Role of Actin and Tubulin in the Regulation of Poly(A) Polymerase-Endoribonuclease IV Complex from Calf Thymus*

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The poly(A) polymerase from calf thymus (Mr = 62,000) is associated with the poly(A)-specific endoribonuclease IV (Mr = 45,000); this complex is dissociable at pH 8.2. The activities of the two enzymes in the complex are strongly inhibited by G-actin and tubulin (both in the subunit-dissociated and in the polymeric form); F-actin was less inhibitory, while myosin and actomyosin were without any effect. Gel filtration experiments revealed a complex formation between poly(A) polymerase/endoribonuclease IV and G-actin or tubulin at an approximately 1:1 molar ratio. The homogeneous poly(A) polymerase was determined to be maximally inhibited by a 5-fold excess of G-actin and by less than a 2-fold excess of tubulin dimer, if calculated on the molecular basis. The inhibitory potency of G-actin on poly(A) polymerase was reduced after co-incubation with DNase I or phalloidin and that of tubulin by oleic acid. It is suggested that actin and/ or tubulin is involved also in regulation of poly(A) metabolism in intact cells.

It is well established that mRNA formation occurs in the nucleus and involves the following steps: capping, poly(A) addition, 1 methylation of internal AMP residues, and RNA: RNA splicing (review Ref. 1). After transportation of mature mRNA into cytoplasm, the poly(A) is shortened by random endonucleolytic cleavage (2). Evidence is available that enzymic poly(A) synthesis, mediated by poly(A) polymerase, occurs not only in the nucleus (3, 4) but also in the cytoplasm (5, 6). Based on an earlier observation (7), we discovered three poly(A)-degrading enzymes, endoribonuclease IV (8, 9), endoribonuclease V (10), and poly(A)-specific exoribonuclease (11), and purified them to homogeneity. The two enzymes poly(A) polymerase and endoribonuclease IV form a complex which can be separated by chromatography on hydroxyapatite only at a pH of 8.2 (5, 8) and which remains stable at pH 6.8 (12).

Efforts have been undertaken to solve the open questions, (a) whether poly(A) is responsible for the transport of poly(A) (+) mRNA from nucleus to cytoplasm and (b) which factors are involved in the control of poly(A) metabolism of mRNA in the cytoplasm. Previous studies (13) suggested that the poly(A)-binding protein P75 might facilitate transport of mRNA. Concerning the control mechanism(s) of poly(A) metabolism, data have been published showing that poly(A) polymerase activity might be modulated by phosphorylation (14) and that poly(A) degradation presumably is regulated by poly(A)-binding proteins (15, 16). In the present report, these problems are studied by a novel approach which is based, firstly, on the observations that the molecular weights of the purified poly(A) polymerase from different sources vary between 48,000 (17) and 140,000 (4), suggesting either the association of a hitherto unknown component with the poly(A) polymerase (or poly(A) polymerase-endoribonuclease IV complexes) or the existence of different distinct forms of poly(A) polymerases, and secondly, on the assumption that the poly(A) polymerase escorts the mRNA through the nucleoplasm into the cytoplasm (6) in an organized and not in a random fashion.

We now report for the first time direct evidence that the poly(A) polymerase-endoribonuclease IV activity is modulated by actin and tubulin, two structural proteins, which are very likely present both in the nucleus and in the cytoplasm (18-20).

EXPERIMENTAL PROCEDURES

Materials—Actinomyosin (from rabbit muscle), myosin (from rabbit muscle; electrophoretically pure), nucleotides, poly(A), colchicine, and DNase I were obtained from Sigma; [3H]ATP (11 Ci/mmol) was from the Radiochemical Centre, Amersham; [3H]poly(A) (37.4 mCi/ mmol of phosphate) was from Miles Laboratories. Phalloidin was a generous gift of Prof. Th. Wieland and H. Faulstich (MPF für Medizinische Forschung, Heidelberg).

