Catabolism of capped (3'-5')- and (2'-5')-adenylates in rat liver nuclei

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Received 17 October 1983

We describe studies concerning the ability of a nuclear dinucleoside triphosphatase to act as a decapping enzyme in RNA catabolism. The enzymatic release of GMP from the Gp_3A moiety was determined in the capped RNA model compounds $Gp_3A3'pA$, $Gp_3A3'pA$-isoprop and $Gp_3A2'pA$ in isolated rat liver nuclei; i.e., in the environment in which the dinucleoside triphosphatase operates in vivo. The Gp_3A cap moiety is hydrolyzed in (3'-5') linked nucleotides only, whereas an extension of the Gp_3A in the 2'-direction prevents the nuclear triphosphatase to operate.

5'-Capped (3'-5')-adenylate *5'-Capped (2'-5')-adenylate* *Capped RNA fragment* *Decapping activity* *Nuclear dinucleoside triphosphatase* *RNA catabolism* *RNA recycling* *Rat liver nucleus*

1. INTRODUCTION

The 5'-terminal cap structures in eukaryotic messenger RNA (mRNA) [1–4] are known to impart stability to these molecules against 5'-3' exoribonucleolytic attack [5–11]. In the degradation pathway of cytoplasmic mRNA as well as nuclear heterogenous RNA (hnRNA), on the other hand, there must exist enzymatic steps for hydrolyzing caps, capped RNA or RNA-fragments. Otherwise, such capped structures would accumulate and affect the biological functioning of the cell.

Such decapping activities were found for instance in different mammalian and vegetable tissues [10,12–15]. The enzymatic cleavage of the cap from either mRNA [10,12,16], capped mRNA-fragments bearing less than 3 [17] or 8 [7] nucleotides in the 3'-direction, respectively, or of cap structured dinucleotides [16,18,19] follows the mode of action of a dinucleoside triphosphatase. This is confirmed by the cleavage pattern as the enzymatic breakdown lead to the formation of m^7GDP [10], m^7GMP [12,16–19] and GMP [16–19] from naturally occurring capped oligoribonucleotides as well as synthetic dinucleoside triphosphates.

In [18] it was reported that a dinucleoside triphosphatase (EC 3.6.1.x) of rat liver nuclei specifically recognizes and hydrolyzes Gp_3A which is the non-methylated parent compound of caps and present in nuclear RNA precursors since cap formation is an early event in hnRNA synthesis [20]. Furthermore, from studies with lymphocyte nuclei it is known that these cells process their hnRNA rather slowly, thereby accumulating capped but not yet methylated species [21]. This paper contains studies concerning the ability of the nuclear triphosphatase to hydrolyze the Gp_3A moiety in $Gp_3A3'pA$, $Gp_3A3'pA$-isoprop and $Gp_3A2'pA$ (fig.1); i.e., when:

(i) Gp_3A bears an additional AMP-moiety adjacent to the cap in the 3'-direction;
(ii) the terminal hydroxyl functions of the additional AMP-moiety are protected by an 2',3'-isopropylidene group;
(iii) Gp_3A is extended by an additional AMP-moiety in the 2'-direction.

We have performed our decapping studies with

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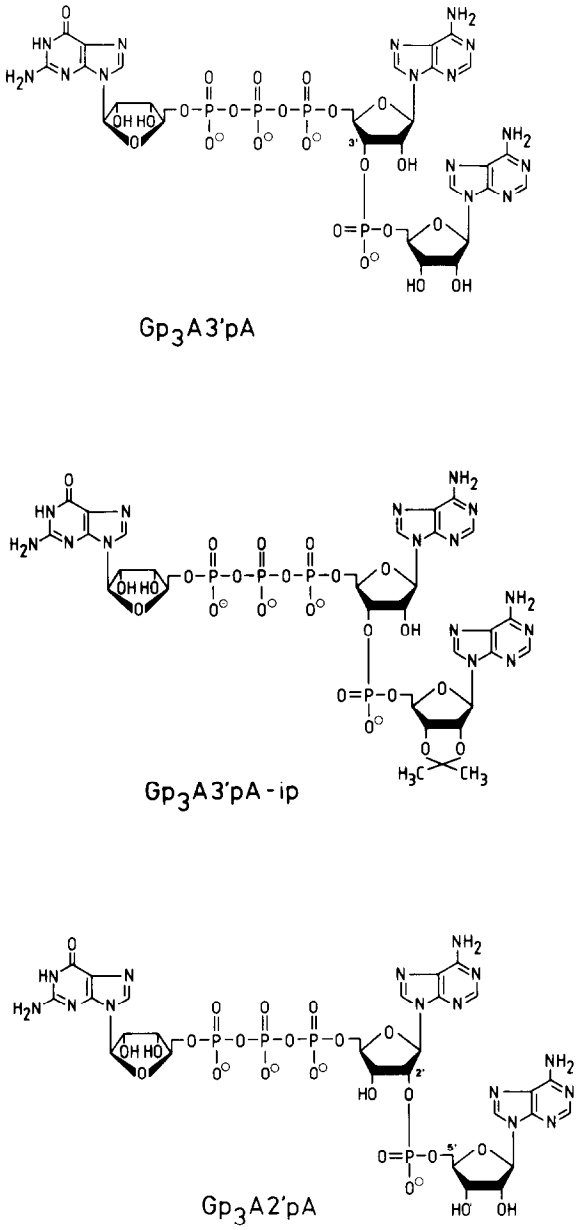

