Ras binding to a C-terminal region of GAP

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Abstract Using fluorescence spectroscopy we have identified a binding region for Ras on the GTPase activating protein (GAP) lying within residues 715–753. A synthetic peptide Y922, corresponding to residues 716–753 of GAP binds to wild type Ras showing 3.3-fold higher affinity for the GTP- over the GDP-bound forms of Ras. Binding is stabilised by Mg^{2+}, although Y922 does not stimulate the GTPase activity of Ras. Peptide binding to the Y32A and Y40F Ras mutants showed equal affinity for both GDP- and GTP-bound forms, with binding to Y32A·GDP abolished in the absence of Mg^{2+}. These results suggest that Y922 mimics the in vivo interactions shown by the intact $p120^{GAP}$ protein and provide the first direct demonstration of Ras interaction with GAP in the region 715–753.

Key words: 5′-(2-((Iodoacetyl)amino)ethyl)naphthalene-1-sulphonic acid fluorescence; GAP peptide; Ras

1. Introduction

The *ras* genes encode 21 kDa proteins (Ras) that bind guanine nucleotides with high affinity and display a low intrinsic GTPase activity [1] that is absolutely dependent upon the presence of divalent cation [2]. Specific point mutations in the cellular forms of Ras impart transforming properties to these mutant proteins and such changes have been found in up to 30% of human tumours [3].

High resolution X-ray crytallographic studies of truncated forms of the wild-type and mutant H-Ras proteins isolated from bacterial expression systems [4–6] suggest that variations in regions of the protein, specifically the structural motifs associated with GTP hydrolysis and effector protein binding, are responsible for the dramatic changes in properties associated with the oncogenic phenotype. Amino acids throughout the N-terminal two-thirds of Ras are involved in nucleotide binding and hydrolysis although an effector binding site appears to occupy a short consecutive stretch of amino acids. Extensive mutagenic analysis [7] has defined the region (residues 32–40) as critical for interactions of Ras with postulated effector proteins [8] in particular the GTPase actvating protein; GAP [7,9–12].

GAP and the homologous protein neurofibromin (the product of the NF1 gene) bind preferentially to Ras in the GTP-bound form. Since both of these proteins increase the GTPase activity of Ras, it has been proposed that they act as negative regulators [13]. However, it is possible that GAP may also play the role of effector molecule for Ras, as, for example, the Ras·GTP·GAP ternary complex is essential for oocyte maturation [14]. The interaction site on GAP for Ras has been approximately mapped by deletional studies to a region towards the C-terminus of the molecule [15]. The C-terminal 344 amino acids of GAP have been shown to stimulate the isomerisation step which precedes GTP hydrolysis by Ras 200-fold [16] whereas the full-length protein causes an increase of about 10^5-fold. This additional increase has been attributed to the presence of the SH_2 domains [11] which are located further towards the N-terminus. Apart from these studies there is little direct information available on the specific amino acid residues of GAP that might contribute to the GTPase stimulation although it has been suggested that GAP contributes a catalytic residue to the Ras·GTPase [10]. Additionally the R903K mutation of GAP in the ^{901}FLR^{903} motif that is conserved amongst the IRA1, IRA2 and NF1 proteins, results in up to a 30-fold decrease in the GAP mediated Ras·GTPase [17,18].

$p120^{GAP}$ is phosphorylated by receptors and non-receptor tyrosine kinases with Y723 being identified as a site of phosphorylation [19,20]. This lies within the region which, has been suggested on the basis of deletional analysis as a site of interaction with Ras [15]. The results of this study have implied that the region 700–750 is important for GAP activity as a negative regulator or as an effector protein for Ras. In order to demonstrate the Ras·GAP interaction by direct physical study, we have employed the peptide mimetic approach. In this work we report upon the binding of Ras·GTP and Ras·GDP to a synthetic peptide which is identical to a region towards the C-terminus of $p120^{GAP}$. We have investigated binding interactions to wild-type Ras as well as proteins with oncogenic and effector site mutations since any binding interactions are likely to be critically dependent upon the correct folding pattern in the Mg·GTP-bound state of Ras.

2. Materials and methods

2.1. Peptides

The chemically synthesised peptides corresponding to residues 700–726 and 715–753 (Y1345 and Y922, respectively; Fig. 1) of the sequence of human $p120^{GAP}$ were prepared, deblocked and purified to homogeneity by HPLC using a Vydac C_{18} column eluted with a linear CH_3CN gradient and lyophilised as described [21]. In order to rule out disulphide bridge formation and at the same time introduce environmentally sensitive extrinsic fluorescent chromophores, Y922 was carbox-

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Abbreviations: $p120^{GAP}$, GAP; GTPase activating protein; mGTP, 2′(3′)-*O*-(*N*-methylanthraniloyl) GTP; mGDP is similarly abbreviated; NF1, the GAP related protein neurofibromin; Ras, the wild-type (G12) protein product of the N-Ras oncogene; G12D, Y32A, Y40F, mutant forms of N-Ras used in this study; IAEDANS, 5′-(2-((iodoacetyl)amino)ethyl)naphthalene-1-sulphonic acid; IAS, 4-(iodoacetamido)salicylic acid; Y1345, Y922 synthetic peptides comprising residues 700–726 and 715–753 of human $p120^{GAP}$, respectively; C·IAEDANS, Y922·IAEDANS and Y922·IAS, cysteine and Y922 labelled with IAEDANS and Y922 labelled with IAS, respectively; ΔF_{max}, the maximum observed fluorescence change in IAEDANS emission intensity.

