Expression of the Kirsten *ras* Viral and Human Proteins in *Escherichia coli*

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The expression vectors pINIII-A and pINIII (*lpp p_s*) were used to construct plasmids which direct the synthesis in *Escherichia coli* of the Kirsten *ras* viral (v-Ki-*ras*) and human cellular (c-Ki-*ras*) oncogene products as fusion proteins containing 9 and 10 extra amino acids, respectively, at their N termini. Authenticity of the bacterially produced proteins was determined by immunoprecipitation and immunoblot analyses with *ras*-specific monoclonal antibodies. After induction with isopropyl- β -D-thiogalactopyranoside, the viral protein represented approximately 20% of the total cellular protein. The majority of the protein was found in the postsonication low-speed centrifugation pellet. The synthesized viral protein was active in GTP binding, as judged by autophosphorylation and photoaffinity labeling assays.

The oncogenes of the Harvey and Kirsten murine sarcoma viruses (v-Ha-*ras*- and v-Ki-*ras*) encode similar proteins of approximately 21 kilodaltons (p21), which differ primarily at their C-terminal ends (6). These viral genes encode substitutions at amino acid positions 12 and 59 of the normal cellular homologs (c-Ha-*ras* and c-Ki-*ras*) which are responsible for their oncogenic activity (6). Activated human cellular homologs have been found that correspond to both the Harvey and Kirsten viral oncogenes. Transformation potential was found to be mediated by changes in amino acid 12 (2, 22, 26, 29, 31, 35) or 61 (35, 36). The c-Ki-*ras* gene possesses two fourth coding exons (exons 4A and 4B) that by a mechanism of alternative splicing result in the synthesis of two p21 proteins that differ in their carboxy-terminal domains (2, 26). While v-Ki-*ras*-encoded p21 contains the amino acid sequence corresponding to exon 4A, the most abundant c-Ki-*ras* p21 species in humans contains the amino acid sequence corresponding to the 4B exon (2, 26).

Purified viral p21 from mammalian cells has been studied physically (8, 23, 33) and biochemically (24, 25). The protein is posttranslationally modified and is found associated with the inner surface of the plasma membrane. These posttranslational modifications result in an altered mobility on sodium dodecyl sulfate (SDS)-polyacrylamide gels. The protein possesses guanine nucleotide-binding activity and undergoes autophosphorylation of the threonine residue at position 59. No other phosphorylated cellular target has been identified in vivo. The proteins encoded by cellular *ras* genes have been shown to possess biochemical activities similar to those of their viral counterparts.

Viral (13, 14, 27) and human normal and transforming (10, 19) Ha-*ras* proteins and the viral Kirsten (30) *ras* proteins have been expressed in *Escherichia coli*. The *E. coli*-synthesized proteins retained their biochemical activities (10, 14, 19, 27). Studies on the *E. coli*-expressed protein have established that transforming *ras* proteins possess slower GTP-hydrolyzing activity than their normal counterparts (9, 15, 19, 28). It is this difference which has been postulated to be responsible for their oncogenicity.

We report here the synthesis of the viral (4A) and the human (4B) K-*ras* p21 proteins in *E. coli*. When induced,

20% of the total cellular protein was v-Ki-*ras*. The protein was judged to be active by autophosphorylation and by GTP photoaffinity labeling. We are now in a position to purify these proteins for biochemical analysis.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, and *E. coli* DNA polymerase I (large fragment) were purchased from Bethesda Research Laboratories or New England Biolabs. Isopropyl-1-thio- β -D-galactopyranoside (IPTG) was obtained from Sigma Chemical Co. Rabbit anti-rat antibodies were bought from Cappel Laboratories. [³⁵S]methionine (1,435 Ci/mmol) and [α -³²P]GTP (420 Ci/mmol) were supplied by Amersham Corp. [γ -³²P]GTP (650 Ci/mmol) was purchased from ICN. Monoclonal antibody Y-259, specific for *ras* proteins, was a generous gift from E. Scolnick (Merck Sharp and Dohme Research Laboratories), and monoclonal antibody anti-p21^{ras} (panreactive) was purchased from Cetus Corp.

