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EXPRESSION OF INTACT Ki-ras p21 PROTEIN IN Escherichia coli

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We have constructed recombinant plasmids capable of expressing in Escherichia coli the intact ras p21 protein encoded by Kirsten murine sarcoma virus. The Ki-ras gene was inserted into an expression vector carrying the E. coli tryptophan promoter and E. coli lipoprotein transcriptional terminator. The resulting plasmids direct the synthesis of large quantities of p21 protein, which represented 20% of the total cellular protein. The Ki-ras p21 protein is immunoprecipitated with monoclonal antibody to p21, and exhibits guanine nucleotide binding activity and autophosphorylation activity. The purified Ki-ras p21 expressed in E. coli has shown to have intact N-terminal and C-terminal amino acid sequences predicted by the nucleotide sequences and migrate as ~23K in SDS/polyacrylamide gels.

Many of the transforming genes of human and rodent tumor cells, identified by NIH3T3 transfection assay, have been shown to be members of the ras family. Molecular cloning studies have identified three members of the ras gene family, Ha-ras, Ki-ras and N-ras, the classification depending on the extent of homology with oncogenes of Harvey (Ha) and Kirsten (Ki) sarcoma viruses and with the transforming gene found in the SK-N-SH neuroblastoma cell line (1). All members of the ras gene family encode immunologically related proteins of molecular weight 21,000 which have been termed p21s and these polypeptides diverge from one another in their carboxy terminal regions. In all cases so far examined, ras proteins are oncogenic when particular amino acid substitution occurs at position 12 or position 61 (2).

The availability of significant amount of purified ras proteins has been required to investigate the mechanism by which ras proteins induce malignant transformation. Expression of Ha-ras genes in E. coli and biochemical properties of the purified Ha-ras p21 proteins have been reported (3-7).

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In this paper, we report efficient expression of intact Ki-ras p21 protein in E. coli and the biochemical activities associated with p21 protein.

MATERIALS AND METHODS

Bacterial strains and media

E. coli K-12 strains, HB101 and SK2284 (8), were used for plasmid construction and p21 expression. LG broth (1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose) and MGC medium (M9 medium supplemented with 0.5% glucose and 0.5% casamino acids) were used for growth of E. coli strains.

Plasmid constructions and DNA sequencing

pHN12 is a derivative of the Kirsten murine sarcoma virus clone HiHi3 (9) provided by E. M. Scolnick. The character of pGEL1 and general method of plasmid construction were described previously (10). Restriction endonuclease fragment containing the SD sequence and 5'coding region were sequenced by M13 dideoxy chain termination method (11).

Expression of Ki-ras p-21 protein in E. coli and protein analysis

E. coli strains were grown overnight in LG broth containing 50μg/ml ampicillin. The culture was diluted 1:100 in MGC medium containing 50μg/ml ampicillin and incubated at 37°C. After growing to A_{550}=4~5, the cells were collected by centrifugation. Total cellular proteins dissolved in Laemmli's buffer (12) were analyzed by SDS/polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue.

Other Methods

Monoclonal anti-p21 antibody Y13-259 (13) was provided by E. M. Scolnick. Immunoprecipitation of lysates was performed as described previously (14). Detailed assay methods for the autophosphorylation activity and guanine nucleotide binding activity are described in the legends of Fig. 3 and 4.

RESULTS AND DISCUSSION

Construction of recombinant plasmids that direct the expression of intact Ki-ras p21 protein

Since the ras gene of Kirsten murine sarcoma virus has a StuI site at 10 nucleotides upstream of the initiation codon, we decided to construct recombinant plasmid which have insertion of intact Ki-ras p21 gene under the transcriptional control of E. coli promoter. The strategy of cloning the Ki-ras gene into an E. coli expression vector, pGEL1, is shown in Fig. 1. As the source of Ki-ras gene, we used pHN12, a derivative of the Ki-MuSV clone pHiHi3: in pHN12, a BamHI site was introduced into a region downstream near the termination codon of ras p21 protein. pHN12 was cleaved with StuI and BamHI, and the resulting 600-bp fragment containing the Ki-ras gene was purified. pGEL1 was cleaved with ClaI, followed by trimming the resulting 5'-protruding ends with E. coli DNA polymerase I. After digestion with Bam HI, the fragment containing the tandem trp promoter, the SD sequence of trp L

