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Comparative Biochemical Properties of p21 *ras* Molecules Coded for by Viral and Cellular *ras* Genes

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In earlier studies, we molecularly cloned a normal cellular gene, c-ras^H-1, homologous to the v-*ras* oncogene of Harvey murine sarcoma virus (v-ras^H). By ligating a type c retroviral promotor to c-ras^H-1, we could transform NIH 3T3 cells with the c-ras^H-1 gene. The transformed cells contained high levels of a p21 protein coded for by the c-ras^H-1 gene. In the current studies, we have purified extensively both v-ras^H p21 and c-ras^H p21 and compared the in vivo and in vitro biochemical properties of both these p21 molecules. The p21 proteins coded for by v-ras^H and c-ras^H-1 shared certain properties: each protein was synthesized as a precursor protein which subsequently became bound to the inner surface of the plasma membrane; each protein was associated with guanine nucleotide-binding activity, a property which copurified with p21 molecules on a high-pressure liquid chromatography molecular sizing column. In some other properties, the v-ras^H and c-ras^H p21 proteins differed. In vivo, approximately 20 to 30% of v-ras^H p21 molecules were in the form of phosphothreonine-containing pp21 molecules, whereas in vivo only a minute fraction of c-ras^H-1 p21 contained phosphate, and this phosphate was found on a serine residue. v-ras^H pp21 molecules with an authentic phosphothreonine peptide could be synthesized in vitro in an autophosphorylation reaction in which the gamma phosphate of GTP was transferred to v-ras^H p21. No autophosphorylating activity was associated with purified c-ras^H-1 p21 in vitro. The results indicate a major qualitative difference between the p21 proteins coded for by v-ras^H and c-ras^H-1. The p21 coded for by a mouse-derived oncogenic virus, BALB murine sarcoma virus, resembled the p21 coded for by c-ras^H-1 in that it bound guanine nucleotides but did not label appreciably with $^{32}P_i$. The forms of p21 coded for by other members of the *ras* gene family were compared, and the results indicate that the guanine nucleotide-binding activity is common to p21 molecules coded for by all known members of the *ras* gene family.

Work in our laboratory has led to the description of a family of normal vertebrate genes, which have been designated *ras* genes (7, 9). The *ras* gene family has been shown thus far to contain two distinct members, c-*ras* Harvey (c-ras^H) and c-*ras* Kirsten (c-ras^K). These two c-*ras* genes are named for their nucleic acid homologies to the oncogene in two retroviruses, Harvey murine sarcoma virus (MuSV) and Kirsten MuSV, isolated originally by passaging mouse retroviruses through rats. Two other oncogenic retroviruses also contain *ras* genes: the Rasheed sarcoma virus (RaSV) isolated from cultured rat cells (19, 27) and the BALB-MuSV isolated from a chloroleukemia of an aging BALB/c mouse (18). The *ras* gene of each of these latter viruses is closely related to the ras^H genes. Both the ras^H and ras^K oncogenes code for related 21,000-dalton proteins (23).

All of the vertebrate cells that we have thus far examined contained at least one copy of a c-*ras* gene homologous to v-ras^H and one copy of a c-*ras* gene homologous to v-ras^K (9). Cells from species of vertebrates contain additional copies of c-*ras* genes homologous to either v-ras^H or v-ras^K (6, 9).

In a variety of normal cells, p21 molecules are detected at relatively low levels as the products of c-*ras* genes (10, 14). Recently, we molecularly cloned members of the c-ras^H gene family from normal rat DNA. By ligating a murine retrovirus long terminal repeat (LTR) to the c-ras^H-1 gene, we could transform NIH 3T3 cells with the c-ras^H-1 gene (8). These cells expressed markedly elevated levels of p21 as compared with most normal cells, and we thus concluded that increased expression of a normal gene product

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was one way in which malignant transformation of cells could occur.

Earlier, we noted differences in phosphorylation between the p21 molecules found in normal cells and those coded for by the v-*ras* genes of Harvey or Kirsten MuSV (14). However, since the levels of p21 expressed in normal cells are low, we were not able to compare unambiguously the biochemical properties of any p21 coded for by a c-*ras* gene with those of a p21 coded for by a v-*ras* gene.

In this manuscript, we report a biochemical comparison in vivo between the v-ras^H p21 and the c-ras^H-1 p21 in transformed NIH mouse fibroblast cells expressing high levels of each of these proteins, and in vitro after purification of each p21 from these cells. Each p21 is a guanine nucleotide-binding protein, but a major qualitative difference in phosphorylation is apparent between the p21 proteins coded for by v-ras^H and c-ras^H-1. The p21 coded for by the BALB-MuSV is similar to that coded for by the c-ras^H-1 gene.

MATERIALS AND METHODS

Cells. NIH 3T3 cells are a contact-inhibited mouse cell line, previously described (12). These cells were transformed by a DNA clone, H-1 (11), of Harvey MuSV, and the resulting transformant, expressing v-ras^H p21, is called NIH-13 A clone 2. NIH cells transformed by c-ras^H-1 are called NIH-18 A clone 404 or NIH clone 17; clones 404 and 17 both synthesize high levels of c-ras^H-1 p21 (8). NIH cells were also transformed by either BALB-MuSV or RaSV, and these transformants have been previously described (1, 19, 27).

For growing cells from which to purify p21, either clone 2 or clone 404 was grown in 690-cm^2 plastic roller bottles (Corning) at 37°C in Dulbecco modified Eagle medium containing 10% fetal calf serum. Each roller bottle yielded between 1.0 and 2.0 g of cells (wet weight). For the purification of v-ras^H p21 we began with 600 g of clone 2 cells, and for c-ras^H-1 p21 we began with 200 g of clone 404 cells.

Metabolic labeling of cells. Cells were labeled with [^{35}S]methionine or $^{32}P_i$ as previously described (23). However, the method for lysing the labeled cells was modified from previously published procedures. After the cells were labeled, the medium containing the isotopes was aspirated, and the cells were washed once on the plastic petri dishes at 4°C with phosphate-buffered saline. The cells were scraped with a rubber policeman into phosphate-buffered saline and collected by centrifugation at 1,000 × *g* at 4°C for 5 min. For approximately 1×10^6 to 2×10^6 cells, 1.0 ml of lysis buffer was added, and the cells were blended with a Vortex mixer to lyse them and to extract the p21. The lysis buffer contained 1.0% Nonidet P-40 (NP40), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5, 5.0 mM magnesium chloride, 1% Trasylol, and 1 mm dithiothreitol (DTT). After the cells were lysed, the extract was spun at 100,000 × *g* for 45 min at 4°C and the supernatant was saved. Labeled lysates were either subjected to immunoprecipitation immediately or frozen in portions at −170°C. Lysates thawed more than once were discarded.