Bacterial strains and plasmids. The *E. coli* strains used were JA221 (*hsdM*⁺ *hsdR* *leuB6* *lacY* *trpE5* F' *lacI*^q) and SB221 (*hsdR* *leuB6* *lacY* *thi* *recA* *trpE5*)/F' (*lacI*^q *proAB* *lacZYA*) (4). Plasmids used include pHiHi3 (6), which contains the viral Ki-*ras* oncogene (a generous gift from E. Scolnick); p3035, a pUC13 derivative containing a human c-Ki-*ras* full-length cDNA (18), obtained from Oncor; pINIII-A1 and pINIII-A2 (16); and pINIII (*lpp p_s*) (11).

DNA manipulations. Plasmid DNA was digested with restriction enzymes under the conditions recommended by the manufacturers. DNA fragments were separated on 5% acrylamide gels and isolated by electroelution. Ligations and transformations were done by published procedures (4). Plasmid isolation of appropriate clones was done as described previously (1).

Construction of Ki-*ras* expression plasmids. Plasmid pHiHi3 was digested with the restriction enzymes *Stu*I and *Hind*III to produce a 592-base-pair (bp) fragment containing the coding region of the viral Kirsten *ras* gene. This fragment contained 10 nucleotides upstream of its first ATG. The expression vector pINIII-A2 was digested with *Eco*RI, and the ends were made flush by treatment with DNA polymerase I (large fragment), and the vector was then digested with *Hind*III. The *ras*-containing fragment was ligated to the digested pINIII-A2, and the ligated DNA was used to

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transform JA221 cells. The A2 reading frame of the expression vector pINIII was chosen to place the gene in the correct reading frame with the initiation codon provided by the vector. The fusion protein contained six amino acids from the vector and three amino acids from the upstream sequences in the *ras* fragment, for a total of nine extra amino acids (see Fig. 2). Transcription of the cloned gene was mediated by the *lpp* and *lacUV5* promoters. Expression of the gene was regulated by the *lac* repressor produced by the vector. Ligation of the blunt *EcoRI* site of the vector to the *StuI* site of the insert reproduced an *EcoRI* site. The resultant plasmid was designated pETN100. pETN100 was digested with *EcoRI*, and the 563-bp fragment containing the Kirsten *ras* gene was isolated and ligated to the expression vector pINIII (*lpp p_s*), which had been digested with *EcoRI*. The pINIII (*lpp p_s*) vector was essentially similar to the pINIII vector used in the first construction, except that the -35 and the -10 regions of the lipoprotein promoter were altered to more closely resemble the consensus sequences for *E. coli* gene promoters (11). The final plasmid was designated pETN200.

p3035 is a pUC13 derivative containing a full-length cDNA corresponding to the 4B version of the human c-Ki-*ras* oncogene from the SW480 colon carcinoma cell line, which contains a valine mutation at codon 12 (2, 18). p3035 was digested with *HindIII* and *BamHI* (sites present in the pUC13 vector polylinker), and the resulting 1.1-kilobase fragment was inserted into the *HindIII* and *BamHI* sites of the pINIII-A1 expression vector. The *HindIII-StuI* fragment corresponding to the 5'-end sequences of the c-Ki-*ras* cDNA was deleted by digestion with these enzymes and ligation after filling in the *HindIII* sticky ends with DNA polymerase I (large fragment). This resulted in a recombinant plasmid which was designated pINIIIA-c-Ki-*ras*, containing an open reading frame for the p21 c-Ki-*ras* protein with 10 additional amino acids at its N terminus (see Fig. 2).

