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Guanine nucleotide-binding activity as an assay for *src* protein of rat-derived murine sarcoma viruses

(Kirsten murine sarcoma virus/transformation)

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ABSTRACT **We have recently identified a 21,000-dalton protein, p21, coded for by Kirsten or Harvey murine sarcoma virus. On the basis of the results obtained with the p21 of a mutant of Kirsten sarcoma virus, temperature sensitive for the maintenance of transformation, we concluded that the p21 was required for the maintenance of transformation induced by either virus. We report herein that when extracts from cells transformed by Kirsten or Harvey sarcoma virus are incubated with [^{3}H]GDP or [α-^{32}P]GTP, picomole quantities of guanine nucleotide can be immunoprecipitated with antisera that contain antibodies to the p21. Previously we have shown that the immunoprecipitability of [^{35}S]methionine-labeled p21 of the temperature-sensitive mutant of Kirsten sarcoma virus is thermolabile. The binding of guanine nucleotide is shown herein also to be thermolabile in extracts of cells transformed by the same mutant. However, the immunoprecipitability of the [^{35}S]methionine-labeled p21 in such extracts of the temperature-sensitive mutant can be preserved if the extracts containing labeled p21 are incubated with added GDP or GTP prior to heating. The results suggest an interaction between p21 and certain guanine nucleotides, and the possible roles of guanine nucleotides and p21 in the maintenance of transformation are discussed.**

We have been interested in three independent sarcoma virus isolates from rats: Harvey sarcoma virus (Ha-MSV) (1), Kirsten sarcoma virus (Ki-MSV) (2), and a rat sarcoma virus (RaSV) of pure rat origin isolated by Rasheed *et al.* (3). We recently identified a protein, p21, coded for by Ki-MSV or Ha-MSV in nonproducer cells transformed by either virus (4). The rat sarcoma virus codes for an immunologically related protein, p29 (5). In studies on the p21 of a mutant of Ki-MSV (ts 371) temperature sensitive (ts) for the maintenance of transformation, we noted that the immunoprecipitability of the p21 coded for by the ts 371 Ki-MSV was markedly more thermolabile than the immunoprecipitability of the p21 of wild-type (wt) Ki-MSV when the [^{35}S]methionine-labeled cell extracts were heated *in vitro* (6). These and other results on the p21 of this mutant (6) and other variants (7) of Ki-MSV (unpublished data and *Results*) suggested strongly that the p21 was the *src* protein of Ki-MSV and was required for the maintenance of virus-induced transformation.

We report here that the immunoprecipitability of the p21 of ts 371 Ki-MSV can be preserved by preincubating [^{35}S]-methionine-labeled extracts with GDP or GTP, prior to heating the extracts *in vitro*. When unlabeled extracts from cells transformed by wt Ki-MSV or wt Ha-MSV are incubated *in vitro* with [^{3}H]GDP, picomole quantities of guanine nucleotide can be immunoprecipitated with antisera that contain antibodies to the p21. The guanine nucleotide binding provides a simple assay for this protein. The possible roles of p21 and guanine nucleotides in transformation are discussed.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from P-L Biochemicals: ATP, GTP, CTP, UTP, ADP, GDP, CDP, UDP, AMP, 5′-GMP, 3′-GMP, CMP, UMP, 3′,5′-cyclic AMP, 3′,5′-cyclic GMP, 3′,5′-cyclic CMP, 3′,5′-cyclic UMP, dATP dGTP, dCTP, dTTP, and guanosine tetraphosphate (ppGpp). Additional compounds were products of Boehringer Mannheim: GDP-mannose, guanosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppG). [^{3}H]GDP (7.8 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [α-^{32}P]GTP (400 Ci/mmol diluted to 12 Ci/mmol prior to use with unlabeled GTP) were obtained from New England Nuclear.

Cell Labeling and Immunoprecipitation. Procedures for labeling with [^{35}S]methionine, immunoprecipitation using *Staphylococcus aureus* containing protein A and sodium dodecyl sulfate/polyacrylamide gel electrophoresis of precipitated proteins were fully described in our previous publications (4).