Immunoprecipitation. Labeled p21 molecules were identified by precipitation with monoclonal antibodies to p21 (10). In these studies, two monoclonal antibodies were used: one, YAG-238, recognizes the p21 molecules coded for by known ras^H genes but not those coded for by ras^K genes. The other monoclonal antibody, YAG-259, recognizes p21 molecules coded for by known ras^H and ras^K genes. Although the specificity of binding in these assays is clear, we have not yet determined affinity constants for the various antibodies for different *ras* gene p21 molecules. The immunoprecipitations were carried out at 4°C in the lysis buffer described above without DTT, and the antibody-p21 complexes were collected with the aid of Formalin-fixed *Staphylococcus aureus* containing protein A, as previously described (13). The p21-*S. aureus* pellets were washed five times with 1.0 ml of a buffer containing 20 mM HEPES (pH 7.5), 1.0 mM EDTA, 1.0% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.1 M sodium chloride. The washed pellets were subjected to SDS-polyacrylamide gel electrophoresis and fluorography as previously described on 10.5% acrylamide gels (23).

Purification of p21. The same procedure was used for purification of v-ras^H p21 and c-ras^H-1 p21. The procedure was originally modeled after procedures published by Spector et al. (24) so that we could compare the properties of p21 which we found with various members of his reported protein kinase cascade. Approximately 100 g per week of either clone 2 or clone 404 cells were grown, and the membranes from these cells were prepared and stored at −170°C. The membranes were prepared by the polyethylene glycol (PEG)-dextran two-phase extraction procedure described by Brunette and Till (5).

The purified membranes from 100 g of cells were suspended in approximately 10 to 20 ml of the suspension buffer (0.25 M sucrose, 30 mM imidazole [pH 7.5], and 1 mM DTT) and stored at −170°C before being used. As detailed below, we later extracted p21 from these membranes. In using the Brunette-Till procedure, we tried various modifications to achieve higher yields or greater purity of the p21. These variations included: performance of the two-phase extraction procedure with no divalent cations, with 1, 10, or 100 mM $CACl_2$ instead of $ZnCl_2$ or 1.0, 10.0, or 100 mM $MgCl_2$ instead of $ZnCl_2$, and with PEG 8000 versus PEG 6000. None of these variations yielded substantially more p21 per 1.0 cm^3 of packed cells or per milligram of protein. In addition, no difference in the yield of p21 extracted was noted whether the membranes were stored at −170°C or extracted immediately. From 600 g of clone 2 cells, we obtained approximately 5,000 mg of total membrane protein. The protein determinations were performed by the method of Lowry et al. (15) in the presence of 1.0% SDS in the phenol reagent to maximize solubilization of membrane proteins. From 200 g of clone 404 cells we obtained 2,500 mg of total membrane protein.

For solubilization of p21 we centrifuged the membranes at 100,000 × *g* for 20 min and washed them once in 40 mm Tris-hydrochloride (pH 7.5)–6 mM DTT. Membranes were then suspended at a concentration of 5.0 mg/ml in 20 mM Tris-hydrochloride (pH

7.5)–1.0% NP40–3 mM DTT and solubilized by being drawn through a 25-gauge needle in a 1.0-ml syringe 10 times. The solubilized material was centrifuged at 100,000 × *g* for 20 min at 4°C, and the supernatant was saved. Approximately 40% of the protein was recovered.

In the course of defining this procedure, we attempted several modifications before solubilizing the p21 with NP40. We washed the membranes in 4.0 M sodium iodide, 2.0 M sodium chloride, or 0.01 or 0.02% deoxycholate. None of these treatments improved the purity of the subsequently solubilized p21, and the high salt washes did not remove any appreciable amount (less than 10%) of the bound p21 molecules or p21 activity.

The p21 solubilized from membranes with NP40 was concentrated and purified by adding sufficient saturated ammonium sulfate to bring the solution to 40% saturation, and the suspension was centrifuged at 100,000 × *g* for 20 min at 4°C. The precipitate floated to the top of the tube. After the precipitate was removed, the protein was suspended in approximately 60 to 70% of the original volume in 0.05% NP40–20 mM Tris (pH 7.5)–3 mM DTT. The resuspended proteins were dialyzed at 4°C against 100 volumes of the same buffer with two changes over 24 h and stored in portions at −170°C.

The dialyzed protein was adsorbed to DEAE-52 by using 1.0 ml of DEAE-52 per 10 to 20 mg of protein in a 50-cm^3 conical plastic test tube. After shaking the protein with the DEAE-52 for 10 to 15 min at 4°C, the resin was washed five times with 0.01% NP40–1 mM EDTA–20 mM Tris-hydrochloride (pH 7.5). Approximately 10 ml of wash buffer was used per 1.0 g of resin. A second set of five washes was carried out with the buffer containing 0.01% NP40, 1 mM EDTA, and 25 mM potassium phosphate (pH 6.8). The p21 was eluted with the same buffer containing 0.2 M potassium phosphate and stored at −170°C after being concentrated by dialysis tubing coated with PEG 8000.

The DEAE-52-purified p21 was dialyzed for 4 to 5 h against 100 volumes of 2 mM EDTA–10 mM HEPES (pH 6.8) to reduce the phosphate concentration before the extract was adsorbed to phenyl-Sepharose. No detergent was added to the p21 samples at this purification step; we cannot exclude the possibility that some NP40 may still have been bound to p21 from the earlier purification steps. The protein solution, approximately 10 mg, was brought to 1.4 M ammonium sulfate, and the solution was then adsorbed to 5.0 to 6.0 cm^3 of phenyl-Sepharose. The column was washed with the same buffer, and the p21 was eluted with 0.1 M ammonium sulfate in the same buffer. Without dialysis, the p21 was concentrated 5- to 10-fold in dialysis tubing coated with PEG 8000. The concentrated protein was brought to 50% glycerol concentration and stored at −20°C, at which temperature it was stable indefinitely.

