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The rat-liver microsomal AP endonuclease. The endoplasmic reticulum is presented as a net thrown into the cytosol to capture newly synthesized karyophilic proteins

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Although most of the rat-liver AP (apurinic/apyrimidinic) endonuclease is in chromatin, some activity is found in microsomes. A quantitative assay of the microsomal AP endonuclease is described. The enzyme is a peripheral membrane protein that is located on the outside surface of microsomes. All the binding sites on the microsomes appear to have the same affinity for the AP endonuclease, suggesting the presence of receptors for the enzyme. The AP endonuclease is displaced from its membrane attachment by submicromolar concentrations of the karyophilic signal of SV-40 T antigen. The AP endonuclease receptors are likely to be on the cytosolic side of the endoplasmic reticulum. It is suggested that binding of the protein to these receptors might be the first step of the transport mechanism that enables the AP endonuclease to penetrate into the nucleus. The same mechanism utilizing the same receptors might be used by other karyophilic proteins, including SV-40 T antigen.

Introduction

The loss of bases, which leaves AP (apurinic or apyrimidinic) sites, is the most common damage in DNA. Enzymes, named AP endonucleases, recognizing AP sites and nicking the DNA strands near these lesions are present in all cells, prokaryotic and eukaryotic [1]. Thibodeau and Verly [2] have studied the cellular localization of the rat-liver AP endonuclease. They found that most of the activity was located in chromatin; however, some activity was bound to the microsomal fraction although the enzyme was practically absent from the cell sap. They proposed that, immediately after synthesis, the enzyme attaches to the cytosolic side of the endoplasmic reticulum to be transported inside the nucleus as on a conveyor belt.

This paper presents a study of the microsomal AP endonuclease of rat liver. The data suggest that the enzyme might be tied to receptors located on the cytosolic side of the endoplasmic reticulum. It is possible that the formation of the enzyme-receptor complex is the first step of the mechanism that transports the enzyme into the nucleus.

Materials and Methods

Rat-liver microsomes

The microsomal fraction from rat liver was prepared as described by Amar-Costesec et al. [3]. The microsomes are suspended in 0.25 M sucrose/0.5 mM PMSF/3 mM imidazole-HCl (pH 7.4) (called sucrose-imidazole hereafter).

Determination of the number of intact AP sites

Our method is derived from the fluorescence assay described by Evans et al. [4]. RF-I DNA from phage ΦX174 is prepared as described by Goffin and Verly [5]; a 1 h incubation at 37 °C in 0.2 M sodium acetate (pH 3.8) produces one apurinic site per molecule. The solution is then neutralized to pH 7.0 with NaOH.

The DNA to be assayed is in 20 mM potassium phosphate/0.5 mM EDTA (pH 11.8), containing 1 μg/ml ethidium bromide (alkaline BET). The solution fluorescence is measured against a control without DNA. In order to measure only RF-I molecules, the DNA is previously submitted to a denaturation–renaturation

Abbreviations: AP, apurinic/apyrimidinic; PMSF, phenylmethylsulfonide fluoride; RF, replicative form; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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cycle; the single-stranded DNA resulting from the irreversible denaturation of RF-II molecules has no secondary structure at pH 11.8 so that it does not contribute to the fluorescence [4].

To determine the number of intact AP sites (i.e., AP sites not associated with breaks), two different denaturation–renaturation cycles are used: one cycle leaves the AP sites intact; the other places a break near each AP site.

To leave AP sites intact, 30 μl DNA (750 ng) solution (pH 7.0) is heated 2 min at 100 °C; the solution is cooled by addition of 2 ml of alkaline BET. This sample will be called '100 °, pH 7'. We have shown that intact AP sites are stable at pH 11.8, at room temperature.

To place a break near each AP site, 2 ml of alkaline BET is added to 30 μl of DNA solution (pH 7.0) before heating for 8 min at 100 °C; renaturation is obtained by placing the tube in a thermostat at 25 °C for 4 min. This sample will be called '100 °, pH 11.8'.