Cells and Viruses Used. All cells and viruses have been described (4–6) and were grown in Dulbecco's modified Eagle's medium with 10% calf serum for mouse, rat, and dog cells, and 10% fetal calf serum for mink cells. The cells used include: mouse cells, NIH 3T3, BALB 3T3, and C127 3T3; rat cells, NRK; a canine kidney cell, MDCK; and a mink lung cell, CCL 64. Viruses include an ecotropic strain of helper-independent Moloney type C virus (Moloney murine leukemia virus, Mo-MuLV) or Friend type C virus; Ki-MSV, Ha-MSV; RaSV; Moloney murine sarcoma virus (Mo-MSV); the Snyder–Theilin strain of feline sarcoma virus; the B77 strain, Schmidt–Ruppin strain, and Prague strain of Rous sarcoma virus. Detailed history of the cell lines infected with these viruses is given in earlier publications (4–6). One cell line, KA-54, has been described by Greenberger and Aaronson (7) and is a revertant of Ki-MSV-transformed BALB 3T3 cells. The cell has high levels of Ki-MSV-specific RNA but has lost many properties of the parental transformed cell. We will report fully on the molecular biology of this cell elsewhere.

Sera. All antisera were prepared by syngeneic transplantation of sarcoma virus-transformed nonproducer rat cells in Osborne–Mendel or Fisher rats. Full details of the sera have been reported (4–6).

Nucleotide Binding Assay. Each extract was incubated at 4°C in siliconized 13 × 100 mm tubes in reaction mixtures of

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Abbreviations: Ha-MSV, Ki-MSV, and Mo-MSV, Harvey, Kirsten, and Moloney murine sarcoma virus; RaSV, rat sarcoma virus; Mo-MuLV, Moloney murine leukemia virus; ts, temperature-sensitive; wt, wild-type; p[CH_2]ppG, guanosine 5′-[β,γ-methylene]triphosphate.

0.30 ml for 10 min. Each reaction mixture contained 20 mM Tris·HCl at pH 7.2, 100 mM sodium chloride, 5 mM magnesium chloride, 1% Triton X-100, 0.026 mM [^{3}H]GDP (specific activity 7.8 Ci/mmol), and various amounts of protein (50–200 μg) as indicated. At 10 min a 0.010-ml aliquot of serum and an additional 0.20 ml of reaction mixture buffer without GDP were added and the incubation was continued for 2 hr more at 4°C. Then 0.05 ml of a 10% suspension of *S. aureus* as a source of protein A was added to each tube. The incubation was then continued for an additional 45 min at the same temperature. The immune complexes were removed by centrifugation at 4°C at 2500 rpm for 5 min in a Beckman J-6 centrifuge and washed three times with 1.0 ml each time of reaction mixture buffer minus GDP. The precipitates were transferred in wash buffer to new tubes and washed twice more. The precipitates were then dissolved by boiling for 3 min in 0.3 ml of 2% sodium dodecyl sulfate/28 mM 2-mercaptoethanol/100 mM Tris·HCl at pH 7.8/40% glycerol. Residual *S. aureus* was removed by centrifugation as above at room temperature and a 0.2-ml aliquot was removed, added to 0.3 ml of water, and assayed for radioactivity in a Beckman LS-350 scintillation counter with 10 ml of Redisolv (Beckman). Under these conditions, 8500 cpm represents 1 pmol of [^{3}H]GDP.

Preparation of Unlabeled Extracts. Cells were grown to confluence in 100-mm plastic petri dishes and were fed with fresh media 24 hr prior to harvest. The medium was removed, and the monolayer was washed once with approximately 10 ml of phosphate-buffered saline at 4°C. Cells were lysed with 2–3 ml of a buffer containing 1% Triton X-100, 100 mM sodium chloride, 5 mM magnesium chloride, and 20 mM Tris·HCl at pH 7.8. The extracts were passed three times through a 5.0-ml plastic syringe with a 22 gauge needle and clarified at 5000 × *g* for 10 min at 4°C. Each extract was then incubated for 2.0 hr at 4°C with 0.5 ml of a 10% suspension of *S. aureus* containing protein A because prior [^{35}S]methionine-labeled extracts had been prepared this way (4). The preincubated extracts were spun for 30 min at 100,000 × *g* at 4°C, and the upper 80% of the extract was stored at −170°C in small aliquots. Extracts were thawed only one time prior to use. Protein concentrations were determined by the Lowry method with bovine serum albumin as a standard.