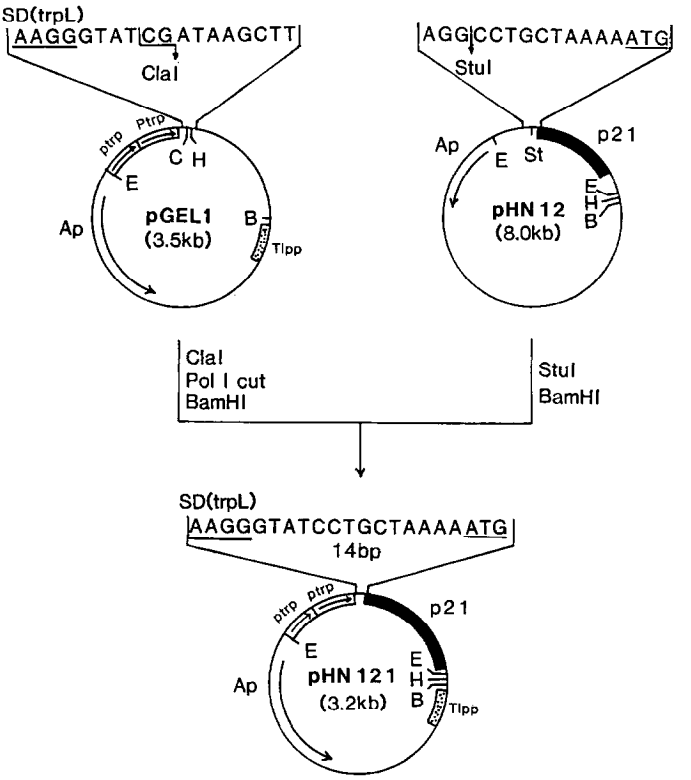

Fig. 1: Construction of pHN121. Restriction endonuclease sites shown are EcoRI, E; BamHI, B; ClaI, C; HindIII, H; StuI, St. Details are given in the text.

and the lpp transcriptional terminator was purified and ligated with the above 600-bp fragment containing the Ki-ras gene. The resulting plasmid, named pHN121, has a structure such that the tandem trp promoter and the SD sequence of trp L are located just upstream of, and the lpp transciptional terminater just downstream of the gene for Ki-ras p21 protein. The nucleotide spacing between the SD sequence and the ATG codon of pHN121 was 14bp which was confirmed by DNA sequencing analysis (data not shown). Another plasmid, pHN122, which has 16 nucleotides spacing between the SD sequence and the ATG codon was constructed in a similar way except that 3'-recessed ends of ClaI-digested pGEL1 were filled in with a Klenow fragment.

Synthesis of intact Ki-ras p21 protein in E. coli

An E. coli strain HB101 was transformed with pHN121 or pHN122. The resulting transformants were grown in MGC medium for 24 h. at 37°C. Cells

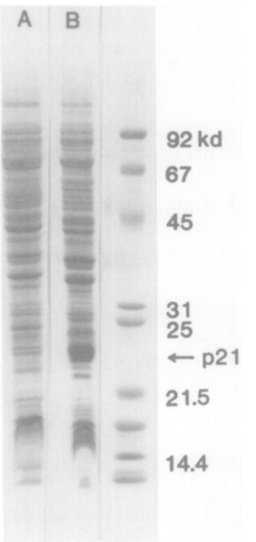

Fig. 2: Expression of Ki-ras gene in E. coli. The cells were analyzed by 12% SDS/polyacrylamide gel electrophoresis, followed by staining with Coomassie brilliant blue. lane A; E. coli with pBR322. lane B; E. coli with pHN121.

were collected and dissolved in Laemmli's buffer and 20μg of proteins were analyzed by SDS/polyacrylamide gel electrophoresis. As shown in Fig. 2, cells transformed with pHN121 gave a prominent band correponding to a protein of about 23K daltons. This protein was also present in the cells transformed with pHN122, while the amount of the protein accumulated was about half of that accumulated in the cells transformed with pHN121 (data not shown). This 23K protein was not detected in the cells transformed with pBR322 suggesting that it represents the product of the Ki-ras gene.

When pHN121 was introduced into an E. coli strain SK2284 in which polynucleotide phosphorylase is defecient (pnp), Ki-ras protein aggregated into inclusion bodies and constituted about 20% of the total cellular protein

Biological properties of Ki-ras protein expressed in E. coli

Supernatant fractions from E. coli cells lysed by freeze-thawing were immunoprecipitated with monoclonal antibody to p21. The ~23K protein was selectively precipitated with the monoclonal antibody suggesting that the ~23K protein expressed in E. coli is a ras protein.

