. The results summarized in Figures 1 and 2 demonstrate that after incubation of liganded

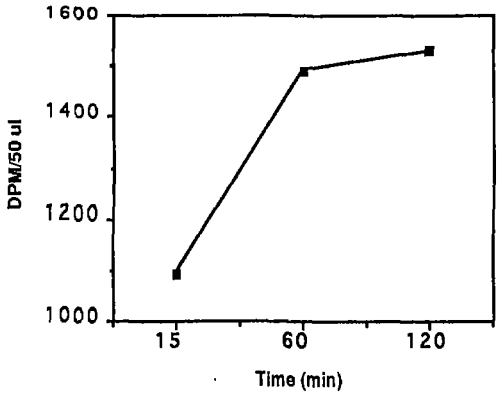


Figure 1. Time-course formation of the nuclear Ah receptor using the reconstituted system.

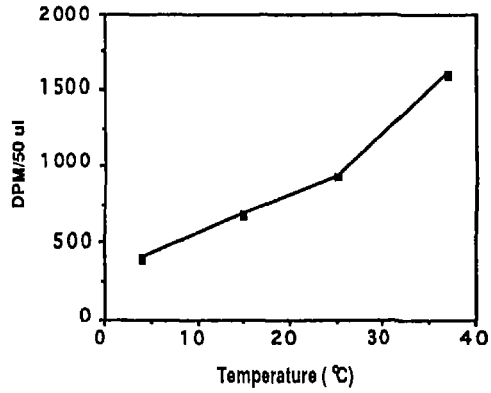
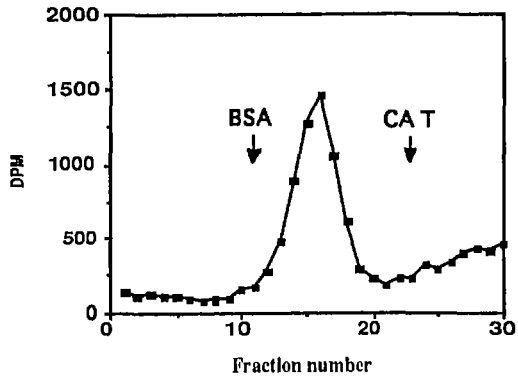


Figure 2. Temperature-dependent formation of the nuclear Ah receptor complex.



Fi gure 3. Velocity sedimentation analysis of the nuclear Ah receptor after *in vitro* translocation of the liganded cytosolic receptor complex.

cytosol and nuclei from Hepa 1c1c7 cells, the cytosolic Ah receptor is translocated into the nuclear compartment in both a time- and temperature-dependent manner. These results are comparable to those previously reported for whole cells[5]. Thus the cell-free system described in this study represents a new model which can be used for investigating the mechanisms associated with the formation of the nuclear Ah receptor complex.

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