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Conformational changes occurring in N-*ras* p21 in response to binding of guanine nucleotide and metal ions probed by proteolysis performed under controlled conditions

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Variations in susceptibility to proteolysis by trypsin and chymotrypsin have been used as indicators of conformational changes taking place in N-*ras* p21 in response to ligand binding. It has been observed that changes occur in undenatured protein, rendering it more resistant to degradation, in the presence of divalent cations such as Mg^{2+} and Ca^{2+} (suggesting direct binding of metals to the polypeptide) and even more markedly in the presence of GDP and/or Mg^{2+} GDP. Monovalent cations (Na^+ or K^+) cannot substitute for Mg^{2+} or Ca^{2+}. Some capacity to bind guanine nucleotide is also retained by p21 treated with 7 M urea, as evidenced by increased resistance to proteolytic degradation, but the ability to bind divalent cations is irreversibly lost following denaturation. Protein prepared under denaturing conditions from a eukaryotic source, however, never regains the resistance to proteolysis shown by the bacterial p21 indicating irreversible changes in secondary and tertiary structure produced under these conditions.

Gene, N-*ras*; Protein, p21; GDP; Mg^{2+} binding

1. INTRODUCTION

ras genes encode a family of widely distributed highly conserved proteins of molecular mass about 21 kDa (p21) (see [1] for review). All these polypeptides bind guanine nucleotide with high affinity and appear to play a central role in the regulation of cell growth. Point mutations in *ras* genes leading to substitutions at amino acids 12, 13, 59, 61 and 63 in p21 have been observed in vivo and it has been found that transfection of these mutant genes causes transformation of cells in vitro (see for example [2,3]). Whilst the mutant proteins have similar affinities for nucleotide, their rates of GTP hydrolysis tend to be appreciably less than those of the wild type protein. It has been suggested that the transforming ability of mutant p21 gested that the transforming ability of mutant p21 may be attributable to this reduced rate of hydrolysis [3–5], although recent evidence has shown that, in some cases at least, there is no direct correlation between GTPase activity and transforming ability [6,7].

It seems reasonable to assume that *ras* proteins exert their influence on effector molecules within the cell through protein-protein interactions. This may be accomplished by the transmission of conformational changes which occur in response to nucleotide binding and/or hydrolysis. Recently, the crystal structures of mutant and wild type H-*ras* proteins have been determined to a resolution of 2.7 Å [8,9]. However, variations in the secondary structure of the polypeptide chain which occur on metal ion and nucleotide binding are not yet well resolved. In the present study we have examined some of these conformational changes occurring in p21 using resistance to proteolysis under closely defined conditions as a probe in an attempt to elucidate more fully the mode of action of p21.

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2. MATERIALS AND METHODS

2.1. *Preparation of* N-ras *p21*

N-*ras* protein was purified to homogeneity by methods outlined previously. p21 mutated at amino acid 61 (Lys → Gln) containing covalently bound lipid, was isolated from Ad2E1A + N-*ras* HER313A cells [10] by the ethanol precipitation method already described [11]. Protein precipitating between ethanol concentrations of 70 and 85% was resuspended in 9 M urea, 50 mM Tris-HCl, pH 7.5, 0.15 M β-mercaptoethanol at a concentration of 2 mg/ml.

Both wild type and mutant (Gly12 → Asp) N-*ras* was purified, after expression in *E. coli*, under non-denaturing conditions using ion-exchange and gel filtration chromatography as described [12]. Purified protein was dialysed extensively against 25 mM NH_4HCO_3, 0.1 mM β-mercaptoethanol and freeze dried. Bacterially expressed protein was also prepared under denaturing conditions, in which case the final gel filtration separation was performed using a column (1.6 × 110 cm) of Sepharose 6BCL equilibrated and eluted with 7 M urea, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.15 M β-mercaptoethanol.

