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FORMATION AND STABILITY OF VINCRISTINE–TUBULIN COMPLEX IN KIDNEY CYTOSOLS

ROLE OF GTP AND GTP HYDROLYSIS*

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Abstract—Vincristine–tubulin complex formed in the 100,000 *g* fraction of mouse kidney dissociated rapidly at 37° in the absence of guanosine-5′-triphosphate (GTP). In the presence of 2 mM GTP, there was a substantial (2.8-fold) increase in complex stability; NaF (100 mM) but not β-glycerophosphate (1 mM) also reduced the rate of dissociation. Further, complex was stabilized by other ribonucleoside-5′-triphosphates (but not their respective 5′-monophosphates), and a nonhydrolyzable analogue of GTP. Stability of the VCR–tubulin complex formed in cytosol from kidney and separated from unbound VCR and GTP by gel filtration was influenced by the concentration of GTP. These results appear not to be a consequence of denaturation of tubulin during incubation, as VCR binding activity remained constant under experimental conditions both in the presence and after the removal of GTP. Further, the rate of formation of the VCR–tubulin complex in kidney was also influenced by the concentration of GTP and was increased by the addition of NaF. In the absence of added GTP, virtually no complex was isolated. ATP, CTP, or ITP has little effect on complex formation, suggesting that the effect may be GTP specific. These data suggest that the destabilizing activity in cytosols prepared from mouse kidney, and the failure to form a stable VCR–tubulin complex in kidney, are in part the consequence of rapid hydrolysis of GTP by a pyrophosphohydrolase. Direct measurement of the hydrolysis of GTP showed that the activity in kidney (9.26 nmol/min/mg protein) was 9.3-fold greater than in tumor extracts.

Studies using human rhabdomyosarcomas growing as xenografts have indicated that the selective action of vincristine (VCR) is due to specific retention of unchanged drug in tumor tissue, with rapid elimination from most non-neoplastic tissues [1]. Noble *et al.* [2] have also shown selective retention of VLB in a sensitive rat tumor *in vivo*. Further, persistence of Vinca alkaloid in cells exposed *in vitro* for short periods of time correlates well with cytotoxicity [3, 4]. However, the mechanism(s) that determines such differential retention between neoplastic and non-neoplastic tissues, and therefore therapeutic selectivity, has received less attention.

The rapid elimination of Vinca alkaloids from many non-neoplastic tissues (of the mouse) may be a consequence of membrane or target phenomena. The former seems unlikely as initial levels of Vinca alkaloid which are cell-associated are often 5- to 15-fold higher than in tumor tissue [1]. The latter would imply differences between target molecules (tubulin) that differentiate neoplastic from non-neoplastic cells. Whereas it is clear that tubulin gene expression may be tissue specific [5, 6] and post-translational modification of both α- and β-tubulin can lead to a large number of isotypes [7], there is only a small amount of published evidence to support the contention that such modifications alter, significantly, the binding of antimitotic agents [8]. However, studies in our laboratories have shown that the stability of VCR–tubulin complexes, formed in membrane-free supernatant fractions, is markedly different in extracts from non-neoplastic tissues when compared to VCR-sensitive neoplastic tissue [1, 9]. Indeed, the stability of complexes formed under these experimental conditions *in vitro* correlates well with retention of Vinca alkaloid *in vivo* [1, 9]. Based upon such data, non-neoplastic tissues were divided into three classes [9]: (a) those that formed stable VCR–tubulin complex (brain); (b) those that formed unstable complex (skeletal muscle, spleen, bone marrow); and (c) those that formed unstable complex, but also destabilized the otherwise stable VCR–tubulin complex formed in tumor extracts (ileum, kidney, liver, lung).

Kidney is a particularly interesting tissue in which to study factors that influence VCR binding and complex stability in that levels of VCR after i.p. injection are 10-fold higher than in tumor, but the drug is eliminated rapidly without apparent metab-

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olism of VCR [1]. Also, the *in vitro* destabilizing effect on complex formed in tumor extract is heat labile and not associated with detectable degradation of tubulin [9]. From these data we reasoned that an additional factor(s) may be involved in the formation and stability of the VCR–tubulin complex in intact tissues. One such candidate was GTP which binds to an exchangeable site on β-tubulin [10–13] prior to incorporation of the subunit into a microtubule. Data derived using membrane-free extracts from human rhabdomyosarcomas suggested that the rate at which VCR–tubulin complex formed was influenced by GTP, as was the stability of this complex [14]. In this article we examine factors which influence binding and stability of VCR–tubulin complex in kidney cytosol, and explore why this complex is unstable.