The phenyl-Sepharose-purified p21 was applied to a TSK 2000 sizing column (Beckman), and the proteins were separated by high-pressure liquid chromatography (HPLC). Samples of 0.20 ml were applied to the column, and fractions of 0.20 ml were collected. The column was equilibrated and eluted with 4°C buffer containing 50 mM HEPES (pH 6.8), 0.2 M sodium chloride, 0.1 mM EDTA, and 5% glycerol. Test tubes chilled to 4°C until just before use were used, and the entire column run was completed within 20 to 25 min. As we collected each set of 10 tubes, the collected fractions were stored at 4°C in an ice water bath. Assays for p21 activity were performed immediately. The p21 activity could be stabilized for 24 to 48 h by adding glycerol to the fractions to a concentration of 20%. Activity was not stable after longer storage. For analyzing the purity of p21 derived from the TSK column fractions, samples of the column fraction were lyophilized, and then the dried samples were dissolved in SDS-containing buffer and subjected to SDS-polyacrylamide gel electrophoresis. The SDS (Pierce Chemicals) was filtered through a 0.45-μm filter (Millipore) before use. Gels were analyzed after being stained with silver nitrate by the procedure of Morrissey (16).

Guanine nucleotide-binding assay. The [^{3}H]GDP binding assay to detect p21 was performed as previously described (20) with a few modifications. Monoclonal antibody YAG-238 was used as a source of antibody to p21, and protein A Sepharose coated with rabbit immunoglobulin prepared against rat immunoglobulin G (Cappel Laboratories) was used. All sera were heated for 30 min at 56°C before use. A 1-pmol amount of [^{3}H]GDP or [^{3}H]GTP equalled 8,200 cpm. Autophosphorylation of p21 with γ-^{32}P-labeled GTP (ICN) was done as previously described (22) at 37°C in 0.10-ml reaction mixtures containing 50 mM Tris-hydrochloride (pH 7.5), 2 mM magnesium chloride, 0.01% NP40, and 5×10^{-6} M γ-^{32}P-labeled GTP (300 Ci/mmol). The reaction was terminated by adding 0.020 ml of 50% trichloroacetic acid (TCA) to a final concentration of 8%; 10 μg of bovine serum albumin was added as carrier. The samples were spun in an Eppendorf centrifuge at 17,000 × *g* for 20 min. The tubes were drained, and the samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. ^{32}P-labeled bands were cut and counted by Cerenkov counting in an LS-250 Beckman scintillation counter.

RESULTS

In vivo studies. In earlier experiments, we showed that v-*ras*^{H} p21 is first synthesized as a prop21 molecule in the cytoplasm of Harvey MuSV-infected cells (22). The prop21 is processed to p21 and to a phosphothreonine form of p21, designated pp21. The p21 and pp21 are bound to the inner surface of the cellular plasma membrane of cells (22, 26). We examined the biosynthesis of c-*ras*^{H}-1 p21 in similar experiments (Fig. 1). Two foci independently induced by c-*ras*^{H}-1 were examined. When cells from each focus were labeled with [^{35}S]methionine for various periods of time, we observed both prop21 and p21 molecules. In other experiments (data not shown), the prop21 was found in the supernatant fraction of centrifuged, non-detergent-treated cell extracts, whereas the p21 remained bound to membranes. These results are similar to what we observed (22) for v-*ras*^{H} prop21 and p21. We next labeled transformed cells with [^{35}S]methionine or $^{32}P_i$ and then examined the lysates for p21 by immunoprecipitation

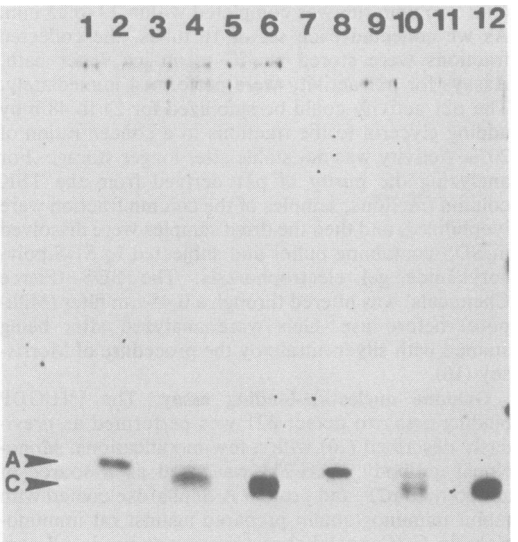


FIG. 1. Biosynthesis of c-*ras*^{H}-1 p21. NIH 3T3 cells transformed by c-*ras*^{H}-1 (8) were labeled for 15 min (lanes 1, 2, 7, and 8), 4.0 h (lanes 3, 4, 9, and 10), or 24 h (lanes 5, 6, 11, and 12) with 3.0 ml of methionine-free Dulbecco medium containing 2% dialyzed fetal calf serum and [^{35}S]methionine at a concentration of 200 μCi/ml (specific activity, 565 Ci/mmol). Extracts of whole cells were prepared, and samples of each extract, containing approximately 10×10^6 TCA-precipitable cpm, were subjected to immunoprecipitation as previously described (21). Immunoprecipitates were analyzed by electrophoresis in SDS-polyacrylamide gels, and bands were visualized by autoradiography for 18 h. Lanes 1–6, Clone 18A; lanes 7–12, clone 17, an independently derived focus from transfections with LTR-linked c-*ras*^{H}-1 DNA (8). A, prop21; C, p21. Odd-numbered lanes were precipitated with control antiserum; even-numbered lanes were precipitated with monoclonal antibody YAG-238 against *ras*^{H} p27 (16).

(Fig. 2). The p21 and pp21 forms of v-*ras*^{H} are apparent (Fig. 2, lanes 4). Only one [^{35}S]methionine band was seen in cells transformed by c-*ras*^{H}-1 or BALB-MuSV (Fig. 2A, lanes 6 and 8). In phosphate-labeled cells (Fig. 2B) the prominent v-*ras*^{H} pp21 was seen (lane 4), whereas only small amounts of pp21 were seen in c-*ras*^{H}-1 cells (lane 6), and no pp21 was detected in BALB-MuSV cells (lane 8). The pp21 in v-*ras*^{H}-transformed cells represented 20 to 30% of the mass of the labeled p21 molecules, whereas the pp21 to p21 ratio in c-*ras*^{H}-1-transformed cells was at least 10-fold less. There was much less p21 in cells transformed by BALB-MuSV, which may account for our inability to detect any pp21. However, it is clear that the p21 coded for by BALB-MuSV incorporated much less $^{32}P_i$ than did the p21 coded for by v-*ras*^{H}. In this respect, the p21 coded for by BALB-MuSV resembled that coded for by c-*ras*^{H}-1.

