Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 6953–6957, November 1984
Biochemistry

Guanosine nucleotide binding by highly purified Ha-*ras*-encoded p21 protein produced in *Escherichia coli*

(GDP/GTP binding/*ras* gene product)

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Communicated by John J. Burns, July 23, 1984

ABSTRACT **High-level expression of the p21 protein product of the BALB murine sarcoma virus v-*ras* gene (similar to the product of the Harvey murine sarcoma virus v-Ha-*ras* gene) has been reported recently, and highly purified preparations of this protein have been obtained. We used a nitrocellulose filter assay for measuring the binding of GDP and GTP to the purified protein. Previously p21 antibodies had been used to precipitate p21–guanosine nucleotide complexes from crude extracts containing the protein. Using the filter assay, we find that the v-Ha-*ras* gene product binds [^{3}H]GDP stoichiometrically. The binding is time-dependent and is faster at 30°C than at 0°C. Optimum binding is obtained in the presence of dithiothreitol and magnesium ions and at pH 7.4. In terms of its GDP binding activity, p21 is heat stable and pronase sensitive. The dissociation constants (K_d) of p21 for [^{3}H]GDP and [^{3}H]GTP, determined by Scatchard analysis, are 6×10^{-8} M and 2.5×10^{-8} M, respectively.**

The *ras* genes code for a family of structurally and immunologically related proteins of 189 amino acid residues termed p21 ras (1, 2). The *ras* genes were initially identified as the oncogenic sequences of certain strains of acute transforming retroviruses (v-*ras*) (3). Their normal counterparts (cellular *ras* genes or c-*ras*) are present in a wide variety of eukaryotic cells (4–10). Cellular *ras* genes acquire transforming properties by single point mutations within their coding sequences (11–20), and the altered *ras* genes are found in a significant fraction of human cancers and in experimentally induced animal tumors (21–23). Oncogenic transformation is believed to be produced by the products of the *ras* oncogenes—i.e., the p21 proteins—but neither the mechanisms by which these proteins bring about transformation nor their normal cellular functions are known. One of the well-characterized biochemical properties of *ras*-encoded proteins is their ability to bind guanine nucleotides, such as GTP, dGTP, and GDP, specifically (24–27). In addition, the p21 proteins encoded by the v-*ras* genes of Harvey and Kirsten murine sarcoma viruses (Ha-MuSV and Ki-MuSV), v-Ha-*ras* and v-Ki-*ras*, contain a threonine residue in position 59 that undergoes autophosphorylation (25–27). Biochemical investigation of the *ras* gene products has been hampered by their very low levels both in normal and tumor cells. Therefore, genes specifying p21 ras proteins have been molecularly cloned and expressed in *Escherichia coli*. Lautenberger *et al.* (28) obtained large yields of a 23,000-dalton protein in which the four amino-terminal amino acids of p21 were replaced by 14 amino acids specified by the vector. As judged by immunoprecipitation, this protein, like p21, had GDP-binding activity.

Lacal *et al.* (29) recently have reported high-level production of the transforming v-*ras* from BALB MuSV in *E. coli* by utilizing a recombinant plasmid with the tightly regulated P_L promoter of bacteriophage λ and purified this protein to virtual homogeneity. This bacterially produced p21 protein is similar in amino acid sequence to that encoded by v-Ha-*ras* in Ha-MuSV. However, mammalian proteins produced in bacterial cells are not subject to the same post-translational modifications as in mammalian cells. Shih *et al.* reported that retroviral p21 is synthesized in a precursor form and processed by cleavage in its carboxyl terminus (30). In addition, some retroviral p21 proteins are phosphorylated (10, 27, 31) and contain covalently bound lipid (32). Thus, it was unknown whether bacterially produced protein that would lack such modifications would be active. Another concern regarding the *E. coli*-produced *ras* gene product was that high concentrations of guanidine hydrochloride were required for solubilization prior to purification, and it seemed possible that such harsh treatments could destroy biological activity. In this report we show that p21 purified from *E. coli* is functional, using the criterion of high-affinity GDP/GTP binding. The only assay previously available for studying p21 binding to guanosine nucleotides required the use of anti-p21 antibodies and prolonged incubation times (24–27). We describe in this paper a much simpler nitrocellulose filtration assay.

