Characterization of antibodies against methyl-pppN cap structure: plant U3 small nucleolar RNA is recognized by these antibodies

Mei-Hua Liu, Rose K. Busch, Becky Buckley and Ram Reddy
Department of Pharmacology and Division of Molecular Virology, Baylor College of Medicine, Houston, TX 77030, USA

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ABSTRACT

In eukaryotes, many small nuclear RNAs contain either a trimethylguanosine cap structure or a ϒ-monomethyl (me) cap structure. Previously, we reported the characterization of anti-mepppG antibodies which recognize methyl-capped RNAs with G as the initiation nucleotide. We report here the preparation of antibodies against mepppN cap structure. Anti-mepppN antibodies recognized only mepppN from a mixture of mepppN and pppN and Immunoprecipitated mepppA-capped U3 small nucleolar RNA from a mixture of cowpea cell RNAs. These anti-mepppN antibodies recognized methylated nucleoside triphosphates (mepppA, mepppC, mepppG and mepppU) with nearly equal efficiency; however, these antibodies did not recognize methyl phosphate or methylated mononucleotides. These antibodies will be useful in the identification and characterization of all methyl-capped RNAs no matter which is the initiation nucleotide.

INTRODUCTION

Many small RNAs in the eukaryotic nuclei contain cap structures on their 5' ends. There are two types of cap structures known; trimethyl guanosine (TMG) cap structure or methyl (me) cap structure (1–4). The antibodies prepared against TMG have been very useful in the identification, isolation and characterization of new small RNAs, small RNP's and also immunoprecipitated mepppA-capped U3 small nucleolar RNA from a mixture of cowpea cell RNAs. These anti-mepppN antibodies recognized methylated nucleoside triphosphates (mepppA, mepppC, mepppG and mepppU) with nearly equal efficiency; however, these antibodies did not recognize methyl phosphate or methylated mononucleotides. These antibodies will be useful in the identification and characterization of all methyl-capped RNAs no matter which is the initiation nucleotide.

MATERIALS AND METHODS

Fine chemicals and Isotopes. Chemicals, including nucleotides, were obtained from Sigma Chemical Co., St. Louis, Mo. [α-32P]-ATP, [α-32P]-GTP, [α-32P]-CTP, [α-32P]-UTP, and [32P]-inorganic phosphate, were obtained from Amersham or ICN. Labeled mepppA was synthesized from [α-32P]-ATP as described below.

Synthesis of mepppA. MepppA was synthesized from ATP as described by Darzynkiewicz et al. (11). Briefly, triethylammonium salt of ATP was dissolved in methanol and incubated at 37°C for 2 hrs in the presence of dicyclohexylcarbodiimide. The mepppA was purified on a DEAE-Sephadex column and lyophilized. The same procedure was used for the synthesis of mepppG, mepppC, mepppU from the corresponding unmethylated nucleoside triphosphates.

Preparation of mepppA-albumin conjugate. MepppA was conjugated to human serum albumin via the periodate oxidized nucleotide (12). 10 mg of mepppA was added to 0.5 ml of 0.1M NaIO4 in water (pH 6.6) and allowed to stand for 10 min. at 25°C in the dark. Excess NaIO4 was decomposed by adding 30 μl of ethylene glycol followed by incubation for 5 min. at 25°C. Then it was added to 28 mg of human serum albumin in 1 ml aqueous solution (pH adjusted to 9-9.5 with 5% potassium
carbonate) with constant stirring and conjugation was allowed to proceed for 1 hr at 4°C. 50 mg of t-butylamineborane was added and left overnight at 4°C. Chromatography was done in the cold on a Sephadex G-25 column and the flow-through fractions were washed with phosphate-buffered saline.

Immunization protocol and purification of the antibodies. Two rabbits were immunized at multiple sites with 1 mg of mepppA-human serum albumin conjugate in complete Freund's adjuvant. In time intervals of 21, 35 and 42 days, equivalent doses were injected using incomplete Freund's adjuvant. One week following the final injection, the rabbits were bled. Booster injections were given monthly and rabbits were bled after 7 days and the sera were tested by immunoprecipitation assay. Immunoglobulins were purified by passing the sera through protein A/G agarose column, eluted with 0.2 M glycine-HCl, pH 2.6, concentrated by precipitation with 50% ammonium sulfate, and dialyzed against phosphate-buffered saline solution. The protein concentration was adjusted to 1 mg/ml and used for further experiments.

