Uptake of Ribopolynucleotides by Mammalian Cells, XIII

DNA, a Component of the Outer Membrane of Ehrlich Ascites Tumor Cells and its Role in RNA Uptake by These Cells

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DNase treatment of Ehrlich ascites tumor cells releases DNA fragments from the membrane surface. This increases the capacity of the cells for homoribopolynucleotide uptake.

Another portion of membrane DNA is embedded in protein structures and is not accessible for DNase. This DNA does not hinder ribopolynucleotide uptake unless it is unwrapped by a subsequent pronase treatment of the cells. We interprete these findings to be the consequence of alterations of the net negative charge brought about by the removal and reestablishment of "open" surface DNA.

Introduction

The uptake of RNA into viable mammalian cells has been reported by numerous workers [1]. The interaction of RNA or ribopolynucleotides and the cellular membrane in the course of this uptake process, however, is still poorly understood. The molecular mechanism of this uptake has been studied using Ehrlich ascites tumor cells *in vitro* and tritium labelled homoribopolynucleotides (M.W. 10^5). This experimental system [2] was particularly suitable for the present work owing to its simplified parameters. The expression "uptake" includes both adsorption at the cellular surface and transport into the cells.

We describe here the role of anionic molecular groups of the cellular membrane in the mechanism of homoribopolynucelotide uptake. These groups were found to be phosphate groups of a membrane-DNA and their function is a reduction of the uptake of homoribopolynucleotides by the cells [3, 4].

In these experiments we were guided by our previous observations that in an incubation medium of pH 5 the intact cells are partially protonated at the surface. In consequence the capacity for polynucleotide uptake is increased [5]. This protonation and its effect on RNA-uptake are reversible. The cells behave analogously when basic groups, which are already protonated at pH 7, are introduced into the surface of intact cells (adsorption of poly-L-lysine at the cellular membrane [6, 7]). The capacity for RNA uptake returns to its original low level, when the poly-L-lysine is removed by protease [7]. The experiments cited above and also the present investigation concern the positive and negative groups of the cellular membrane.

Methods and Materials

Special methods are described in the legends of Table I and Fig. 1. Ehrlich ascites tumor cells were obtained from NMRI mice 8 days after inoculation. The incubation and measuring techniques for ribopolynucleotide uptake were described before [2]. The DNase used in this study was obtained from Worthington. Treatments of the cells with DNase and pronase were described before [4]. The treatment of the cells with RNase (E.C. 2.7.7.16) or phosphodiesterase (E.C. 3.1.4.4) was described previously [2]. The cells were incubated in a solution of thymus DNA (Worthington) 0.4 mg/ml or 20 μg/ml at 30 °C for 30 min.

Results and Discussion

Ehrlich ascites tumor cells were incubated with DNase *in vitro*. Subsequently the cells showed an increased uptake-capacity for poly U, poly A, poly A · poly U and poly A · 2 poly U (Table I). The difference between adsorption of the ribopolynucleo-

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Table I. Uptake of homoribopolynucleotides by Ehrlich ascites tumor cells. The cells were pretreated with deoxyribonuclease dissolved in Hank's solution (30 °C, 30 min). The cells were then exposed to the polynucleotide dissolved in Hank's solution (30 °C, 30 min). This process was followed by two washings with 0.9% NaCl solution. Then the cells were filtered on glass-fiber filters for counting in a liquid scintillation counter. Some cell samples were pretreated with DNase and then treated with pronase dissolved in Hank's solution (5 μg/ml) at 30 °C for 30 min. These pronase treated cells were then washed once and exposed to the polyribonucleotides. Treatment with DNA was carried out at 0 °C for 30 min. Ribonuclease treatment: Finally the cells were treated with RNase (E.C. 2.7.7.16) 1 mg/ml or phosphodiesterase (E.C. 3.1.4.4) 0.1 mg/ml (poly A) at 30 °C for 15 min [2]. The data are representative for 4 duplicate experiments.

Pretreatment of the cells [$4 \cdot 10^6$ cells/sample]	Ribopolynucleotide [0.035 μmoles, $6 \cdot 10^5$ dpm/ml]	Uptake of polynucleotide at 0 °C and 30 min [dpm]	Ribopolynucleotides remaining after RNase (phosphodiesterase) treatment of the cells [dpm]
Incubation of cells with DNase * (0.5 mg/ml), washing	Poly[^{3}H]U	8 800	320
	Poly A·Poly[^{3}H]U	10 100	9 300
	Poly[^{3}H]A	5 200	4 800
Control without DNase	Poly[^{3}H]U	3 400	180
	Poly A·Poly[^{3}H]U	4 500	4 200
	Poly[^{3}H]A	2 300	2 050
Incubation of cells with DNase (0.5 mg/ml), which were pretreated with *N*-bromosuccinimide	Poly[^{3}H]U	3 200	250
	Poly A·Poly[^{3}H]U	4 600	4 400
	Poly[^{3}H]A	2 500	2 100
Incubation of cells with DNase (0.5 mg/ml), which where preatreated with 2-hydroxy-5-nitrobenzylbromide	Poly[^{3}H]U	9 300	380
	Poly A·Poly[^{3}H]U	11 000	10 800
	Poly[^{3}H]A	5 400	4 900
Incubation of cells with DNase (0.5 mg/ml) and afterwards with pronase	Poly[^{3}H]A	4 800	4 300
Incubation of cells a. with DNase (0.5 mg/ml) b. with pronase c. with DNase (0.5 mg/ml)	Poly[^{3}H]A	9 400	8 700
Incubation of cells with DNase (0.5 mg/ml) and afterwards with DNA (0.4 mg/ml)	Poly[^{3}H]A	2 500 **	2 600
Incubation of cells with DNase (0.5 mg/ml) and afterwards with DNA (20 μg/ml)	Poly[^{3}H]A	4 900 **	4 600