METHODS

Materials. [G-^{3}H]Vincristine (sp. act. 7–12 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA, and was purified by HPLC prior to use [15]. [8-^{3}H]GTP (sp. act. 10.6 Ci/mmol) was obtained from the Amersham Corp. (Arlington Heights, IL). Guanilyl imidodiphosphate (GMP-PNP) was obtained from Pharmacia (Piscataway, NJ). All other chemicals were obtained from the Sigma Chemical Co., St. Louis, MO. DE-81 discs were purchased from Whatman, Inc., Clifton, NJ, and Sephadex G-25 columns (PD10, 1.4 × 5 cm) from Pharmacia.

Preparation of cytosols. Kidneys from female CBA/CaJ mice were excised rapidly and placed on ice. They were washed in ice-cold 10 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6.8, containing 10 mM $MgCl_2$ (Buffer A). Cytosols were prepared by homogenization in 5 vol. of ice-cold Buffer A using a Potter–Elvehjem homogenizer with a motor-drive Teflon pestle followed by centrifugation at 100,000 *g* for 60 min at 4°.

Quantitation of [^{3}H]*VCR–tubulin complexes.* Aliquots of reaction mixtures were assayed by filtration through two DE-81 discs held within a Millipore filter manifold and washing with 16 ml of ice-cold Buffer A [9, 16]. Protein-bound drug but not free [^{3}H]VCR was retained on the filters, which were incubated in 2 ml of 1.5 M NaCl solution for 16 hr at room temperature to elute bound complex. Fifteen milliliters of ACS scintillant was added, and radioactivity was determined.

Stability of vincristine binding activity in cytosols. Bound and unbound GTP was removed from 1 ml of kidney cytosol by two treatments with 100 μl of a 10% charcoal suspension containing 2% bovine serum albumin (BSA) [17, 18]. Cytosol was incubated with charcoal for 15 min on ice and centrifuged at 12,000 *g* for 15 min at 4°. The supernatant fraction was again incubated with charcoal and centrifuged. In control experiments, 82.4% of [^{3}H]GTP was removed from cytosols. Cytosol (250 μl) was subsequently incubated at 37° in buffer with or without 0.1 mM GTP (total volume 1 ml). After prewarming reaction mixtures for 2 min, 180 μl was removed and incubated for 15 min at 37° with [^{3}H]VCR (308 nM) and 0.1 mM GTP. Vincristine binding was determined by DE-81 disc filtration of 60-μl samples (126.7 μg protein). Cytosols were incubated at 37°, and samples were assayed for VCR binding at 30, 60, and 90 min. Protein was determined by the method of Bradford [19].

Stability of kidney [^{3}H]*VCR–tubulin complexes in the presence of phosphatase inhibitors or GTP.* Kidney cytosol (2 ml) was incubated with [^{3}H]VCR (412.1 nm) and 0.1 mM GTP for 15 min at 37°. [^{3}H]VCR–Tubulin complex was isolated from unbound drug by Sephadex G-25 filtration [14]. Aliquots of complex (900 μl) were incubated at 37° in foil-wrapped tubes. Phosphatase inhibitors were added as follows: NaF (100 mM), β-glycerophosphate (1 mM), or GTP (2 mM). The final volume of the reaction mixtures was 1.44 ml. Aliquots (110 μl, 175.2 μg protein) were assayed by DE-81 disc assay at intervals over 90 min.

$$\%\ \text{Complex remaining} = \frac{\text{dpm retained at time } t}{\text{dpm retained at time } 0} \times 100$$

Stability of kidney [^{3}H]*VCR–tubulin complexes in the presence of nucleotides.* Kidney cytosol (4.1 ml) was incubated for 15 min at 37° with [^{3}H]VCR (344.3 nM) and 0.1 mM GTP. Protein-bound drug was separated from unbound VCR by Sephadex G-25 gel filtration; this also removed 98.5% of unbound GTP [14]. Aliquots (720 μl) of pooled [^{3}H]VCR–tubulin complex were incubated at 37° in foil-wrapped tubes in a total volume of 1.44 ml. Ribonucleotides were added at the following concentrations: GTP, GDP, ATP, ADP, CTP, CDP (2 mM), GTP, GDP, CDP (10 mM); GMP, AMP, CMP, GMP-PNP (a non-hydrolyzable GTP analogue) (20 mM). Aliquots (110 μl, 202.5 μg protein) were assayed at intervals over 90 min by DE-81 disc filtration.

Relationship between stability of kidney [^{3}H]*VCR–tubulin complex and GTP concentration.* Kidney cytosol (2.1 ml) was incubated at 37° for 15 min with [^{3}H]VCR (269 nM) and 0.1 mM GTP in a foil-wrapped tube. Protein-bound drug was separated from unbound [^{3}H]VCR and GTP by Sephadex G-25 gel filtration as before. Radiolabeled complex was pooled, and 550 μl of complex in a total volume of 1.1 ml of Buffer A was incubated at 37° for 90 min in the presence of 10, 5, 1 or 0.1 mM GTP. Aliquots (60 μl, 118 μg protein) were assayed by DE-81 disc filtration.