Synthesis of Kirsten viral *ras* protein in *E. coli*. SB221 cells containing the pETN200 plasmid were grown at 37°C in M9 medium (20) supplemented with 2% Casamino Acids, 2% glucose, thiamine (2 µg/ml), leucine and tryptophan (20 µg/ml each), 0.02% MgSO₄, and ampicillin (50 µg/ml). At a Klett-Summerson colorimetric reading of 10 or 40 with a blue filter, IPTG was added to a final concentration of 2 mM. When the cells reached a Klett-Summerson colorimetric reading of 100 or at the appropriate time points, the cells were harvested by centrifugation. Subcellular fractionation was done by procedures described previously (4). Briefly, the cells were pelleted, washed once in 10 mM phosphate buffer (pH 7.1), and suspended in 1 ml of the same buffer. The lysate was sonicated for 6 min in 30-s pulses. This suspension was subjected to low-speed centrifugation to remove aggregated material. The supernatant from this centrifugation was then subjected to a high-speed centrifugation at 105,000 × *g* for 2 h. The pellet contained the total membrane fraction, and the supernatant contained the soluble fraction.

Immunoblot analyses. Cells harboring the pETN200 or the pINIII (*lpp p_s*) vector were grown in supplemented M9 medium and induced with 2 mM IPTG for 1 h at an OD₆₀₀ of 1. One milliliter of the culture was spun down and suspended in 0.1 ml of sample buffer (62 mM Tris hydrochloride, pH 8.7, 0.7 M 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.005% bromophenol blue). The samples were boiled and analyzed on a 15% SDS-polyacrylamide gel and then electrophoretically transferred to nitrocellulose filters as described previously (32). The filter was washed twice for 20

min each with 3% milk in TBS (50 mM Tris hydrochloride, pH 7.5, 0.2 M NaCl). Anti-p21^{ras} panreactive monoclonal antibody was added to the solution and incubated overnight at 4°C. The filter was subsequently washed four times for 5 min each in 1% milk in TBS. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (IgG) was added to the solution and incubated for 90 min at room temperature. The immunoblot was then washed four times for 5 min each in TBS, and the bands were visualized in TBS containing 20% methanol, 0.06% chloro-1-naphthol, and 0.033% hydrogen peroxide.

Immunoprecipitations. Overnight cultures of *E. coli* JA221 harboring the pINIIIA c-Ki-*ras* vector were grown in supplemented M9 medium. Portions (1 ml) of the cultures were induced with 2 mM IPTG for 1 h at an OD₆₀₀ of 0.2 in the presence of 150 µCi of [³⁵S]methionine. The labeled cells were suspended in 5 ml of phosphate-buffered saline with 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride and sonicated six times for a total of 3 min. The samples were centrifuged, and samples containing 10⁶ cpm from the supernatant were taken for each immunoprecipitation. Incubations with antibodies were performed on ice. Forty microliters of rabbit anti-rat IgG was added and incubated for 1 h, and the unspecific precipitated material was cleared by incubation with protein A-Sepharose (Pharmacia) and low-speed centrifugation. Four microliters of monoclonal antibody Y-259 was added to the supernatant and incubated overnight; 40 µl of rabbit anti-rat IgG was then added and incubated for 1 h, followed by incubation with protein A-Sepharose (40 µl) for 40 min. After centrifugation, the pellets were washed and boiled for 3 min in sample buffer, and the supernatants were analyzed in a 12.5% SDS-polyacrylamide gel. The gel was fixed, stained with Coomassie blue, dried, and exposed to X-ray film at -70°C.

Biochemical activity of bacterially expressed Kirsten *ras* protein. Autophosphorylation of the p21 protein was done essentially as described previously (27). Briefly, the 50-µl reaction mixture contained 10 µl of cell lysate fractions, 25 µCi of [α-³²P]GTP, 1 mM ATP, 50 mM Tris hydrochloride (pH 8.0), 100 mM NaCl, 1% Triton X-100, and 5 mM MgCl₂. After incubation for 60 min at 37°C, the reaction was stopped by the addition of sample buffer. The samples were boiled and loaded onto a 17.5% polyacrylamide gel. The gel was stained, dried, and autoradiographed with XAR film as before.

GTP binding to p21 protein was carried out as described previously (27). Binding was assayed in the same reaction mixture used for autophosphorylation, but 25 µCi of [γ-³²P]GTP was used. Preincubation was allowed to proceed at 4°C for 1 h in the absence of light. The reaction mixture was then exposed to UV light for 30 min. Samples were treated with sample buffer, boiled, and loaded onto a 17.5% polyacrylamide gel. The gel was dried and exposed to X-ray film as described above.