RESULTS

Prior studies had indicated that the ability to immunoprecipitate the [^{35}S]methionine-labeled p21 of ts 371 mutant of Ki-MSV was destroyed by heating *in vitro* of extracts of cells transformed by ts 371 Ki-MSV. To try to find a potential substrate that had affinity for the p21, various nucleotides were added *in vitro* to such labeled extracts, and the extracts were heated, returned to 4°C, and then immunoprecipitated to visualize the [^{35}S]methionine-labeled p21 on acrylamide gels. The results are shown in Fig. 1. As previously noted (6) the immunoprecipitability of the p21 of wt Ki-MSV was not affected by heating the extract containing p21 at 42°C for 5 min (Fig. 1*A*). In contrast (Fig. 1*C*, lanes 1–4), extensive loss of the p21 band occurred when the extract from cells transformed by ts 371 Ki-MSV was subjected to the same heating. Addition of either GTP or GDP to the extract from the ts mutant, prior to heating *in vitro*, protected the immunoprecipitability of the p21 of ts 371 Ki-MSV (Fig. 1*C*, lanes 7 and 8, 20 and 21, and 27 and 28). Extensive protection was obtained with either 1.0 or 0.1 mM either GTP or GDP, and slight protection with 0.01 mM GDP was also discernible (lane 22). ATP, ADP, CTP, CDP, UDP, orthophosphate, or pyrophosphate had no discernible effect. At high levels of CTP or ADP, marked general precipitation of the proteins was noted and these lanes were difficult to evaluate. (Fig. 1*C*, lanes 10 and 18). Slight protection was obtained with 1.0 mM UTP (Fig. 1*C*, lane 12) but not with 0.1 mM UTP (Fig. 1*C*, lane 13). In other studies, not shown, protection was also obtained with dGTP and p[CH_2]ppG, the β,γ-methylene analogue of GTP. No protection was obtained with cyclic GMP, cyclic AMP, cyclic UMP, GDP-mannose, 3′-GMP, 5′-GMP, CMP, AMP, or UMP. Neither GTP nor GDP had any effect on the background in the cells not infected with Ki-MSV (Fig. 1*B*) or on the stability of the p21 of the wt Ki-MSV (Fig. 1*A*). In other controls, extracts of ts 371 Ki-MSV were heated first (Fig. 1*C*) and returned to ice, and then GDP was added. Under these conditions, no p21 was precipitable if extracts subsequently remained at 4°C or were reheated with GDP and then immunoprecipitated (Fig. 1*C*, lanes 29–31). The results suggest that guanine nucleotides containing more than a single phosphate might interact directly or indirectly with p21.

Next, unlabeled extracts of C127 cells infected with Mo-MuLV or Ki-MSV and Mo-MuLV were incubated with [^{3}H]GDP. Antiserum or control serum was then added and the resulting immune complexes were collected by centrifugation. The complexes were dissolved by boiling in sodium dodecyl sulfate-containing buffer and radioactivities were measured. The results are shown in Table 1. Extracts of C127 cells infected with Mo-MuLV showed no appreciable binding in the presence of control serum or antiserum. Extracts of cells infected with Ki-MSV and Mo-MuLV bound appreciable amounts of the label in the presence of antiserum. Up to 1 pmol of nucleotide was bound at the highest level of extract. In studies not shown, when the same extract was tested for binding of GTP labeled in the α phosphate with ^{32}P, again up to 1 pmol of nucleotide was bound. The results suggest that a guanine containing nucleotide is bound by the extract and is immunoprecipitated by the antiserum. Further identification of the product bound will be necessary, but on the basis of these results we feel that it is justified to refer to the bound radioactive material as "guanine nucleotide." When the incubation with extract and labeled [^{3}H]GDP was carried out for 1 hr, and then a 100-fold molar excess of unlabeled GDP was added for the remainder of the assay, no [^{3}H]GDP was bound, suggesting that the linkage of the guanine nucleotide to the protein(s) in the immune complex was reversible.