The fluorescence of the two samples is measured; the average number, n, of intact AP sites per RF molecule is given by the equation:

$$n = \ln \frac{\text{fluorescence '100 °, pH 7'}}{\text{fluorescence '100 °, pH 11.8'}}$$

Fluorescence measurement

The solution (2 ml) is placed in a quartz cell with an optical path of 1 cm. The spectrofluorimeter KONTRON SEM-23 has a Xenon lamp of 150 W, the excitation monochromator is set at 305 nm, and the emission monochromator at 595 nm. The photon flux is measured with an EMI 9781 A photomultiplier. The cell containing the sample must be left in the spectrofluorimeter for 2 min to stabilize the record.

Experiments and Results

A method for assaying the microsomal AP endonuclease

The microsome suspension was diluted with 50 mM Hepes-KOH (pH 7.5)/50 mM NaCl/20 mM $MgCl_2$/0.01% Triton X-100; Triton was found to be necessary in order to have a reproducible determination of the microsomal AP endonuclease. To 30 μl of microsomes in Triton solution was added 30 μl ΦX174 RF DNA (700 ng; depurinated or not) solution, and the mixture was incubated at 37 °C for 15 min. Controls without microsomes were run in parallel. The reaction was stopped by heating 10 min at 78 °C; the tubes were subsequently dipped in ice. The samples were then submitted to a denaturation–renaturation cycle that left the AP sites intact. The average number, n', of breaks per RF molecule due to microsomal enzymes is given by the equation:

$$n' = \ln \frac{\text{fluorescence 'control'}}{\text{fluorescence 'sample'}}$$

TABLE I

Assay of microsomal AP endonuclease

The microsome suspension was diluted with the assay buffer containing Triton X-100. To 30 μl of this suspension (the amount of microsomes is arbitrarily given by 10^6/dilution factor), was added 30 μl ΦX174 RF-I DNA (1.00 intact AP site per molecule) solution, and the mixture was incubated at 37 °C for 15 min before measuring the fluorescence as described in the text. n', average number of breaks per molecule produced by the microsomal enzyme; S_0 and S, average numbers of intact AP sites per molecule in the substrate RF-I DNA at time 0 and after the 15 min incubation, respectively.

Amount of microsomes	Fluorescence	n'	$S = S_0 - n'$	$\ln(S_0/S)$
0	50.5	0.00	1.00	0.00
208	35.0	0.37	0.63	0.45
313	30.5	0.50	0.50	0.68
417	27.0	0.62	0.38	0.96
625	23.5	0.77	0.23	1.44
833	21.5	0.85	0.15	1.89

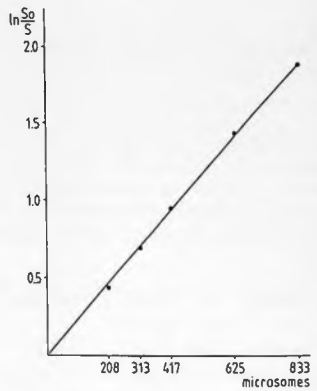
When ordinary ΦX174 RF DNA was used, n' was negligible, showing that the amount of unspecific endonucleases in microsomes was very small. We can thus conclude that the action of microsomes on depurinated ΦX174 RF DNA was due to its AP endonuclease activity. Table I, which gives n' for different dilutions of the microsome suspension, shows that microsomes indeed contained an AP endonuclease activity, confirming the results of Thibodeau and Verly [2].

The AP site concentration in the incubation medium is $3.9 \cdot 10^{-9}$ M; we do not know the K_m of the microsomal AP endonuclease, but it might be much higher. If this were the case, the reaction rate would be first-order to the substrate concentration, and for an amount, E, of enzyme one could write: $\ln S_0/S = kE$, where S_0 and S are, respectively, the average numbers of intact AP sites per RF molecule at 0 min ($S_0 = 1.00$) and after 15 min of incubation at 37 °C.

