Communication

Two Forms of Purified m\textsuperscript{7}G-cap Binding Protein with Different Effects on Capped mRNA Translation in Extracts of Uninfected and Poliovirus-infected HeLa Cells*

(Received for publication, April 17, 1981)

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Eukaryotic mRNA cap binding proteins were purified from ribosomal salt wash in the presence of protease inhibitors by sucrose gradient sedimentation and m\textsuperscript{7}GDP-Sepharose affinity chromatography. Rabbit reticulocyte and erythrocyte proteins with sedimentation constants of <6 S yielded a ~24,000-dalton cap binding protein. It stimulated capped mRNA translation in extracts of uninfected HeLa cells but did not restore capped mRNA function in extracts prepared from poliovirus-infected cells. Restoring and stimulatory activities both were associated with a larger, ~8-10 S complex that included the ~24,000-dalton polypeptide and several higher molecular mass components. The same two translational activities were also obtained in a slightly smaller ~5-7 S complex from uninfected HeLa cells but were absent from poliovirus-infected cell preparations.

Poliovirus is one of a small number of eukaryotic viruses that contain RNA with messenger activity and uncapped 5'-termini (12). Thus, if the change in initiation factors that accompanies poliovirus infection leads to a block in the activity of cap binding protein, then replacement of cellular protein synthesis by cap-independent poliovirus RNA translation presumably would be facilitated.

Previous studies showed that capped mRNA translation could be "restored" in extracts of poliovirus-infected HeLa cells by addition of partially purified eIF-4B (13), an initiation factor involved in mRNA binding to ribosomes. However, eIF-4B was found to contain cap binding protein (14). Subsequently, "restoring activity" was purified from rabbit reticulocyte ribosomal salt wash (15). The major polypeptide in the purified restoring activity corresponded to cap binding protein, but the most highly purified activity was labile, suggesting that other proteins might also be involved. Consistent with this possibility, we report that preparations of cap binding protein that stimulate capped mRNA translation in extracts of uninfected HeLa cells do not restore capped mRNA translation in extracts of poliovirus-infected cells. Restoring activity was associated with a protein complex that included cap binding protein and several higher molecular weight polypeptides.

EXPERIMENTAL PROCEDURES

Sources of materials were: L-[\textsuperscript{35}S]methionine (>1,000 Ci/mmol), New England Nuclear; DEAE-cellulose (DE52), Whatman, Inc.; Ul- trol grade Hepes, Calbiochem; ultrapure grade sucrose and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, Schwarz/Mann; acrylamide and bisacrylamide, Bio-Rad; phenylmethanesulfonyl fluoride and soybean trypsin inhibitor, Sigma; and aprotinin (Trasylol), Mobay Chemicals, Inc.

Sindbis virus mRNA was isolated from infected chick embryo fibroblasts (16) and encephalomyocarditis virus RNA was extracted with phenol from virions purified from infected mouse L cells (17).

Reticulocyte lysates were prepared from phenylhydrazine-treated, anemic rabbits as described previously (18) except that the buffers contained 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mg/mL of soybean trypsin inhibitor, 50 units/mL of aprotinin, and 1 mM ethyleneglycol bis(\textalpha-aminoethyl ether) N,N',N'-tetraacetic acid (19) in all steps prior to resuspension of the (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} precipitates.

Ribosomal salt wash was prepared from reticulocyte crude ribosomes (18). (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, was slowly added to attain 70% saturation (43.6 g/100 ml) followed by stirring for 60 min at 0 °C. Precipitate was collected by centrifugation (10,000 × g for 30 min) and dissolved in buffer consisting of 20 mM Hepes (pH 7.5), 0.5 mM KCl, 1 mM EDTA, and 5 mM 2-mercaptoethanol.

Dialyzed material was applied to m\textsuperscript{7}GDP-Sepharose (6). This protein differentially stimulates the translation of capped mRNAs as compared to uncapped viral RNAs in HeLa cell extracts (7).