G-actin was prepared from rabbit skeletal muscle according to the method described by Spadich and Watt (21). G-actin was converted into F-actin by dialysis against 2 mM Tris-HCl (pH 6.0), 0.5 mM 2-mercaptoethanol, 90 mM KCl, 2 mM MgCl2, 2 mM ATP, 0.2 mM CaCl2 (22, 23). The polymerization of actin was followed by the increase in optical density at 232 nm (25). Tubulin dimer was isolated from rat brain (19) including two cycles of polymerization and depolymerization. The preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described earlier (16, 26) with the exception that the running buffer was adjusted to pH 9.1. By comparing with protein standards, the major two bands were identified as α- and β-tubulin (Fig. 1). The purity was about 90%; the other 10% of the protein distributed in about nine minor bands which are most likely microtubule-associated proteins which were copurified by the procedure applied (27). The concentration of tubulin dimer (Mr = 115,000; Ref. 28) was determined spectrophotometrically at 290 and 230 nm (29). Tubulin dimer was converted into the polymerized form by incubation in the presence of 4 M glycerol at 30 min at 37°C (25). The formation of microtubules was monitored turbidimetrically at 350 nm (30). Oligo(pA)10 was prepared from poly(A) by degradation with endoribonuclease IV (9).

Enzymes—Poly(A) polymerase-endoribonuclease IV complex was

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isolated from calf thymus by the method of Tsaiapalis et al. (5), modified by Müller (8). The specific activities of the enzymes in purification step V (8) were 75 units (= 75 nmol of AMP incorporated during a 30-min incubation period)/mg of protein in the assay for poly(A) polymerase and 1250 units/mg of protein in the assay for endoribonuclease IV. One unit of endoribonuclease IV is defined as described earlier (8). The protein concentration was 2 mg/ml. Pure poly(A) polymerase was obtained by chromatography on hydroxyapatite (8); the specific activity was determined to be 1070 units/mg (protein concentration, 0.12 mg/ml). Prior to use, the enzymes were dialyzed against 10 mM Tris-HCl (pH 8.0), containing 0.2 mM MgCl2, 5 pg of oligo(pA),0, 0.2 mM [3H]ATP (200 dpm/pmol), 20 µl of enzyme (containing 0.15 units) in a final volume of 100 µl. Products of the reaction were detected as acid-insoluble radioactivity (11).

**Endoribonuclease IV Assay**—The assay measures the conversion of high molecular weight radioactive poly(A) to low molecular weight products. The standard assay mixture (100 µl) contained 100 mM Tris- HCl (pH 8.7), 30 µg/ml bovine serum albumin, 0.5 mM MnCl2, 0.2 mM dithiothreitol, 1 mM [3H]poly(A) (1200 dpm/nmol of phosphate) and 10 µl of enzyme (containing 2.5 units). Incubation was performed for 10 min at 37°C. Oligo(A) fragments with more than 15 AMP units were collected on DE81 discs and counted (8). The extent of enzymatic poly(A) hydrolysis is indicated as the amount of oligomers (in nanomoles of phosphate), converted into a soluble form (not bound to DE81 discs) after a 10-min incubation period.

Complex formation was performed under the following conditions (1 h at 20°C) (31). 20 µl of enzyme preparations were preincubated with 20 µl of tubulin dimer (dissolved in buffer 1: 100 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.8, 1 mM ethylene bis(oxyethyl)enedinitrilo)tetraacetic acid, 0.1 mM GTP, 0.5 mM MgCl2; Refs. 19 and 27), polymerized tubulin in the same buffer as used for tubulin dimer but supplemented with 4 mM glycerol, buffer 2; Ref. 19), G-actin (in buffer 3: 2 mM Tris-HCl, pH 8.0, 2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM CaCl2; Refs. 22 and 25), F-actin (in buffer 4: buffer 3 supplemented with 60 mM KCl, 2 mM MgCl2; Refs. 22 and 25), actomyosin (in buffer 5: 10 mM Tris-HCl, pH 8.0, 50 mM KCl), or myosin (in buffer 5). Under the buffer conditions used, the critical protein concentrations are for tubulin approximately 0.2 mg/ml (1.8 µM) (27) and for actin approximately 0.04 mg/ml (1 µM) (23, 25). In the control assays, the enzyme samples were preincubated in those buffers which were used for the dissolution of the contractile proteins.