Table I gives the calculation for different amounts of microsomes, and Fig. 1, the graphic presentation of the final data, shows a linear relationship between $\ln(S_0/S)$ and the amount of AP endonuclease. Thus we have a method to assay quantitatively the microsomal AP endonuclease; our assumption that the concentration of AP sites in the assay was much lower than the K_m seems to be correct.

Side-localization of the microsomal AP endonuclease and nature of its binding to the membrane

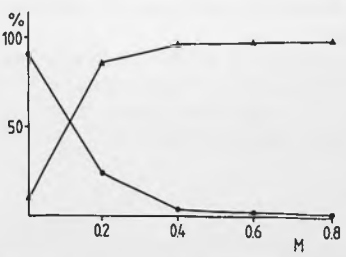
When they are derived from the rough endoplasmic reticulum, microsomes have their ribosomes on the outside. When depurinated DNA is added to a suspension of such microsomes, breaks appear in the DNA strands; since the macromolecular substrate cannot penetrate inside the microsomes, the conclusion is that at least a part of the enzyme is located on the cytosolic side of the endoplasmic reticulum [2].



1. Assay of microsomal AP endonuclease. $\ln(S_0/S)$ is given versus the amount of microsomes. The data are from Table I.

Triton X-100, at a 0.5% concentration, completely ociates the microsomes so that the microsomal AP onuclease can no longer be sedimented by high-speed rifugation. The actions of a suspension of intact rosomes on depurinated DNA, and of the corre- ding solution of Triton-dissociated microsomes are same (not shown), suggesting that the enzyme might located exclusively on the cytosolic side of the endo- mic reticulum.

Aliquots from the microsome suspension were ex- ed to different KCl concentrations; after 15 min at C, the suspensions were centrifuged for 30 min at 00 rpm (rotor 50 Ti; Beckman L5-65). The AP onuclease activities of the supernatants and of the ets were measured after dilution with the Triton 00 containing buffer. Fig. 2 shows that, with a iciently high ionic strength, all the AP endonuclease vity can be released in the supernatant. This indi- s that the microsomal AP endonuclease is not an gral membrane protein but rather a peripheral one



2. The microsomal AP endonuclease is a peripheral membrane tein located on the outside of the microsomes. KCl was added to microsome suspension to reach the recorded concentration (M). er 15 min at 0 °C, the suspension was centrifuged and the AP onuclease activities were measured in the supernatant (▲) and the pellet (●); the results are presented in percent of the total activity.

TABLE II

Reassociation of free microsomal AP endonuclease and AP endonuclease-free microsomes

Microsomes were treated with 0.8 M KCl to release the AP endonuclease completely. Different volumes (ml) of the free microsomal AP endonuclease solution were added to 1 ml of AP endonuclease-free microsome suspension; the total volume was brought to 11 ml in each case and the KCl concentration was 0.15 M. After 2 h at 37 °C, the suspension was centrifuged and the AP endonuclease activities of pellet and supernatant were measured. The AP endonuclease unit is the amount of enzyme giving, in the standard assay, $\ln(S_0/S) = 1$. The standard assay was carried out on 30 μl of the enzyme solution (supernatant or dissociated pellet), so that the number of units found in the assay is multiplied by the volume of enzyme solution (ml)/0.03 to give the figures in the table.

Free AP endonuclease (ml)	Pellet (enzyme units)	Supernatant (enzyme units)
1.0	3200	2730
2.5	4400	5240
5.0	5880	8250
7.5	6230	10500

Further study of the AP endonuclease binding to microsomes

KCl was added to a microsome suspension, to reach 0.8 M (the concentration that releases the AP endonuclease completely); after 15 min at 0 °C, the suspension was centrifuged as in the preceding experiment. The supernatant was diluted with sucrose-imidazole to lower the KCl concentration to 0.15 M the volume was then reduced by ultrafiltration on a YM 5 membrane (Amicon). The pellet was resuspended in sucrose-imidazole containing 0.8 M KCl; sucrose-imidazole was then added slowly to reach 0.15 M KCl in 1 h. The AP endonuclease-free microsomes were again centrifuged and resuspended in sucrose-imidazole containing 0.15 M KCl. All these procedures were carried out at 4 °C.