The phosphoamino acid(s) labeled in the c-*ras*^{H}-1 p21 was examined (Fig. 3). The phosphoamino acid comigrated with the phosphoserine marker in a two-dimensional analysis. We previously showed and have reconfirmed (data not shown) that the phosphoamino acid of v-*ras*^{H} pp21 is phosphothreonine. Thus, these results indicate a clear qualitative difference between the p21 proteins coded for by v-*ras*^{H} and c-*ras*^{H}-1. In other studies (data not shown), we examined the phosphoamino acid in the p29 of RaSV and again detected only phosphoserine. Thus, the p21 coded for by the v-*ras*^{H} and v-*ras*^{K} genes can be converted to a phosphothreonine, pp21, which represents a high proportion of p21 molecules in cells transformed by Harvey or Kirsten MuSV. In contrast, this form of p21 was not detected in cells transformed by c-*ras*^{H}-1 BALB-MuSV or RaSV. We have not yet been able to obtain sufficient c-*ras*^{H}-1 pp21 to compare its labeled serine phosphopeptide with the phosphothreonine peptide of v-*ras*^{H} p21.

In vitro properties of v-*ras* ^{H}p21. To understand the basis for the difference in phosphorylation observed between v-*ras*^{H} and c-*ras*^{H}-1 p21, we undertook purification of each p21 from NIH 3T3 cells transformed by each of these two genes.

As p21 represents a relatively small percentage of the total cell protein (see below), we elected to purify membranes from cells transformed by v-*ras*^{H}, since virtually all of the p21 molecules have been detected in association with the plasma membrane. Putative p21 molecules were solubilized with nonionic detergent from whole cells or from membranes prepared from an equal portion of cells, and the extracts were assayed for p21 by the GDP binding assay with a monoclonal antibody which detects *ras*^{H} p21 molecules (Fig. 4). The specific binding activity of p21 solubilized from membranes was approximately 10-fold higher than that of p21 solubilized from whole cells. The total yield of p21 prepared from membranes varied from 50 to 80% of that solubilized from a comparable amount of whole cells. Thus, we elected to prepare and store (see above) membranes from large amounts of cells and then to purify v-*ras*^{H} p21 from these membranes.

The summary of the purification procedure is shown in Table 1. We purified v-*ras*^{H} p21 between 1,000- and 3,000-fold from whole cells. The overall yield of activity was approximately 20%. We employed a relatively simple purification scheme which used batch elutions from resins until the last step of purification. The hydroxylapatite step which we had used previously was omitted because of great variability in quantitatively adsorbing p21 to hydroxylapatite.