2.2. *Proteolytic degradation of* N-ras *p21*

N-*ras* p21, at a concentration of approx. 1 mg/ml, was dialysed against appropriate buffers, as outlined in section 3 and then against 50 mM NH_4HCO_3 pH 7.9, 0.1 mM EDTA. Additions of $MgCl_2$, $CaCl_2$, NaCl, KCl, GDP or EDTA were made as described below followed by TPCK-treated trypsin (Worthington) or chymotrypsin (Sigma Chemical Co. Ltd.) at the appropriate concentrations. Digests were incubated at 30°C and aliquots (15 μl) withdrawn after the times indicated and added to 3% SDS, 6 M urea, 50 mM Tris-HCl, pH 7.5, 0.1 M β-mercaptoethanol. Samples were subjected to SDS-PAGE on 15% acrylamide gels run in the presence of 0.1 M Tris, 0.1 M Bicine, 0.1% SDS followed by Western blotting. N-*ras* p21 was visualised using monoclonal (E5.46) or polyclonal antibodies, both raised against eukaryotic N-*ras* p21 [11], and the Amersham International biotin-streptavidin horse radish peroxidase detection systems. In all cases similar results were obtained with monoclonal and polyclonal antibodies.

2.3. *GDP binding to* N-ras *p21*

2.3.1. Ultraviolet spectroscopy

Guanine nucleotide bound to N-*ras* p21 was detected using a Pye Unicam SP8-400 UV/VIS scanning spectrophotometer. Protein samples were adjusted to a concentration of 1 mg/ml, centrifuged at 10000 × *g* for 10 min and scanned.

2.3.2. GDP binding to p21 on nitrocellulose filters

Protein obtained after proteolytic digestion of p21 was subjected to SDS-PAGE as described above and then electrophoretically transferred to nitrocellulose filters. After extensive washing in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Tween 20 (buffer A), the filters were incubated in buffer A containing 1 mg/ml BSA for 1 h and then in buffer A/BSA containing 3 μCi/ml [α-^{32}P]GTP (~3000 Ci/mmol).

3. RESULTS

Incubation of N-*ras* p21, purified under non-denaturing conditions, with limited amounts of trypsin or chymotrypsin, results in limited proteolysis such that the larger peptide products may be detected using Western blotting. It was observed that appreciable differences were apparent between samples depending on whether metal ions or guanine nucleotide were bound to the apoenzyme. In order to undertake these experiments it was necessary to remove Mg^{2+} and GDP from p21. This was achieved by prolonged dialysis (16 h) against 10 mM EDTA, 50 mM NH_4HCO_3, pH 7.9 at 4°C. It can be seen from the ultraviolet spectra presented in fig.1 that incubation of p21 with EDTA results in the loss of a peak observed at 260 nm in the untreated sample (cf. panels A and B). Addition of GDP back to p21 leads to a reappearance of the peak and a spectrum identical to that shown in panel A suggesting rebinding of nucleotide. Dialysis against urea (panel C) produces further changes in the spectrum between 250 and 260 nm which may be attributable to either removal of residual GDP or gross conformational changes undergone by the protein. When GDP is added to the urea-denatured p21 (cf. panels D and E) a peak is apparent at 260 nm (as in the undenatured protein) but this is removed on dialysis (panel F) indicating that urea-denaturation irrevocably destroys the protein's ability to bind nucleotide. These observations are consistent with results obtained previously by Poe et al. [13], who suggested that a peak on their spectrum of H-*ras* p21 at 258 nm was due to bound nucleotide. Hattori et al. [14] noted a spectral change in p21 after a denaturation with 3 M guanidine hydrochloride presumably due to nucleotide release.

For proteolysis experiments GDP- and Mg^{2+}-free p21 was produced by dialysis against EDTA. The protein was then dialysed against 50 mM NH_4HCO_3, pH 7.9 to remove EDTA and incubated with trypsin or chymotrypsin, together with various additions as described in the legend to fig.2. Aliquots were withdrawn at the stated times and subjected to Western blotting.

At a trypsin/p21 ratio of 1:40 no degradation could be observed over at least 3 h (data not shown). At a higher protease concentration (trypsin/p21 1:4) degradation occurred rapidly in the absence of added divalent cations or nucleotide (panel A). In the presence of Mg^{2+}, Ca^{2+} or GDP the protein was appreciably more resistant to