Stability of kidney [^{3}H]*VCR–tubulin complex with the addition of GTP at 30 min.* Kidney cytosol (2.1 ml) was incubated with [^{3}H]VCR (544 nM) and 0.1 mM GTP for 15 min at 37°. [^{3}H]VCR–tubulin complex was isolated from unbound drug by Sephadex G-25 gel filtration. Radiolabeled complex was pooled, and 550 μl of complex in a total volume of 1.1 ml of Buffer A was incubated at 37°. Three reaction mixtures were prepared: one of pooled complex alone, and two with 1 mM GTP. To the second 1 mM GTP reaction mixture, 1.1 μmol of GTP was added at 30 min (the amount of GTP present in the reaction mixture at time 0). Aliquots (171 μg protein), with the third sample volume corrected for dilution, were analyzed by DE-81 disc filtration.

Rate of formation of [^{3}H]*VCR–tubulin complex in kidney cytosol.* Endogenous GTP was separated

from the protein fraction of kidney cytosol by Sephadex gel filtration of 1-ml samples using ice-cold Buffer A as the eluent. Fractions containing protein were pooled (1.5 ml), and [^{3}H]VCR (86.9 nM) was added to each of five reaction mixtures containing 550 μl of kidney cytosol in a total volume of 1.1 ml of Buffer A. These were incubated at 37° alone or in the presence of 0.1 mM GTP, 100 mM NaF, 0.1 mM GTP + 100 mM NaF, or 10 mM GTP. Aliquots (60 μl, 135 μg protein) were assayed on DE-81 discs to determine the amount of radiolabeled complex formed at intervals over 9 min.

In a separate experiment, kidney cytosol was charcoal treated to remove bound and unbound GTP, as described previously. [^{3}H]VCR (96.9 nM) was added to each of five reaction mixtures containing 275 μl charcoal-treated kidney cytosol in a total volume of 1.1 ml of Buffer A. These were incubated at 37° alone or in the presence of a 2 mM concentration of GTP, ATP, CTP, or ITP. Aliquots (80 μl, 199.8 μg protein) were assayed on DE-81 discs at intervals over 9 min.

HPLC analysis of [^{3}H]GTP degradation by HxRh18 and kidney cytosols. For assay of [8-^{3}H]GTP hydrolysis, HxRh18 tumor cytosol was prepared; kidney cytosol was diluted 1:1 (v/v) with Buffer A. Endogenous GTP was removed by charcoal adsorption as before. The reaction mixture contained, in a final volume of 100 μl, nucleotide-depleted cytosol (90 μl, prewarmed at 37° for 2 min) and 795 μM [8-^{3}H]GTP (sp. act. 22.51 mCi/mmol). Samples were incubated at 37° for up to 5 min (during which period reactions were linear). At the appropriate time, an aliquot (30 μl) was mixed with 9 μl of 4 M perchloric acid, and placed on ice for 5 min. The sample was neutralized with 10.6 μl of 1 M KOH, and centrifuged to remove the precipitate (12,000 *g*, 4 min, 4°). To 30 μl supernatant, 20 μl of a mixture containing GTP, GDP and GMP was added, and a 20-μl reaction was analyzed by HPLC. Zero time samples were prepared by mixing [8-^{3}H]GTP (3 μl) with perchloric acid, and subsequently adding 27 μl of prewarmed cytosol. These samples were subsequently processed as above. Samples were eluted from a Partisil 10/25 SAX analytical column (Whatman) using a linear gradient from 227 to 750 mM ammonium phosphate at pH 3.5 over 30 min. The flow rate was 1 ml/min, fractions (0.5 ml) were collected into minivials, and radioactivity was determined. Retention times for guanosine, GMP, GDP and GTP were 4.2, 6.6, 14.8, and 26.5 min respectively. To detect non-enzymic hydrolysis of [8-^{3}H]GTP, an aliquot in buffer A was incubated at 37° for 5 min and processed as above. Analysis by HPLC showed that [8-^{3}H]GTP was stable under these conditions.