To investigate the specificity of this binding, various extracts and sera were tested; the results are shown in Tables 2 and 3. Extracts of cells transformed by Ki-MSV, Ha-MSV, or RaSV bound a significant amount of label, whereas extracts of similar cells transformed by a variety of other sarcoma viruses (not derived from rats) did not bind the label. Importantly, extracts from a revertant of Ki-MSV-transformed BALB cells, KA-54, did not give binding of [^{3}H]GDP. We have shown in studies to

Table 1. Requirements for binding guanine nucleotides

Extract added	Serum used	[^{3}H]GDP bound, pmol
None	Anti-p21	0.05
Ki-MSV (100 μg)	Anti-p21	1.10
Ki-MSV (50 μg)	Anti-p21	0.70
Ki-MSV (25 μg)	Anti-p21	0.38
Control (100 μg)	Anti-p21	0.05
None	Control	0.05
Ki-MSV (100 μg)	Control	0.05
Control (100 μg)	Control	0.05

The Ki-MSV extract was prepared from a C127 mouse cell infected with Ki-MSV and Mo-MuLV, and the control extract was from C127 cells infected with Mo-MuLV. The specific activity of [^{3}H]GDP was 8500 cpm/pmol.

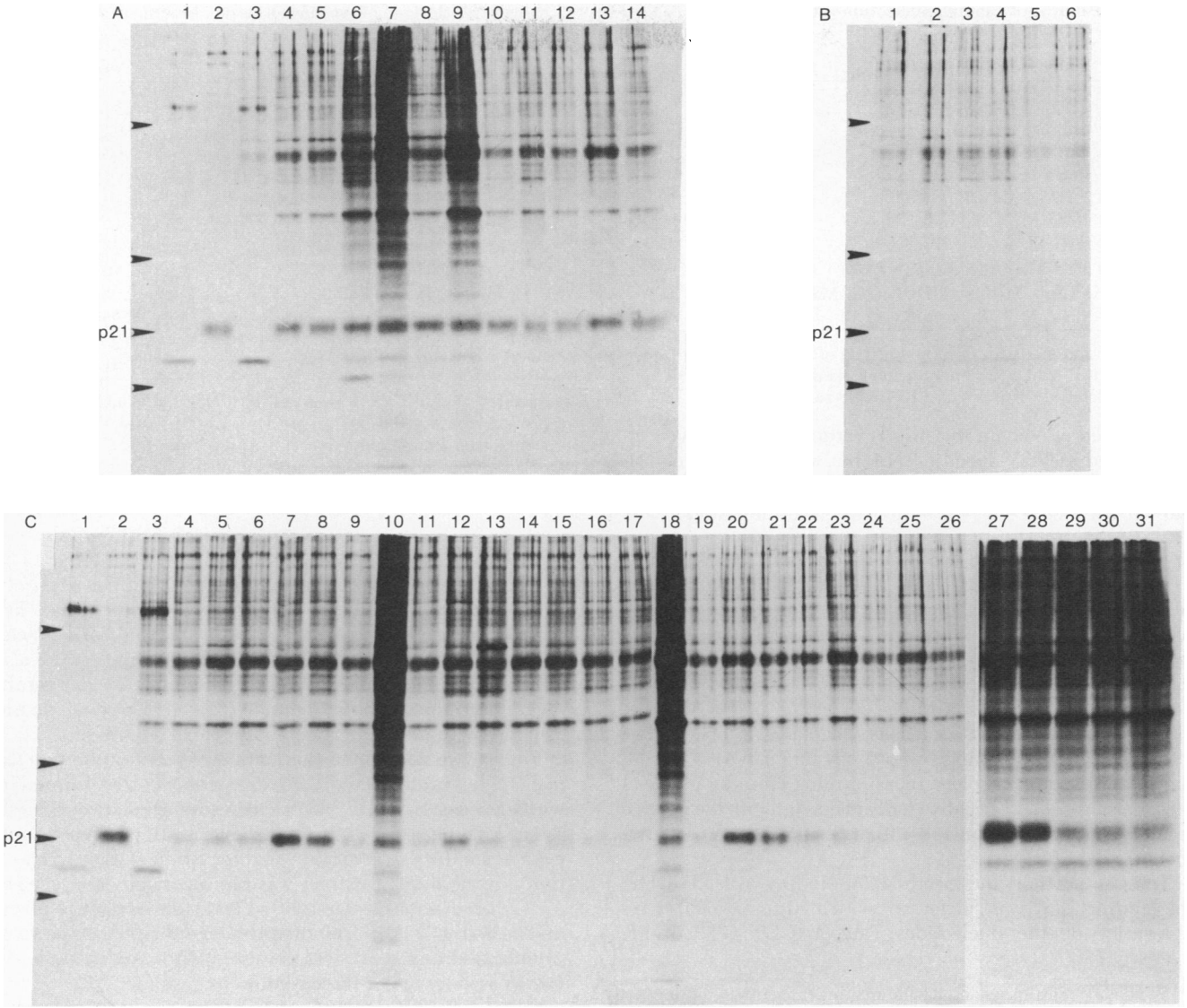

FIG. 1. Stabilization against thermal inactivation of the p21 of ts 371 Ki-MSV by GDP and GTP. C127 cells infected with wt Ki-MSV/wt Mo-MuLV or ts 371 Ki-MSV/wt Mo-MuLV or wt Mo-MuLV alone were labeled with [^{35}S]methionine at 34°C and lysates were prepared at 4°C. Portions of extracts containing 10^7 or 1.5×10^7 incorporated cpm were heated in the presence of indicated nucleoside di- or triphosphates at 42°C for 5 min in 100 μl of buffer containing 5 mM $MgCl_2$, 100 mM NaCl, 20 mM Tris·HCl at pH 7.2, 1% Triton X-100, and Trasylol at 200 units/ml. After being quenched on ice, the extracts were diluted with an equal volume of buffer containing 2% (vol/vol) Triton X-100, 1% deoxycholate, 0.2% sodium dodecyl sulfate, 20 mM phosphate buffer at pH 7.5, 200 mM NaCl; 2 mM EDTA, Trasylol at 200 units/ml, and bovine serum albumin at 2 mg/ml. The extracts were then incubated with 10 μl of Ha-MSV antiserum or normal rat serum overnight. The antigen–antibody complexes were precipitated with 50 μl of *S. aureus* suspension (10% vol/vol). All the procedures were performed at 4°C. After washings, the precipitated proteins were subjected to electrophoresis in 11.5% polyacrylamide gel. The locations of molecular weight markers are indicated by arrows. From top to bottom: 68,000 bovine serum albumin, 30,000 carbonic anhydrase, and 14,000 lysozyme. Fluorography was for 18 hr. Full details are given in earlier publications (4, 6).

(*A*) Lysates (10^7 cpm) of C127 cells infected with wt Ki-MSV/wt Mo-MuLV. Lanes 1 (normal serum) and 2 (antiserum) were extracts without heating. Lanes 3 (normal serum) and 4 (antiserum) were extracts heated at 42°C. Lanes 5 to 14 were extracts heated at 42°C in the presence of the following compounds and were precipitated with Ha-MSV antiserum: lane 5, 1 mM ATP; lane 6, 1 mM GTP; lane 7, 1 mM CTP; lane 8, 1 mM UTP; lane 9, 1 mM ADP; lane 10, 1 mM GDP; lane 11, 1 mM CDP; lane 12, 1 mM UDP; lane 13, 1 mM orthophosphate; lane 14, 1 mM pyrophosphate.

