Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 282-288

COMPARISON OF THE CONFORMATION AND GTP HYDROLYSING ABILITY OF N-TERMINAL RAS p21 PROTEIN SEGMENTS

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Received February 6, 1989

SUMMARY: Conformational, GTP binding, and GTP hydrolytic studies are carried out with synthetically prepared N-terminal 34 residue segments (residues 2-35) of p21 ras oncogenic (12-Val) and non-oncogenic (12-Gly) proteins. It was found that these N-terminal regions bind nucleotides through their phosphate groups, and that substitution of valine for glycine produces a more pronounced α-helical structure and decreases the conformational flexibility. The glycine containing peptide, when compared to the valine containing analog, catalyses the hydrolysis of GTP 6 times more efficiently. Results suggest that restriction of conformational adaptation may contribute to the transforming capacity of the Val-12 p21 protein.

The ras gene family comprises three distinct genes: H-ras, K-ras, and N-ras. When activated, these genes are capable of inducing morphological and tumorigenic transformation of the NIH 3T3 cell line. The proteins encoded by the ras gene family with molecular weights of approx. 21,000 D (p21), reside in the plasma membrane, bind guanine nucleotide with high affinity, and possess a weak guanosine-5'-triphosphate (GTP) hydrolytic activity. In addition, viral ras proteins containing a threonine residue at position 59 exhibit a GTP dependent autokinase activity (For review, see reference 1). However, neither the normal cellular function of the p21 proteins nor the mechanisms underlying their transforming ability are clearly understood.

Although the GTP binding site in the ras p21 protein involves the two noncontiguous segments containing amino acid residues 5-22 and 109-120, the p21 proteins become oncogenic only when particular amino acid substitutions occur at positions 12, 13, 59 or 61 (cited in reference 1). Thus, it has been postulated that the replacement of amino acid at these positions alters the protein structure to a more transformationally active conformation, which, in addition, results in a reduction of the GTP hydrolytic activity (2-6). Furthermore, it has been proposed that this alteration in GTP hyd-

0006-291X/89 $1.50

rolytic activity may be an underlying mechanism involved in the acquisition of oncogenic potential (7-10). In order to examine the conformational difference between "non-transforming" and "transforming" ras p21 proteins, and to correlate the conformational change with hydrolytic activity, two 34 amino acid residue (residues 2-35) N-terminal segments, one having glycine and the other valine at position 12 were synthesized [T-E-Y-K-L-V-V-V-G-A-G(or V)-G-V-G-K-S-A-L-T-I-Q-L-I-Q-N-H-F-V-D-E-Y-D-P-T] and studied.

Experimental Procedures

Peptide Synthesis: Chemical synthesis of two 34 amino acid residue N-terminal segments, the Gly-peptide (12-Gly) and the Val-peptide (12-Val), were performed by the stepwise solid-phase approach and using HOBT (11). The Gly-peptide was purified on a Sephadex G-25 column eluted with 40% acetic acid, followed by semi-preparative HPLC on a C-8 reverse phase column. The Val-peptide was purified on a high pH-stable reverse phase column with an aqueous 0.1 M ammonium acetate buffer at pH 9 / acetonitrile gradient. Their structures were confirmed by peptide sequencing and amino acid analysis.

Equilibrium Dialysis: Measurements were performed in separate cells with a 2.5 ml half-cell volume (Spectrum Medical Industries, Inc.) using Spectrapore 6 membranes (1,000 dalton cutoff). Peptide concentrations from 3.94 nM to 13.7 nM with added (8-^{3}H)-GTP or (2,8-^{3}H)-ATP were placed in one of the chambers and allowed to stand for one hour at 37 °C against pH 7.4 buffer (50 mM Tris-HCl or 63 mM ammonium acetate). After equilibration at 21 °C for 12 hours, aliquots were taken from each chamber and tritium counts determined by liquid scintillation counter. Experiments were repeated twice and the results were averaged.

P-31 Nuclear Magnetic Resonance: In binding studies between the Gly-peptide and GTP the P-31 signals were monitored with and without the peptide in 0.1 M Tris-HCl at pH 7.4. For the Val-peptide 63 mM ammonium acetate buffer at pH 7.4 was used in similar experiments. Due to the poor solubility of the peptides the GTP / peptide ratio was kept at approximately 6. The relative hydrolysis rates of GTP in the presence of the peptides was monitored by integrating the increasing inorganic phosphate signal at 3.30 ppm as a function of time. The uncatalysed hydrolysis of GTP with time was subtracted for data presented in Figure 2.

Circular-Dichroism Spectropolarimetry: Spectra of the peptides, both in the presence and in the absence of GTP, were measured by a JASCO J-500A/DP-501N CD Spectropolarimeter. Eight scans were accumulated for each spectrum. Cell length was 2 mm. GTP/peptide molar ratio was approximately 25. Mean residue ellipticities were calculated using the CONTIN CD computer program (12).

Results and Discussion

Binding between Nucleotides and Peptides: Two methods were employed.