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ymethylated at the -SH group of C745 with either IAEDANS (Y922·IAEDANS) or IAS (Y922·IAS). Typically 5 mg lyophilised peptide was resuspended in 1.0 ml of 6.0 M guanidine-HCl; 25 mM Tris-HCl, pH 8.0. A 3-fold molar excess of either probe was then added and the reaction incubated at 30°C for 2 h in darkness. The labelled peptides were purified by HPLC as described above and characterised by absorption and fluorescence spectroscopy. It was subsequently observed that Y922·IAS was photo-unstable, whilst Y922·IAEDANS demonstrated photostability over 6 months. As a control for non-specific probe·Ras interactions, cysteine labelled with IAEDANS was prepared as for Y922·IAEDANS described above. The concentration of Y922·IAEDANS stock solutions were routinely determined by absorption at 337 nm ($\varepsilon = 5.7\ mM^{-1} \cdot cm^{-1}$).

2.2. Proteins

The wild-type (G12), and G12D, Y32A and Y40F mutants of N-Ras were overexpressed in *Escherichia coli* DH5α using a *trp* promotor construct system and purified as described [22]. The typical yield from 4.0 litre cultures was 12–14 mg purified protein. Metal ion-free Ras was prepared from Ras·Mg·GDP by passing the protein (routinely 2–6 mg) through a Chelex-100 column eluted with 50 mM Tris-HCl, pH 7.4; 0.5 mM DTT at 4°C. Nucleotides were added to the protein solutions at 1:1 mole stoichiometry and where indicated, $MgCl_2$ was added to a final concentration of 10 mM.

2.3. Fluorescence spectroscopy

Fluorescence measurements were made in triplicate using a thermostatically controlled Perkin Elmer fluorimeter at 23°C. For all experiments excitation of the IAEDANS moiety was at 337 nm and emission monitored at 520 nm. For experiments involving titrations with the Mg·nucleotide complexes of Ras, measurements were obtained in 50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 0.5 mM DTT (buffer A) using matched 1 cm path-length quartz fluorescence cuvettes. For experiments with the metal ion-free Ras·nucleotide complexes, $MgCl_2$ was omitted from the buffer (buffer B). Ras protein complexes were added to give final concentrations between 3 and 8 μM from stocks of 100 μM to minimise dilution effects (< 12% initial volume). All data were corrected for instrument variations by comparing the ratio of sample emission to that of a fluorescent standard (IAEDANS), as well as for small dilution effects and background emission. At low Y922·IAEDANS concentrations (0.1–0.3 μM) the observed change in emission intensity in the presence of Ras was negligible in some cases, ruling out a direct determination of dissociation constants for the Y922·IAEDANS·Ras complex. Therefore, approximations of binding affinities ($K_{d \cdot app}$) were evaluated by observation of the fluorescent signal (ΔF) of 1 μM Y922·IAEDANS in the presence of varying Ras concentrations (0.1–3.0 μM). With increasing Ras concentration (in most cases up to 3 μM) the observed changes in Y922·IAEDANS emission signal changes were larger and approached, or became equal to an end-point (ΔF_{max}), where the change in fluorescence signal of Y922·IAEDANS remained constant for a given increase in Ras concentration. Thus, apparent dissociation constants ($K_{d \cdot app}$), which are critically dependent upon the observation of an end point, could be evaluated by a non-linear least-squares analyses of data to a one binding site function using the programs Enzfitter and Fig P.

2.4. NMR spectroscopy

All ^1H NMR spectra were recorded on a Bruker AMX500 spectrometer on peptide samples (~100 μM) in 50 mM [^2H]Tris in ^2H_2O adjusted to pH 7.4 with ^2HCl. A gated presaturation pulse of 1.1 s was used for accumulation over a 5000 Hz sweep at an ambient probe temperature of 23°C with a 90°C pulse of 5 ms. Spectra were collected as 1028 free induction decays and Fourier transformed.

3. Results

3.1. Covalent modification of peptides

For the intrinsic GTPase of Ras to be stimulated by $p120^{GAP}$ a binding interaction between the two proteins must first occur. This interaction has been attributed to the C-terminal domain of GAP. While the sequence 702–1044 was shown to stimulate the Ras GTPase, residues 751–1044 comprised an inactive protein [15]. This suggests that the structural elements on GAP required for interaction with Ras, prior to GTP hydrolysis may be located within the region 702–751. Thus, a starting point for analysis has been to examine the binding kinetics between Ras and synthetic peptides of GAP (Y1345 and Y922 (Fig. 1)) that encompass this region.

Preliminary examination of Y1345 by ^1H NMR spectroscopy indicated that there was little or no interaction with Ras and this region of GAP even at the 1:1 mole stoichiometry (data not shown), suggesting that binding events with Ras may involve residues further towards the C-terminus of the 700–753 region of GAP. Therefore, the Y922 peptide was examined by NMR spectroscopy; however, it was observed that the peptide underwent aggregation at concentrations above 100 μM as determined by the increase in NMR signal line-width, which precluded further examination by two-dimensional spectroscopy required for structural analysis. Hence, a more intrinsically stable and sensitive approach was adopted to examine the interactions of Ras and the GAP peptide Y922.