RESULTS

Stabilization of VCR–tubulin complex by nucleotides. In a previous study [14], it was shown that both the rate of formation of [^{3}H]VCR–tubulin complex and the stability of complex formed in tumor extracts are influenced by the concentration of GTP. It was of interest, therefore, to determine whether the instability of complex formed in kidney extracts was due to degradation of GTP. [^{3}H]Vincristine–tubulin complex was formed in kidney cytosols and isolated free of unbound drug by gel filtration. Under the experimental conditions used, this complex was unstable at 37° with a $t_{\frac{1}{2}}$ of 18 min (Fig. 1). NaF (100 mM), an inhibitor of alkaline phosphatase, partially stabilized the complex under the same conditions with 47% complex remaining at 30 min (32% remaining in the control); GTP (2 mM) stabilized further, with 87% complex remaining at 30 min. However, β-glycerophosphate (1 mM), an inhibitor of acid phosphatase, had no stabilizing effect (Fig. 1). As shown in Fig. 2, complex incubated with 1 mM GTP was stable for 60 min, after which loss of

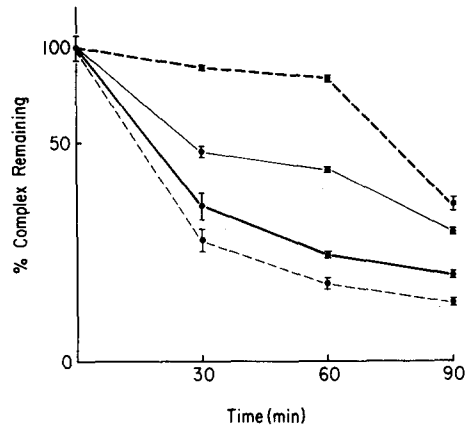

Fig. 1. Stability of the [^{3}H]VCR–tubulin complex from kidney in the presence of GTP or phosphatase inhibitors. [^{3}H]VCR–Tubulin complex was formed in kidney cytosol and separated from unbound [^{3}H]VCR by Sephadex gel filtration. Pooled complex was incubated at 37° alone (———), or in the presence of 100 mM NaF (——), 1 mM β-glycerophosphate (---), or 2 mM GTP (---). Samples were assayed at intervals over 90 min by DE-81 disc filtration to determine the percent of radiolabeled complex remaining. Results represent the mean ± SD of three determinations.

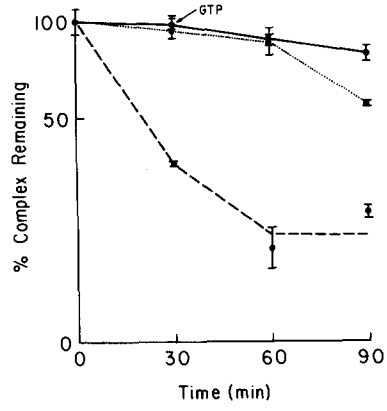

Fig. 2. Stability of the kidney [^{3}H]VCR complex with the addition of GTP during incubation. Nucleotide-depleted complex was incubated at 37° in the absence of added GTP (---), after addition of GTP to 1 mM at time zero (.....), or at zero time and 30 min into the incubation (——). Samples were assayed at intervals over 90 min by DE-81 disc filtration (mean ± SD, N = 3).

Table 1. Stability of kidney [^{3}H]VCR complex in the presence of nucleotides

Ribonucleotides	% Complex remaining*
GTP (2 mM)	83.7 ± 8.2
(10 mM)	119.9 ± 1.3
ATP (2 mM)	99.2 ± 2.1
CTP (2 mM)	72.7 ± 0.9
GDP (2 mM)	73.1 ± 5.4
(10 mM)	98.8 ± 3.7
ADP (2 mM)	74.8 ± 3.2
CDP (2 mM)	47.4 ± 1.9
(10 mM)	65.4 ± 4.7
GMP (20 mM)	44.5 ± 4.0
AMP (20 mM)	30.0 ± 0.7
CMP (20 mM)	47.0 ± 3.5
GMP-PNP† (20 mM)	73.9 ± 4.7
Kidney-[^{3}H]VCR alone	33.6 ± 1.0

* [^{3}H]VCR–Tubulin complex was formed in kidney cytosol by incubation with [^{3}H]VCR and 0.1 mM GTP for 15 min at 37° and separated from unbound drug and GTP by Sephadex gel filtration. Pooled complex was incubated at 37° in the presence of ribonucleotides. Radiolabeled complex was determined at intervals over 30 min by filtration of aliquots through DE-81 discs. The results represent the mean ± SD of three determinations.

† Guanilyl imidodiphosphate, a non-hydrolyzable GTP analogue.

