Vol. 190, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1993 Pages 186-191

NUCLEAR TRANSLOCATION OF AFLATOXIN B_1 - PROTEIN COMPLEX

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Received December 2, 1992

SUMMARY: The in vitro binding of [^{3}H]-AFB_1 to various proteins was studied by equilibrium dialysis. At 23 ± 1°C, [^{3}H]-AFB_1 binding activity (mmol/mol) decreased as follows: pyruvate kinase > albumin - NLS > albumin > carbonic anhydrase > RNase > histones. The nuclear translocation and activation of AFB_1 and AFB-protein complexes was investigated using isolated rat liver nuclei in the presence of ATP and a NADPH regenerating system. Proteins containing NLS such as histones and albumin-NLS facilitated AFB_1 translocation into the nucleus where activation and adduct formation took place.
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Aflatoxin B_1 (AFB_1), a secondary metabolite isolated from the mold Aspergillus flavus, is mutagenic, hepatotoxic, and hepatocarcinogenic in a wide variety of animal species (1,2). Epidemiological studies suggest that AFB_1 exposure is related to the occurrence of hepatocellular carcinoma in humans (3). Considerable studies have concentrated on the activation, deactivation, and adduct formation of AFB_1, and the current concept is that before the formation of the AFB_1-DNA adduct, AFB_1 must be converted to AFB_1-8,9 epoxide by cytochrome P_{450} isozymes in the cell. The formation of AFB_1 adduct with nuclear DNA has been implicated in tumor formation, however, the mechanism by which AFB_1 or AFB_1-8,9-epoxide is translocated into the nucleus is still unclear (4-7).

The existence of cytosolic Ah receptors and binding proteins of lipophilic carcinogens involved in the metabolism of xenobiotics has been documented (8-10). Their role in translocating carcinogens into the nucleus and interaction with xenobiotic response elements of the DNA has been recently suggested (11,12). Earlier reports indicate that serum proteins and some liver cytosolic proteins are capable of binding AFB_1 non-covalently (13-15), however, their role in AFB_1 metabolism remains unknown.

In this report, we utilized equilibrium dialysis to prepare a number of AFB_1-protein complexes and studied their translocation into isolated nuclei. Results indicate that the

0006-291X/93 $4.00
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ligand protein complex is selectively translocated into the nucleus and the presence of nuclear localization sequence (NLS) is obligatory.

MATERIAL AND METHODS

Materials: Male Wistar rats weighing 251 ± 16 g each were obtained from Harlan-Olac (Indianapolis, IN). The animals were housed and cared for by the animal facility of the University. The rats were fed Purina laboratory chow and water ad lib up until 18 hr before experiments, and were maintained on a 12 hr light/dark cycle. The rats were allowed to adjust to the facility for at least 48 hr before experiments were conducted. G-[^{3}H]-aflatoxin B_1 (18 Ci/mmol) in methanol was obtained from Moravek Biochemical, Inc. (Brea, CA). Sigma Chemical Company (St. Louis, MO) provided carbonic anhydrase, pyruvate kinase, RNase, glucose-6-phosphate dehydrogenase, Triton X-100, DEAE-cellulose, metyrapone, RNase-free sucrose, glucose-6-phosphate, and $NADP^+$. CytoScint was purchased from ICN Radiochemicals (Irvine, CA). DuPont Biotechnology (Boston, MA) was the source of the Rapid Multiple Peptide Synthesis (RaMPS) system. All other reagents were of the highest purity commercially available.

Isolation of Nuclei: The nuclei used in this study were isolated from Triton X-100 (0.1% v/v) treated liver homogenate as described by Blobel and Potter (16) modified by Rothblum et al. (17). The crude nuclei were further purified by sedimenting through 1.74 M and 2.4 M sucrose solutions at 112,000 g for 45 min each. The freshly isolated nuclei were used for AFB_1 translocation and activation assays.

Isolation of Various Proteins: Rat liver cytosol was obtained by differential centrifugation and a fraction containing AFB_1 binding proteins were partially purified by Sephadex G-25 column chromatography, ammonium sulfate fractionation (0-70%), and anion-exchange column chromatography using DEAE-cellulose (fraction eluted with 0.1 M NaCl) as described by Taggart et al. (15). Histones were extracted from the chromatin with ice-cold H_2SO_4 (17). Rat plasma albumin was isolated from an ammonium sulfate (2.15 M) supernatant fraction by isoelectric precipitation at pH 4.6.(18).

Preparation of Albumin - NLS Conjugate: Synthesis of the NLS having the sequence GGKRKKKPGGC was carried out using the RaMPS system based upon the method of Merrifield (19). The peptide was synthesized from the carboxyl terminus to the amino terminus as described by Carpino and Han (20). The synthetic NLS was cross-linked to rat plasma albumin with m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) using the method of Lerner et al (21).