In poliovirus-infected HeLa cells, initiation of host cell protein synthesis is abruptly shut off in favor of viral protein synthesis (8). The molecular basis for this striking regulatory phenomenon has been studied extensively. It was shown that cellular mRNA is not degraded in poliovirus-infected cells (9), and capped mRNA extracted from them is translated in cell-free protein-synthesizing systems (10). In contrast to mRNA, protein synthesis initiation factors are altered after poliovirus infection. Factors isolated from infected cells stimulate the translation of poliovirus RNA but not cellular mRNAs (11). These observations have been interpreted on the basis that

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1The abbreviations used are: eIF, eukaryotic initiation factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
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Fig. 1 (left). Polyacrylamide gel electrophoresis profiles of affinity-purified proteins. Sucrose gradient fractions of ribosomal high salt wash from rabbit reticulocytes (A-D) and erythrocytes (RBC) were pooled according to sedimentation position as indicated in the text. Proteins were further purified by chromatography on mGDP-Sepharose (6). Samples eluted with mGDP were analyzed by electrophoresis in 12.5% (pool A) or 10% polyacrylamide gels and stained with Coomassie blue (21). Micrograms of protein applied were (left to right): pool A, 2.2, 4.4, 6.6; pool B, 0.9, 1.8, 2.7; pool C, 0.4, 0.8, 1.2; pool D, 0.35, 0.7, 1.0; and RBC, 0.8, 1.7, 3.4. Relative amounts of constituent polypeptides were determined by densitometric tracing of photographic negatives. Standard proteins used for molecular weight estimations were soybean trypsin inhibitor (20,000), carbonic anhydrase (30,000), ovalbumin (45,000), bovine serum albumin (67,000), phosphorylase (94,000), and myosin (210,000). Position of cap binding protein is indicated by arrows.

Fig. 2 (right). Stimulation of Sindbis capsid protein synthesis by affinity-purified proteins added to extracts of uninfected HeLa cells. Sindbis virus mRNA was translated as described under “Experimental Procedures,” and the 3H-labeled products were analyzed by autoradiography after electrophoresis in 10% polyacrylamide gels. The position of the M, = 33,000 capsid (24) in gel profiles of three separate experiments is indicated by the arrow. Samples in lanes 1–3 and 4–8 each contained 1 μg of Sindbis virus mRNA. Affinity-purified proteins were also present as follows: lane 2, 0.15 μg of pool A; lane 3, 0.14 μg of pool C; lanes 5 and 6, 0.18 and 0.36 μg of pool B; lanes 7 and 8, 0.14 and 0.36 μg of pool D. Lane 9, no mRNA added; lane 10, 0.25 μg of Sindbis mRNA; lane 11, 0.25 μg of Sindbis mRNA and 1.3 μg of erythrocyte cap binding protein.

RESULTS AND DISCUSSION

Since highly purified restoring activity as obtained previously (15) was not stable, it seemed possible that polypeptides intrinsic to the activity were degraded during purification. Consistent with this possibility, cap binding protein in buffers of low ionic strength, e.g., 0.1 M KCl, associates with eIF-3 (15), an initiation factor containing multiple polypeptides that may result from proteolysis (22). Furthermore, polypeptides of M, > 24,000 that appear to be structurally related to the cap binding protein have been detected in reticulocyte lysate and in partially purified eIF-3.2 In an effort to obtain undegraded restoring activity, we purified cap binding protein from ribosomal salt wash in the presence of the four protease inhibitors as described under “Experimental Procedures.”

Previous analyses of reticulocyte initiation factors indicated that cap binding protein of M, ~ 24,000 (identified by cross-linking to 3H-labeled 5'-G in oxidized mRNA and polyacrylamide gel electrophoresis) sediments in a large complex of M, ~ 200,000 (23). To determine if stable restoring activity is related to the presence of cap binding protein in a high molecular weight complex, initiation factor preparations were fractionated according to size by sedimentation in sucrose gradients containing 0.5 M KCl. Sedimentation rate markers included creatine kinase (4.9 S), fumarase (8.5 S), catalase (11.3 S), and β-galactosidase (15.9 S). Fractions pooled from several gradients on the basis of the marker protein positions