RESULTS

Expression of the Kirsten viral and cellular *ras* proteins in *E. coli*. With the cloning strategy shown in Fig. 1, plasmid pETN200 was constructed. This clone was grown on minimal medium and induced with IPTG. Cells harboring pETN200 gave smaller colonies on IPTG agar plates, and induction in liquid medium slightly affected the doubling time. A new protein band was detected by SDS-polyacrylamide gel electrophoresis of whole cell lysates after a 1-h induction (see Fig. 3). An increase in molecular weight was

FIG. 1. Cloning strategy for construction of pETN200. Diagonal lines represent v-Ki-ras sequences; solid lines, pBR322 sequences; pINIII sequences are as described in reference 16. A^r, Ampicillin resistance gene.

FIG. 3. Expression in *E. coli* of v-Ki-ras protein. *E. coli* cells harboring the vector pETN200 were induced with IPTG at a Klett-Summerson colorimetric reading of 40. One-milliliter samples were taken at the indicated time points after induction and analyzed in a 17.5% acrylamide gel, followed by staining with Coomassie blue. The amount of each sample loaded onto the gel was normalized to the Klett-Summerson reading of 40. Lanes 1 through 7, Time zero and 1, 2, 4, 8, 12, and 24 h, respectively. Lane 8, molecular size markers: phosphorylase B (94.7 kDa), bovine serum albumin (65 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

expected due to the nine added amino acids at the N terminus (Fig. 2), and the new band had an apparent molecular weight of 26,000. This value was slightly larger than expected and may be due to a conformational or modification difference between the mammalian *ras* protein and the bacterial *ras* protein (19). After 24 h of induction, the amount of *ras* protein in the cell represented about 20% of the total cellular protein. This was calculated by integration of the scanned stained gels with a Shimadzu densitometer.

To determine the cellular location of the *ras* protein, *E. coli* cells harboring either pETN200 or the pINIII (*lpp p5*) vector were induced with IPTG as described in Materials and Methods. The cells were harvested, disrupted by sonication, and subjected to differential centrifugation. The majority of the *ras* protein was found in the low-speed centrifugation pellet, indicating that the protein occurs as an aggregate within the cell (Fig. 4). A small amount was found in the membrane fraction, but this was probably due to contamination of the low-speed centrifugation pellet with the membrane fraction. A comparison between the total cell lysate in lane 2 and the low-speed centrifugation in lane 4 showed that substantial purification could be achieved by this simple step.

To confirm the authenticity of the bacterially expressed protein as viral Kirsten *ras*, antibodies specific for mammalian p21^{ras} were used (Fig. 5). Total cellular *E. coli* protein extracts were analyzed by immunoblotting after SDS-

	vector	p21 ^{ras}
v-Ki-ras	Met lys gly lys ... glu phe leu leu lys met thr ----	
5'	---- <u>ATG</u> AAA GGG AAG ... GAA TT C CTG CTA AAA <u>ATG</u> ACT ----	3'
	---- <u>ATG</u> AAA GGG AAT TCC AAG CT C CTG CTG AAA <u>ATG</u> ACT ----	
c-Ki-ras	Met lys gly asn ser lys leu leu leu lys met thr ----	

FIG. 2. Expected nucleotide and amino acid sequence at the amino termini of the *ras* fusion proteins. The vector and p21^{ras} ATG codons are underlined. The nucleotide sequences were confirmed by DNA sequencing of the recombinant plasmids.