Preparation of AFB_1-Protein Complex: The binding of AFB_1 to various proteins was achieved by a 5-cell equilibrium dialysis apparatus (Bel-Art Products, Pequannock, NJ). [^{3}H]-AFB_1 was added to a final volume of 0.9 ml of Tris-HCl buffer (pH 7.5) on one side of a cell, whereas the other side contained proteins in 0.9 ml of the buffer. The apparatus was mounted on a multi-purpose rotator and rotated at a speed of 30 rpm. Diffusion of [^{3}H]-AFB_1 across the dialysis membrane (6,000-8,000 MW cutoff) was thus allowed to equilibrate at 23° ± 1° C. Triplicate aliquots of 5 μl samples from each side of the well were removed at various times. The amount of AFB_1 bound was determined by subtracting the unbound form of AFB_1 present on the side of the well without protein from the AFB_1 on the side where protein was added. In some experiments, the dialysis was carried out in a dialysis bag (6,000-8,000 MW cut off) at room temperature for 36 hr or 48 hr.

Nuclear Translocation and Activation Assays: Nuclear translocation assay was carried out essentially as described by Markland et al. (22). Nuclei were incubated in 5.0 ml of 0.1 M

sodium phosphate buffer (pH 7.4) containing 5 mM ATP, and a NADPH regenerating system (glucose-6-phosphate, 3.4 mM; glucose-6-phosphate dehydrogenase, 10 units; $NADP^+$, 0.5 mM). [^{3}H]-AFB was added directly or as a noncovalent protein complex. The incubation was conducted under a steady stream of 95% O_2/5% CO_2 with constant shaking. Upon completion, the nuclei were reisolated from the mixture by centrifugation at 900 g for 10 min or purified by centrifugation through a 2.4 M sucrose as described above. The amount of AFB_1 translocated into the nucleus was determined from the total radioactivity present in aliquots of the purified nuclei. The remainder of the nuclei were extracted by the phenol/chloroform technique for the isolation of nuclear DNA (23). The specific radioactivity (fmol/mg DNA) was calculated and taken as the amount of AFB_1 activated.

Analytical Methods: Proteins were determined by the method of Lowry et al. (24) with crystalline bovine serum albumin as the standard. DNA was determined spectrophotometrically (14). Samples containing radioactivity were mixed with 10 ml of CytoScint and radioactivity was determined using a Beckman LS-7800 liquid scintillation system (Beckman Instrument, Irvine, CA). The counting efficiency was 47-49% with a counting error of ± 2%. Disintegrations per min (dpm) was calculated from cpm using a quench correction curve.

RESULTS AND DISCUSSION

Preliminary experiments were performed to determine the conditions for the binding of AFB_1 to various proteins. Using proteins such as liver cytosolic proteins purified from the DEAE-cellulose column, nuclear histones, rat serum albumin and albumin-NLS, the formation of the AFB_1-protein complex by equilibrium dialysis was not affected by pH's ranging from 6.0 - 8.8. The time necessary for the ligand, AFB_1, to equilibrate across the dialysis membrane (6,000-8,000 MW cutoff) was about 30 hr. Furthermore, the binding was reversible and non-specific.

Under standardized conditions, 10 mM Tris-HCl (pH 7.4) at 23° ± 1°C, AFB_1 (220 pmol), and protein (500 μg), and dialysis for 36 hr, the binding of AFB_1 with different proteins was not uniform (Table 1). The highest binding was exhibited by pyruvate kinase, a tetrameric cytosolic enzyme followed by albumin-NLS, albumin, carbonic anhydrase, pancreatic RNase and rat liver histones. Binding to these proteins was dependent on the concentrations of both AFB_1 and the protein. (data not shown).

Nuclear activation of AFB_1 was studied with nuclei isolated from homogenate treated with 0.1% Triton X-100 (16). During the 2 hr period, 7% of the added AFB_1 was found intranuclearly and the majority of the AFB_1 radioactivity was associated with the acid - insoluble fraction suggesting that nuclear activation of AFB_1 took place. This was further supported by the decreases seen with either the omission of the NADPH- regenerating system or the addition of metyrapone, an inhibitor of cytochrome P_{450} (Table 2). When the partially purified cytosolic AFB_1 binding protein was added, nuclear AFB_1 content was decreased further to 10% of the complete system. Since the protein exhibited strong AFB_1 binding and moderate glutathione-S-transferase activity (15), the observed decrease of AFB_1

Table 1. The In Vitro Binding of [^{3}H]-AFB$_1$ to Various Proteins [1]