(*B*) Lysates (10^7 cpm) of C127 cells infected with wt Mo-MuLV alone. Lanes 1 (normal serum) and 2 (antiserum) were extracts without heating. Lanes 3 (normal serum) and 4 (antiserum) were extracts heated at 42°C. Lanes 5 and 6 were extracts heated at 42°C in the presence of 1 mM GTP (lane 5) or 1 mM GDP (lane 6) and were precipitated with Ha-MSV antiserum.

(*C*) Lysates (1.5×10^7 cpm) of C127 cells infected with ts 371 Ki-MSV/wt Mo-MuLV. Lanes 1 (normal serum) and 2 (antiserum) were extracts without heating. Lanes 3 (normal serum) and 4 (antiserum) were extracts heated at 42°C. Lanes 5 to 31 were extracts heated at 42°C in the presence of the following compounds and were precipitated with Ha-MSV antiserum: lane 5, 1 mM ATP; lane 6, 0.1 mM ATP; lane 7, 1 mM GTP; lane 8, 0.1 mM GTP; lane 9, 0.01 mM GTP; lane 10, 1 mM CTP; lane 11, 0.1 mM CTP; lane 12, 1 mM UTP; lane 13, 0.1 mM UTP; lane 14, 1 mM orthophosphate; lane 15, 0.1 mM orthophosphate; lane 16, 1 mM pyrophosphate; lane 17, 0.1 mM pyrophosphate; lane 18, 1 mM ADP; lane 19, 0.1 mM ADP; lane 20, 1 mM GDP; lane 21, 0.1 mM GDP; lane 22, 0.01 mM GDP; lane 23, 1 mM CDP; lane 24, 0.1 mM CDP; lane 25, 1 mM UDP; lane 26, 0.1 mM UDP. Lanes 27 to 31 were different sets of experiments demonstrating the lack of GDP protection when the compound was added after heat inactivation of extracts: lane 27 (1 mM GDP) and lane 28 (0.1 mM GDP), extracts heated at 42°C in the presence of GDP; lane 29, 1 mM GDP added after heating; lane 30, 0.1 mM GDP added after heating; lane 31, 1 mM GDP added after heating, and the mixture was reheated at 42°C for another 5 min.

Table 2. Binding of guanine nucleotide with different extracts

Source of cellular extract	[^{3}H]GDP bound, pmol
NIH 3T3, NIH 3T3/F-MuLV	0.05
NIH 3T3/SR-RSV	0.05
NIH 3T3/Ha-MSV	1.22
NIH 3T3/RaSV (F-MuLV)	0.44
BALB 3T3, BALB 3T3/B77-RSV	0.07
BALB 3T3/Mo-MSV	0.07
BALB 3T3/Ki-MSV	1.13
BALB 3T3/Ki-MSV revertant (KA-54)	0.07
XC, NRK, Mink, MDCK, MDCK (Mo-MSV)	0.13
Mink/Ki-MSV, MDCK/Ha-MSV	0.83

Each reaction mixture contained 100 μg of protein from the indicated extract. F-MuLV, Friend murine leukemia virus; SR-RSV and B77-RSV, Schmidt–Ruppin and B77 Rous sarcoma virus.

be published elsewhere that this revertant has high levels of undegraded Ki-MSV-specific RNA, but we cannot detect p21 in [^{35}S]methionine-labeled extracts of the KA-54 cells by immunoprecipitation and acrylamide gel electrophoresis. With the Ha-MSV-transformed cells and this antiserum, up to 1.2 pmol of label was bound with 100 μg of cellular protein.

When various sera were tested for their ability to precipitate the label in the presence of a Ha-MSV extract, again a high degree of specificity was observed (Table 3). Only sera that had previously been noted to precipitate [^{35}S]methionine labeled Ha-MSV p21 yielded precipitation in the assay using [^{3}H]GDP. Sera prepared against Ha-MSV-, Ki-MSV-, or RaSV-transformed cells yielded positive results, whereas a variety of other rat sera from rats bearing tumors carrying Mo-MSV or the Lilly–Steeves strain of spleen focus-forming virus did not yield positive results. These negative sera did not diminish the positive result when they were mixed with a positive serum (data not shown).