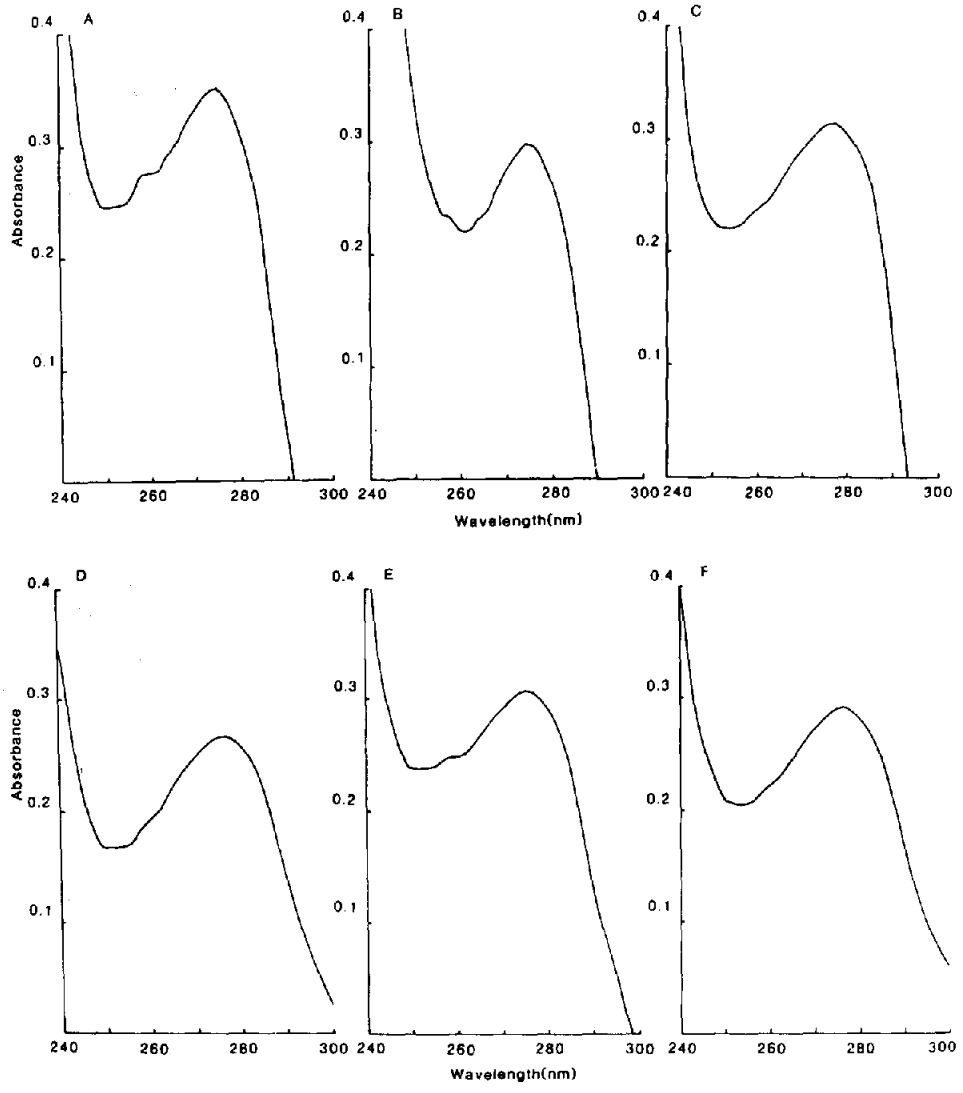

Fig.1. Ultraviolet absorption spectra of purified N-*ras* p21. N-*ras* p21 was purified after expression in *E. coli* as described in section 2. Freeze-dried protein was re-dissolved in 50 mM NH_4HCO_3, pH 7.9, 1 mM DTT, 2 mM $MgCl_2$ (buffer B). Spectra were recorded after the following treatments: (A) dialysis against buffer B; (B) dialysis against 50 mM NH_4HCO_3, pH 7.9, 5 mM EDTA, 1 mM DTT followed by buffer B; (C) dialysis against 7 M urea, 50 mM NH_4HCO_3, pH 7.9, 5 mM EDTA, 1 mM DTT; (D) gel filtration in the presence of 7 M urea as outlined in section 2, followed by a dialysis against buffer B; (E) p21 treated as in D in the presence of 0.05 mM GDP; (F) treated as in E but dialysed against buffer B.

hydrolysis, indicating that conformational changes result from binding of all three of these ligands, giving rise to a more highly structured molecule and increased resistance to proteolysis. The protein was more susceptible to degradation by chymotrypsin than trypsin (panels B and C) but at an enzyme/substrate ratio of 1:40 it can be seen that the addition of Mg^{2+}, Ca^{2+} or Mg^{2+} GDP resulted in protective conformational changes. At the higher chymotrypsin concentration (panel C) no p21 peptides were detectable after incubation in the presence of EDTA or GDP. The results presented in panel D show that the monovalent cations (Na^+ or K^+) would not substitute for calcium or magnesium. Because different patterns of proteolysis occur (with both enzymes) depen-