(A) Equilibrium dialysis experiments. Data are presented in Table I. Results show evidence for complex formation between GTP and the Val-peptide, as well as for the Gly-peptide. The binding constants with the 2 peptides are similar, being in the range of 5×10^{6} M^{-1}. They are, however, four orders

Table I

Binding Constants Obtained From Equilibrium Dialysis Experiments

Peptide	GTP (M^{-1})	ATP (M^{-1})
Gly-Peptide	$4.9 \pm 0.1 \times 10^6$	$6.1 \pm 1.0 \times 10^6$
Val-Peptide	$6.5 \pm 0.6 \times 10^6$	$1.2 \pm 0.4 \times 10^6$

of magnitude less than for the case of the native p21 protein (13). This difference indicates that the synthetic peptide segments contain some but not all the residues for proper binding to GTP. In line with this observation recent X-ray structure studies of the GDP-H-ras p21 complex (14) indicate that the C-terminal segment (amino acid residues 109-120) of the p21 proteins contributes to the formation of hydrogen bonds between guanine and the amino acid residues of the protein. In addition, regions of the synthetic N-terminal segments of p21 proteins will have greater flexibility than in the native protein, where restriction of mobility is more likely. Our finding, that the peptides bind GTP and ATP to a similar degree, are consistent with the crystal structure and related reports (15), in which the N-terminal domain of the p21 proteins is required for binding phosphate groups, with the C-terminal region contributing the nucleotide specificity.

Recently Fry et al. (16) used NMR spectroscopy to demonstrate that the interaction between ATP and the 44 residue segment of adenylate kinase was identical to those between intact adenylate kinase and ATP. Sequence similarity between the N-terminal segment of adenylate kinase (residues 1-44) and that of the ras p21 protein (residues 1-35) is not high, but both peptides contain a glycine-rich flexible loop and a group of hydrophobic amino acid residues. In case of adenylate kinase the hydrophobic pocket, formed by residues Ile-28, Val-29, His-36, and Leu-37, is known to interact with the adenine-ribose moiety of nucleotides, and the similar hydrophobic pocket containing residues Leu-23, Ile-24, His-27, and Phe-28 of the N-terminal segment of the ras p21 proteins may serve a similar role. In this regard, the crystal structure of the GDP-H-ras p21 protein complex reveals that the phenyl ring of Phe-28 is close and it is perpendicular to the guanine base. Apparently that type of non-bonding interaction contributes substantially to the structural stability and ligand binding capacity (17). Also, the Asp-31 is located proximately to the ribose sugar of GDP, as indicated by the x-ray data on the GDP-H-ras p21 protein complex. These factors may explain the larger binding constant (5×10^6 M^{-1}) of the p21 N-terminal segment (residues 2-35) with GTP

in comparison with that of adenylate kinase with ATP (binding constant = $3x10^4$ M^{-1}).

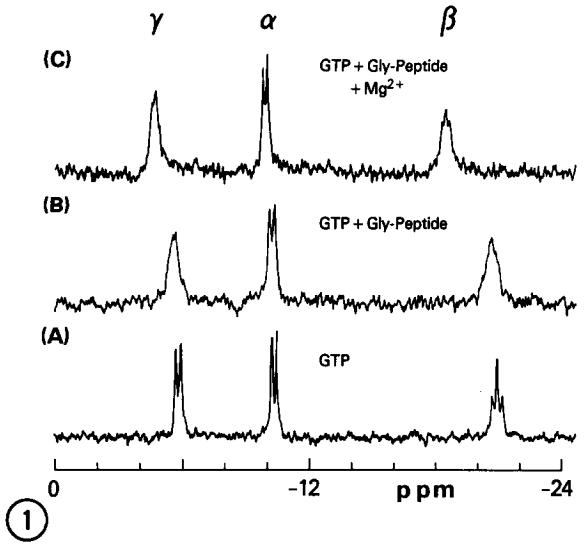
(B) NMR Spectroscopy. Upon addition of the Gly-peptide or Val-peptide to a solution containing GTP or ATP, the line widths of all three phosphorus-31 NMR resonances were broadened as shown in Figure 1 (similar data for Val-peptide not shown). These results, in agreement with the equilibrium dialysis data, suggest that a complex is formed between the synthetic peptides and the nucleotide, and also that the free nucleotide is in fast exchange with the bound form. Simultaneously, downfield shifts were observed for the β and γ phosphate resonances upon binding with the peptides. The larger changes in chemical shift were observed for the β phosphate resonance. These chemical shift changes can be interpreted as resulting from an interaction between a basic amino acid, such as Lys-16, which is in the vicinity of the β and γ phosphate groups of the bound GTP. Our interpretation is in agreement with the finding of Sigal at al. (18), that the substitution of Asp-16 for Lys-16 reduced the GTP binding affinity but not the base specificity of native length p21 proteins, and is also supported by the crystallographic structure of the GDP-H-ras p21 complex, in which the Lys-16 in the L1 loop lies close to the phosphate of the bound GDP (14).