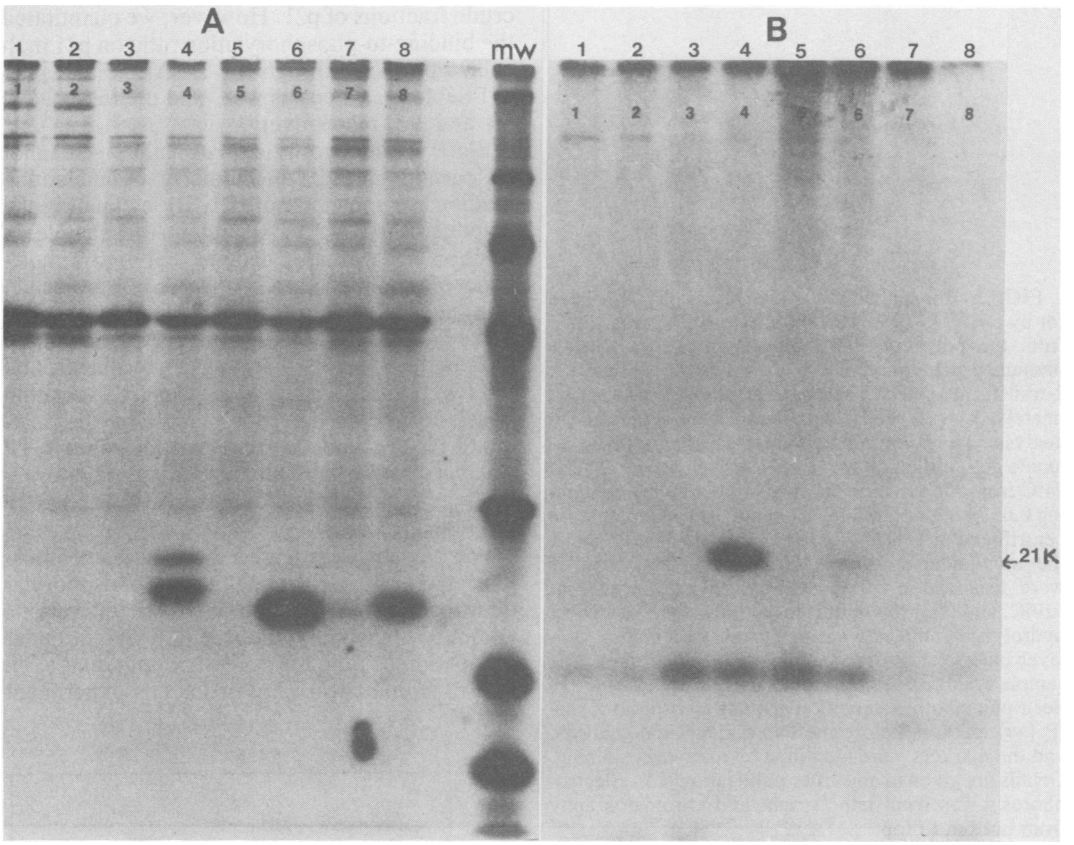


FIG. 2. Analysis of p21 and pp21 coded for by v-*ras* and c-*ras*^{H}-1 genes. NIH 3T3 cells or NIH 3T3 cells transformed by v-*ras*^{H}, c-*ras*^{H}-1, or BALB-MuSV were labeled for 24 h at 37°C with [^{35}S]methionine or $^{32}P_i$ as described in the text. Approximately 10×10^6 TCA-precipitable cpm of each extract was precipitated with 0.005 ml of a control rat monoclonal antibody against a retroviral *env* gene product (kindly supplied by Linda Wolff, (National Cancer Institute) or with rat monoclonal antibody 238, which precipitates p21 coded for by *ras*^{H} genes (10). (A) Extracts labeled with [^{35}S]methionine; (B) extracts labeled with $^{32}P_i$. Lanes: 1, NIH 3T3 cells, control serum; 2, NIH 3T3 cells, antiserum 238; 3, v-*ras*^{H} cells, control serum; 4, v-*ras*^{H} cells, antiserum 238; 5, c-*ras*^{H}-1 cells, control serum; 6, c-*ras*^{H}-1 cells, antiserum 238; 7, BALB-MuSV cells, control serum; 8, BALB-MuSV cells, antiserum 238. Molecular weight markers (mw) are shown. The markers above and below p21 (21K) are 25,000 and 18,000, respectively.

This step was replaced with a DEAE-cellulose step. Major purification was achieved with phenyl-Sepharose chromatography; in other studies (not shown) with octyl-Sepharose, elution of active p21 was not reproducible. The final step in the current procedure was an HPLC molecular sizing column (Fig. 5). We monitored each fraction of the column for p21 molecules with silver stain (Fig. 5A) and for p21 activity by both guanine nucleotide-binding activity (Fig. 5C) and p21-associated, GTP-specific autophosphorylating activity (Fig. 5B). In the fractions obtained from this sizing column, the p21 could be visualized as a doublet on the silver-stained gel (Fig. 5A, lanes 9, 10, and 11; fractions 9, 10, and 11 represent column fractions 62, 63, and 64 shown in Fig. 5C).

The level of pp21 was greatest in the fractions, where it was partially separated from the p21. Two prominent high-molecular-weight bands (Fig. 5A) were not present in the p21 preparation, since they were only present in the lane which contained only sample buffer. We therefore believe that they represent contaminants in the SDS. The molecular weight markers represent 50 ng of each protein visualized with the silver stain. Based on the intensity of the stained marker, we estimate that we were seeing between 5 and 20 ng of v-*ras*^{H} p21 (Fig. 5A, lanes 9, 10 and 11). Importantly, all of the GDP-binding activity detected from the column (Fig. 5C) and all of the p21 GTP-specific phosphorylating activity (Fig. 5B) comigrated with the stained p21 (Fig. 5A). The molecular weight

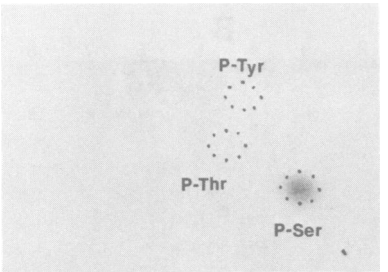


FIG. 3. Phosphoamino acid analysis of pp21 coded for by c-*ras*^{H}-1. pp21 coded for by c-*ras*^{H}-1 was eluted from the polyacrylamide gel shown in Fig. 2 after immune precipitation of ^{32}P-labeled extracts of c-*ras*^{H}-1-transformed cells. The pp21 detectable from approximately 5×10^7 cells was obtained as sufficient for analysis. The immunoprecipitated and SDS-polyacrylamide gel-purified c-*ras*^{H}-1 pp21 was eluted for 24 h at 24°C from the crushed gel slices into a buffer containing 0.05 M ammonium bicarbonate. The samples were centrifuged at 17,000 × *g* for 15 min at 4°C to remove the acrylamide and then lyophilized. The samples were dissolved in 6.0 M HCl, hydrolyzed for 4.0 h at 110°C, and then relyophilized to remove the acid. The hydrolyzed samples were analyzed on cellulose thin-layer plates for phosphoamino acids. The radioactive sample was mixed with unlabeled phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) markers before the two-dimensional analysis, and the markers were identified by ninhydrin staining. Details are given in an earlier publication (23). Electrophoresis was from left to right and chromatography from bottom to top.

calibration of the HPLC column in this region is not fully linear, and thus we have not shown molecular weight markers. However, p21 eluted in the range of 20,000 to 25,000 daltons. It is difficult to be certain about the relative biochemical activity of p21 versus pp21, but the binding and phosphorylating activity peaks seemed to comigrate with the p21 rather than with the pp21. When fractions were assayed for GTP-binding activity, the activity also comigrated with the GDP-binding activity (data not shown).

To further quantitate the binding and phosphorylation activity, we excised the phosphorylated p21 bands from the gel, and the label incorporated by the phosphorylated bands was counted by Cerenkov counting. For each sample of p21, the ratio of [^{3}H]GDP to $^{32}P_i$ in pp21 was calculated for each fraction in the peak. The ratio was 3.6 in fraction 60, 2.9 in fraction 61, 3.4 in fraction 62, and 3.0 in fraction 63. These results for the HPLC-purified p21 indicate that the binding and phosphorylating activities are intrinsic properties of v-*ras*^{H} p21. We could detect the autophosphorylating activity in preparations of p21 that had not been passed through the DEAE-52 column, but we could not obtain linear kinetics for autophosphorylation in earlier crude fractions of p21. However, we quantitated the binding-to-phosphorylation ratio on p21 molecules purified through the DEAE-52 and phenyl-Sepharose steps as well, and the ratios were 3.1 and 3.5, respectively.

Since we had so little v-*ras*^{H} p21, we cannot be certain of the stoichiometry of nucleotide binding per molecule of p21. By estimating the amount of p21 (5 to 10 ng) in the sample of fraction 62, we can calculate that 1 to 2 molecules of nucleotide are bound per p21 molecule. The half-maximal extent of binding was observed at 5×10^{-9} M GDP or GTP. A Scatchard plot analysis of this binding was not linear, and thus we cannot calculate a formal dissociation constant. We do not understand why the Scatchard plot was not linear. However, when GTP was used as the substrate at 4°C, more than 90% of the bound nucleotide was GTP, not GDP (unpublished data).