The determination of the molecular weights was performed by gel filtration on Sephadex G-200; calibration of the column was performed with proteins of known molecular weights (aldolase, Mr = 158,000; phosphorylase a, Mr = 98,000; bovine serum albumin, Mr = 68,000; ovalbumin, Mr = 43,000; 4, chymotrypsinogen a (Mr = 25,000). SG, stacking gel; BPB, bromphenol blue.

### RESULTS

**Effect of Actin and Tubulin on Poly(A) Polymerase-Endoribonuclease IV Complex**—The activities of poly(A) polymerase and of endoribonuclease IV, isolated from calf thymus, are strongly inhibited after preincubation with tubulin dimer, polymerized tubulin, and G-actin (Table I). The inhibition studies were performed with and activin tubulin concentrations which were 50-fold or 10-fold higher than the critical concentrations for these contractile proteins (see under "Experimental Procedures"). The highest inhibitory effect was measured after preincubation with tubulin dimer (64% reduction of the activity of poly(A) polymerase and 47% reduction of endoribonuclease IV activity); somewhat lower was the inhibitory potency of polymerized tubulin and G-actin (Fig. 2). F-actin which had been from G-actin by treatment with 60 mM KCl and 2 mM MgCl2 (dialysis against buffer 4) exerted only a small influence on the activity of the two enzymes (Table I). Detailed dose-response experiments re-

### TABLE I

<table>
<thead>
<tr>
<th>Enzyme (plus contractile protein)</th>
<th>Complex (0.8 mg/ml)</th>
<th>Homogeneous enzyme (0.056 mg/ml)</th>
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<tbody>
<tr>
<td>Poly(A) polymerase</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Plus G-actin (2 mg/ml)</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>Plus F-actin (2 mg/ml)</td>
<td>78</td>
<td>81</td>
</tr>
<tr>
<td>Plus tubulin dimer (2 mg/ml)</td>
<td>36</td>
<td>21</td>
</tr>
<tr>
<td>Plus polymerized tubulin (2 mg/ml)</td>
<td>41</td>
<td>28</td>
</tr>
<tr>
<td>Plus myosin (2 mg/ml)</td>
<td>98</td>
<td>103</td>
</tr>
<tr>
<td>Plus actomyosin (2 mg/ml)</td>
<td>96</td>
<td>100</td>
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</table>

### Endoribonuclease IV

Control

<table>
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<tr>
<th></th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plus G-actin (2 mg/ml)</td>
<td>71</td>
</tr>
<tr>
<td>Plus F-actin (2 mg/ml)</td>
<td>84</td>
</tr>
<tr>
<td>Plus tubulin dimer (2 mg/ml)</td>
<td>53</td>
</tr>
<tr>
<td>Plus polymerized tubulin (2 mg/ml)</td>
<td>55</td>
</tr>
<tr>
<td>Plus myosin (2 mg/ml)</td>
<td>104</td>
</tr>
<tr>
<td>Plus actomyosin (2 mg/ml)</td>
<td>95</td>
</tr>
</tbody>
</table>
Fig. 2. Effect of G-actin and tubulin on the activity of poly(A) polymerase-endoribonuclease IV complex. Different concentrations of G-actin or tubulin dimer were preincubated at 20 °C (1 h) with 40 µg of poly(A) polymerase-endoribonuclease IV complex in a final volume of 40 µl. A concentration of contractile proteins in the preincubation assays are given on the abscissa. Poly(A) polymerase activity (expressed in picomoles of AMP incorporated/30 min/assay) and endoribonuclease IV activity (expressed in nanomoles of oligomers released from poly(A)/10 min/assay) were measured after preincubation (see under “Experimental Procedures”). Influence of G-actin (●-●-●) and tubulin dimer (○-○-○) on poly(A) polymerase; effect of G-actin (▲-▲-▲) and tubulin dimer (△-△-△) on endoribonuclease IV.