By virtue of the cysteine residue at position 745 in the sequence of Y922, the peptide could be covalently modified. The probes chosen for attachment to Y922 included IAEDANS and IAS. The emission properties of each probe bound to the peptide was examined and it was observed that Y922·IAS was intrinsically unstable, being readily susceptible to photobleaching. However, Y922·IAEDANS was found to be stable and possessed a Stokes shift of 183 nm (Fig. 1B). Therefore, with excitation at 337 nm and emission monitored at 520 nm, the contribution to sample emission from intrinsic protein background is likely to be minimal and thus Y922·IAEDANS provided an appropriate tool for probing the interactions between GAP and Ras.

3.2. The Y922 GAP peptide discriminates between Ras·GDP and Ras·GTP

To investigate any binding interactions, Y922 labelled with IAEDANS was titrated with wild-type Ras in varying nucleotide and metal ion states. If the Y922 peptide contains structural elements that allow for correct folding in solution, any binding events may be observed as a change in the fluorescence of IAEDANS in the presence of Ras. Also, the Mg·GTP-bound state of Ras should bind more tightly to Y922·IAEDANS as Mg^{2+} is critical for GTPase activity. Additionally, it has recently been shown that there is little or no interaction between Ras·GDP and GAP344 [16]. Fig. 2A shows that upon titration of Y922·IAEDANS with wild-type Ras·Mg·GTP there is a decrease in the emission intensity of the fluorophore with a maximum change in fluorescence intensity of 19.8% observed at a saturating protein to peptide ratio of 3:1. This result suggests that a binding event is being monitored and analysis of this datausing a non-linear least-squares fit to a one-binding site model determined the $K_{d \cdot app}$; 0.86×10^{-6} M (Table 1). In comparison, titrations of Y922·IAEDANS with the metal ion-free GTP-bound form of wild-type Ras also resulted in a decrease in IAEDANS emission intensity. However, the maximum change in emission intensity was found to be 9.1% at the protein to peptide ratio of 1:1, and this remained relatively constant up to a ratio of 3:1 (Fig. 2A). In the absence of Mg^{2+} the $K_{d \cdot app}$ for the Y922·IAEDANS·Ras·GTP complex was 1.1×10^{-6} M (Table 1). These data suggest that both the Mg-bound and the Mg-free forms of Ras·GTP bind to