Radioimmunoassay. The immunoprecipitations were carried out as described by Lerner and Steitz (13). For immunoprecipitation, 50 µl of antibody was added to antigen (50 µl volume) and allowed to stand for 20 min. on ice. 200 µl of Pansorbin (Calbiochem) was added and again incubated on ice for 20 min. The immunoprecipitates were washed 5 or 6 times with NET-2 buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 2mM EDTA and 0.05% NP 40) and the radioactivity in the immunoprecipitates was quantitated. Whenever necessary, the nucleotides were extracted from the immunoprecipitates by heating for 10 min. with 0.1% sodium dodecyl sulfate and separated by electrophoresis on DEAE-cellulose paper. The immunoprecipitated RNAs were phenol extracted, precipitated with ethanol and fractionated on 10% polyacrylamide gels. For competition assays, antibodies were preincubated with different concentrations of nucleotides in a volume of 50 µl for 30 min. on ice.

Labeling of HeLa and cowpea cell RNAs. For preparation of uniformly labeled RNA, HeLa cells were incubated at 37°C or cowpea protoplasts were incubated at 37°C with [³²P]-orthophosphate for 16 hrs in phosphate-free medium (10). The 4-8S RNA was prepared by centrifugation of the whole cell RNA on a sucrose density gradient and pooling the fractions corresponding to 4-8S RNAs (14).

RESULTS
Characterization of the mepppA-albumin conjugate
The optical density patterns of albumin, and mepppA-albumin conjugate were determined at 260 and 280 nm and from the A260/A280 ratio (15), the number of mepppA residues conjugated to each human serum albumin molecule was calculated to be between 15 and 16 (data not shown).

Specificity of mepppA antibodies
To test the specificity of anti-mepppA antibodies by radioimmunoassay, a mixture containing labeled pppA and mepppA (Fig.1, lane 1) was used for immunoprecipitation by three batches of anti-mepppA antibodies obtained from two different rabbits. From this mixture, one batch of anti-mepppA antibodies immunoprecipitated mepppA but not pppA (Fig. 1, lane 4); the other two batches contained relatively low titer of antibodies (Fig. 1, lanes 2 and 3). There was no detectable radioactivity in the immunoprecipitates when pre-immune serum was used (Fig. 1, lane 5). These results showed that the anti-
mepppA antibodies are specific enough to distinguish between pppA and mepppA. Further studies were carried out only with the sera with the highest antibody titer. From the amount of labeled mepppA immunoprecipitated, it was calculated that 1 ml (—1 mg of protein) of anti-mepppA antibodies were capable of immunoprecipitating 16 pmoles of mepppA.

In addition to mepppA, with all the batches of sera that were tested, there were minor spots observed with electrophoretic mobility slightly faster, as well as slower, than mepppA (Fig. 1, lanes 3 and 4). In other experiments, the minor spot with the slower migration co-migrated with mepppG (data not shown). These results suggested that the commercial labeled ATP may have some GTP as a contaminant and this labeled GTP got converted to mepppG used as the starting material for immunoprecipitation (Fig. 3) and show that these anti-mepppN antibodies specifically precipitated mepppC (lane 6) from a mixture of mepppC and pppC (lane 5) and precipitated mepppU (lane 4) from a mixture of mepppU and pppU (lane 3). In addition, mepppG, mepppA and mepppC and mepppU were precipitated (lane 8) from a mixture containing all four nucleotide triphosphates and methylated nucleotide triphosphates (lane 7). These data showed that the type of the base is not critical for precipitation with these antibodies; therefore, these antibodies are referred to as anti-mepppN antibodies instead of anti-mepppA antibodies.