MATERIALS AND METHODS

Preparations. The bacterially synthesized p21 protein was purified as described earlier (29) with the following modifications to solubilize p21 from the particulate fraction. Ten grams of *E. coli* cells (pJCL-E30) (29) were sonicated in 50 mM Tris·HCl, pH 8.0/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride (5 ml/gm of cells), and the sonicated cell extract was centrifuged for 10 min at 4000 × *g* to remove unbroken cells and cell debris. The particulate fraction was pelleted by ultracentrifugation for 2 hr in a Beckman 35 Ti rotor at 30,000 rpm. The pellet was homogenized in an excess of buffer A (50 mM Tris·HCl, pH 7.4/5 mM 2-mercaptoethanol/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride) in a Dounce homogenizer, and the suspension was centrifuged as above. The pellet was suspended in buffer A (2 ml/gm of cells), and p21 protein was solubilized by the addition of an equal volume of 7 M guanidine·HCl. The clear supernatant obtained after centrifugation for 2 hr in a Beckman 35 Ti rotor at 30,000 rpm is designated p21(a). In some cases p21(a) was purified further by high-performance liquid chromatography (HPLC) on a C_8 reversed-phase column as described earlier (29). The pooled fractions containing p21 were evaporated *in vacuo* to dryness. The residue was suspended in 1 ml of water and again evaporated to dryness *in vacuo* to insure complete removal of trifluoroacetic acid. The residue was suspended in a small volume of 3.5 M guanidine·HCl; it is referred to as p21(b). Attempts to remove guanidine·HCl resulted in precipitation of p21. Both unlabeled GTP and [^{3}H]GTP were purified from contaminating

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Abbreviations: MuSV, murine sarcoma virus; Ha-MuSV and Ki-MuSV, Harvey and Kirsten MuSV; v-*ras*, viral *ras* gene; v-Ha-*ras* and v-Ki-*ras*, v-*ras* of Ha-MuSV and Ki-MuSV.

GDP by chromatography on DEAE-Sepharose CL-6B as described by Moffatt (33). Concentrations of guanosine nucleotides were determined by absorbance at 252 nm.

Assays. The GDP binding assay used for p21 was similar to the one used earlier for measuring the binding of [^{3}H]GDP to eukaryotic initiation factor 2 (eIF-2) (34). Reaction mixtures (50 μl) contained 20 mM Tris·HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM NaCl, 2 μg of bovine serum albumin (Bethesda Research Laboratories), and 2 μM [^{3}H]GDP (10.8 Ci/m mol, Amersham; 1 Ci = 37 GBq) and were incubated at 30°C for 30 min. Aliquots (40 μl) were filtered on 0.45-μm nitrocellulose filters and washed at once with 10 ml of ice-cold buffer containing 20 mM Tris·HCl (pH 7.4), 1 mM dithiothreitol, 5 mM magnesium chloride, and 100 mM sodium chloride. Filters were dissolved in 1 ml of methyl cellosolve, and the amount of [^{3}H]GDP retained on the filter was determined by liquid scintillation counting in 10 ml of hydrofluor. In all of the experiments, 1 pmol of [^{3}H]GDP represents 6000 cpm. Protein was determined by the method of Bradford (35) or by the fluorascamine procedure (36) with bovine serum albumin as the standard. One unit of p21 is taken as the amount of protein that binds 1 pmol of [^{3}H]GDP under standard assay conditions. Polyacrylamide gel electrophoresis was performed as described (37).

RESULTS

Specific Activity and Purity of Bacterial p21. Fig. 1 shows the $NaDodSO_4$/polyacrylamide gel electrophoresis pattern of p21(a) (lane 1) and p21(b) (lane 2). Guanidine·HCl at 3.5 M was able to extract all of the p21 from the washed particulate fraction, along with a few high-molecular-weight contaminants. A densitometric scan of several such gels showed that p21 represents 60–80% of the total protein in p21(a) preparations. Most of these minor contaminants were removed when p21(a) was purified by HPLC. Densitometric scanning of the gels indicated that these preparations are at least 90% pure. The specific GDP binding activities of p21(a) and p21(b) were approximately 24,000 and 37,300 units/mg of protein, respectively. From these values, the binding appears to be stoichiometric with one molecule of nucleotide bound per p21 molecule.