To assess partially the specificity of binding with [^{3}H]GDP, the binding assay was carried out with a 20-fold molar excess of a variety of other nucleotides (Table 4). GDP, GTP, dGTP, or ppGpp effectively competed with the binding. The only other nucleotide tested that competed appreciably was UTP. It is noteworthy that guanine mononucleotides did not compete. In the thermal stability studies guanine mononucleotides also did not protect the p21 of ts 371 Ki-MSV (Fig. 1). The results suggest that the binding is relatively specific for guanine nucleotides with more than one phosphate.

Table 3. Serum specificity for guanine nucleotide binding

Serum added	Known anti-p21 titer	[^{3}H]GDP bound, pmol
None	—	0.05
Control rat	<1:10	0.05
Anti-Ha-MSV/NRK	1:600	1.23
Anti-Ha-MSV/NRK	1:200	0.63
Anti-Ki-MSV/NRK	1:100	0.51
Anti-Ha-MSV/NRK	<1:10	0.08
Anti-Mo-MSV/NRK	<1:10	0.05
Anti-Mo-MSV/NRK	<1:10	0.05
Anti-SFFV/NRK	<1:10	0.05
Anti-RaSV/FRE	1:50	0.38

A 0.01-ml aliquot of each serum was tested in a binding assay mixture that contained 100 μg of protein from an extract of NIH cells nonproductively transformed by Ha-MSV. The anti-p21 titer was determined in prior immunoprecipitation experiments using an [^{35}S]methionine-labeled extract of the same Ha-MSV transformed cells. The immunoprecipitation in those studies was performed in 0.30-ml reaction mixtures and a 0.010-ml aliquot of serum in that reaction would be defined as a 1:30 dilution of serum. The [^{35}S]methionine p21 was visualized by fluorography on polyacrylamide slab gels and the lowest dilution of serum that yielded p21 on the gel is defined as the anti-p21 titer. Conditions for this immunoprecipitation have been described (4, 5). The control sera are from either an Osborne–Mendel or a Fisher rat. All the sera except the anti RaSV/FRE are from Osborne–Mendel rats and the latter is from a Fisher rat. SFFV, spleen focus-forming virus.

Table 4. Effects of other nucleotides on binding of [^{3}H]GDP

Competitor added	[^{3}H]GDP bound, pmol
None	1.10
GDP	0.08
GTP	0.08
dGTP	0.08
ppGpp	0.10
p[CH_2]ppG	0.15
GMP (3′ or 5′)	1.10
3′,5′-Cyclic GMP	1.10
ATP, ADP, CTP, CDP	0.95–1.10
UDP, phosphate, pyrophosphate	0.95–1.10
dTTP, dATP, dCTP, CMP, AMP, UMP	0.95–1.10
UTP	0.42

Each reaction mixture was incubated at 4°C and contained 100 μg of protein from C127 cells infected with Ki-MSV and Mo-MuLV in 0.30 ml; other components of the assay are as detailed in *Materials and Methods*. The potential competing nucleotide was added 5 min prior to the [^{3}H]GDP and was in 20-fold molar excess. In the absence of Ki-MSV extract, 0.05 pmol of nucleotide was present in the immune complex. This value is not subtracted from the above values.

Finally, we performed thermal inactivation studies on the nucleotide-binding activity in extracts of cells transformed by wt Ki-MSV or ts 371 Ki-MSV. The results are shown in Table 5. The binding activity was quite stable in the wild-type extract even when this extract was heated for 5 min at 41°C. The activity in the mutant extract was rapidly inactivated even at 37°C. When equal amounts of wt and ts extracts were mixed and heated at 37°C, the binding observed was 60% of the value of unheated mixed extracts, consistent with no destruction of the wt activity under these conditions.