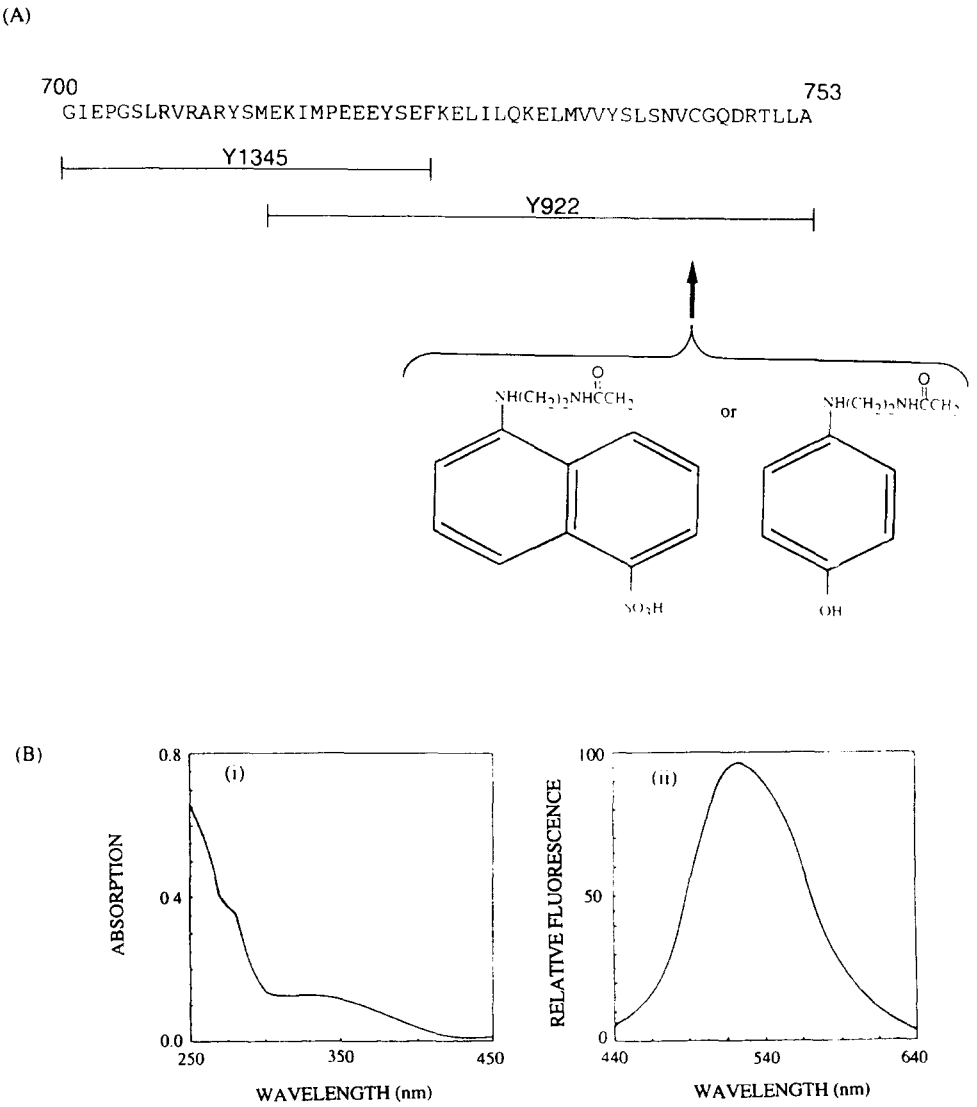

Fig. 1. Amino acid sequences of the synthetic peptides of human p120GAP and spectral characteristics of Y922·IAEDANS. (A) Peptides Y1345 (residues 700–726) and Y922 (residues 715–753) are shown along with the fluorescent probes IAEDANS and IAS covalently attached to Y922 at C745. (B) Absorption spectrum of 11 μM and uncorrected emission spectrum of 2 μM Y922·IAEDANS in 50 mM Tris-HCl, pH 7.4; 10 mM $MgCl_2$; 0.5 mM DTT at 23°C. The emission spectrum was recorded with excitation at 337 nm and emission scanned between 400 and 600 nm with an emission maximum at 520 nm. Excitation and emission band passes were 2.5 nm.

Y922·IAEDANS and that Mg^{2+} contributes approximately 1.3-fold to the stability of the binding interaction.

Suprisingly, titrations of Y922·IAEDANS with Ras·Mg·GDP were also observed as a decrease in the emission intensity of the fluorophore, although saturation was not attained at the protein-to-peptide ratio of 3:1 (Fig. 2(A)). The maximum fluorescence change in IAEDANS emission in the presence of Ras·Mg·GDP was found to be 17% at a protein to peptide ratio of 8:1 (data not shown) and the evaluated $K_{d\cdot app}$ was 2.8×10^{-6} M (Table 1). These observations suggest that Y922·IAEDANS binds to GDP-bound Ras, but owing to the 3.3-fold higher affinity for GTP-bound Ras, the peptide interacts preferentially with triphosphate-bound form of the protein. This observation suggests that the fluorescent derivative of Y922 possesses structural elements which allow an interaction with Ras and that the 10.7% difference observed in the fluorescence intensity of Y922·IAEDANS binding to Ras·Mg·GTP and Ras·Mg·GDP (at the protein to peptide ratio of 3:1) is a direct result of the difference in intrinsic peptide structure resulting after interaction with these two states of Ras.

In order to eliminate the possibility that these results derive from non-specific peptide·protein or non-specific IAEDANS interactions, which might influence evaluation of $K_{d\cdot app}$, control experiments were undertaken. First, C·IAEDANS was titrated with Ras·Mg·GTP (Fig. 2B). No change in the emission intensity was observed at the protein to derivatised amino acid ratio of 3:1. This control indicates that IAEDANS bound to the Y922 peptide imparts information due to changes in the environment of the Y922 peptide and not as a result of non-specific interactions of Ras with the C·IAEDANS moiety of the labelled peptide. Second, Y922·IAEDANS was titrated