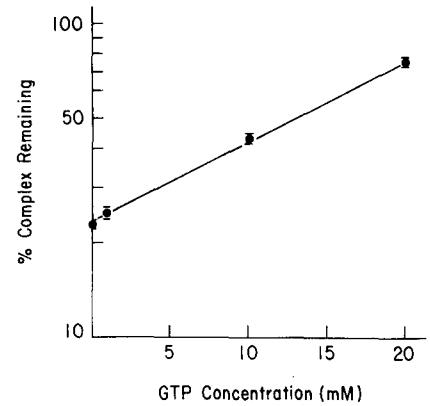

Fig. 3. Effect of GTP concentration on the stability of kidney [^{3}H]VCR complex. [^{3}H]VCR–Tubulin complex was formed in kidney cytosol and isolated from unbound [^{3}H]VCR and GTP by Sephadex gel filtration. Pooled complex was incubated at 37° in the presence of 20, 10, 1 or 0.1 mM GTP, and samples were assayed at intervals over 90 min by DE-81 disc filtration. Data show the percent of radiolabeled complex remaining at 90 min (mean ± SD, N = 3).

complex was measured. If additional GTP (1.1 μmol) was added 30 min after the start of incubation, destabilization was prevented.

The effects of other ribonucleoside 5′-phosphates were examined subsequently (Table 1). GDP, ATP, ADP and CTP (2 mM) all increased the amount of complex remaining at 30 min. Also, increasing the concentration to 10 mM further increased stability; there was an increase in complex remaining with 10 mM GTP and CDP relative to the 2 mM concentrations of each (10 mM ATP, ADP, or CTP caused precipitation in the reaction mixture). GMP-PNP (20 mM), a non-hydrolyzable GTP analogue, stabilized the kidney [^{3}H]VCR complex, with 73.9% remaining at 30 min. Complex was not stabilized by GMP, AMP, or CMP, even at concentrations of 20 mM. Thus, 79% complex remained at 90 min after the addition of GTP at 30 min, whereas 55% remained when no further GTP was added over the 90-min incubation period (Fig. 2). The effect of GTP concentration on the stability of [^{3}H]VCR–tubulin complex formed in kidney cytosol after incubation at 37° for 90 min is shown in Fig. 3. Complex was stabilized by GTP in a linear fashion, with 13 nM GTP required to maintain 50% kidney [^{3}H]VCR complex at 90 min in this experiment.

Stability of VCR binding site. These data suggested that, in cytosols from kidney, as we had shown previously in tumor, the stability of VCR–tubulin complex was modulated by GTP. An alternative possibility was that depletion of GTP caused denaturation of tubulin, with concomitant loss of VCR binding activity. It is known, for example, that GTP stabilizes the colchicine binding site [20]. To test this possibility, cytosols were prepared from mouse kidney, depleted of GTP by charcoal adsorption and incubated at 37°, with or without 0.1 mM GTP for 90 min. At various times aliquots were removed, and [^{3}H]VCR (230 nM) and GTP (0.1 mM) were added. After a further 15 min at 37°, the formation of [^{3}H]VCR–tubulin complex was assayed (Fig. 4). Under these conditions, cytosols depleted of GTP and incubated at 37° in the absence of GTP retained full VCR-binding capacity for at least 90 min. These data indicated that the observed instability of complex could not be explained by a decrease in the binding capacity for VCR in these preparations.

Effect of GTP on the rate of formation of the VCR–tubulin complex. Previous data indicated that GTP influences the rate of formation of VCR–tubulin complex in tumor cytosols [14]. To extend these observations, the influence of GTP on complex formation in kidney cytosol was determined, as was the

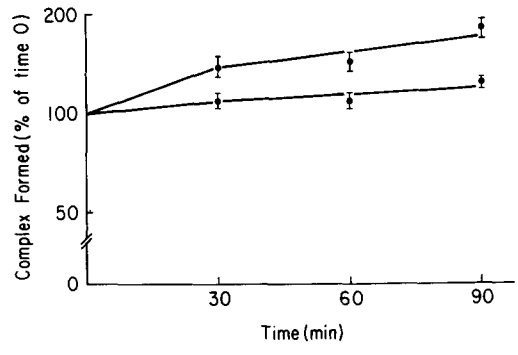

Fig. 4. Stability of vincristine binding activity in kidney cytosols. Mouse kidney cytosol was depleted of GTP by two cycles of charcoal treatment and incubated at 37° in the presence (upper line) or absence (lower line) of 0.1 mM GTP. Vincristine binding activity was determined at intervals over 90 min (mean ± SD, N = 3).