Fig.1. Capped (3'-5')- and (2'-5')-adenylates.

the aforementioned capped RNA model compounds in isolated nuclei; i.e., in the environment in which the dinucleoside triphosphatase operates in vivo. Some of our findings have been published in part as a poster contribution [22].

2. MATERIALS AND METHODS

[^{3}H]Gp$_3$A3'pA and [^{3}H]Gp$_3$A3'pA-isoprop as well as [^{3}H]Gp$_3$A2'pA were prepared by the reaction of carbonyldiimidazole-activated pA3'pA, pA3'pA-2',3'-isopropylidene as well as pA2'pA, respectively with [^{3}H]GDP which was purchased from New England Nuclear, Dreieich. pA3'pA and pA3'pA-2',3'-isopropylidene were synthesized by use of the triester-phosphite method [23]; the preparation of pA2'pA was performed by condensation of adenosine 5'-phosphorimidazolide in aqueous solution by using lead nitrate as a catalyst as in [24]. The syntheses of the compounds employed will be described in detail elsewhere [25]. The preparation of Gp$_3$[^{14}C]A has been published [26]. All the compounds used were characterized by ^{31}P NMR spectroscopy (Bruker WP 250 F8). The ^{31}P NMR (D_2O, pH 7) signals centered at: δ = +1.2 (3'-5' P), −0.1 (2'-5' P), −11 (α-,γ-P), −22 (β-P) ppm (external standard: 85% H_3PO_4). Product analysis after enzymatic degradation was done by convenient chromatographic techniques including high-pressure liquid chromatography as in [27].

Isolation of liver nuclei from male rats (Wistar rats Bor: WISW, SPF TNO, 150–200 g) was performed as in [28] and [18]. DNA was determined as in [29]. Protein was measured according to the Biuret method.

The degradation studies of the ^{3}H-labeled compounds Gp$_3$A3'pA, Gp$_3$A3'pA-isoprop, Gp$_3$A2'pA and Gp$_3$[^{14}C]A were carried out using the following assay conditions: a 50-μl suspension of rat liver nuclei (4 mg protein·ml^{-1}; 0.7 mg DNA·ml^{-1}) in buffer (0.44 M sucrose, 0.01 M K_2HPO_4, 0.003 M $MgCl_2$, 0.005 M NaN_3) was added to 100 μl 0.05 M triethanolamine–HCl buffer (pH 7.2) containing 32 nmol of the employed compound in a total volume of at least 150 μl at 25°C. All the degradation experiments were performed at saturating conditions over a period of 20 min (fig.2). Twenty-μl-aliquots of the incubation mixture taken during assaying were denatured with 5 μl perchloric acid (15%) and neutralized after centrifugation. The determination of the ^{3}H- or ^{14}C-labeled reaction products was performed by thin-layer chromatography (TLC) on PEI-cellulose plates (Macherey and Nagel, Düren) using 0.8 M KNO_3 (pH 5.7) as the mobile phase. The radioac-

tive spots of the catabolites [^{3}H]GMP (R_f = 0.25) and [^{3}H]guanosine (R_f = 0.65) or [^{14}C]AMP (R_f = 0.37) and [^{14}C]adenosine (R_f = 0.67), respectively, as well as the non-degraded capped compounds (R_f = 0.18) were detected by a TLC-Linear Analyzer LB 2821 (Berthold, Wildbad).