Fig. 3. Change of the elution pattern of poly(A) polymerase-endoribonuclease IV complex after incubation with tubulin dimer, polymerized tubulin, or G-actin on Sephadex G-200. Equal volumes of poly(A) polymerase-endoribonuclease IV complex (2 mg/ml) were mixed with tubulin dimer (2 mg/ml), polymerized tubulin (2 mg/ml), G-actin (2 mg/ml), or buffer 3 under conditions which are described under “Experimental Procedures.” After 1 h at 20 °C; aliquots containing 20 µg of the enzyme complex were taken and applied to a Sephadex G-200 column (1 x 20 cm) equilibrated at a flow rate of 3 ml/h with either buffer 1, 2, or 3, each containing 10 µg/ml bovine serum albumin. Fractions (0.5 ml) were collected and 20-µl aliquots were used for determination of poly(A) polymerase (●-●-●) and endoribonuclease IV activity (○-○-○) (the total amount of enzyme activity/fraction is indicated). Elution pattern of the control poly(A) polymerase-endoribonuclease IV complex (○); elution was performed with buffer 1 at 4°C. Pattern of the enzyme complex preincubated with polymerized tubulin (□); elution with buffer 2 at 25°C. Pattern of the enzyme complex preincubated with G-actin (△); elution with buffer 3 at 4°C. DB, dextran blue. The bar marks the positions of the authentic compounds: d-T, tubulin dimer; p-T, polymerized tubulin; G-A, G-actin. The buffer systems are described under “Experimental Procedures.”

In a final volume of molecular weights of heteroprotein complexes, we determined after their preincubation with G-actin (Fig. 3D). From the elution volume of the enzymes, a shift to a V, of 9.5 ml (corresponding M, = 136,000) had been found, indicating again a 1:1 molar ratio between the contractile protein and the enzyme complex. Very striking was the shift after preincubation of the enzyme complex with polymerized tubulin (Fig. 3C); the two enzyme activities were now detected near the void volume. It remains to be studied whether the tubulin eluting at the void volume consists exclusively of polymerized tubulin (microtubuli and 36S tubulin) or contains in addition a minor percentage of a 6S fraction (34).

Inhibition of Purified Poly(A) Polymerase—The activity of poly(A) polymerase was also affected by G-actin and tubulin in the assays using the homogenous enzyme (Table I). As in the complex form, the homogeneous enzyme was most sensitively inhibited by tubulin dimer (by 79%), followed by polymerized tubulin (72%) and G-actin (49%). To approach the problem of stoichiometry between the number of contractile protein molecules and the number of enzyme molecules which form a complex, dose-response experiments with homogeneous poly(A) polymerase were performed. The final concentration of the enzyme which has M,
Actin's and Tubulin's Influence on Poly(A)-specific Enzymes

...Figure 4. Effect of actin and tubulin on purified poly(A) polymerase. Equal volumes of the poly(A) polymerase (0.12 mg/ml) were preincubated with different concentrations of G-actin (O-0), F-actin (---), tubulin dimer (---) and polymerized tubulin (---) in a final volume of 40 µl; the final concentrations of contractile proteins in the preincubation assays are given on the abscissa. Subsequently, aliquots were taken and poly(A) polymerase activity was determined in the standard assay.

**Table II**

<table>
<thead>
<tr>
<th>Contractile protein</th>
<th>Modifier (mole ratio to protein)</th>
<th>Poly(A) polymerase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-actin</td>
<td>None</td>
<td>%</td>
</tr>
<tr>
<td>DNase I (5:1)</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Phalloidin (5:1)</td>
<td></td>
<td>77</td>
</tr>
<tr>
<td>Tubulin dimer</td>
<td>None</td>
<td>%</td>
</tr>
<tr>
<td>Colchicine (1:1)</td>
<td></td>
<td>62</td>
</tr>
<tr>
<td>Colchicine (5:1)</td>
<td></td>
<td>53</td>
</tr>
<tr>
<td>Polymerized tubulin</td>
<td>None</td>
<td>%</td>
</tr>
<tr>
<td>Colchicine (1:1)</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Colchicine (5:1)</td>
<td></td>
<td>34</td>
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</table>