To 1 ml of the final AP endonuclease-free microsome suspension, a variable quantity of the free microsomal AP endonuclease solution was added, and the total volume was brought to 11 ml with sucrose-imidazole containing 0.15 M KCl. After a 120 min incubation at 37 °C, the mixture was centrifuged in the usual conditions. The AP endonuclease activity was measured in the supernatants and the pellets as was described above. Table II gives the results, which are presented in a Scatchard plot in Fig. 3 according to the equation:

$$\frac{1}{ME} = \frac{1}{E}\frac{K_d}{M_t} + \frac{1}{M_t}$$

where ME is the amount of enzyme bound to microsomes in 11 ml of suspension, and E the amount of unbound enzyme. As shown in Fig. 3, $1/ME$ is a linear function of $1/E$; thus it seems that all the binding sites have the same affinity ($1/K_d$) for the AP endonuclease.

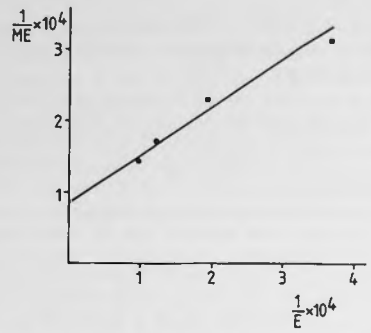


Fig. 3. Scatchard plot of Table II data. ($1/ME$) is plotted vs. ($1/E$), where ME is the enzyme bound to microsomes, and E the free enzyme.

One can thus suppose that these binding sites are receptors for the enzyme.

If the unit of microsomal AP endonuclease is defined as the amount of enzyme, giving, in the standard assay, $\ln(S_0/S) = 1$, the intersection of the straight line passing through the experimental points with the ordinate axis ($1/M_t = 0.85 \cdot 10^{-4}$) enables us to calculate that the microsomes in the 11 ml of the incubation medium have a number of receptors capable of binding 11 800 units of AP endonuclease (M_t). Furthermore, the slope 0.67 of the straight line passing through the experimental points enables us to calculate the dissociation constant of the enzyme-receptor complex:

$$K_d = \frac{0.67 \times 11\,800}{11} = 720 \text{ units} \cdot \text{ml}^{-1}$$

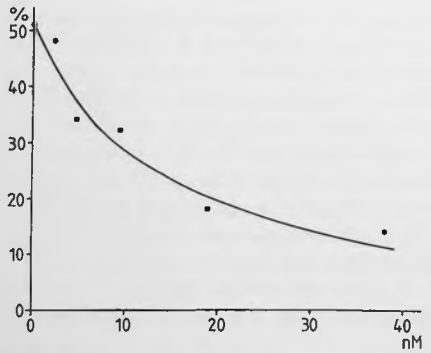


Fig. 4. Action of Pro-Lys-Lys-Lys-Arg-Lys-Val on the binding of the microsomal AP endonuclease to its membrane receptors. Mixtures of 2 ml AP endonuclease-free microsome suspension and 8 ml of free microsomal AP endonuclease solution containing different amounts of the heptapeptide, were incubated with mild shaking 120 min at 37°C. After centrifugation, the AP endonuclease activities of the supernatants and the pellets were measured. The graph gives the percentage of bound activity as a function of the heptapeptide concentration (nM).

TABLE III

Action of two heptapeptides differing by one aminoacid on the binding of the microsomal AP endonuclease to its membrane receptors

The experiment was carried out as described in the legend of Fig. 4, except that the same volumes of free microsomal AP endonuclease solution and AP endonuclease-free microsome suspension were used in all cases, and that increasing amounts of two different peptides, Pro-Lys-Lys-Lys-Arg-Lys-Val (Lys in the table) and Pro-Lys-Asn-Lys-Arg-Lys-Val (Asn in the table) were added. The table gives the amount of bound activity (units) as a function of the peptide concentration (nM).