To quantitate the cross-reactivity of the anti-mepppN antibodies, competitive radioimmunoassay was done and the concentration of nucleotide analogs required for 50% inhibition of mepppA binding to the anti-mepppN antibodies was determined (Fig. 4). Approximately, 15 picomoles of mepppA was sufficient for 50% competition; surprisingly, similar concentrations (about 80—120 picomoles) of mepppG, mepppC and mepppU were sufficient to give 50% competition (see, Fig. 4 and Table 1). These data are consistent with the results obtained by immunoprecipitation (Fig. 3) and show that these anti-mepppN antibodies bind to all the methylated four commonly occurring nucleotide triphosphates. The specificity of anti-mepppN antibodies with respect to other compounds structurally related to mepppA is shown in Table 1. These data show that the anti-mepppN antibodies cross react to some extent with mepppG but do not react at all with methylphosphate or with S-adenosyl methionine.

The specificity of anti-mepppN antibodies was also tested by direct immunoprecipitation of labeled nucleotide analogs. Methyl
phosphate was prepared by methylation of inorganic phosphate as described in the Methods Section. After this methylation reaction, one can visualize three components in this mixture: inorganic phosphate, methyl phosphate and dimethyl phosphate (Fig. 5A, lane 1). None of these compounds were immunoprecipitated by anti-mepppN antibodies (Fig. 5A, lane 2). To rule out the possibility that methyl phosphate was recognized but was lost during the experimental manipulations, the sample used in Lane 1 was mixed with other labeled nucleotides (Fig. 5A, lane 3) and then immunoprecipitated with anti-mepppN antibodies. There was no detectable methyl phosphate in the immunoprecipitates and, as expected, mepppA was immunoprecipitated (Fig. 5A, lane 4). In addition to mepppA, there were three other minor radioactive spots observed in Fig. 5A, lane 4; One of them co-migrated with inorganic phosphate. When purified mepppG was subjected to identical extraction procedure, the same spots were also observed (data not shown). Therefore, we presume that these minor spots are derived from mepppA during the extraction of mepppA from Pansorbin by heating at 60°C in 0.1% SDS. From these data, it is concluded that the antibodies do not recognize methyl phosphate and require something more than a simple methyl phosphate for binding.

Methylated mononucleotides are not recognized by anti-mepppA antibodies

We also tested whether anti-mepppN antibodies would immunoprecipitate methylated mononucleotides. Whole HeLa cell 4-8S RNA was digested with nuclease P1 and the digest was methylated as described in the Methods Section. This mixture, when analyzed on DEAE-cellulose paper, contained mononucleotides, methylated mononucleotides, as well as methylated nucleoside triphosphates (Fig. 5B, lane 1). When this mixture was immunoprecipitated with anti-mepppN antibodies, only methylated nucleoside triphosphates were found in the precipitate; mononucleotides or methylated mononucleotides were not found in the immunoprecipitates (Fig. 5B, lane 2). Since nucleoside monophosphates and triphosphates were derived from the same uniformly labeled HeLa RNA, it is likely that the specific activity of the mononucleotides or methylated mononucleotides is similar, if not the same. In spite of this, the methylated mononucleotides, which are present in excess compared to methylated triphosphates, were not precipitated by these antibodies. These data show that the spacing between the methyl group and the nucleoside is critically important for the antibody binding.

Immunoprecipitation of HeLa cell RNAs with anti-mepppN antibodies

We immunoprecipitated uniformly labeled HeLa cell 4-8S RNA, to see whether there are RNAs with cap structure other than mepppG in HeLa cells. Fig. 6, lane 1 shows the 4-8S RNA used as the starting material for immunoprecipitation. As controls, preimmune serum, anti-TMG antibodies and anti-mepppG antibodies were used. The pre-immune serum did not precipitate any of the small RNAs (Fig. 6, lane 2). Immunoprecipitates obtained with anti-TMG antibodies contained U1-U5 snRNAs (Fig. 6, lane 3) and the anti-mepppG antibodies immunoprecipitated U6 and 7SK snRNAs (Fig. 6, lane 4). In addition to these major snRNAs, several minor RNA bands were also observed with these two antibodies (lanes 3 and 4). In some experiments small amounts of 5S RNA and 5.8S RNA were also found in the immunoprecipitates (Fig. 6, lane 4); immunoprecipitation of these RNAs was not competed by preincubation of the anti-mepppG antibodies with mepppG (data not shown; ref. 8) indicating that these RNAs do not contain the mepppG cap structure. When the HeLa cell
Figure 6. Analysis of in vivo labeled HeLa cell small RNAs immunoprecipitated by anti-mepppN antibodies. HeLa cells uniformly labeled with \[^{32}P\]orthophosphate were sonicated and RNAs isolated from a 10,000×g supernatant. Lane 1: the starting (start) material; RNAs found in immunoprecipitates obtained with anti-trimethyl guanosine antibodies (lane 3); pre-immune antibodies (lane 2); anti-meGTP antibodies (Lane 4); anti-mepppN antibodies (lane 5); and anti-mepppA antibodies pre-incubated with mepppA (lane 6). The RNAs were fractionated on a 10% polyacrylamide gel, dried and subjected to autoradiography.