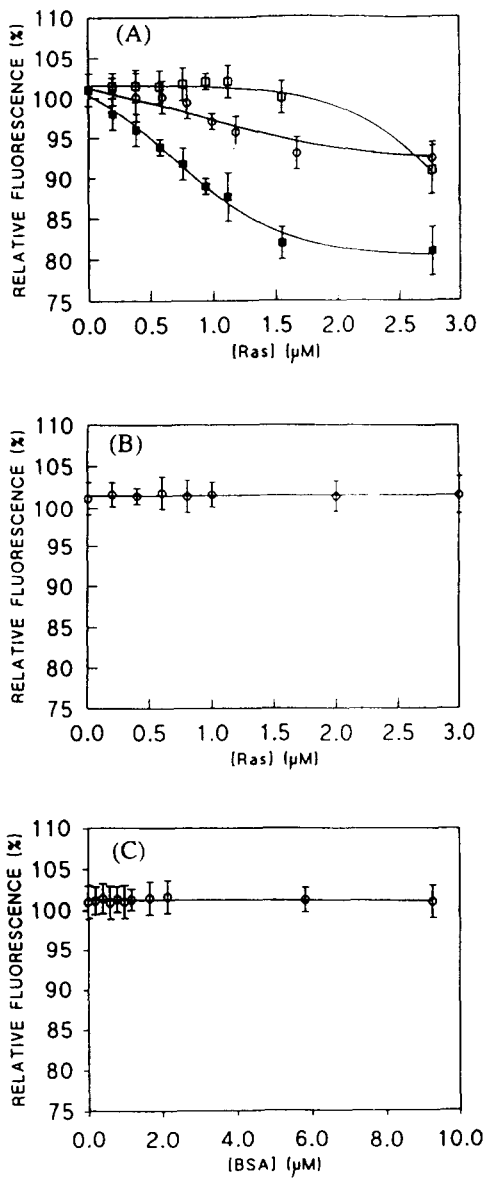

Fig. 2. Interaction of Y922·IAEDANS with wild-type Ras. (A) Titration of Y922IAEDANS with Ras complexed to Mg·GTP (■), metal ion-free Ras·GTP (○) and Ras·Mg·GDP (□). (B) Titration of C·IAEDANS with Ras·Mg·GTP. (C) Titration of Y922·IAEDANS with BSA. In each case the initial concentration of Y922·IAEDANS or C·IAEDANS was 1 μM in 1.6 ml of buffer A (see section 2) at 23°C. Ras and BSA were added to give final concentrations between 3 μM and 9 μM from stocks of 100 μM to minimise dilution effects (< 12% of initial volume). For the titration with metal ion-free wild-type Ras complexed to GTP buffer B was used. For all titrations, the excitation of the IAEDANS moiety was at 337 nm and emission was monitored at 520 nm. Excitation and emission band passes were 2 nm and 15 nm, repsectively. Solid lines represent best fit of the averaged data from three experiments (±standard deviation; summarised in Table 1) to a one binding-site function. Data were corrected for background emission of buffer and intrinsic protein emission. Variations in lamp stability was corrected for by comparision of sample emission to that of free IAEDANS to give relative fluorescence (%).

with BSA, which like Ras is acidic, but does not bind to either Mg^{2+} or GTP (Fig. 2C). Once again, no change in the emission intensity of IAEDANS was observed, even at the protein-to-peptide ratio of 10:1. These results confirm the observation that the fluorescence changes of Y922·IAEDANS in the presence of Ras·Mg·GTP and Ras·Mg·GDP are indicative of specific binding events and are not the result of non-specific protein·peptide interactions.

3.3. *Influence of Ras·GTPase activity on evaluations of* $K_{d\cdot app}$ *for the Y922·IAEDANS·Ras·Mg·GTP complex*

The rate of GTP hydrolysis by Ras was examined using a fluorescent analogue of GTP, namely mGTP, since this compound is well characterised in terms of binding affinity and fluorescence enhancements upon binding to Ras [16]. It is also of interest to determine whether Y922 contains structural elements that contribute to a stimulation, if any, in the GTPase reaction following the binding events described above. Fig. 3 shows the hydrolysis of mGTP by Ras at 23°C. It was observed that mGTP hydrolysis in the presence of Ras occurs in a biphasic manner with an overall decrease in emission intensity of 8.6% on hydrolysis of mGTP to mGDP. A non-linear least-squares analysis of the data evaluated a double exponential decay with rate constants to be 1.62×10^{-4} and 1.40×10^{-4} s^{-1} for the first and second phases of hydrolysis in the absence of GAP peptide. Neither of these rates was effected by the presence of GAP peptide (open symbols in Fig. 3; rate constants of 1.62×10^{-4} and 1.28×10^{-4} s^{-1} for the first and second phases, respectively). Taking into account the observation that the Ras·mGTPase is 3-fold higher than the Ras·GTPase [16], this experiment provides a control as GTP is likely to represent greater than 98% of the total nucleotide at the end of the experimental titrations of Y922·IAEDANS titrations with Ras·GTP (ca. ~30 min; similar results were obtained by monitoring the direct hydrolysis of [γ-^{32}P]GTP; data not shown). This observation strongly indicates that the influence of GDP upon the derived $K_{d\cdot app}$ for the GTP-bound Ras interaction with Y922·IAEDANS is negligible.

The examination of mGTP hydrolysis also provides a reproducible fluorescence standard (ca. ~10%) for the observed

Table 1
Fluorescence changes and apparent dissociation constants of Ras proteins binding to Y922·IAEDANS

Ras complex		Decrease in emission intensity (%)	$K_{d\cdot app}$ ($\times 10^{-6}$ M)
Wild-type	Mg·GTP	19.85 ± 2.0	0.86 ± 0.1
	Mg·GDP[a]	9.12 ± 1.8	2.81 ± 0.2
	GTP	7.50 ± 1.8	1.10 ± 0.2
G12D	Mg·GTP	7.50 ± 1.5	0.31 ± 0.1
	GTP	4.41 ± 2.0	0.90 ± 0.1
Y32A	Mg·GTP	6.70 ± 1.5	0.21 ± 0.1
	Mg·GDP	12.62 ± 1.0	0.30 ± 0.2
	GTP	5.70 ± 1.0	0.36 ± 0.2
	GDP[b]	–	–
Y40F	Mg·GTP[c]	12.50 ± 1.3	0.85 ± 0.1
	Mg·GDP[c]	12.50 ± 2.0	0.70 ± 0.1
	GTP[c]	12.40 ± 1.8	0.38 ± 0.2
	GDP	10.60 ± 2.0	0.97 ± 0.1

[a] Maximum fluorescence change at a protein to peptide ratio of 3:1 (overall decrease in emission intensity was 17 ± 1.3% at a protein to peptide ratio of 8:1; see text).
[b] There was no observable change in Y922·IAEDANS emission intensity, therefore $K_{d\cdot app}$ is not determined.
[c] Binding reaction was greater than 95% saturated at a protein to peptide ratio of 3:1.