Peptide concentration (nM)	Bound AP endonuclease (units)	
	Asn	Lys
0	17000	17000
8	18900	
16	18500	
32	16300	
64	17400	8700

The microsomal receptor for the AP endonuclease

Microsomes prepared from 6 g of rat liver were treated as in the previous section; a free microsomal AP endonuclease solution (48 ml) and a suspension of AP endonuclease-free microsomes (12 ml), both in sucrose-imidazole/0.15 M KCl, were obtained.

Mixtures were prepared containing 2 ml of AP endonuclease-free microsome suspension and 8 ml of free microsomal AP endonuclease solution, containing various amounts of the heptapeptide Pro-Lys-Lys-Lys-Arg-Lys-Val (IAF, Montreal, Canada). After a 120 min incubation with mild shaking at 37°C, the mixtures were centrifuged and AP endonuclease activities were measured in the supernatants and the pellets. Fig. 4 shows that the heptapeptide displaced the AP endonuclease from its receptors.

The experiment was repeated using Pro-Lys-Asn-Lys-Arg-Lys-Val (IAF), which differs from the preceding peptide by one amino acid. Table III shows that this 'mutant' peptide, in contrast to the 'wild-type' peptide, did not displace the microsomal AP endonuclease from its receptors.

The apparent K_i of Pro-Lys-Lys-Lys-Arg-Lys-Val for the binding of the AP endonuclease to its receptors has the same order of magnitude in the two experiments, in the range of 10–100 nM.

Discussion

Our aim was to determine whether the microsomal AP endonuclease was located exclusively on the outside of the microsomes, and to investigate the nature of the enzyme binding to the membrane. To reach these objectives, we first had to devise a method for assaying quantitatively the microsomal AP endonuclease. Trials

with the method of Paquette et al. [7] using depurinated [^{3}H]DNA and determination of the acid-soluble radioactivity failed for two reasons: the method was not-sensitive enough, and a high exonuclease activity made the results totally unreliable. We then adapted the method of Evans et al. [4] to obtain a quantitative assay: ΦX174 RF-I DNA containing an average of one apurinic site per molecule was prepared; nicking of this substrate was followed by fluorescence measurements, in the presence of ethidium bromide, of the remaining RF-I molecules. The method is thus specific for endonucleases; it does not show exonucleases, since these enzymes are not active on RF-I molecules, and the degradation of RF-II molecules remains unnoticed.

We have shown that rat-liver microsomes are not active on ΦX174 RF-I DNA unless it contains AP sites. This confirms the observation of Thibodeau and Verly [2] that microsomes contain an AP endonuclease.

The average number of breaks near AP sites per RF molecule was not proportional to the amount of microsomal enzyme, indicating that, in the conditions of the assay, the reaction rate was far from V_{max}. In fact, the AP site concentration was much lower than the K_m, so that the reaction rate was first-order to the substrate concentration: the amount of enzyme in the assay was proportional to $\ln(S_0/S)$, where S_0 is the average number of intact AP sites per RF-I molecule in the substrate at time 0, and S the number left intact after a 15 min incubation at 37°C with the microsomal AP endonuclease.

Using this quantitative assay, we have shown that the AP endonuclease, which is located on the external side of the microsomes, can be completely released with a high enough ionic strength, indicating that the microsomal AP endonuclease is not an integral membrane protein, but rather a peripheral one. On the other hand, since hepatic microsomes come predominantly from the endoplasmic reticulum and since the vesicles formed from the rough endoplasmic reticulum have their ribosomes on the outside, the result suggests that the microsomal AP endonuclease is located mostly on the cytosolic side of the endoplasmic reticulum.