DISCUSSION

The current results provide a first step toward the development of a biochemical assay to measure a biologically relevant function of the *src* protein of Kirsten or Harvey sarcoma virus. The binding of guanine nucleotides seems to be a specific assay for the p21 coded for by each of these viruses. High levels of

Table 5. Thermal stability for guanine nucleotide binding of extracts of wt and ts Ki-MSV-transformed cells

Extract	Temperature, °C	[^{3}H]GDP bound, pmol
wt Ki-MSV	0	1.10
	30	1.15
	37	1.05
	41	1.00
ts Ki-MSV	0	0.65
	30	0.50
	37	0.13
	41	0.10

Aliquots of each extract were heated for 5 min at the indicated temperatures, quenched on ice, and assayed at 4°C for [^{3}H]GDP binding. Each assay contained approximately 100 μg of cellular extract. A control of C127 cells infected with Mo-MuLV bound 0.06 pmol of [^{3}H]GDP even if preheated to 41°C.

binding are observed only with extracts of cells transformed by Ki-MSV or Ha-MSV and only with antisera that have previously been shown to precipitate the p21. Relatively high levels of nucleotide are bound, and the amounts of nucleotide bound can be accurately quantitated and are proportional to the amount of extract added. We cannot yet measure the kinetics of the reaction because we have not found a way to terminate the reaction and maintain the GDP in a form that will immunoprecipitate. However, because the binding can be studied in solution prior to the addition of antibody, it should be possible to find conditions for measuring the kinetics. We have not yet fully characterized the bound product or determined the number of possible components needed for the stable binding, any components that might inhibit binding, or whether stable binding requires the presence of antibody.

It is of interest to try to interpret earlier immunoprecipitation results with the p21 of Ki-MSV ts 371. In the current studies, the immunoprecipitability of the ts p21 can be preserved by preincubating ts extracts with GDP or GTP prior to heating. These results suggest that the antibodies to p21 are recognizing determinants more complex than a primary amino acid sequence of the p21. There seem to be three main possibilities for what the antibodies recognize. (*i*) The anti-p21 antibodies may recognize a hapten– (GDP or GTP) protein (p21) complex. (*ii*) In the presence of GDP or GTP, some conformational change in p21 may occur and the antibodies might recognize this conformation of the p21. (*iii*) In the presence of GDP or GTP, formation of higher molecular weight complexes of p21 with itself, or p21 with another protein(s) might occur, and the antibody would recognize some part of the complex. In this model, it is also possible that the GDP or GTP interacts primarily with the putative second protein, and that the complex then interacts with p21. It will be important to ascertain what the antibody recognition sites are because this knowledge might lead to a useful approach to the function of the p21, and it is also important to determine if nucleotide is binding directly to p21.

It is also of interest to consider possible functional roles for the p21 and GDP, GTP, or guanosine polyphosphates. Guanine nucleotides are known: to be involved in the stability of different forms of microtubules (8), to function as precursors in the biosynthesis of various nucleotide sugars (9), or in the biosynthesis of RNA and DNA, and to act as donors in the capping of mRNA (10), as energy sources in various steps in protein biosynthesis, in the biosynthesis of cyclic GMP, in the regulation of the activity of adenylate cyclase and the responsiveness of this enzyme system to hormones, and in bacterial systems in the synthesis of "magic spots," guanosine tetraphosphate and guanosine pentaphosphate (11). We cannot discern in which metabolic pathway p21 and guanine nucleotides might function. Nevertheless, the interaction of p21 and guanine nucleotides could represent a biochemical intermediate, which would regulate the activity of one of the pathways or possibly be converted to a product that might be a proximal mediator of transformation. Lastly, it should be useful to compare the biochemistry of this *src* protein to the *src* protein of Rous sarcoma virus, which has been observed to have protein kinase activity associated with it (12, 13). The interaction of both *src* proteins with nucleotides containing high-energy phosphate bonds might be an important common feature. Such comparative biochemistry of proteins required for cellular transformation should provide major insights into the metabolic pathways that control this complex biological process.

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