We were unable to observe the separate entities of α, β, γ phosphate resonances for the free GTP versus the GTP-peptide complex, in contrast to the case of GTP-ras p21 complex reported by Rosch et al. (19). Thus, the rate of exchange in the peptide-GTP complex is faster than that of GTP-ras p21 complex within the NMR time scale (20). That is, the dissociation constant of the peptide GTP complex should be at least several orders of magnitude larger than 10^{-10}, reported earlier for the GTP-ras p21 complex (13).

Conformational Changes of Peptides by CD Spectroscopy:

Peptide conformational changes upon nucleotide binding were studied by CD spectroscopy, as reported in our preliminary account (21). The Gly-peptide in pH 7.4 buffer was found to exist mainly in the β-sheet conformation (51%) with no α-helical content. The mutant Val-peptide demonstrated a β-sheet/α-helical content of 39% / 13%. In experiments with GTP, nucleotide binding increased the α-helical content by 6-8% for both peptides. Whereas the β-sheet content of the Val-peptide was not changed significantly in the presence of GTP, for the Gly-peptide GTP binding reduced the β-sheet content from 51% to 32%.

These results indicate that the Gly-peptide is highly flexible in solution, and conformation will be easily changed in response to substrate binding. Substitution of glycine with valine at position 12 in the N-terminal segment



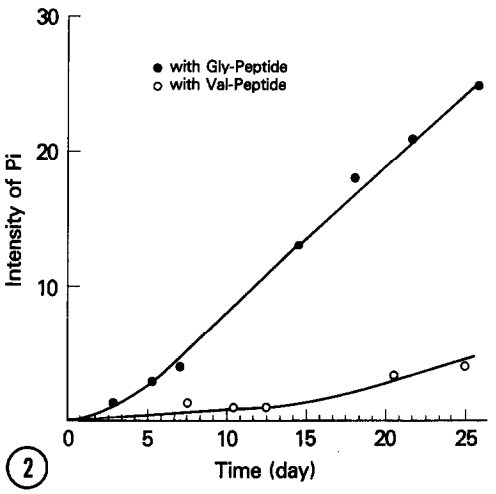


Figure 1. Phosphorus-31 NMR spectra at 80.99 MHz in 0.1 M Tris-HCl buffer at pH 7.4 at 37 °C. A. GTP alone; B. GTP with the Gly-peptide; and C. GTP with the Gly-Peptide in presence of Mg^{++}. GTP and Mg^{++} concentrations: 1.67×10^{-3} M. Peptide concentration: 2.53×10^{-4} M. Instrument: Varian XL-200 Spectrometer with 10 mm probe; 90° pulse time, 20 usec; 6000 transients per spectrum; pulse delay, 15 sec.; proton noise decoupled; and chemical shifts referenced to 80% aqueous phosphoric acid.

Figure 2. Relative rates of GTP hydrolysis in presence of the synthetic 34 residue Gly-peptide or the Val-peptide at 37 °C, at pH 7.4 in 0.1 M Tris-HCl buffer or in 63 mM ammonium acetate buffer, respectively. GTP and peptide concentrations are as described in Figure 1. The production of inorganic phosphate was monitored by P-31 NMR spectroscopy at 80.99 MHz.

of p21 proteins changes the conformation to a more α-helical structure, as predicted on the basis of the algorithms of Chou and Fasman (22). However, this conformational restriction upon substitution of glycine with valine does not weaken the binding strength of the Val-peptide to GTP as born out by the equilibrium dyalysis experiments (Table I). A common feature of the Gly-peptide and the Val-peptide on binding with GTP is that a part of the peptide has to adopt an α-helical conformation. Such a conformational change may bring the lysine residue, which is common in several functionally similar proteins (16), to interact with the phosphate group of bound GTP.

Hydrolysis of GTP:

It has been reported that the GTPase activity of p21 protein is high when glycine or proline is present in position 12 (5). Lacal et al. suggested that the catalytic site of p21 proteins may reside near residue 12 (23). Analysis of the x-ray structure of the GDP-p21 protein complex reveals that the loop L1, comprising residues 10-16 and located just below the β phosphate of GDP, make it the prime candidate for the GTPase catalytic site (14). In order to investigate whether the synthetic N-terminal segments of p21 proteins possess

GTPase activity and whether substitution of glycine at position 12 to valine will effect the hydrolytic activities, GTP was incubated with the Gly-peptide and separately with the Val-peptide. It was found that the Val-peptide hydrolysed GTP six times slower than the Gly-peptide (Figure 2). Although the rate of hydrolysis of GTP by these peptides is much slower than the rate observed with the native p21 proteins, the ratio of GTP hydrolytic activity of the Gly-peptide and the Val-peptide is comparable to that of the native p21 protein and of its mutant (10,15,17,24). These results, in conjunction with results from CD studies, strongly suggest that the transforming ability of p21 proteins may be enhanced because the conformation of the mutant p21 protein is locked into the "GTP bound mode" that then activates processes necessary for cell proliferation (25). Our results constitute the first experimental demonstration that differences exist in the solution conformation of the N-terminal segments non-transforming and transforming ras p21 proteins.

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