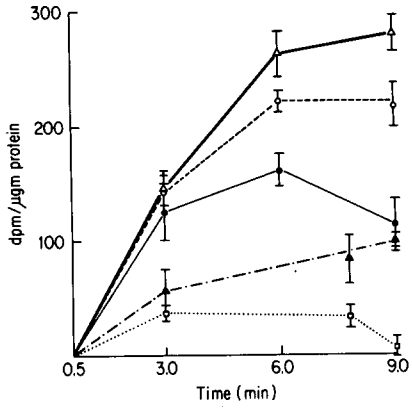

Fig. 5. Importance of GTP for formation of the [^{3}H]VCR–tubulin complex in kidney cytosol. Unbound endogenous GTP was separated from the protein fraction of mouse kidney cytosol by Sephadex gel filtration. Pooled protein was incubated at 37° with 10 mM GTP (———), 0.1 mM GTP (———), 0.1 mM GTP + 100 mM NaF (---), 100 mM NaF (–·–·–), or alone (.....). After prewarming reaction mixtures for 2 min at 37°, [^{3}H]VCR was added, and complex formation was examined over 9 min by DE-81 disc filtration. Sample mixing and disc application required 30 sec. The results presented are the mean ± SD of three determinations at each time point after subtraction of the 30-sec point as background.

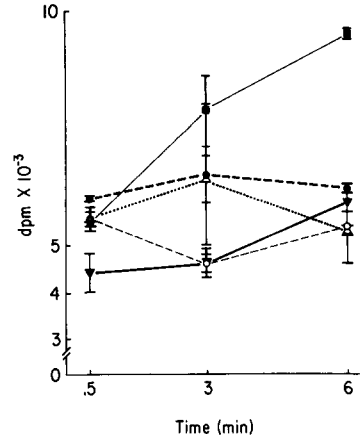

Fig. 6. Formation of [^{3}H]VCR–tubulin complex in kidney cytosol in the presence of ribonucleoside triphosphates. Bound and unbound GTP were removed from kidney cytosol by two incubations with activated charcoal. Nucleotide-depleted cytosol was incubated at 37° with 2 mM GTP (——), CTP (——), ITP (---), ATP (.....), or alone (---). After prewarming reaction mixtures for 2 min at 37°, [^{3}H]VCR was added, and complex formation was examined over 9 min by DE-81 disc filtration. The results represent the mean ± SD of three determinations at each time point.

effect of NaF (Fig. 5). Mixing [^{3}H]VCR with cytosol and applying samples to DE-81 discs required 30 sec. Therefore, initial rates were determined between 0.5 and 3 min after addition of [^{3}H]VCR. The initial time point (0.5 min) was subtracted as background from the values at 3, 6 and 9 min. Formation of complex in Sephadex G-25 filtered extracts (for the removal of free GTP) was more rapid in the presence of 0.1 mM GTP (3.3-fold) and 10 mM GTP (3.9-fold). The addition of NaF (100 mM) with 0.1 mM GTP increased the rate of formation to that measured in the presence of 10 mM GTP. Also, addition of NaF prevented the loss of complex between 6 and 9 min that occurred in the presence of 0.1 mM GTP alone. In the absence of GTP, the rate of formation of complex was low, and virtually no complex was present at 9 min (Fig. 5). Addition of NaF alone allowed complex to form at a relatively slow rate. ATP, ITP and CTP had little effect in promoting complex formation (Fig. 6). In several experiments, CTP inhibited complex formation, but ATP occasionally enhanced formation slightly, compared with the reaction with no added nucleotide.

Data derived above suggested that the intrinsic instability of kidney complex (and perhaps the observed destabilizing effect of kidney cytosol on tumor complex) may be due to the hydrolysis of GTP. We therefore examined the "GTPase" activity of kidney cytosol. For comparison, the hydrolysis of GTP was examined in a human rhabdomyosarcoma xenograft (HxRh18) which forms a relatively stable VCR–tubulin complex under these conditions [14]. [^{3}H]GTP was incubated with kidney cytosol and with HxRh18 tumor cytosol, and degradation was monitored over time at 37° (Table 2). It was evident that a greater than 9-fold difference in GTPase activity existed between the two tissues, with a rate of [^{3}H]GTP degradation of 9.26 nmol/min/mg protein in kidney and 0.99 nmol/min/mg protein in the HxRh18 preparation. After a 5-min incubation at

Table 2. [^{3}H]GTP hydrolysis by kidney and tumor cytosols

	GMP (%)	GDP (%)	GTP (%)	Activity (nmol/min/mg protein)
Rh18 tumors	5.8	4.6	87.6	0.99
Kidney	43.1	1.1	55.8	9.26
[^{3}H]GTP	6.9	3.9	89.2	

[^{3}H]GTP alone (795 μmol) or GTP-depleted membrane-free supernatant fractions from HxRh18 tumor or mouse kidney were prepared in Buffer A. Kidney cytosol was diluted 1:1 with Buffer A. A reaction mixture containing 90 μl cytosol and 795 μM [8-^{3}H]GTP (sp. act. 22.51 mCi/mmol) was incubated at 37° for 5 min, during which time reactions were linear. Aliquots were obtained, and [8-^{3}H]GTP degradation was determined by HPLC analysis. The percent of each nucleotide present at 5 min is shown.