RNA was immunoprecipitated with anti-mepppN antibodies, again we observed only U6 and 7SK snRNAs as the major RNAs recognized by these antibodies (Fig. 6, lane 5). We did not observe any RNAs with anti-mepppN antibodies that were not precipitated by anti-mepppG antibodies (compare lanes 4 and 5). When the anti-mepppN antibodies were preincubated with mepppA and then used for immunoprecipitation, the precipitation of U6 and 7SK snRNAs was mostly competed out; the precipitation of 7SK RNA was reduced by about 60% (Fig. 6, lane 6). These data indicate that HeLa cell 4-8S RNA does not contain detectable quantities of RNAs with cap structures different from mepppG that can be immunoprecipitated by these antibodies.

U3 snRNA from plant cells is recognized by anti-mepppN antibodies

Since our recent data showed that plant U3 snRNA contains mepppA cap structure (10), we labeled cowpea cells with \[^{32}P\]orthophosphate and 4-8S RNA was isolated and used as the starting material for immunoprecipitation (Fig 7, lane 1). When this RNA was precipitated with anti-mepppN antibodies, several RNA bands were observed and the major RNAs corresponded to U6 and U3 snRNAs (Fig. 7, lane 3). In addition, there were two other bands; a minor band slightly larger than 55 RNA (designated 130 based on its approximate length of 130 nts) and another RNA of about 300 nucleotides long in the vicinity of 7SK snRNA (Fig. 7, lane 3). None of these RNAs were precipitated when the anti-mepppN antibodies were first incubated with mepppA and then used for precipitation (Fig. 7, lane 4); pre-immune sera also did not precipitate any RNAs (Fig. 7, lane 2). These data show that anti-mepppN antibodies recognize and immunoprecipitate mepppA cap containing U3 snoRNA from a mixture of cellular RNAs. As expected from the lack of requirement for a particular nucleotide (Fig. 4), anti-mepppN antibodies also immunoprecipitated mepppG cap-containing U6 and/or 7SK snRNAs (Figs. 6 and 7).

DISCUSSION

Data presented in this study show that the anti-mepppN polyclonal rabbit antibodies are capable of recognizing and immunoprecipitating mepppA as well as mepppG cap-containing RNAs from a mixture of cellular RNAs. Previously, we prepared and characterized anti-mepppG antibodies (8). These anti-mepppG antibodies were not competed by mepppA (8) and precipitated mepppG cap-containing RNAs but not mepppA capped RNAs (10). Thus, these anti-mepppN antibodies characterized in this study will be useful in identifying mepppA as well as methyl capped RNAs initiating with G, C or U. Although most of the capped snRNAs initiate with either A or G (3,5,9), some RNAs are known to initiate with pyrimidines (16). These RNAs, if transcribed by RNA polymerase III, will have mepppU or mepppC cap structures. The antibodies characterized in this study will be useful in identifying these capped RNAs also.

There were two RNAs that were consistently detected in cowpea cells with approximately 130 and 300 nucleotides in length (Fig. 7); RNAs of similar size were also observed in HeLa cells (Fig. 6). The 330 nucleotide-long 7SK RNA in human cells contains a methyl cap structure (8,17), and it is an abundant nuclear RNA of unknown function (18). The 300 nucleotide-long
RNA observed in the cowpea cells may be the homolog of human 7SK RNA. Further characterization is needed to test this possibility. When bean cell RNA was immunoprecipitated with anti-mepppG antibodies, several RNAs in the size range of 100–190 nucleotides were observed (8). The cowpea cells appear to be different in this respect since RNAs in this size range, except for the 130 nt-long RNA, were not evident.

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