FIG. 4. Subcellular localization of the *E. coli*-expressed v-Ki-*ras* protein. *E. coli* cells were inoculated into medium and grown to a Klett-Summerson colorimetric reading of 10. At that time 2 mM IPTG was added. At a Klett-Summerson colorimetric reading of 100 the cells were centrifuged and fractionated as described in the text. The different cellular fractions were analyzed as described in the legend to Fig. 3. Lanes: 1, 3, 5, and 7, pINIII (*lpp p_s*); lanes 2, 4, 6, and 8, pETN200. Lanes: 1 and 2, total cell lysate; 3 and 4, aggregate fraction; 5 and 6, soluble fraction; 7 and 8, total membrane fraction; 9, molecular size markers as described in the legend to Fig. 3.

polyacrylamide gel electrophoresis. The 26-kilodalton (kDa) protein was recognized by the *ras*-specific antibody in IPTG-induced cells (Fig. 5B, lane 2), but not in the cells containing the vector alone (Fig. 5B, lane 1).

An expression vector (pINIII-c-Ki-*ras*) for the human c-Ki-*ras* protein was also constructed. After induction with IPTG of *E. coli* cells harboring this vector, a protein of 24-kDa was synthesized which was recognized by the Y-259 *ras*-specific monoclonal antibody (Fig. 6, lane 1). In contrast, this protein was not detected before induction (lane 3) or in cells containing the pINIII vector alone (data not shown).

GTP-binding activity of *E. coli*-expressed v-Ki-*ras* protein. To test the autophosphorylation activity of the 26-kDa protein, cell lysates were incubated with [α - 32 P]GTP (Fig. 7). Lane 2 represents the autophosphorylation activity found in

FIG. 5. Western blot of bacterially synthesized v-Ki-*ras* p21. Total cellular extracts of *E. coli* harboring the pETN200 (lanes 2) or pINIII (*lpp p_s*) (lanes 1) vector were electrophoresed and transferred to a nitrocellulose filter as described in the text. After the electroblot, the polyacrylamide gel was stained with Coomassie blue (A). The nitrocellulose filter was cut, and the area corresponding to the cell protein extracts was incubated with the *ras*-specific antibody, while the region corresponding to the protein molecular size markers (lanes M) was stained with amido black (B). The numbers indicate the sizes (in kilodaltons) of the protein standard markers.

FIG. 6. Immunoprecipitation of *E. coli*-expressed human c-Ki-*ras* p21. Total cellular extracts of *E. coli* harboring the pINIII-c-Ki-*ras* vector were analyzed by immunoprecipitation with (lanes 1 and 3) or without (lanes 2 and 4) Y-259 monoclonal antibody as described in the text, before (lanes 3 and 4) or after (lanes 1 and 2) induction with IPTG. Numbers indicate the position and size (in kilodaltons) of the size markers ovalbumin (43 kDa), α -chymotrypsinogen (25.7 kDa), β -lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa). The arrow points to p21.

the total cell lysate. Lane 4 represents the activity found in the soluble fraction, and lane 6 that of the insoluble low-speed pellet. The majority of the activity present in the total cell lysate was contributed by the activity in the insoluble low-speed pellet.

To directly show that the 26-kDa protein bound GTP, [γ - 32 P]GTP was incubated with the postsonication fraction of the cell lysates which contained the expressed *ras* protein. The reaction mixture was then exposed to UV light, which covalently crosslinked the GTP to the protein (Fig. 8, lane 2). From the results of the previous experiment, we knew that the majority of the activity would reside in this fraction. No GTP binding was observed when the extract was not exposed to the UV light (lanes 3 and 4) or when the extract was from cells containing the vector alone (lane 1).

FIG. 7. Autophosphorylation activity of *E. coli*-expressed v-Ki-*ras* p21. Autoradiogram of *E. coli* lysates treated as described in the text. Lysates were incubated with 25 μ Ci of [α - 32 P]GTP for 1 h at 37°C. Lanes 1 and 2, Total cellular lysates; lanes 3 and 4, cytoplasmic fraction; lanes 5 and 6, aggregate fraction. Lanes 1, 3, and 5 represent cellular fractions from cells harboring pINIII (*lpp p_s*). Lanes 2, 4, and 6 represent cellular fractions from cells harboring pETN200. Size markers are shown (in kilodaltons).