The p21 ras proteins encoded by the ras genes of Harvey and Kirsten murine sarcoma viruses contain a threonine residue in position 59 that serves as the substrate for phosphorylation from the γ-position of GTP. We examined the autophosphorylation activity of the Ki-ras protein expressed in E. coli. The autophosphorylation activity was assayed according to the method of Lautenberger et. al.(3). The 23K protein expressed in E. coli with pHN121 retained the ^{32}P label indicating a covalent attachment of the γ-phosphate to the protein which is absent in extracts of cells transformed with pBR322 (Fig. 3).

The common biochemical property of ras proteins is their ability to bind guanine nucleotide, such as GTP, GDP, dGTP and dGDP. The polypeptide in E. coli cells were solublized in 7M urea solution and then renatured in buffer which contains 140mM urea. The guanine nucleotide binding activity was

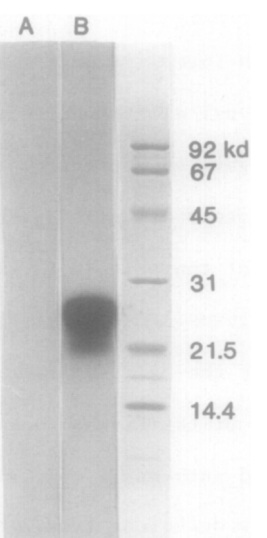

Fig. 3: Autophosphorylation activity of Ki-ras protein expressed in E. coli. Ki-ras protein was purified from the inclusion bodies inside E. coli cells. The inclusion bodies were separated by centrifugation after sonication of the cells. The pellet was then dissolved in Tris-HCl buffer containing 7M urea, and then proteins were renatured by dilution with 50mM HEPES buffer, pH7.5. The renatured Ki-ras protein (5μg) was added to a 100μl reaction mixture containing 50mM Tris-HCl buffer, pH8.0; 5mM $MgCl_2$; 1mM DTT and 1.5μM γ-^{32}P-GTP (19Ci/mmol). The reaction mixture was immuno-precipitated with monoclonal anti-p21 antibody Y13-259 and resolved by 14% SDS/polyacrylamide gel electrophoresis, followed by detecting with autoradiography. lane A; E. coli with pBR322. lane B; E. coli with pHN121.

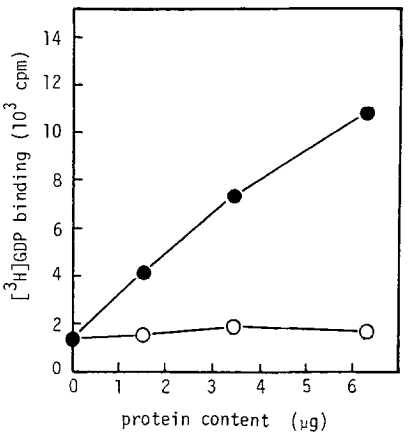

Fig. 4: ^{3}H-GDP binding activity of E. coli p21. The reaction mixture contained; 50mM Tris-HCl buffer, pH8.0, 5mM $MgCl_2$, 0.1M NaCl, 1mM DTT, 4μM ^{3}H-GDP (7.8Ci/mmol) and the renatured Ki-ras protein, and was incubated at 4°C for 10 min. After immuno-precipitation with monoclonal antibody Y13-259, the precipitates were washed three times with 10mM sodium phosphate buffer, pH7.5, containing 0.1M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100. The GDP-protein complexes were solubilized in aqueous counting scintillant ACS II (Amersham). ●; E. coli with pHN121. ○; E. coli with pBR322.

assayed in the presence of 30mM urea. As shown in Fig. 4, [^{3}H]GDP binding was proportional to the amount of protein solublized from the E. coli transformed with pHN121, whereas proteins from E. coli with pBR322 showed no detectable GDP binding activity.

These biochemical properties of the 23K protein expressed in E. coli with pHN121 are consistent with that of ras protein of Kirsten murine sarcoma virus.

Purification of Ki-ras protein

E. coli strain SK2284 containing pHN121 were grown in MGC medium containing ampicillin (50μg/ml) at 37°C for 9 h. Glucose (0.5%) was then added and the culture was incubated further for 15 h.