37° in kidney cytosol, virtually all of the degraded [^{3}H]GTP was present as GMP, whereas in tumor cytosol there was an approximately equal distribution of GMP and GDP.

DISCUSSION

The goal of these studies was to examine factors which contribute to the intrinsic instability of the VCR–tubulin complex formed in kidney cytosol and to the heat labile destabilizing effect of kidney cytosol on VCR–tubulin complex formed in tumor cytosols from rhabdomyosarcoma xenografts. [^{3}H]VCR–tubulin complex formed in HxRh18 tumor cytosols is completely stable at 37° for 2 hr in the presence of 0.1 mM GTP [14]. Under the same conditions, kidney [^{3}H]VCR complex dissociated rapidly. Previously, we reported [14] that both the formation and stability of the [^{3}H]VCR–tubulin complex formed in cytosols from tumor HxRh18 are modulated by the presence of GTP. It was of interest, therefore, to determine whether the instability of the kidney VCR complex was a consequence of GTP degradation. In the absence of GTP, kidney VCR complex dissociated rapidly. The addition of 2 mM GTP to the reaction mixture increased stability by 2.8-fold. NaF stabilized 1.5-fold, but β-glycerophosphate had no effect. This would suggest that instability may be a consequence of an alkaline phosphatase rather than an acid phosphatase activity. The fact that ribonucleoside monophosphates failed to stabilize the complex argues against complex instability or the destabilizing effect of kidney on tumor complex being mediated by an orthophosphoric monoester hydrolase. However, nucleoside di- and triphosphates did increase complex stability, suggesting that this effect may be a consequence of a pyrophosphohydrolase. Consequently, stabilization of the [^{3}H]VCR–tubulin complex may be a consequence of nucleoside pyrophosphates acting as competitive substrates or as phosphatase donors through an NDP kinase or myokinase, hence maintaining the concentration of GTP. In addition, adding GTP to the reaction mixture after a period of incubation, thus repleting GTP, enhanced complex stability. GMP-PNP, a non-hydrolyzable GTP analogue, also increased stability 2.3-fold, further suggesting the importance of GTP hydrolysis in complex dissociation.

The $\alpha\beta$-tubulin heterodimer contains 2 mol of GTP. One of these is tightly bound (N-site) and is non-exchangeable [11]. The second binding site (E-site) is located on the β-subunit, where GTP binds but is readily exchangeable [10–13]. The E-site GTP may be hydrolyzed by exogenous alkaline phosphatase [11], and during polymerization is hydrolyzed by a microtubule-associated GTPase [21, 22]. The E-site nucleotide may influence the binding of several classes of antimitotic agents. Colchicine binding activity in platelet lysates was increased by 75% in the presence of GTP (2 mM) and magnesium ion (1 mM) [23]. Colchicine binding in cytosol from brain or from purified tubulin was also stabilized by GTP, but this effect was a consequence of inhibition of the loss of colchicine binding activity that occurs with aging of tubulin [24, 25]. Colchicine binding activity of tubulin may also be preserved by VCR and VLB [26], and certain data support the binding of Vinca alkaloids near to the E-site [27].

Our studies indicate that in cytosols prepared both from human rhabdomyosarcoma [14] and mouse kidney (Fig. 4) the rate at which VCR binds to tubulin was increased 2- to 3-fold in the presence of GTP. Data presented argue against GTP stabilizing the VCR binding site since this was stable in cytosols in which >82% of GTP had been removed. Thus, the situation is not the same as that reported for colchicine. In kidney, the amount of [^{3}H]VCR–tubulin complex formed was 2.2-fold greater in the presence of 10 mM GTP than at 0.1 mM, and virtually no complex was isolated in the absence of exogenous GTP. In addition, other nucleoside triphosphates had little effect on promoting complex formation, although ATP occasionally enhanced formation slightly. This may be secondary to slight residual contamination with GTP with ATP acting as a phosphate donor, and suggests that in cytosols GTP may be a specific requirement for formation of a complex that can be isolated by DE-81 disc filtration. Further, addition of NaF stimulated [^{3}H]VCR complex formation, and prevented loss of complex between 6 and 9 min. Therefore, increasing the concentration of GTP 100-fold or preventing GTP degradation enhanced both the rate and extent of complex formation and further stabilized complex over the time studied. These data suggest that hydrolysis of GTP influences both the rate of formation and stability of complex in kidney cytosol. Clearly, this nucleotide stabilizes the complex formed in tumor cytosol [14], or in kidney cytosol. Data showed that GTPase activity in kidney cytosol was 9-fold greater than in similar preparations from HxRh18 xenografts, and also that a greater proportion of GTP was hydrolyzed to GMP during incubation with kidney cytosol. Further, GMP did not stabilize the [^{3}H]VCR complex. Thus, GTP hydrolysis correlated inversely with the stability of the [^{3}H]VCR–tubulin complex *in vitro*, and retention of VCR in these two tissues *in vivo* after administration to tumor-bearing mice [1, 9].