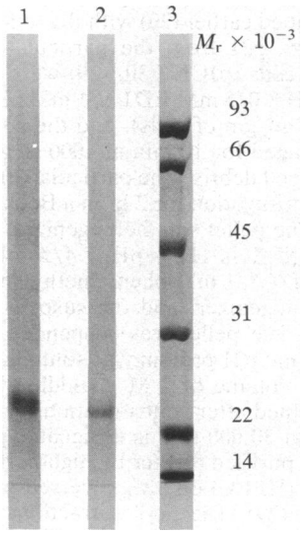

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of bacterial p21(a) and p21(b) preparations. p21(a) (5 μg) and p21(b) (3 μg) were subjected to electrophoresis under denaturing conditions through a 12–17% $NaDodSO_4$/polyacrylamide gel (Separation Sciences), and the gel was stained with Coomassie brilliant blue. Lanes: 1, p21(a) (p21 solubilized from washed particulate preparation by 3.5 M guanidine·HCl); 2, p21(b) (p21 purified by HPLC on C_8 reverse-phase column); 3, protein molecular weight markers: phosphorylase B (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Kinetics of Guanosine Nucleotide Binding. In our system, p21 expressed in *E. coli* is soluble only in the presence of guanidine·HCl or urea. For this reason, the p21 protein was kept in 3.5 M guanidine·HCl solution, and all assays were performed in the presence of 0.35 M guanidine·HCl, which did not seem to interfere with GDP binding. Higher concentrations of guanidine·HCl completely inhibit the formation of the p21–GDP complex. In very low concentrations of guanidine·HCl, the p21 protein precipitates and GDP binding is lost. For the concentration of p21 used in the present experiments, 0.35 M guanidine·HCl in the assay mixture gave optimal binding. Purified p21 protein from *E. coli* solubilized by guanidine·HCl exhibited different GDP binding properties compared to that reported for $p21^{v\text{-}has}$ and $p21^{c\text{-}has}$ produced in rodent cells (24–27). Whereas GDP binding was rapid at 4°C with $p21^{v\text{-}has}$ and $p21^{c\text{-}has}$, bacterial p21 binding kinetics are rather slow and only reached saturation in 30 min at 30°C; at 0°C, almost no GDP binding was detected (Fig. 2). The binding kinetics were the same whether p21(a) or p21(b) was used in the assay. One possible explanation for the slow kinetics might be gradual renaturation of p21 molecules after guanidine·HCl is diluted from 3.5 to 0.35 M in the reaction mixture. Were this the case, renaturation would require the presence of GDP because preincubation of p21 at 30°C for 30–60 min after diluting guanidine·HCl to 0.35 M did not alter the binding kinetics (data not shown).

Binding Requirements. [^{3}H]GDP binding to p21 had an optimum pH of about 7.4–7.6 (Fig. 3*A*) and was markedly increased by $MgCl_2$ (Fig. 3*B*) and a sulfhydryl compound such as dithiothreitol or 2-mercaptoethanol (Fig. 3*C*). The requirement for Mg^{2+} is not absolute because there was some specific [^{3}H]GDP binding (which could be blocked by competition from excess unlabeled GDP) even in the presence of EDTA (not shown). Dithiothreitol was much more effective than 2-mercaptoethanol. [^{3}H]GDP binding was a linear function of the amount of highly purified p21(b) present in the

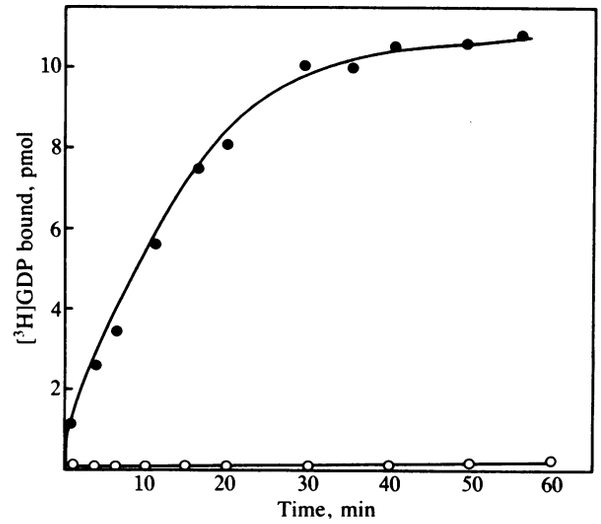

FIG. 2. Kinetics of [^{3}H]GDP binding to bacterial p21. A reaction mixture (500 μl) containing 20 mM Tris·HCl (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM NaCl, 5 μg of p21(a), and 2 μM [^{3}H]GDP were incubated at 30°C (●) or 0°C (○). All of the components were preincubated at the indicated temperature for 5 min prior to initiation of the reaction with [^{3}H]GDP. At the indicated intervals, 40-μl aliquots of the reaction mixture were removed and immediately filtered through 0.45-μm nitrocellulose filters. Filters were washed at once and processed as described for the determination of the bound radioactivity.