FIG. 8. Photoaffinity labeling with GTP of *E. coli*-expressed v-Ki-ras p21. Autoradiogram of *E. coli* lysates affinity labeled with [γ - 32 P]GTP for 1 h at 4°C and UV-irradiated for 30 min on ice. Lanes 1 and 2, low-speed centrifugation pellet; lanes 3 and 4, total cellular lysates not exposed to UV irradiation. Lanes 1 and 3 represent cellular fractions from cells harboring the pINIII (*lpp p_s*) vector. Lanes 2 and 4 represent cellular fractions from cells harboring pETN200. Size markers are shown (in kilodaltons).

DISCUSSION

We report here the synthesis of the Kirsten *ras* viral and human cellular proteins in *E. coli* by using expression vectors derived from the lipoprotein bacterial gene (16). When both genes were inserted into the pINIII-A vectors (pINIII-A-c-Ki-ras and pETN100), the *ras* proteins accounted for only about 1% of the total protein after induction with IPTG (Fig. 6, and data not shown). The expression vector pINIII (*lpp p_s*), which contains a promoter upmutation in the *lpp* gene sequences (11), was used to increase the expression level of the viral *ras* protein. After induction, up to 20% of the total bacterial protein was represented by the viral *ras* protein.

Owing to the vectors used, the expected location of the proteins was the cytoplasm. However, when cells harboring pETN200 were subjected to differential centrifugation, the majority of the protein was found in the aggregate pellet from the low-speed centrifugation.

Others working with the Harvey *ras* protein in *E. coli* have had various solubility problems. Fusion between *lacZ* and the *ras* protein produced a protein found in the soluble fraction (27). Authentic p21 protein was found in the soluble fraction by using a *p_L* promoter vector (10). However, a *p_L* vector resulted in the synthesis of an insoluble fusion cII-*ras* protein (14). In another two cases, authentic Ha-*ras* proteins were also found in the insoluble fraction within the cell (13, 19). The fusion v-Ki-*ras* protein synthesized by our vector could be found in the insoluble low-speed pellet 15 min after induction (data not shown). The added nine amino acids at the N terminus in our construction may have contributed to its insolubility. Alternatively, the Kirsten *ras* protein could be intrinsically more insoluble than the Harvey *ras* protein.

Due to its insoluble state, it was originally thought that the protein would not be active. It was assumed that activity would lie in the soluble fraction within the cell. However, this was not the case. The bulk of the activity present in the total cell lysate was contributed by the activity of the insoluble postsonication pellet. These results were in contrast to those of others, who reported that solubilization was necessary to directly examine GTP binding (15). The exact reasons why we found activity in the particulate fraction are unclear. Perhaps the differences lie in the intrinsic properties of the Harvey and Kirsten *ras* proteins. Alternatively, the

extensive sonication in our purification method may have disrupted the inclusion bodies enough to allow activity.

Both the autophosphorylation and GTP-binding activities were unique to the *E. coli* cells harboring pETN200. Cells harboring the vector alone did not exhibit significant activity. It was also clear that the bacterially produced protein was unable to transfer phosphate to any other bacterial protein to a significant extent. The conserved activities of the v-Ki-*ras*-synthesized protein are in agreement with the results obtained with bacterially produced viral Harvey *ras* protein (27). The nine extra amino acids at the amino terminus of the protein made in our studies did not affect its enzymatic activity.

Based on structural (amino acid sequence) and functional (GTP-binding and hydrolytic activities) homologies with other guanine nucleotide-binding proteins (elongation factor Tu, transducin, and G proteins) (17), it has been hypothesized that *ras* proteins could represent a class of signal-transducing proteins involved in the cellular response to external factors (21). The amino acid sequences that form the GTP-binding domain (17) as well as the carboxy-terminal cysteine which is required for membrane binding (34) are conserved among the different *ras* proteins. Yet the *ras* proteins all differ at their C terminus. Therefore, it may be reasonable to postulate that the normal *ras* proteins possess intrinsic differences in their in vivo physiological function(s) that could be determined by the dissimilarity at their carboxy terminus. This variance would lead to coupling with specific external signal membrane receptor systems. The availability of the 4A and 4B carboxy-terminal versions of the Kirsten *ras* proteins by expression in *E. coli* should allow analysis of the putative intrinsic differences between *ras* proteins.

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