These data extend previous studies demonstrating the importance of GTP in the formation and stability of VCR–tubulin complexes in neoplastic tissue cytosols. They also indicate that the *in vitro* instability of [^{3}H]VCR–tubulin complex formed in kidney cytosols is a consequence, at least in part, of GTP hydrolysis. Vincristine binding to purified tubulin from tumors was more rapid than complex formation in tumor cytosols and was not modulated by GTP (unpublished observations). Thus, the influence of GTP on VCR binding may be mediated through some interaction with tubulin itself (perhaps at the E-site) or through an interaction with another substance present in cytosol that may modulate VCR binding. Endogenous factors have been reported in brain and liver cytosols [28–30] that modulate the binding of colchicine to microtubule protein. Further studies are needed to elucidate more clearly the relationship between GTP and VCR binding in tissues and to determine its possible influence on the sensitivity of neoplastic and non-neoplastic tissues to VCR.

REFERENCES

1. J. A. Houghton, L. G. Williams, P. M. Torrance and P. J. Houghton, *Cancer Res.* **44**, 582 (1984).
2. R. L. Noble, P. W. Gout, L. L. Wijcik, F. Hebden and C. J. Beer, *Cancer Res.* **37**, 1455 (1977).
3. P. J. Ferguson, J. R. Phillips, M. Selner and C. E. Cass, *Cancer Res.* **44**, 3307 (1984).
4. P. J. Ferguson and C. E. Cass, *Cancer Res.* **45**, 5480 (1985).
5. I. Gozes and V. Z. Littauer, *Nature, Lond.* **276**, 411 (1978).
6. I. Gozes and C. J. Barnstable, *Proc. natn. Acad. Sci. U.S.A.* **79**, 2579 (1982).
7. D. J. Field and J. C. Lee, *Analyt. Biochem.* **144**, 584 (1985).
8. G. Piperno, M. LeDizet and X-j. Chang, *J. Cell Biol.* **104**, 289 (1987).
9. J. A. Houghton, L. G. Williams and P. J. Houghton, *Cancer Res.* **45**, 3761 (1985).
10. S. M. Penningroth and M. W. Kirschner, *Biochemistry* **17**, 734 (1978).
11. E. J. Manser and P. M. Bayley, *Biochem. biophys. Res. Commun.* **131**, 386 (1985).
12. R. L. Geahlen and B. E. Haley, *J. biol. Chem.* **254**, 11982 (1979).
13. J. P. Nath, G. R. Eagle and R. H. Himes, *Biochemistry* **24**, 1555 (1985).
14. L. C. Bowman, J. A. Houghton and P. J. Houghton, *Biochem. biophys. Res. Commun.* **135**, 695 (1986).
15. J. A. Houghton, P. M. Torrance and P. J. Houghton, *Analyt. Biochem.* **134**, 450 (1983).
16. B. Bhattacharyya and J. Wolff, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2375 (1976).
17. M. Kirsch and L. R. Yarbrough, *J. biol. Chem.* **256**, 106 (1981).
18. I. V. Sandoval, E. MacDonald, J. L. Jameson and P. Cuatrecasas, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4881 (1977).
19. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
20. R. C. Wiesenberg, G. G. Borisy and E. W. Taylor, *Biochemistry* **7**, 4466 (1968).
21. M. Caplow, J. Shanks and B. P. Brylawski, *Can. J. Biochem.* **63**, 422 (1985).
22. H. D. White, B. A. Coughlin and D. L. Purich, *J. biol. Chem.* **255**, 486 (1980).
23. D. M. Kenney, F. C. Chao, J. L. Tullis and G. S. Conneely, *Thomb. Diath. haemorrh.* **34**, 780 (1975).
24. R. F. Luduena and M. C. Roach, *Archs Biochem. Biophys.* **210**, 498 (1981).
25. J. K. Wang and A. J. Morris, *Archs Biochem. Biophys.* **161**, 118 (1974).
26. L. Wilson, *Biochemistry* **9**, 4999 (1970).
27. A. B. Huang, C. M. Lin and E. Hamel, *Biochem. biophys. Res. Commun.* **128**, 1239 (1985).
28. P. Sherline, K. Schiavone and S. Brocaid, *Science* **205**, 593 (1979).
29. A. Lockwood, *Proc. natn. Acad. Sci. U.S.A.* **76**, 1184 (1979).
30. E. Baraona, F. Finkleman, Y. Matsuda and C. S. Lieber, *Analyt. Biochem.* **130**, 302 (1983).