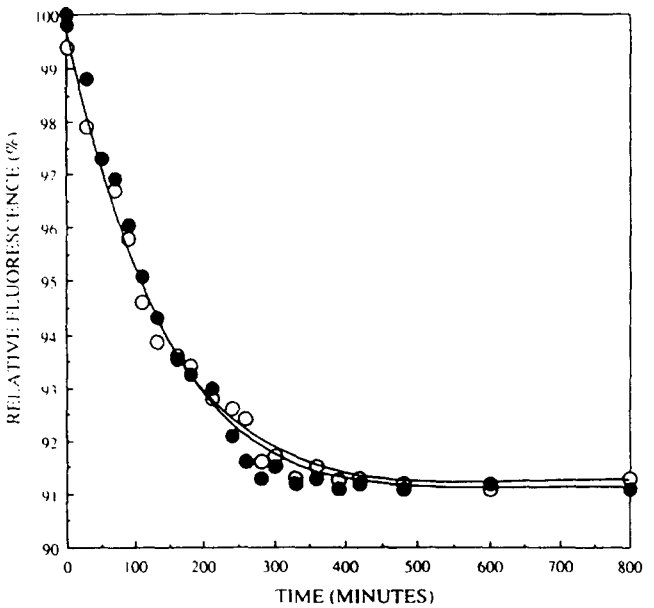

Fig. 3. Intrinsic GTPase activity of wild-type Ras. 1 μM Ras·mGTP in 1.6 ml of buffer A was equilibrated in a 1.0 cm quartz fluorescence cuvette at 23°C in the absence (●) or presence of 1 μM Y922 (○). With excitation at 356 nm the emission intensity of samples was monitored with time at 440 nm. The excitation and emission band passes were 2.5 and 10 nm, respectively. Data represent the average of three separate mesurements for each condition and were corrected for instrument variation by comparing sample emission to that of a fluorescent standard; in this case free mGTP. Solid lines represent best fit of the data to a double exponential with rate constants of 1.62×10^{-4} s^{-1} ($\pm 0.8 \times 10^{-5}$) and 1.40×10^{-4} s^{-1} ($\pm 1.0 \times 10^{-5}$) in the absence of GAP peptide and 1.60×10^{-4} s^{-1} ($\pm 1.2 \times 10^{-5}$) and 1.28×10^{-4} s^{-1} ($\pm 0.95 \times 10^{-5}$) in the presence of GAP peptide.

changes in Y922·IAEDANS emission monitored in the above titrations. The change in fluorescence intensity of the mant fluorophore upon hydrolysis of mGTP to mGDP in the presence of Ras is comparable to the findings of other groups [16] and thus the observed changes in Y922·IAEDANS emission (between 4% and 19%) and evaluated apparent dissociation constants are reliable indications of the interaction of the Y922 peptide with wild-type Ras·Mg·GTP, as variations in fluorescence signal due to machine artefacts and instrument variation can be ruled out.

Overall the results from the previous two sections confirm the observation that this peptide contains sufficient structural elements for binding to occur to the wild-type protein in a discriminatory fashion such that Mg·GTP is favoured over Mg·GDP, but that this peptide does not stimulate the GTPase activity of Ras (presumably because amino acid sequences outside 715–753 are also required). These findings are also consistent with the observation that GAP binds preferentially to the GTP-bound state of Ras. To assess the effect of biologically significant Ras mutantions on the interaction with Y922 an additional set of experiments were performed.

3.4. *Altered binding affinities for the GAP peptide and the oncogenic G12D mutant of Ras*

To investigate the influence of mutations in N-Ras upon the Ras·GAP interaction the behaviour of the oncogenic G12D protein was examined. Although this protein has been shown to display near wild-type affinity for the intact GAP protein [25] the GTPase activity is reduced 90% (cf. wild-type) [6,24]. In the presence of G12D·Mg·GTP a decrease of 7.5% in the emission intensity of Y922·IAEDANS was observed at a protein to peptide ratio of 3:1 (Fig. 4A) whilst in the absence of Mg^{2+} the decrease in emission intensity of Y922·IAEDANS was 4.4% (Fig. 4A). Using the same criteria as for the wild-type protein, the observed fluorescence intensity change allowed the apparent dissociation constants to be evaluated; 0.31×10^{-6} and 0.9×10^{-6} M for the G12D·Mg·GTP and the G12D·GTP complexes, respectively (Table 1). These results suggest that the affinity of Y922·IAEDANS for the G12D protein is not only 2.7-fold higher than for the wild-type protein, but also demonstrates a greater dependence (3-fold) upon the presence of Mg^{2+}, indicating that Mg^{2+} plays an important role in the binding interaction. These results strongly indicate a change in the effector binding domain of the G12D protein in comparison to the wild-type protein, specifically relating to Mg^{2+} coordination and therefore the effect of substitution in the effector domain of Ras was examined.