In an experiment performed to better understand the nature of the enzyme binding, the AP endonuclease was released from the microsomes with a high ionic strength. The free microsomal AP endonuclease and the AP endonuclease-free microsomes in 0.15 M KCl were mixed: various amounts of free enzyme were added to a constant amount of enzyme-free microsomes. After 2 h at 37°C, the remaining free enzyme and the enzyme that had bound to the microsomal membranes were measured, and the results were plotted in Scatchard coordinates. A straight line was obtained, suggesting that all the binding sites had the same affinity for the enzyme.

We could thus hypothesize that receptors for the AP endonuclease were present on the cytosolic side of the endoplasmic reticulum. Since the enzyme is located mainly in chromatin, one could also speculate that the enzyme, synthesized on free polysomes, was attached to these receptors to be transported into the nucleus, passing through the nuclear pores, due to the continuity of the membranes of the endoplasmic reticulum, the external and internal nuclear envelopes.

The next question was whether such a transport mechanism was unique for the AP endonuclease, or whether it could be a general mechanism used by all the karyophilic proteins. It is well known [8,9] that the transportation in the nucleus of karyophilic proteins depends on a sequence of amino acids. Our suggestion is that this karyophilic signal is recognized by a receptor on the cytosolic side of the endoplasmic reticulum; the receptor-protein complex would then be carried into the nucleus by the membrane system as on a conveyor belt.

Transportation into the nucleus of SV-40 T antigen depends on the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val [10] changing the third amino acid into asparagine is sufficient to prevent the penetration of the mutated protein into te nucleus [11]. We mixed a constant amount of AP endonuclease-free microsomes from rat liver, a constant amount of free microsomal AP endonuclease, and various amounts of the normal T peptide (Pro-Lys-Lys-Lys-Arg-Lys-Val) or of the mutated T peptide (Pro-Lys-Asn-Lys-Arg-Lys-Val): the normal T peptide displaced the microsomal AP endonuclease from its microsomal receptors (Fig. 4), whereas the mutated T peptide did not (Table III). The K_i of the normal T peptide is very low, of the order of 10–100 nM. This strongly suggests that SV-40 T antigen and AP endonuclease might utilize the same receptors to be transported into the nucleus. It is likely that, when the primary structure of rat-liver AP endonuclease is known, a karyophilic signal very similar if not identical to that of SV-40 T antigen will be found.

Several authors [12,13], using karyophilic proteins labelled either with a fluorescent probe (for optical microscopy) or gold particles (for electron microscopy), have postulated that the nuclear protein import is divided into two steps: accumulation at the nuclear pores, which is dependent on the karyophilic signal and entry within the nucleus, which is ATP-dependent. We propose a third step which is indeed the first one: fixation to the receptors on the endoplasmic reticulum; in this model, the endoplasmic reticulum is a large net thrown into the cytosol to collect the karyophilic proteins.

Electron micrographs [14] show that the karyophilic protein labelled with gold particles accumulates at the nuclear pores, and several authors [15,12,13] have postulated that the receptors for the karyophilic signal are located at the nuclear pores. Our hypothesis is different. According to us the receptors are on the endoplasmic reticulum and the external nuclear membrane. The ac-

cumulation at the nuclear pores, which would be the second step in our model, might be explained in the following way: the empty receptors are free to move on the membranes of the endoplasmic reticulum and the external nuclear envelope because they have no affinity for the glycoproteins of the pore complex; however, when the receptor has caught a karyophilic protein, it is recognized by the pore complex and blocked there. The two first steps would be energy-independent but, of course, signal-dependent.

The known sequences of karyophilic signals show a great homology [16]. This does not exclude the possibility that there might be several different receptors, each for a different class of karyophilic proteins. The hypothesis of membrane receptors, necessary for nuclear import of karyophilic proteins, provides an explanation for the observation that some proteins remain in the cytoplasm in the early embryogenesis of *Xenopus*, and then appear later in the nucleus [17]: the development program might prescribe an early expression of the proteins and a later one of the endoplasmic-reticulum receptors needed for their travel from cytoplasm into the nucleus.

Acknowledgments

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