* DNase used in this study is free from RNase contaminations (determination according to Thatch [8]).
** This value is in accord with 110% of the result of the corresponding control experiment.

tides to the cellular surface and their entry into the cells was also determined (Table I). DNase treated cells were exposed to homoribopolynucleotides and subsequently treated with RNase or phosphodiesterase which remove adsorbed ribopolymers [2]. These enzyme incubations were carried out under conditions similar to the initial DNase incubation. Ribopolynucleotides which are protected against enzyme degradation and removal, therefore, are unlikely to have occupied such places of the membrane surface where DNA was removed by DNase action initially. Even poly A·poly U cannot replace DNA at this membrane position.

Our incubation conditions (0.5 mg/ml DNase, 30 °C 30 min) are completely different from those of a standard DNase incubation. Both the interpretation of an increased capacity of ribopolynucleotide uptake and also the identification of a DNA as a constituent of the cellular membrane, however, depend strongly on an evidence for the DNA-specifity of the enzyme under our conditions. Therefore, we correlated the DNase-membrane "reaction" directly with the enzymatic activity (Fig. 1). According to Poulos *et al.* [9] the enzyme contains a tryptophane residue which is essential for the nucleolytic activity and two other tryptophane residues which are not

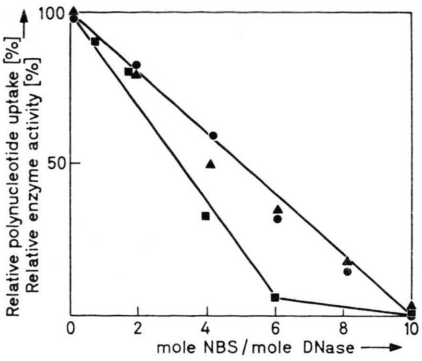

Fig. 1. Dependence of the DNase-caused ribopolynucleotide uptake characteristics from the enzymatic activity of DNase. 1. Decrease of enzymatic activity with rising *N*-bromosuccinimide concentration [9], –■–. 2. Decrease of uptake capacity for ribopolynucleotides with rising *N*-bromosuccinimide concentration, –▲– poly U and –●– poly A. The reaction of DNase with *N*-bromosuccinimide and the characterisation of the "active" tryptophane residue is taken from Poulos' paper. This reaction was carried out with $10^{-2}-10^{-3}$ M *N*-bromosuccinimide at 25 °C [9]. Cell samples were identically pretreated with DNase which was modified with different concentrations of *N*-bromosuccinimide [9]. The data were representative for 3 duplicate experiments.

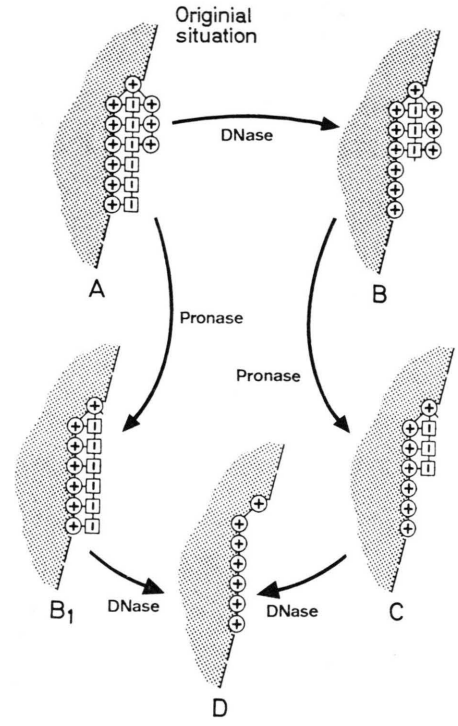

Fig. 2. Scheme of a section of a cellular membrane. This scheme illustrates our theory and shows a part of the membrane of an intact cell. The spatial arrangement is based on the accessibility of membraneous structures for enzymes and on the interactions of these structures with extracellular RNA. This scheme reflects the data of Table I.
□, Negative charge which can be removed by DNase (DNA); ⊕, positive charge which can be removed by pronase (protein).