The present report describes the novel phenomenon that the enzyme activities of the poly(A) polymerase-endoribonuclease IV complex are strongly inhibited by G-actin and tubulin dimer; the polymerized forms of these contractile proteins are less effective. From the experiments, it cannot be ruled out unequivocally that the polymerized contractile proteins are devoid of any inhibitory potency because the dose-response experiments were performed also with doses near the critical concentrations of F-actin and polymerized tubulin. However, in the present experiments with phalloidin, which lowers the critical concentration by a factor of 90 (23), it seems to be very likely that at least F-actin exerts some inhibitory activity on the poly(A) polymerase-endoribonuclease IV complex. Without effect on enzyme activities were myosin and actomyosin. The poly(A) polymerase, separated from the complex and purified to homogeneity, showed the same sensitivity toward the contractile proteins as the enzyme associated with endoribonuclease IV. A further interesting feature of the interaction between actin and tubulin and purified poly(A) polymerase is the finding that 5 molecules of G-actin and only 2 molecules of tubulin dimer/1 molecule of poly(A) polymerase are required to cause maximal inhibitory activity. From the gel filtration experiments analyzing the approximate molecular weights of the complexes between poly(A) polymerase-endoribonuclease IV and G-actin or tubulin dimer, we got strong evidence that each 1:1 complex between the enzymes and the contractile proteins is formed. The findings that myosin and actomyosin had no influence on the enzyme activities suggest that the binding is structure- and conformation-dependent. Hence, the poly(A) anabolic and the poly(A) catabolic enzyme behave in a way similar to deoxyribonuclease I (39).

The findings showing that DNase I and phalloidin exert a reduction of the inhibitory potency of G-actin on poly(A) polymerase indicate that DNase I competes with the polymerase for the binding site on G-actin. The influence of phalloidin on the G-actin-caused inhibitory effect is explained by the known activity of this toxin to convert G-actin to F-actin (23, 36); the latter protein was only slightly inhibitory on poly(A) polymerase activity (this paper). Furthermore, colchicine, which binds specifically to tubulin (37), also reduced the inhibitory activity of tubulin to poly(A) polymerase, suggesting a lower affinity of the colchicine-tubulin complex to the enzyme compared to the free tubulin dimer.

These findings, obtained in in vitro systems, may also lead to the elucidation of the possible biological role of the complex formation between G-actin or tubulin and the poly(A) metabolic enzymes in the intact cell system. In a series of papers, it is shown that G-actin is a genuine constituent of nuclei of several cell types (for review, see Ref. 39); however, its func-
bundles of actin filaments will form in situ in nuclei, e.g. of HeLa cells (40), suggesting an important role in nuclear functions (41). This finding led to the discovery of a reversible translocation of actin from the cytoplasmic actin stress fibers to the nuclear filamentous actin bundles via the monomeric state of actin. On the other hand, tubulin (α, β-heterodimer) is likewise characterized by the remarkable property of assembly to higher entities, the mitotic spindles and the cytoplasmic microtubules (42), a process which is highly reversible (review, Ref. 43). Recently, Spiegelman et al. (44) presented some evidence indicating that up to 10 initiation centers for the synthesis of microtubules exist which are located near the nucleus. In some biological models, the cytoplasmic microtubules are obviously connected with the chromatin strands (45). In one report, it is even suggested that tubulin together with actin and myosin is one major component of the nonhistone protein fraction (46). This assumption is supported by reports demonstrating a high affinity of tubulin and/or microtubules with actin and myosin is one major component of the nonhistone protein fraction (46). This assumption is supported by reports demonstrating a high affinity of tubulin and/or microtubules with DNA (47-49), RNA (50), and poly(A) (50). Based on the cited published data and the results described above showing a direct influence of G-actin and tubulin on the activity of poly(A) polymerase-endoribonuclease IV complex, we propose that G-actin and/or tubulin is involved in regulation of poly(A) metabolism and perhaps also in the transport of poly(A) (+) mRNA through the nuclear membrane.

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