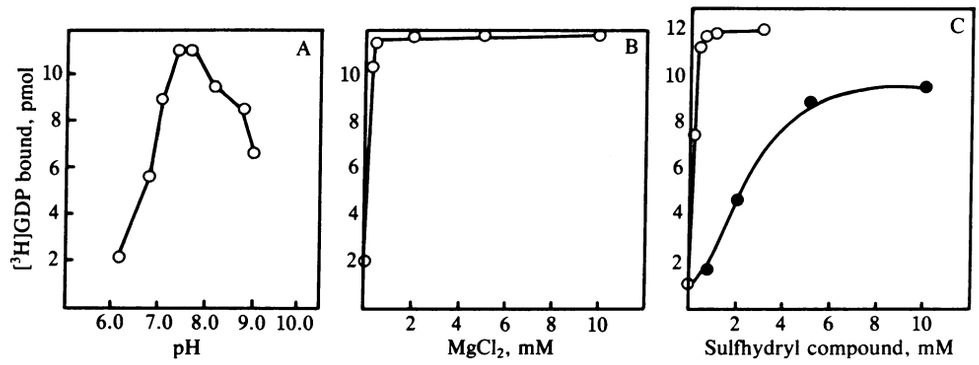

FIG. 3. Components required for stable and maximal binding of [^{3}H]GDP to bacterial p21. (*A*) [^{3}H]GDP binding to 0.5 μg of p21(a) was determined in the presence of 20 mM Tris·HCl adjusted to the indicated pH. (*B*) [^{3}H]GDP binding to 0.5 μg of p21(a) was determined as a function of $MgCl_2$ concentration. (*C*) [^{3}H]GDP binding to 0.5 μg of p21(a) was determined under standard assay conditions except that the concentrations of dithiothreitol (○) or 2-mercaptoethanol (●) were varied as indicated.

assay (not shown). p21 retained about 80% of its GDP binding activity after incubation at 100°C for 30 min. However, treatment with Pronase resulted in total loss of the [^{3}H]GDP binding activity of p21 (not shown).

Scatchard Analysis of Guanine Nucleotide Binding. Fig. 4*A* shows the binding of [^{3}H]GDP of p21 as a function of the GDP concentration. Saturation was reached at about 0.6 μM GDP. A Scatchard plot (38) of the data (Fig. 4*B*) is consistent with single-site binding kinetics, with a calculated K_d of 6.0 $\times 10^{-8}$ M. The binding of [^{3}H]GTP as a function of the GTP concentration is shown in Fig. 5*A*, and the Scatchard plot is shown in Fig. 5*B*. The saturation value of GTP binding (Fig. 5*A*) was about 0.3 μM. From the Scatchard plot, a K_d value of GTP of 2.5×10^{-8} M was tentatively calculated. The data for GTP are less rigorous because of the nonlinearity of the Scatchard plot. Similar results were obtained with [γ-^{32}P]GTP.

Nucleotide Binding Competition. The competition of GTP and GDP binding to p21 was studied by using either [^{3}H]GDP or [^{3}H]GTP and increasing the concentrations of unlabeled nucleotide as competitor. Approximately 3-fold more unlabeled GDP than GTP was required for parallel displacement of [^{3}H]GDP from the nucleotide binding site (Fig. 6*A*). When [^{3}H]GTP was used, 3-fold greater concentrations of unlabeled GDP than GTP were again required for displacement (Fig. 6). Thus, the nucleoside binding site on p21 shows preference for GTP, with an affinity approximately 3-fold greater than that for GDP. We tested several nucleoside triphosphates, diphosphates, monophosphates, and 3′,5′-cyclic phosphates for their ability to bind P21 either by direct nitrocellulose filter assay with [γ-^{32}p]- or [α-^{32}p]-labeled nucleotides or by competition of [^{3}H]GDP binding by unlabeled nucleotides. When [α-^{32}p]-labeled dATP, dGTP, dCTP, or TTP were used, only dGTP was found to bind to p21. [γ-^{32}p]ATP did not bind. ATP, CTP, dATP, TTP, ADP, CDP, dCDP, 5′-AMP, 3′-AMP, 5′-UMP, 5′-GMP, 2′,5′-cAMP, 2,3′-cCMP, 2′,3′-cGMP, and adenosine 2′,5′-diphosphate were unable to competitively inhibit the binding of [^{3}H]GDP to p21. When added in excess, GDP, dGDP, GTP, dGTP, GMPNP, and guanosine-5′-O-(2-thiodiphosphate) completely inhibited the binding of [^{3}H]GDP to p21. Thus, the nucleotide specificity of bacterial p21 is comparable to the viral and cellular p21 proteins produced in rodent cells (24–27).