3.5. *Ras effector binding domain mutations disrupt the Ras·GAP peptide interaction*

A major site of interaction between Ras and $p120^{GAP}$ has been mapped to the region 25–46 of Ras [25]. It is therefore of interest to investigate how mutations in this region result in deviations from the wild-type interactions with the GAP peptide Y922. The Y32A mutant was chosen as Y32 is proposed to interact directly with GAP in the GTP-bound form of wild-type Ras [4,26] and therefore mutation of this tyrosine is likely to alter the interaction of the GTP-bound state of Ras with the GAP peptide.

Titrations of Y922·IAEDANS with Ras carrying the Y32A substitution are shown in Fig. 4B. In all cases except for the metal ion-free Y32A·GDP, a change in the emission intensity of Y922·IAEDANS was observed. For Y32A·Mg·GTP the overall percentage change in emission intensity was 6.7% compared to 12.6% for Y32A·Mg·GDP binding. This represents a reversal in signal intensity compared to the wild-type protein although there is not a concomitant reversal in affinity for these different nucleotide states of Ras. Instead, both Y32A·Mg·GTP and Y32A·Mg·GDP bind to Y922·IAEDANS with approximately equal affinity (Table 1). This is suprising since the Y32A substitution was expected to alter only the GTP-bound state interactions. One explanation for this is that Y32 is not solely required for interactions with GAP, but is also required for nucleotide binding. The higher affinities for Y32A·Mg·GTP and Y32A·Mg·GDP over wild-type are likely to be a consequence of an alteration in the structure of the nucleotide binding domain of Ras as a result of this mutation in the effector domain. In contrast to the wild-type and G12D proteins, the removal of Mg^{2+} from the GDP-bound state of the Y32A protein abolishes any interactions with the GAP peptide as no change in IAEDANS emission intensity was observed; even at the protein to peptide ratio of 3:1, suggesting that Mg^{2+} coordination is also altered by the Y32A substitution. These findings also provide a control demonstrating that Mg^{2+} is critical for the interaction between Ras and $p120^{GAP}$ to occur and confirm the specificity of the interaction between Ras and the GAP peptide.

In contrast to the Y32A protein, mutation at the C-terminus

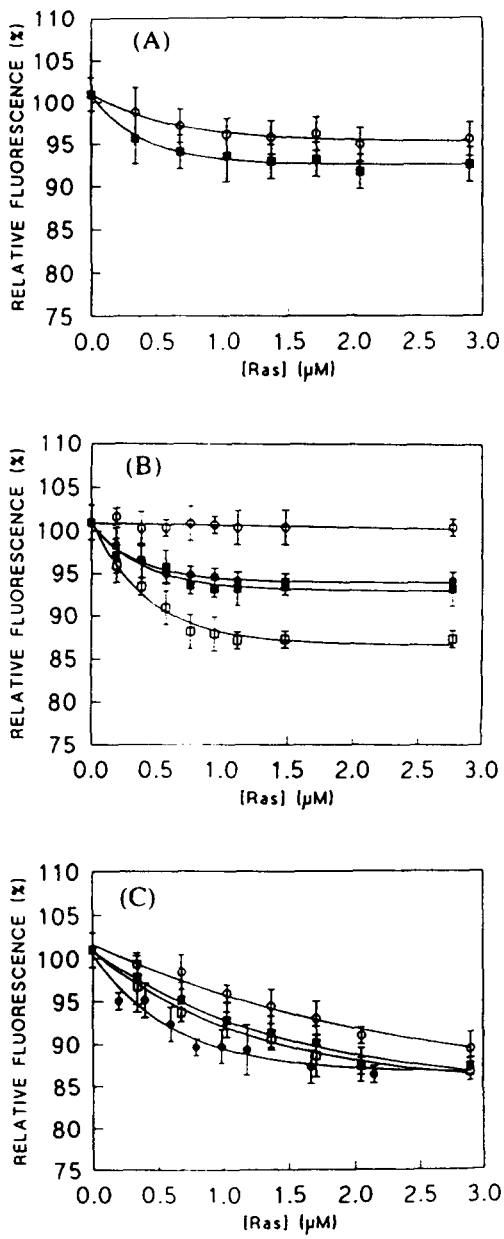

Fig. 4. Interaction between Y922·IAEDANS and mutant Ras proteins. (A) Titration of Y922·IAEDANS with G12D protein complexes: Mg·GTP in buffer A (■) and metal ion-free GTP in buffer B (○). (B) Titration of Y992·IAEDANS with Y32A protein complexes: Mg·GTP (■) and Mg·GDP (□) in buffer A, and GTP (●) and GDP (○) in buffer B. (C) Titration of Y922·IAEDANS with Y40F protein complexes: Mg·GTP (■) and Mg·GDP (□) in buffer A, and metal ion-free A32·GTP (●) and A32·GDP (○) in buffer B. Conditions as for Fig. 2A. Solid lines represent best fit of averaged data from three experiments and are summarised in Table 1.

of the effector domain has an opposite effect upon the interaction with the GAP peptide. Although Y40 has been deemed to be essential for the orientation of the whole 32–40 region of Ras [26], it is reasonable to suppose that substitution of Y40 for another aromatic residue should not dramatically alter the conformation of this loop. However, titrations of Y922·IAEDANS with Y40F indicate that changes to the Ras effector domain must occur (Fig. 4C). Although a decrease in the fluorescence intensity of IAEDANS was observed at saturating concentrations of the Y40F protein in all cases examined, the distinction between Ras·Mg·GTP and Ras·Mg·GDP observed in the wild-type protein is reversed with this mutant. Inspection of the $K_{d\cdot app}$ for the Y40F protein with Y922 reveals the affinities of the Mg·GTP and Mg·GDP-forms of the protein are approximately the same (0.85×10^{-6} and 0.7×10^{-6} M, respectively). These values are also close to that for the wild-type protein in the presence of GTP and magnesium. However, the 4-fold higher affinity for Y40F·Mg·GDP (cf. wild-type·Mg·GDP) suggests that in comparison to the Y32A mutant, the Y40F substitution also alters nucleotide interactions and Mg^{2+} coordination in Ras with greatest effect being upon the GDP-bound form of the protein.