3. RESULTS AND DISCUSSION

The cleavage of the unmethylated cap moiety Gp_3A in the compounds [^{3}H]$Gp_3A3'pA$, [^{3}H]$Gp_3A3'pA$-isoprop and [^{3}H]$Gp_3A2'pA$ was investigated in rat liver nuclei in comparison to Gp_3[^{14}C]A as the standard substrate. From previous studies it is known that cap-type structured dinucleoside triphosphates as Gp_3A, m^7Gp_3A and other guanosine-modified analogs were hydrolyzed by a dinucleoside triphosphatase (EC 3.6.1.x) in rat liver nuclei [18]. Similar nucleoside triphosphatase activities have been found in extracts of HeLa cells [7], tobacco [19], potato [16] and more recently in human placenta [17]. The human enzymes [7,17] recognize and hydrolyze m^7Gp_3N structures (N is any base); i.e., these enzymes are specific with respect to N7-methylation of the 5'-terminal guanosine and do not hydrolyze unmethylated Gp_3N compounds. Unlike mammalian cytoplasmic cap-splitting activities, the vegetable enzymes [16,19] and the nuclear triphosphatase [18] hydrolyze unmethylated Gp_3N structures as well. From the partially enriched HeLa cell activity as well as the 1400-fold purified human placental enzyme it is well known, that both failed to cleave the cap moiety when extended in the 3'-direction by the addition of 3 [17] or more than 8 [7] nucleotides, respectively.

As the nucleotide chain length adjacent to the cap was shown to be important for the cap-degrading activity we have extended Gp_3A by adding nucleotides in the 3'- as well as the 2'-direction. The extension in both directions was performed to clarify whether or not a RNA-type 3'-5'-phosphodiester linkage is prerequisite for the decapping activity. Moreover, we have protected the 3'-terminus by a 2',3'-isopropylidene group ($Gp_3A3'pA$-isoprop) to exclude Gp_3A-liberation by 3'-exoribonucleolytic degradation.

The results obtained are given in fig.2 and table 1. The hydrolysis rate of the Gp_3A moiety in

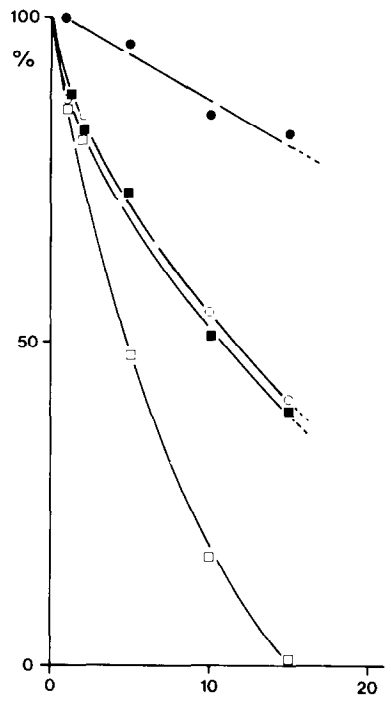

Fig.2. Enzymatic decapping of cap structured compounds in nuclei. [^{3}H]$Gp_3A2'pA$ (●—●) not corrected for [^{3}H]GMP release due to [^{3}H]Gp_3A liberation from [^{3}H]$Gp_3A2'pA$; [^{3}H]$Gp_3A3'pA$ (■—■); [^{3}H]$Gp_3A3'pA$-isoprop (○—○); Gp_3[^{14}C]A (□—□). Data are given from one typical experiment. For details in assaying see section 2.

Table 1

Nuclear dinucleoside triphosphatase activity

Compound	Release of labeled	Degradation rate (nmol·min^{-1}·mg nuclear protein^{-1})[a]
Gp_3[^{14}C]A	AMP,ADP	17.3 (± 2.0)[b]
[^{3}H]$Gp_3A3'pA$	GMP	8.4 (± 0.4)
[^{3}H]$Gp_3A3'pA$-ip	GMP	9.2 (± 2.6)
[^{3}H]$Gp_3A2'pA$	GMP	0.7 (± 0.5)[c]

[a] The degradation rates were determined during the linear phase of assaying (up to 5 min) and are mean values of at least 3 independent experiments

[b] Standard deviations are given in parentheses

[c] Corrected for [^{3}H]GMP release due to [^{3}H]Gp_3A liberation from [^{3}H]$Gp_3A2'pA$ by a (2'-5')-phosphodiesterase activity