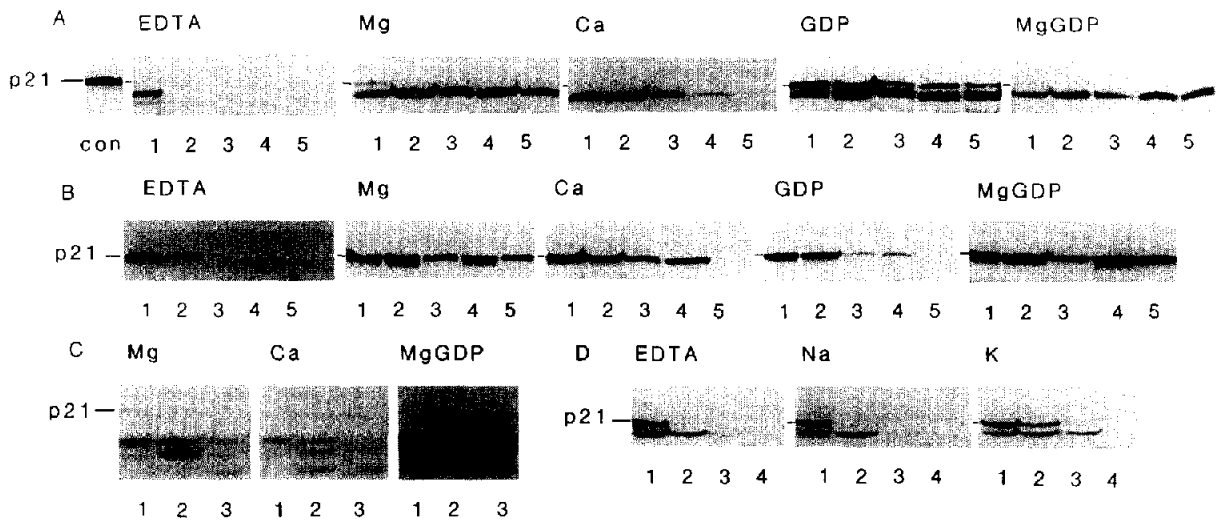

Fig.2. Proteolysis of purified undenatured N-*ras* p21. N-*ras* p21 was purified after expression in *E. coli* as described in section 2. Bound GDP was removed by dialysis against 10 mM EDTA, 50 mM NH_4HCO_3, pH 7.9. Protein was then dialysed against 50 mM NH_4HCO_3, pH 7.9. Additions were made as follows as indicated in the figure: 5 mM EDTA; 10 mM $MgCl_2$; 10 mM $CaCl_2$; 1 mM GDP; 1 mM GDP; 10 mM $MgCl_2$; 10 mM NaCl or 10 mM KCl. p21 (20 μg) was incubated with: (A and D) trypsin (5 μg); (B) chymotrypsin (0.5 μg) or (C) chymotrypsin (5 μg). Aliquots were removed after the following times and subjected to SDS-PAGE and Western blotting: (1) 10 min; (2) 25 min; (3) 65 min; (4) 160 min; (5) 310 min; con, control p21 before treatment with protease.

ding on the added ligands it is reasonable to conclude that there exists (quite separately) in p21 a divalent cation-binding site as well as a nucleotide-binding site. It is also worth noting that p21 even in the apo form is remarkably resistant to attack by proteases, suggesting a very highly structured protein molecule.

It can also be seen from panel A of fig.2 that there is a particular peptide bond (or bonds) in Mg^{2+}-*ras* p21 which is readily cleaved by trypsin at high concentrations resulting in a reduction in molecular mass of about 1500 Da, the remainder of the protein being stable to proteolysis even after prolonged treatment. To ascertain whether material was lost from the N- or C-termini of the molecule two approaches were adopted. Firstly, nitrocellulose filters similar to those shown in fig.2 were incubated with [α-^{32}P]GTP as described in section 2. The data in fig.3 shows that the cleaved protein was equally capable of binding nucleotide as the intact molecule, suggesting that no reduction had occurred in the nucleotide-binding site, which encompasses the N-terminus. A second approach to the determination of cleavage site was to isolate the larger protein product after tryptic treatment and subject it to amino acid analysis. Thus p21 was incubated with trypsin in the presence of Mg^{2+} for 60 min. The reaction was stopped by the addition