We further investigated the kinetics of phosphorylation by performing the autophosphorylation reaction in reaction mixtures of various volumes. Phosphorylation of p21 was in linear proportion to time for at least 60 min, and a 30-min time point was chosen for this experiment

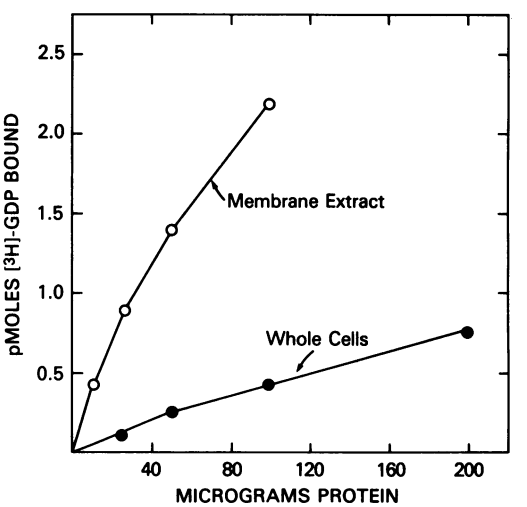


FIG. 4. p21 activity in whole cells and membranes. Clone 2 cells were grown to 90 to 100% confluence in plastic roller bottles. After the cells were collected, the p21 was extracted from 2.0 ml of packed cells and the protein concentration was determined by the method of Lowry et al. (5). Membranes were prepared by the PEG-dextran procedure (5) from a parallel sample of 2.0 ml of cells. The p21 was extracted from 25 mg of membranes, and the protein concentration of the resulting extract was determined. Procedural details for each step and for the [^{3}H]GDP binding assay carried out on each extract are described in the text. One picomole represents 8,200 cpm. Symbols: ●, whole cells extract; ○, membrane extract.

TABLE 1. Purification of v-*ras* p21 and c-*ras* p21

p21 source[a] and purification step	Total amt of p21 (mg)	Sp act (U)[b]	Degree of purification	Total units[c] (% recovery of activity)
v-*ras*^{H}				
Membrane extract	2,294	3.4	1.0	7,799 (100)
Ammonium sulfate (0–40%)	816	10.8	3.2	8,812 (110)
DEAE-52	55	56.3	16.5	3,097 (39)
Phenyl-Sepharose	~1.5	~1,020	~300	1,530 (20)
c-*ras*^{H}-1				
Membrane extract	1,000	2.0	1.0	2,000 (100)
Ammonium sulfate	480	6.6	3.3	2,948 (130)
DEAE-52	35	51.5	25.7	1,802 (90)
Phenyl-Sepharose	<1.0	ND[d]	ND	830 (42)

[a] The amount of membrane protein used as starting material was 5,000 mg for v-*ras*^{H} p21 and 2,500 mg for c-*ras*^{H}-1 p21.

[b] The specific activity (1 U) of p21 is expressed as picomoles of [^{3}H]GDP bound per milligram of protein.

[c] Total units equals total milligrams of protein times units of p21. The total units of c-*ras*^{H} p21 after the phenyl-Sepharose step was calculated by the activity per volume and the total volume of p21 recovered.

[d] ND, Not determined: protein concentration too low.

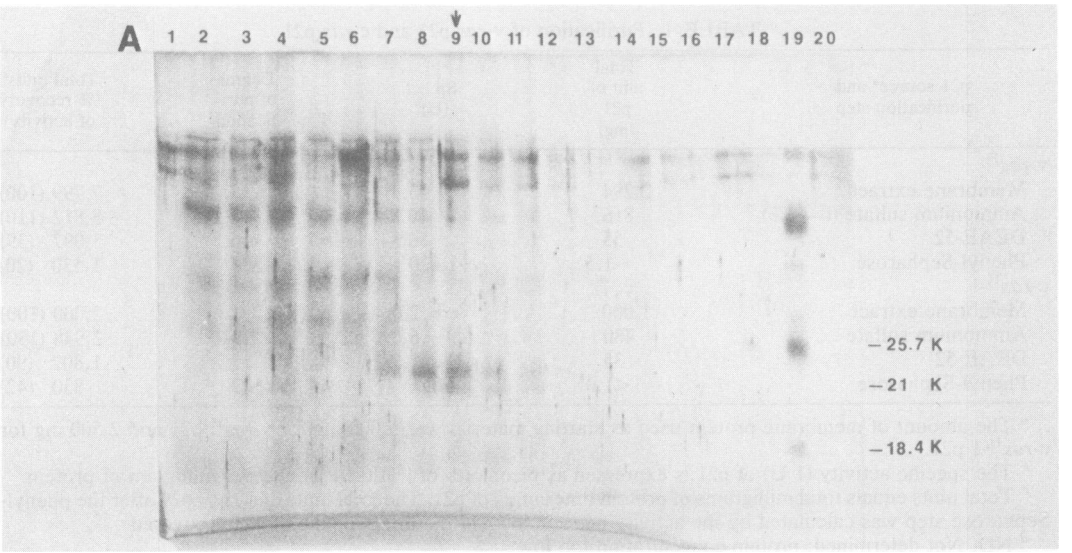
(Fig. 6). Equivalent rates of phosphorylation were obtained whether phosphorylation was performed in a volume of 0.05, 0.2, or 0.8 ml. The bands were excised and counted by Cerenkov counting, and the actual values of incorporation were 182 cpm/0.05 ml, 215 cpm/0.2 ml, and 225 cpm/0.8 ml (averages of triplicate determinations). Since p21 migrated as a monomer on the HPLC sizing column, the results suggest that the autophosphorylation reaction is an intramolecular reaction. We confirmed that a single phosphothreonine peptide is phosphorylated in vitro (22) and that the same peptide carries the phosphate as in vivo-labeled v-*ras*^{H} pp21 (21) (data not shown). Thus, we conclude that the formation of v-*ras*^{H} pp21 from v-*ras* p21 is a GTP-specific autophosphorylation. Only a small fraction of p21 was converted to pp21 in vitro, and we do not understand what the rate-limiting variable is in this reaction. In addition, we have not yet found an exogenous substrate for the GTP autokinase activity of v-*ras*^{H} p21.