DISCUSSION

We describe a simple nitrocellulose filter assay for measuring [^{3}H]GDP and [^{3}H]GTP binding to purified p21 protein, and we were able to study the stoichiometry and kinetics of the binding reaction. Previous attempts to analyze GDP/GTP binding by the antibody precipitation method gave nonlinear Scatchard plots (27). Our assays gave essentially linear plots for both GDP and GTP, and we were able to obtain K_d values for the binding of GDP and GTP to p21, although at high fractional saturations the Scatchard plot for GTP binding deviated slightly from linearity. As discussed by Cuatrecasas and Hollenberg (39), this deviation may be due to inaccuracy in estimating the true free-ligand concentrations when only a very small fraction of the total ligand is bound. Dahlquist (40) has noted also that deviations from

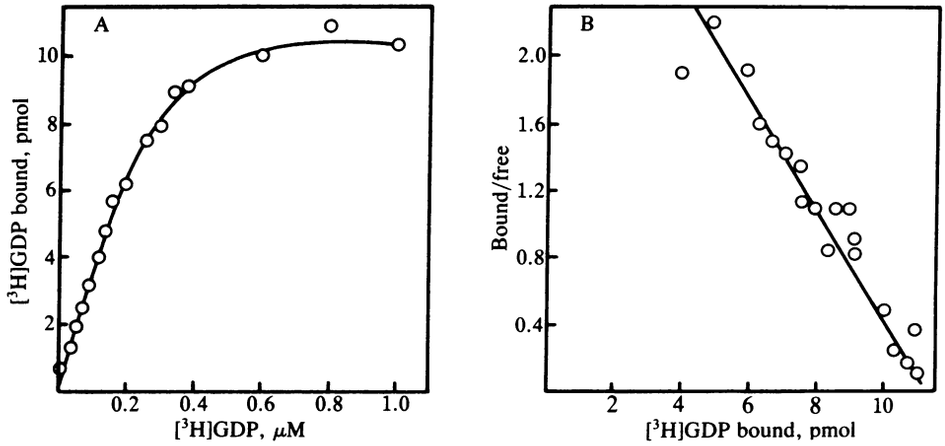

FIG. 4. [^{3}H]GDP binding to bacterial p21 as a function of the GDP concentration. [^{3}H]GDP binding was determined as described with 0.3 μg of HPLC-purified p21 and [^{3}H]GDP as indicated. (*A*) [^{3}H]GDP bound as a function of GDP concentration. (*B*) Scatchard plot.

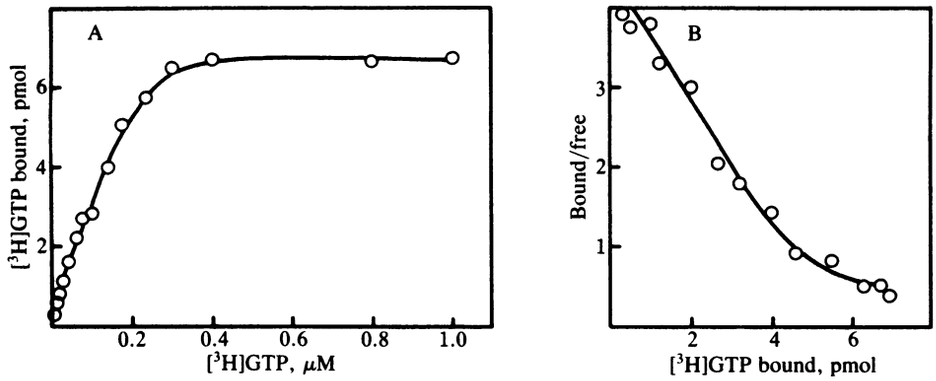

FIG. 5. Binding of [³H]GTP to bacterial p21. (*A*) [³H]GTP binding to 0.3 μg of HPLC-purified as a function of the GTP concentration. (*B*) Scatchard plot.

linearity may occur when studying the binding of a single macromolecular species that is partially denatured. In addition, we consistently have observed that a given amount of p21 protein bound less GTP than GDP at their respective saturating concentration.