Ki-ras proteins expressed under these conditions are aggregated into inclusion bodies inside the E. coli cells and constituted about 20% of the total protein. These inclusion bodies were separated by centrifugation after sonication of cells for 30 min at 0°C. The washed pellet was then dissolved in Tris-HCl buffer containing 7M urea, and the proteins were renatured by dilution with 50mM HEPES buffer, pH7.5. Final purification by DEAE Sephacel column chromatography yields homogeneous Ki-ras protein as judged by

SDS/polyacrylamide gel electrophoresis. As described in a previous section, the Ki-ras protein expressed in E. coli migrates with an apparent molecular weight of ~23K. Direct amino-terminal amino acid sequence analysis of the purified Ki-ras protein expressed in E. coli revealed that the first 15 amino acid residues correspond completely with the predicted Ki-ras p21 sequence. In addition, carboxypepeptidase A treatment in 0.2M N-ethyl-morpholine-acetate buffer, pH8.0 at room temperature for 5 min yield methionine, isoleucine and valine which agreed with the amino acids predicted in the carboxy terminus of Ki-ras p21 sequence. Thus, the slower migration of Ki-ras p21 expressed in E. coli may represent the inability of post translational modification of p21 as suggested by McGrath et. al. in their studies: human- Ha-ras proteins produced in E. coli migrate more slowly than p21 from mammalian cells (5). Shih et. al. reported that p21 is synthesized in a precursor form, designated pro-p21, which migrated more slowly in acrylamide gels and may be processed by cleavage in its carboxy terminus (15). Shimizu et. al. proposed an interesting hypothesis that the particular sequence BNBB (basic amino acid, any amino acid, basic amino acid, basic amino acid), which is the common structure of the carboxy terminus of the ras gene products, is a proteolytic cleavage site (16). Purified ras proteins expressed in E. coli should make it possible to investigate the post-translational modification of p21 and their role in cell transformation.

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REFERENCES

1. Land, M., Parada, L. F. and Weinberg, R. A. (1983) Science 222, 771-778.
2. Taparowsky, E., Shimizu, K., Goldfarb, M. and Wigler, M. (1983) Cell 34, 581-586.
3. Lautenberger, J. A., Ulsh, L., Shih, T. Y. and Papas, T. S. (1983) Science 221, 858-860.
4. Stein, R. B., Robinson, P. S. and Scolnick, E. M. (1984) J. Virology 50 343-351.
5. McGrath, J. P., Capon, D. J., Goeddel, D. V. and Levinson, A. D. (1984) Nature 310, 644-649.

6. Lacal, J. C., Santos, E., Notario, V., Barbacid, M. Yamazaki, S., Kung, H., Seamans, C., McAndrew, S. and Crowl, R. (1984) Proc. Natl. Acad. Sci, USA 81, 5305-5309.
7. Gross, M., Sweet, R. W., Sathe, G., Yokoyama, S., Fasano, O., Goldfarb, M., Wigler, M. and Rosenberg, M. (1985) Mol. Cell. Biol. 5, 1015-1024.
8. Hantala, J. A., Bassett, C. L., Giles, M. H. and Kushner, S. R. (1979) Proc. Natl. Acad. Sci. USA 76, 5774-5778.
9. Ellis, R. W., DeFeo, D., Shih, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowry, D. R. and Scolnick, E. M. (1981) Nature 292, 560-511.
10. Sekine, S., Mizukami, T., Nishi, T., Kuwano, Y., Saito, A., Sato, M., Itoh, S. and Kawauchi, H. (1985) Proc. Natl. Acad. Sci. USA 82 4306-4310.
11. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5466.
12. Laemmli, U. K. (1970) Nature 227, 680-685.
13. Furth, M. E., Davis, L. J., Fleurdelys, B. and Scolnick, E. M. (1982) J. Virology 43, 294-304.
14. Shih, T. Y., Weeks, M. O., Young, H. A. and Scolnick, E. M. (1979) Virology 96, 64-79.
15. Shih, T. Y., Weeks, M. O., Gruss, P., Dhar, R., Oruszlan, S. and Scolnick, E. M. (1982) J. Virol. 42, 253-261.
16. Shimizu, K., Birnbaum, D., Ruley, M. A., Fasano, O., Suard, Y. Edlund, L., Taparowsky, E., Goldfarb, M. and Wigler, M. (1983) Nature 304, 497-500.