Protein	Molecular Weight (10^{-3})	[^{3}H]-AFB$_1$ Bound (pmol/500 μg protein)	(mmol/mol)
Rabbit pyruvate kinase	237.0	6.9 ± 5.0 (2)	3.28
Rat albumin-NLS	77.2	16.4 ± 6.5 (12)	2.53
Rat albumin	67.1	15.8 ± 5.4 (12)	2.12
Bovine carbonic anhydrase	31.0	9.2 ± 1.4 (2)	0.50
Rat histones	15.2	4.0 ± 2.0 (10)	0.12
Bovine pancreatic RNase	13.7	7.5 ± 3.8 (3)	0.21

1. Data presented are mean ± SD of the number of experiments given in parentheses.

uptake by nuclei suggest that the liver cytosolic AFB$_1$ binding protein is not associated with translocation of AFB$_1$ into the nucleus, instead the AFB$_1$ is sequestered outside the nucleus. This sequestration could provide two other possible functions: (a) it could act as a reservoir of toxin, similar to the reversible and nonspecific binding of estradiol to cytosolic proteins (28); and (b), the binding could be involved in detoxification, particularly if AFB$_1$ is bound to one or more isozymes of glutathione-S-transferase.

Table 2. Nuclear Translocation of AFB$_1$ [1]

Incubation Conditions	Nuclear AFB$_1$ (%)
Complete	100
- NADPH regenerating system	24
+ metyrapone, 1.0 mM	26
+ cytosolic AFB$_1$ binding protein[2]	10

1. Nuclei (15 mg) were incubated with [^{3}H]-AFB$_1$ (55.5 pmol) in a final volume of 7.5 ml for 120 min. At the end of incubation, nuclei were reisolated, washed, and nuclear AFB$_1$ content determined; 100% represents 3.94 pmol of AFB$_1$ and values presented are mean of 4-8 experiments.
2. 11.4 mg protein of the 0.1 M NaCl fraction from DEAE-cellulose column was added to the incubation mixture at zero time.

Table 3. Nuclear activation of AFB_1-protein complex[1]

Protein	AFB_1-DNA Adducts[2] (fmol/mg DNA)
Albumin	1.77 ± 0.88 (7)
Albumin-NLS	13.69 ± 0.20 (4)
Histones	5.51 ± 1.16 (4)
Pyruvate Kinase	0.28 ± 0.10 (3)

1. The preparation of AFB_1-protein complex was described in "Methods". Nuclei (10 mg) were incubated with 25 pmol of AFB_1 bound to protein for 120 min at 37 °C with skaking.
2. At the end of incubation nuclei were reisolated, washed, and DNA isolated. Data presented are mean ± SD with the number of experiments given in parentheses.

Since AFB_1 is lipophilic and known to bind to a number of proteins, it is unlikely that AFB_1 enters the nucleus mainly as an unbound form. It is possible that AFB_1 translocates across the nuclear pore as a ligand-protein complex. To test this possibility, the nuclei were incubated with AFB_1 bound to albumin, albumin-NLS, histones and pyruvate kinase (Table 3). Although pyruvate kinase exhibited highest AFB_1 binding (Table 1), it was unable to translocate the complex into the nucleus due to the lacking of NLS. Rat histones, however, which exhibited the lowest AFB_1 binding (Table 1), were able to translocate the complex because histones are nuclear proteins containing NLS. When albumin-NLS was used in the assay, AFB_1-DNA adduct formed was 7-8 fold higher than albumin without NLS. It is clear that the accumulation of AFB_1-DNA adduct in the nucleus is the result of translocation of AFB_1-protein complex. Since the search for a specific AFB_1 binding protein by a number of laboratories has not been successful, AFB_1 translocation by a specific nuclear translocator is highly unlikely. Nuclear translocation of AFB_1, unlike the translocation of steroid or thyroid hormones, polycyclic aromatic hydrocarbons, and other heterocyclic amines (8-10), differs in that there is apparently no specific receptor required to form the ligand-receptor complex before nuclear translocation. On the other hand, it is possible that some AFB_1 molecules are bound to proteins destined for the nucleus (25-27). Therefore, the translocation and activation of AFB_1, as well as the formation of AFB_1-DNA adducts are opportunistic in nature. Studies are underway to further evaluate the binding of AFB_1 to nuclear proteins other than histones and their role in AFB_1-induced carcinogenesis. Nuclear translocation of protein is essential for cell survival. Binding of AFB_1 to a protein destined

for the nucleus would increase the uptake and activation of the toxin, lead to damage of DNA, and increase the risk of developing cancer.

ACKNOWLEDGMENT: We thank Mrs. Colleen M. Healy for the preparation of the manuscript.

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