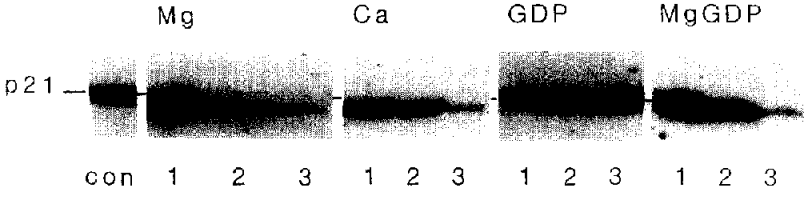

Fig.3. GDP binding to purified N-*ras* p21 after treatment with trypsin. Bacterially expressed N-*ras* p21 (20 μg) was incubated with trypsin (5 μg) under the conditions listed in the legend to fig.2. Aliquots were removed after the following time intervals: (1) 5 min; (2) 25 min; (3) 125 min, and subjected to SDS-PAGE followed by transfer to a nitrocellulose filter. After incubation with [α-^{32}P]GTP, as described in section 2, the filter was autoradiographed.

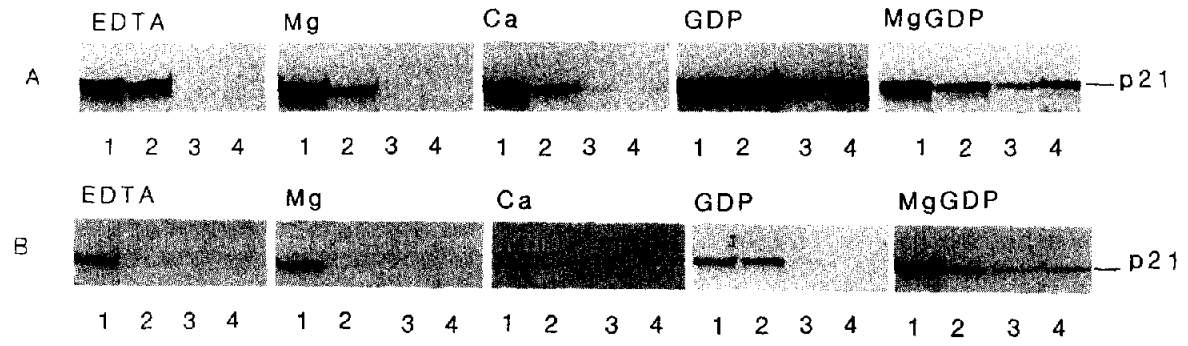

Fig.4. Proteolysis of denatured purified N-*ras* p21. N-*ras* p21 was purified, as described in section 2, after expression in *E. coli*. The protein was then denatured by chromatography in the presence of 7 M urea as described, dialysed against 50 mM NH_4HCO_3, pH 7.9, and incubated with protease. EDTA, $MgCl_2$, $CaCl_2$, GDP or GDP $MgCl_2$ were present in the same concentrations given in the legend to fig.2. N-*ras* p21 (20 μg) was incubated with: A, trypsin (0.5 μg) or B, chymotrypsin (0.5 μg). Aliquots were removed after the following times: (1) 5 min; (2) 20 min; (3) 80 min and (4) 135 min and subjected to SDS-PAGE followed by Western blotting.

of soya bean trypsin inhibitor and the mixture dialysed extensively against water. The resulting truncated p21 was analysed and found to have a composition approximately consistent with residues 1–170 (data not shown). This polypeptide might be expected if cleavage occurred at peptide bonds C-terminal to Arg 167 or Lys 169 or 170, all of which should be susceptible to tryptic attack.