4. Discussion

In this work we have demonstrated that a synthetic peptide comprising residues 715–753 of human $p120^{GAP}$ binds to wild-type N-Ras. The reproducible fluorescence changes and control experiments undertaken militate against a non-specific protein·peptide binding scenario. The larger ΔF_{max} and concomitant higher binding affinity for Ras·Mg·GTP over Ras·Mg·GDP indicate that this interaction is a specific binding event mimicing the in vivo situation. The observation that GDP-bound Ras is recognised by Y922 is surprising given that there is no identifiable physiological role for the Ras·Mg·GDP·GAP quaternary complex in cellular signalling pathways. However, the discriminatory interaction where the GTP-bound form of Ras is favoured over the GDP- bound form is entirely consistent with the observation that GAP344 [16] as well as the intact GAP protein bind preferentially to the GTP-bound form of Ras [27]. The apparent dissociation constants for Ras·Mg·GTP and Ras·Mg·GDP binding to Y922 are higher than that found for Ras·Mg·GTP binding to the intact GAP protein (K_d = 5 μM; [27]). This difference presumably arises because the relatively short Y922 peptide may not possess the entire complement of secondary sites required for full activity of $p120^{GAP}$. However, the GAP peptide must contain sufficient structural elements to mediate an interaction with Ras, since Y922 can distinguish both the nucleotide-bound state and the presence of Mg^{2+} for the wild- type protein. In principle this could be achieved by two different mechanisms. First, Y922 might undergo a conformational change leading to environmental variations between the GDP- and GTP-bound interactions with Ras. Second, the conformation of Ras may differ between the GDP- and GTP-bound forms, giving rise to differences in protein·peptide docking patterns.

Given the observed differences in fluorescence intensity and apparent dissociation constants it is likely that interactions of the peptide with the protein (which contains the same structural elements regardless of whether Ras·GTP or Ras·GDP is present) is driven by the alteration to the effector domain of Ras dependent upon the nucleotides bound. This is exemplified by the NMR spectroscopic study of Y1345 (which does not possess a cysteine and therefore could not be modified with IAEDANS) which revealed little or no interaction with Ras. This suggests that the elements required for docking with Ras are located further toward the C-terminus of the 700–753 sequence. The fluorescence changes observed suggest that a change in the environment of the Y922 peptide allows residue C745 to report

upon the interaction with Ras. However, this interaction may be mediated by other residues in Y922, most notably three glutamic acid residues (aa's 720–722) which may form non-covalent interactions with Ras. Although these are present within Y1345 (which did not bind to Ras), the binding of Ras to GAP is likely to involve the C-terminal tail as well as central core amino acids. Such a motif is conserved in the GAP related NF1 protein [28] implying that the GTPase activating proteins may bind by a common mechanism.

Magnesium plays a role in Ras signalling since the GTPase activity is absolutely dependent upon the presence of Mg^{2+} [2]. We have observed that Mg^{2+} is also required for binding to the Y922 GAP peptide. With the wild-type protein, the loss of Mg^{2+} results in only a 1.28-fold lower binding affinity for the GTP-bound form of Ras, whereas with the G12D protein this difference is 3-fold. Additionally, with the Ras effector domain mutants, Y32A and Y40F, the effect of the removal of Mg^{2+} is greatest upon the Ras·GDP interactions. Most notably, the selectivity of GTP over GDP observed with the wild-type protein is lost in these two mutants, and the binding of Y32A·GDP is abolished in the absence of Mg^{2+}. These findings are likely to result from changes in magnesium ion coordination and imply that the protein·peptide docking pattern (orientation of Ras relative to the GAP peptide) and therefore proximity of GAP to the nucleotide and Mg^{2+} is also changed. Magnesium is presumably required to maintain a stable conformation in Ras for interaction with GAP, which is thereby critically dependent upon the presence of tyrosine residues at 32 and 40.

It is concluded that amino acids 715–753 of $p120^{GAP}$ mediate the interaction with Ras whilst residues 901–903 identified by mutational analysis as necessary for full GAP activity [18] are therefore most likely to constitute the catalytic domain of GAP. It is not suprising therefore that the peptide used in this study (aa's 715–753) does not enhance the GTP activity of Ras. Thus Y922 mimics fully the binding of GAP to Ras, discriminating between GTP- and GDP- bound Ras and requiring Mg^{2+} to stabilise the binding interaction. Y922 also demonstrates the predicted interactions with mutant forms of Ras, indicating further that Y922 mimics the true GAP·Ras interaction.

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