[^{3}H]Gp$_3$A3'pA as well as [^{3}H]Gp$_3$A3'pA-isoprop decreases to about 50% when compared to Gp$_3$[^{14}C]A. The identical rate in releasing [^{3}H]GMP from both components shows that the nuclear triphosphatase is able to cleave the Gp$_3$A moiety in Gp$_3$A3'pA and Gp$_3$A3'pA-isoprop without previous liberation of Gp$_3$A. The rate reduction measured in the capped adenylates compared to Gp$_3$A may be based on the following fact: Gp$_3$A is in a way a symmetric dinucleotide with regard to the central β-phosphorous. The enzymatic attack, therefore, should be possible from both sides of the molecule. This assumption is in line with the release of ^{14}C-labeled ADP and AMP as degradation products. In this view, Gp$_3$A3'pA as well as Gp$_3$A3'pA-isoprop are asymmetric compounds which interact with the dinucleoside triphosphatase in an obviously side directed manner. Due to statistical means, therefore, the rate of cleavage of the Gp$_3$A moiety decreases about one half in both capped (3'-5')-adenylates compared to Gp$_3$A.

In contrast to capped (3'-5')-adenylates an extension of Gp$_3$A in the 2'-direction as realized in [^{3}H]Gp$_3$A2'pA prevents the nuclear dinucleoside triphosphatase to hydrolyze the Gp$_3$A moiety (fig.2, table 1). The small [^{3}H]GMP release measured (fig.2) is mainly due to [^{3}H]Gp$_3$A liberation from [^{3}H]Gp$_3$A2'pA during the course of incubation by a (2'-5')-phosphodiesterase activity obviously present in nuclei [30]; i.e., Gp$_3$A2'pA is not or, if any, a very poor substrate of the nuclear triphosphatase. The different behaviour of capped (3'-5')- and (2'-5')-adenylates shows that both the cap- as well as the (3'-5')-nucleotide moieties interact specifically with the nuclear enzyme. Only a RNA-type 3'-5'-linked nucleotide adjacent to the cap contacts the nuclear triphosphatase in the adequate way necessary to trigger cap degradation (fig.3). Thus our results stress the assumption in [17] that the placental m^{7}Gp$_3$N-phosphatase appears to attack the cap from the penultimate base side of the molecule due to the fact that the addition of 3 or more nucleotides adjacent to the cap prevents the interaction with the enzyme.

The data presented support the idea that rat liver nuclear dinucleoside triphosphatase acts in vivo as a decapping enzyme. This nuclear triphosphatase seems to be involved in the nuclear degradation cascade of hnRNA. The rates of post-

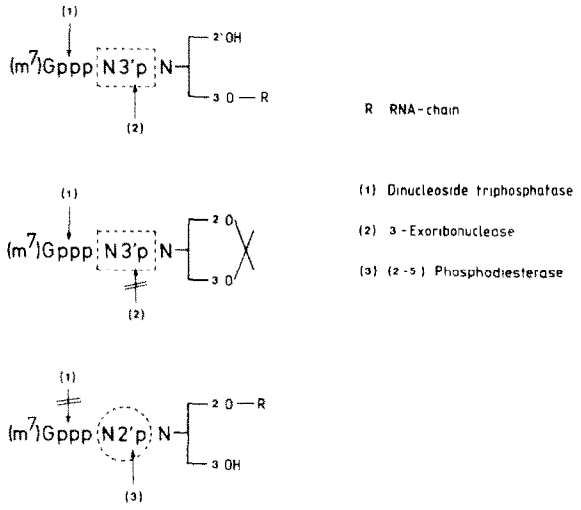

Fig.3. Schematic representation of enzymatic attack on capped (3'-5')- and (2'-5')-adenylates.

transcriptional RNA maturation or degradation depend on the physiological state of the cell; i.e., the half-life of RNA species differs as, for example, shown for resting and stimulated lymphocytes [31]. The degradative function of the nuclear triphosphatase is further substantiated by the ability of this enzyme to attack unmethylated caps which represent early species during RNA processing [21,31–33]. These results confirm the findings in [34] showing the importance of the nucleus for RNA degradation. In conclusion, the nuclear dinucleoside triphosphatase is involved in vivo in the nuclear degradation cascade of hnRNA and thereby recycles excess capped RNA precursors.

ACKNOWLEDGEMENTS

We are grateful to Drs K.-S. Boos and S. Bornemann, Paderborn as well as Professor K.P. Schäfer, Bochum for fruitful discussions, and to the government of Nordrhein-Westfalen and the Fonds des Verbandes der Chemischen Industrie for financial support.

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