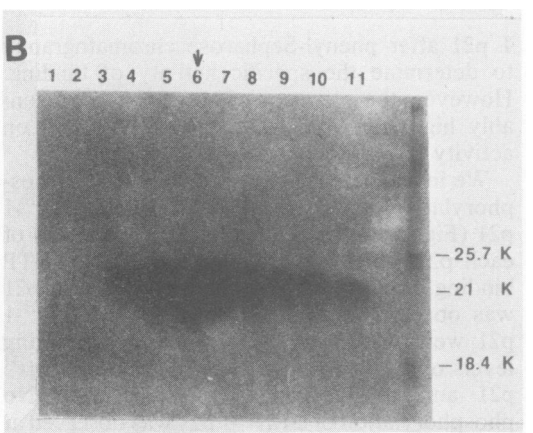
In vivo properties of c-*ras*H p21. We next turned our attention to the p21 coded for by the c-*ras*^{H}-1 gene. Membranes were prepared from NIH 3T3 cells or NIH 3T3 cells transformed by a strain of Rous sarcoma virus or by c-*ras*^{H}-1 p21. The membranes were solubilized with NP40 as described above and assayed for GDP-binding activity (Fig. 7). The p21 of c-*ras*^{H}-1 could also be detected in the guanine nucleotide-binding assay by using the monoclonal antibody to *ras*^{H} p21. We purified the c-*ras*^{H}-1 p21 through the ammonium sulfate, DEAE-52, and phenyl-Sepharose column steps (Table 1) and found that the c-*ras*^{H}-1 p21 could be purified by the same procedure as, and with yields virtually identical to, v-*ras*^{H} p21. We had too little c-*ras*^{H}-1 p21 after phenyl-Sepharose chromatography to determine the specific activity of binding. However, the recovery of activity was reasonably high. Recovery was calculated based on activity of p21 per volume of each extract.

We investigated the binding versus autophosphorylating activity of v-*ras*^{H} p21 and c-*ras*^{H}-1 p21 (Fig. 8). Over a range of concentrations of each p21 which gave equivalent GDP or GTP binding, no autophosphorylation of c-*ras*^{H}-1 p21 was observed. When v-*ras*^{H} p21 and c-*ras*^{H}-1 p21 were mixed in the presence of saturating levels of GTP substrate, no inhibition of v-*ras*^{H} p21 autophosphorylation was observed. No phosphorylation of c-*ras*^{H}-1 p21 was observed at 0 or 24°C or when [γ-^{32}P]ATP was used. We conclude that c-*ras*^{H}-1 p21 does not possess the autophosphorylating activity of v-*ras*^{H} p21, although each ras gene product does have guanine nucleotide-binding activity. These in vitro results correlated with the in vivo labeling results in that no phosphothreonine pp21 form of c-ras^H-1 p21 was detected. We do not know what enzyme phosphorylates c-ras^H p21 to form the small amounts of c-ras^H-1 phosphoserine pp21 (Fig. 2B and 3).

DISCUSSION

The transforming ability of many oncogenic retroviruses has been shown to be due to their acquisition of normal host cell genes (2, 3, 7). These viral genes are commonly referred to as oncogenes, and a number of normal host cell genes homologous to viral oncogenes have been molecularly cloned. We will refer to the host cell homologs of viral oncogenes as protooncogenes (25) for the rest of the discussion. In two cases,





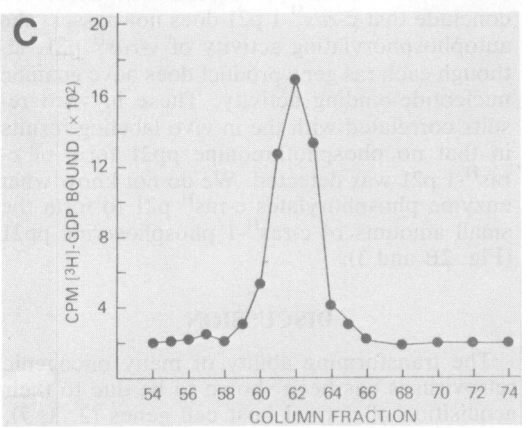


FIG. 5. Molecular sieve analysis of v-*ras*^{H} p21 and its biochemical activities. (A) Silver stain. An 0.20-ml portion of p21 purified through the phenyl-Sepharose purification step, containing approximately 30 μg of protein, was applied to a TSK 2000 molecular sieve chromatography column, and the column was eluted with 4°C buffer as detailed in the text. A flow rate of 0.6 ml/min was used, and fractions of 0.2 ml were collected. The samples were then electrophoresed on a 10.5% polyacrylamide gel, and the proteins were visualized by being stained with silver nitrate. Samples (0.08 ml) of each of the fractions were lyophilized and then dissolved at 95°C for 5 min in 0.04 ml of a mixture containing 0.1 M Tris-hydrochloride (pH 6.8), 20% glycerol, 1.0% SDS, 0.14 M β-mercaptoethanol, and 0.1% bromophenol blue. Gel fraction 9 (arrow) represents fraction 62 from the column run shown in panel C; the column fractions in lanes 1 through 8 represent fractions 54 through 61, and those in lanes 10 through 13 represent fractions 63 through 70. Lane 20 had no added sample. Lane 19 contains molecular weight markers. (B) Autophosphorylation. Samples of 0.02 ml of the same fractions were assayed for GTP-specific phosphorylation as described in the text. The samples were then electrophoresed on SDS-polyacrylamide gels, and the bands were identified by autoradiography. The arrow indicates fraction 62 from the column shown in panel C. Lanes 1 through 5 represent column fractions 57 through 61, and lanes 7 through 11 represent column fractions 63 through 67. (C) [^{3}H]GDP binding. Samples of 0.05 ml of the same fractions were assayed for [^{3}H]GDP-binding activity as described in the text. One picomole represents 8,200 cpm.

the protooncogenes were ligated in vitro to a retroviral LTR, and this LTR-protooncogene chimeric molecule can induce malignant transformation of NIH 3T3 cells (4, 8, 17). For the LTR c-*mos*, the results showed that a normal protooncogene can function as an oncogene. However, normal expression of the c-*mos* gene has not been detected. Therefore, the positive re-

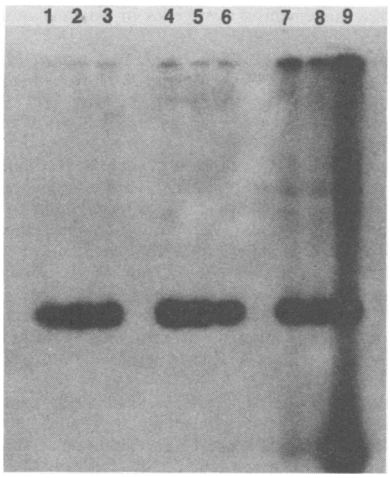


FIG. 6. Effect of reaction mixture volume on phosphorylation of v-*ras* p21. Approximately 1.0 to 2.0 μg of protein containing 0.01 ml of v-*ras* p21 purified through the phenyl-Sepharose purification step was assayed for GTP-specific autophosphorylation at 37°C for 30 min in reaction mixtures of either 0.05, 0.2, or 0.8 ml. The phosphorylated pp21 was recovered by TCA precipitation and centrifugation and then subjected to electrophoresis in an SDS-polyacrylamide gel as described in the text. Lanes: 1, 2, and 3, pp21 from 0.05-ml reaction mixture; 4, 5, and 6, pp21 from 0.2-ml reaction mixture; 7, 8, and 9, pp21 from 0.8-ml reaction mixture.