Although the insolubility of the p21 protein could have been an obstacle for studying its binding properties, the binding of guanine nucleotides did not appear to be seriously disturbed by the rather high concentrations of guanidine·HCl required to maintain the protein in solution but may have slowed it down and may have been responsible for the very slow rate observed. In any case, we found that with sufficiently long incubation, the binding of GDP to p21 was stoichiometric, suggesting that at least this property of the protein remained more or less intact. It is highly unlikely that some minor contaminants in the p21 preparation are responsible for the observed GDP binding because (*i*) HPLC-purified p21 seems to be electrophoretically homogeneous and gave stoichiometric binding, and (*ii*) other recombinant proteins of similar size (e.g., immune interferon) made in *E. coli* under the control of the same expression plasmid and solubilized from the particulate fraction of bacterial cell extracts in a manner similar to that used for p21 had minor contaminants similar to those in the p21(a) preparation and yet exhibited no GDP/GTP binding.

The system described here should allow the comparative biochemical studies of normal and mutant p21s because the *ras* coding region can be modified by recombinant DNA techniques and reintroduced into the expression vector. Preliminary results indicate that there are quantitative differences of GDP binding between normal and transforming p21 proteins. In addition, we have detected GTPase activity associated with p21 protein. The transforming p21 protein seems to have approximately 1/10th of the GTPase activity of its normal counterpart.

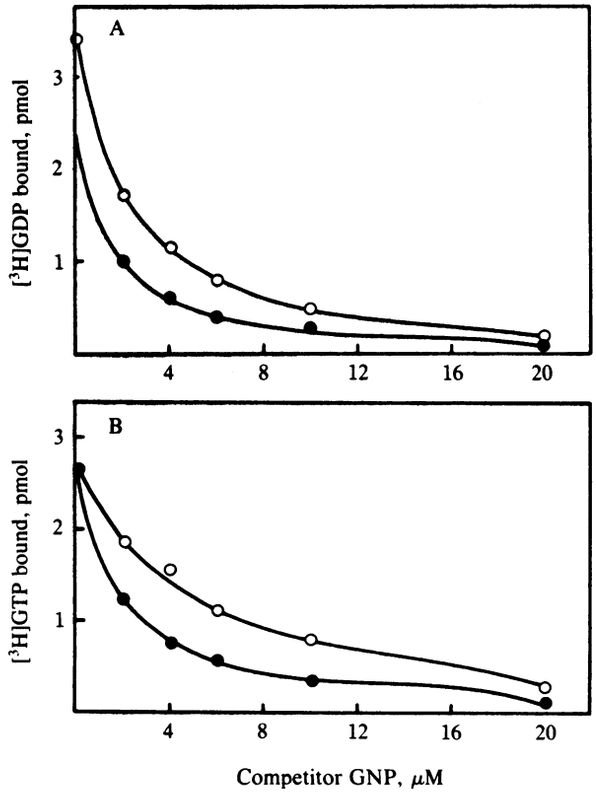

FIG. 6. Competitive inhibition of the binding of [³H]GDP and [³H]GTP to bacterial p21 by GDP and GTP, respectively. Binding was determined as described with 2 μM [³H]GDP (*A*) or [³H]GTP (*B*) and unlabeled GDP or GTP as indicated. Reactions were initiated with 0.1 μg of HPLC-purified p21, which bound 3.65 pmol of [³H]GDP (*A*) and 2.65 pmol of [³H]GTP (*B*) in the absence of the unlabeled nucleotides GDP (○) and GTP (●).

We thank Dr. H. Weissbach, Dr. A. Skalka, and Dr. S. Ochoa for critically reviewing the manuscript, Dr. Ed Lee's group for fermentation of *E. coli* cells, and Ms. Juli Farruggia for typing the manuscript.

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