essential [9]. Following directly the Poulos procedure the tryptophane residue of the "active center" was oxidized selectively with *N*-bromosuccinimide. Consequently the nucleolytic activity [9] and also the enhancement of ribopolynucleotide uptake [3] were abolished. In another experiment neither the nucleolytic activity [9] nor the enhancement of ribopolynucleotide uptake were influenced when a chemical modification of DNase, specific for a non functional tryptophane residue, was carried out using Poulos' reaction with 2-hydroxy-5-nitrobenzylbromide [9]. It is very likely, therefore, that DNase alters the cellular membrane *owing to its DNA-specifity*. These experiments suggest, that DNA is a constituent of the cellular membrane and that this DNA is accessible to DNase. The degradation of this DNA brings about the stimulations of ribopolynucleotide uptake by the cells (Fig. 2 A → B).

The cells were removed from the DNase incubation medium and DNA fragments were determined in this supernatant. From $4.5 \cdot 10^7$ cells, fragments corresponding to ca. 10 µg DNA were obtained, in comparison with 0.0 µg DNA from a control experiment without DNase. For these measurements a quantitative colorimetric assay for DNA fragments (deoxyribose) with diphenylamine under acid conditions was used [10].

DNA was also isolated from the membrane fraction of these cells [11] and the amounts extracted correspond to the values recorded above. We cannot exclude, however, the possibility of an adsorption of other cellular DNA-species at the membrane fraction during the homogenizing and separation procedures. The function of membrane DNA in ribopolynucleotide uptake presented in this paper, is lost once the cells are disruptured.

The scheme in Fig. 2 outlines a series of experiments carried out with DNase treated cells (B). Cells of type (B) having an increased capacity for ribopolynucleotide uptake were treated with pronase (B → C). This treatment restores in the cells the original low capacity for RNA uptake which was found in cells of type (A). Apparently further membrane DNA which was originally embedded in membrane protein structures, was uncovered by the protease. This DNA appears to have the same function as the "open" surface DNA of cells (A). The

cells of type (C) were carried one step further and were again treated with DNase (C → D). This procedure again increases the capacity for ribopolynucleotide uptake and again DNA-fragments were found in the supernatant of cells (D).

The data indicate a similarity of the "open" DNA of cells (A) and the "open" DNA of cells (C). This is based on two criteria: i. The sensitivity for DNase and ii. the hindrance of ribopolynucleotide uptake.

Cells of type (D) were also obtained by treating normal Ehrlich ascites tumor cells initially with pronase (A → B_1) and afterwards with DNase (B_1 → D). This was demonstrated by an increase of the capacity for ribopolynucleotide uptake (data see Table I) and also by the presence of DNA fragments in the supernatant solution corresponding to ca. 10 μg DNA.

The viability of cells which had been submitted to the treatments A → D in Fig. 2 was controlled. Enzyme treated cells and untreated controls had under our special conditions [4] the same proliferation behaviour when implanted into mice. Dye inclusion tests (eosine) gave nearly identical data for treated and untreated cells (viable cells > 90%). On the other hand the viability of overtreated cells was lost and their ribopolynucleotide uptake behaviour was not reproducible, even in the same experiment. 0 – 10% of dead cells, therefore, cannot be made responsible for the results of Table I and Fig. 2.

The experiments indicate that one part of the DNA is in an exposed position at the surface whereas another part is shielded off by membraneous proteins. These hypothetical DNA-protein complexes could be compared with polynucleotide-poly-L-lysine particles which were integrated into the cellular membrane of intact Ehrlich ascites tumor cells [6]. These polynucleotide-poly-L-lysine particles could be removed by a successive nuclease-protease treatment of the cells and this technique was the prerequisite for the present enzyme treatments.

The effect of DNase on the cells was cancelled by a subsequent incubation of the cells in thymus DNA solution 0.4 mg/ml (Table I). Apparently this DNA can restore the function of the membrane DNA which was removed from the cells in the first instance [12].

This result might suggest that an adsorption of DNA from the ascites fluid could have occurred which originates from decayed cells. However, the data of Table I show that small DNA concentrations (20 μg/ml) cannot restore the original RNA uptake capacity of the cells although the adsorption rate and relative adsorption efficiency of DNA increase with dilution [12]; only this incubation with small DNA concentrations can be regarded a simulation of the possible composition of the ascites fluid. The findings imply, that DNA can easily be adsorbed to the cellular membrane but has to be forced into such membrane positions from which membrane DNA was removed initially.

We believe, therefore, that membrane DNA is inherent in the cells or at least is a regular component of the cellular membranes.

It can be assumed that the negatively charged phosphate groups of the DNA at the cellular surface, impede an uptake of poly-nucleotides which are also negatively charged. Our results suggest that the membrane DNA might be arranged in a certain order. Presumably these positions of maximal negative charge are directly responsible for the RNA uptake capacity but they are not identical with defined uptake positions on the cellular membrane. These positions are more likely basic groups on the membrane.

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