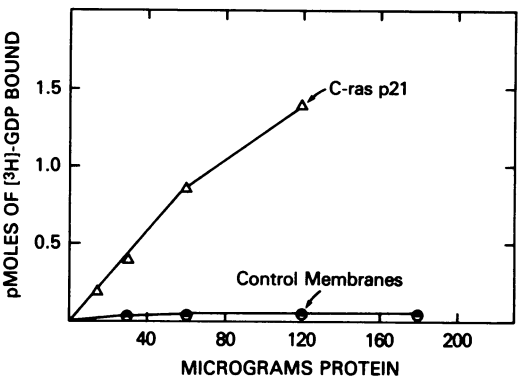


FIG. 7. [^{3}H]GDP-binding activity of c-*ras*^{H}-1 p21. Membranes were prepared from NIH 3T3 cells, Rous sarcoma virus-transformed NIH 3T3 cells, and c-*ras*^{H}-1 transformed NIH 3T3 cells (8), and the p21 was extracted as described in the text. The Rous sarcoma virus-transformed NIH 3T3 cells were a gift from Geoff Cooper, Boston, Mass. The concentration of protein was determined in each sample, and the samples were assayed for [^{3}H]GDP-binding activity. One picomole represents 8,200 cpm. Symbols: △, c-*ras*^{H}-1 NIH 3T3 membrane extract; ○, Rous sarcoma virus-transformed NIH 3T3 membrane extract; ●, NIH 3T3 membrane extract.

sults obtained with LTR c-*ras*^{H}-1 have further implications, since c-*ras*^{H}-1 is expressed as RNA and p21 molecules in a wide variety of normal cells. The results with c-*ras*^{H}-1 thus have indicated that enhanced expressed of a protooncogene normally expressed at low levels can lead to malignant transformation. These results, then, are consistent with a dosage model of oncogenesis, a hypothesis which argues that an increased level of a given oncogene product by itself can cause oncogenesis.

In the current studies, we have examined the biochemical properties of the p21 gene products of c-*ras*^{H} and v-*ras*^{H} genes to discern what biochemical properties of p21 are necessary for its oncogenic activity and to compare the properties of v-*ras*^{H} p21 and c-*ras*^{H} p21. Our results indicate that the p21 coded for by either v-*ras*^{H} or c-*ras*^{H} is a guanine nucleotide-binding protein. This conclusion is based on the facts that the binding activity copurifies with p21 molecules and that the activity is detectable with monoclonal antibodies to p21. Thus, we have confirmed our earlier results which relied on crude preparations of p21 and polyclonal anti-p21 antibodies contained in rat serum (23). We can also conclude that the p21 molecules coded for by each of the cellular or viral *ras* genes thus far examined are guanine nucleotide-binding proteins. This list so far includes p21 coded for by v-*ras*^{K}, v-*ras*^{H}, RaSV (20), BALB-MuSV (E. Scolnick, unpublished data), and rat c-*ras*^{H}-1.

Earlier studies indicated that a GTP-specific

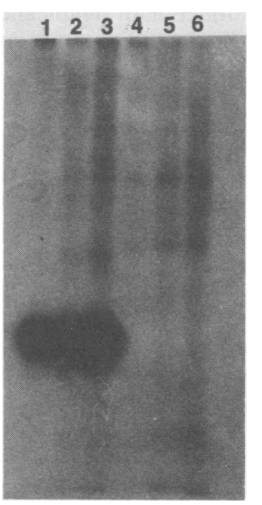


FIG. 8. Comparison of in vitro phosphorylation of c-*ras*^{H}-1 p21 and v-*ras* p21. Various amounts of each p21 were assayed for [^{3}H]GDP- or [^{3}H]GTP-binding activity, and amounts of each p21 which bound 0.25, 0.5, or 1.0 pmol of GTP or GDP were assayed for GTP-specific autophosphorylation as described in the text. The samples were then collected by precipitation with 10% TCA and centrifugation and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. Lanes: 1–3, v-*ras* p21; 4–6, c-*ras*^{H}-1 p21.

autophosphorylating activity was associated with v-*ras*^{H} p21. The current studies have confirmed this and also indicated that the p21-to-pp21 GTP-autophosphorylating activity is an intrinsic property of v-*ras*^{H} p21 and is probably an intramolecular reaction. The fact that the same phosphothreonine residue is phosphorylated both in vivo and in vitro supports the hypothesis that the GTP-specific autophosphorylation reaction occurs in vivo with v-*ras*^{H} p21. However, the physiological role of the p21-to-pp21 autophosphorylation reaction and the functional role of v-*ras* pp21 remain obscure. The c-*ras*^{H} p21 does not carry out the p21-to-pp21 reaction, and the p21 of BALB-MuSV does not label appreciably with $^{32}P_i$. Thus, neither this autophosphorylation reaction nor phosphothreonine pp21 can be required for the oncogenic activity of p21. A serine form of p21 representing a very small molar amount of p21 has been detected on p21 coded for by rat c-*ras*^{H}-1 and RaSV and with v-*ras*^{H} and v-*ras*^{K} (unpublished data). The GTP-specific autophosphorylating activity and phosphothreonine form of p21 have only been detected in v-*ras*^{K} p21 and v-*ras*^{H} p21.

Thus, the only biochemical activity presently known to be common to all *ras* p21 molecules is the guanine nucleotide-binding activity. The binding activity is a convenient assay by which to measure and purify *ras* p21 molecules. However, to assess the biological significance of this activity we will need to determine the nucleotide binding site(s) on *ras* p21 molecules and to assess their functional significance by genetic approaches. Such studies are in progress.

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