2 H. Trachsel, personal communication.
were: A, <6 S; B, 2–8 S; C, 7–10 S; and D, 8–16 S. Pools A through D were each applied to m'GDP-Sepharose, and the bound material eluted with m'GDP (6) was analyzed by polyacrylamide gel electrophoresis (Fig. 1). Pool A contained a polypeptide in the position of cap binding protein (arrow) and little material of higher molecular weight. The same pattern was obtained with cap binding protein purified from erythrocytes (Fig. 1, RBC). In each case, the ~24,000-dalton polypeptide comprised >95% of the stained material as determined by densitometry. The predominant component in pool B was also a band of apparent \( M_r \) ~ 24,000, but two minor polypeptides of \( M_r \) = 48,000 and 55,000 were evident in addition. Pool C contained a 48,000-dalton band, a lower level of the cap binding protein, and prominent polypeptides migrating near phosphorylase (\( M_r = 94,000 \)) and myosin (\( M_r = 210,000 \)). Similar high molecular weight bands were also present in the fast sedimenting, large complexes comprising pool D. Identity of the \( M_r \) ~ 24,000 stained band and cap binding protein was confirmed on the basis of specific, i.e. m'GDP-inhibited, cross-linking to oxidized reovirus mRNA (14, data not shown). The results indicate that cap binding protein associates with higher molecular weight polypeptides to form a fast sedimenting, large complex (8–10 S) that is retained on m'GDP-Sepharose and specifically eluted with m'GDP. The 8–10 S estimate from the overlapping fractions of pools C and D was confirmed by assaying individual gradient fractions for translational activity before chromatography.

Affinity-purified cap binding protein and its higher molecular weight complexes were tested for the ability to enhance capped mRNA translation in vitro (7). All four pools stimulated Sindbis virus capsid protein formation in HeLa cell extracts (Fig. 2, lanes 1–8), and the same results were obtained in a separate test with erythrocyte-derived cap binding protein (Fig. 2, lanes 9–11). The stimulatory activities of the different protein pools were concentration-dependent.

Although pools A and B contained cap binding protein and stimulated uninfected HeLa cell extracts, they were essentially devoid of restoring activity. As shown in Fig. 3 A, the capacity of HeLa cell extracts to translate Sindbis mRNA (lane 2) was abruptly shut off in extracts prepared at 3 h after poliovirus infection (lane 4). Translation was not restored by addition of cap binding protein affinity-purified from pool A (Fig. 3 B, lane 2) or pool B (Fig. 3 C, lanes 2 and 3). Restoring activity was also absent from the erythrocyte-derived cap binding protein tested in the same way (data not shown). By contrast, pools C and D, i.e. the samples that contained the high molecular weight components in addition to cap binding protein, effectively restored Sindbis capsid synthesis (Fig. 3, B, lane 3 and C, lanes 4 and 5, respectively). The results were not dependent on the use of Sindbis virus mRNA since similar

![Fig. 3. Restoration of Sindbis virus mRNA translation in extracts of poliovirus-infected HeLa cells by affinity-purified proteins.](image)
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(results were obtained with rabbit globin mRNA; furthermore, translation of uncapped RNA of encephalomyocarditis virus in extracts of uninfected and poliovirus-infected HeLa cells was not stimulated by any of the affinity-purified fractions (data not shown). The purified restoring activity in pools C and D was stable to prolonged storage at −196 °C and repeated freeze-thawing. Thus, the polypeptides associated with cap binding protein in a large, 8-10 S complex may simply stabilize restoring activity; alternatively, they may have separate activities related to cap recognition. In any case, affinity-purified cap binding protein was obtained in two distinct forms: one which stimulates capped mRNA translation in extracts of uninfected HeLa cells and a more complex form which also has stable restoring activity in extracts of poliovirus-infected cells.

To determine if stimulatory and restoring activities analogous to those in rabbit blood cells are also present in other mammalian cells, initiation factors were prepared from HeLa cell cultures. The 70% (NH4)2SO4 precipitate of ribosomal salt wash was sedimented in 15-30% sucrose gradients containing 0.6 M KCl, and gradient fractions were tested for stimulatory and restoring activities in extracts of uninfected and poliovirus-infected HeLa cells, respectively. Synthesis of Sindbis virus coat protein by extracts of poliovirus-infected cells could be restored by addition of a fast sedimenting complex (Fig. 4A, gradient fractions 18-22 correspond to ~5–7 S position). The same gradient fractions effectively stimulated Sindbis RNA translation in uninfected cell extracts (Fig. 4B). Similar analyses were carried out with initiation factors prepared from HeLa cells at 3 h after infection with poliovirus. As expected on the basis of previous reports (8, 11, 13), they had no restoring activity and did not increase the translation of capped mRNA in extracts of uninfected HeLa cells (data not shown). Although the loss of capped mRNA translation in poliovirus-infected HeLa cells is correlated with inactivation of cap binding protein translational effects, a causal relationship remains to be established. However, recent studies indicate that both stimulatory and restoring activities can be neutralized by a monoclonal antibody prepared against purified erythrocyte 24,900-dalton cap binding protein.3

Acknowledgment—We thank A. LaFiandra for providing help with the HeLa cell systems.

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