To examine the effects of denaturation on the structure of N-*ras* p21, as shown by resistance to proteolytic attack, purified protein was subjected to gel filtration in the presence of 7 M urea, followed by dialysis against 50 mM NH_4HCO_3. Treatment with trypsin and chymotrypsin was carried out in a similar manner to that already described for the undenatured p21. A comparison of the data presented in figs 2 and 4 indicates appreciable differences between the results obtained with denatured and undenatured *ras*. The former protein is considerably more susceptible to trypsin and chymotrypsin treatment, being degraded at concentrations approximately ten-fold less than those required for digestion of the p21 prepared under non-denaturing conditions. However, the urea-treated polypeptide appears to be still capable of binding guanine nucleotide and presumably, as a result, effecting a conformational change making it less susceptible to digestion. Even so it should be noted that no evidence of GDP binding could be detected using UV spectroscopy (fig.1). It is possible that the nucleotide binds weakly to the protein and may therefore be removed by dialysis yet is still capable of altering the susceptibility to proteolysis. The ability to undergo a conformational change on binding divalent cations appears to have been lost completely however.

A final study was performed using N-*ras* p21 purified from cultures of the transformed human retinoblast cell-line Ad2E1A + N-*ras* HER313A. The data presented in fig.5 indicates that this protein is very readily degraded at enzyme/substrate ratios of between 1:100 and 1:1000. No differences were obtained in the presence or absence of divalent cations or of added guanine nucleotide.

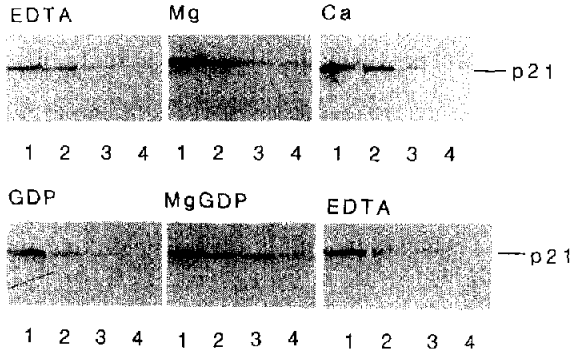

Fig.5. Proteolysis of eukaryotically expressed N-*ras* p21. N-*ras* p21 was purified from Ad2E1A + N-*ras* HER313A cells as described in section 2 and dialysed against 50 mM NH_4HCO_3, pH 7.9. Protein (25 μg) was incubated with trypsin (0.1 μg) or chymotrypsin (0.1 μg) in the presence of 1 mM EDTA, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM GDP or 1 mM GDP, 5 mM $MgCl_2$ as indicated. Aliquots were removed after: (1) 10 min; (2) 25 min; (3) 60 min; (4) 120 min; and subjected to SDS-PAGE followed by Western blotting. The final set of samples (EDTA) were incubated with chymotrypsin, the remainder with trypsin.

4. DISCUSSION

It has long been known that large conformational changes in proteins occurring as a result of metal ion binding, are detectable as increased (or decreased) resistance to proteolytic cleavage (see, for example, the Ca^{2+}-binding proteins, troponin C and calmodulin). We have shown here that the removal of nucleotide and Mg^{2+} from N-*ras* p21 (prepared under non-denaturing conditions after expression in *E. coli*) renders the polypeptide much more susceptible to digestion by trypsin and chymotrypsin; even so very high levels of the enzymes are required for degradation suggesting a highly structured form of the molecule. More interestingly conformational changes seem to occur on the addition of Mg^{2+} (or Ca^{2+}) suggesting that the metal ion binds directly to the protein and is not simply involved in nucleotide binding. This is in agreement with recent suggestions by Feuerstein et al. [15] and Grand et al. [12] who have presented other evidence to support the proposition that *ras* p21 is a metal ion-binding protein. Other changes in conformation occur in the presence of GDP such that certain susceptible peptide bonds become less available to the proteases. Whilst there are undoubtedly differences in secondary structure between mutant and wild type p21 [9,12] these appear to be rather too subtle to be detected using this technique since identical patterns of proteolysis were always obtained between the 2 forms of *ras* regardless of whether they were denatured or not (data not shown).

Denaturation (with urea) and renaturation of bacterially expressed p21 allows the recovery of a limited ability to bind nucleotide but not Mg^{2+}. Resistance to proteolysis never approaches the untreated level however. This is a further result which must be added to the growing weight of evidence which suggests that denaturants should be avoided in the preparation of p21 if the full biological activity of the protein is to be retained. The *ras* protein obtained after purification from the eukaryotic source appears to retain no ability to bind metal ions or GDP (or at least to undergo conformational changes when so doing). This is probably because rather more stringent conditions were employed in the preparation (9 M urea in the presence of high concentrations of organic solvents). It is hoped that the data presented here will be employed in future to prepare defined peptides for use in ligand-protein and protein-protein interaction studies involving p21.

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