Raf is a serine/threonine kinase that binds through its amino-terminal regulatory domain to the GTP form of Ras and thereby activates the mitogen-activated protein kinase pathway. In this study, we have characterized the interaction of the Ras-binding domain of Raf with Ras using equilibrium binding methods (scintillation proximity assay and fluorescence anisotropy), rather than with more widely used nonequilibrium procedures (such as enzyme-linked immunosorbent assay and affinity precipitation). Initial studies using glutathione S-transferase fusion proteins with either residues 1-257 or 1-190 of Raf showed that although it was possible to detect Ras binding using an enzyme-linked immunosorbent assay or affinity precipitation, it was substochiometric; under equilibrium conditions with only a small excess of Raf almost no binding was detected. This difference was probably due to the presence of a high percentage of inactive Raf protein. Further studies used protein containing residues 51-131 of Raf, which expressed in Escherichia coli as a stable glutathione S-transferase fusion. With this protein, binding with Ras could readily be measured under equilibrium conditions. The catalytic domain of neurofibromin inhibited binding of Ras to Raf, and Raf inhibited the binding of Ras to neurofibromin showing that Raf and neurofibromin cannot be bound simultaneously to Ras. The affinities of interaction of neurofibromin and Raf with Harvey-Ras were similar. The rate constant for dissociation of Raf from Ras was estimated to be >1 min⁻¹, suggesting that Ras, Raf, and neurofibromin may be in rapid equilibrium in the cell. In contrast to previous reports, under equilibrium conditions there was no evidence for a difference in affinity between the minimal Ras binding domain of Raf (residues 51-131) and a region containing an additional 16 carboxyl-terminal amino acids, suggesting that residues 132-147 do not form a critical binding determinant. The proto-oncogene ras encodes a membrane-associated guanine nucleotide binding protein (M, 21,000) that is activated by mutation in approximately 30% of human tumors (for reviews, see Refs. 1-4). It acts as a molecular switch in signaling from growth factor receptors to the cell membrane to nuclear transciption factors via the cytoplasmic mitogen-activated protein kinase cascade (5, 6). Activation of a growth factor receptor by ligand binding results in the conversion of Ras from its inactive GDP-bound to active GTP-bound state. This occurs through nucleotide exchange catalyzed by exchange factors. Signaling is generally thought to be terminated by the binding of the GTPase-activating proteins, p120GAP and neurofibromin (NF1-334), which increase the intrinsically low rate of GTP hydrolysis on Ras (7, 8). GAPs, like other putative effectors, bind to the Switch 1 region of Ras (8).

The craf-1 proto-oncogene encodes a serine/threonine kinase (Raf) that is a 648-amino acid protein with an apparent M, of 74,000 (9). Comparison of the sequence of Raf with that of invertebrate homologues identified three conserved regions, CR1, CR2, and CR3 (10). CR1 (residues 61-192) consists of the Ras-binding domain (11-13) followed by a cysteine-rich region (residues 139-184) with a characteristic zinc finger motif (14). CR2 (residues 251-266) is rich in serine and threonine residues, some of which are phosphorylated on activation (15), and CR3 (residues 333-625) is the catalytic kinase domain. Ras binds directly to the amino-terminal region of Raf in a GTP-dependent manner (11-13, 16). Since Raf activates the mitogen-activated protein kinase cascade that is regulated by Ras (17-19), and dominant-negative forms of Raf block Ras-dependent activation of mitogen-activated protein kinase (20), it seems clear that Raf is an important effector of Ras signaling. A minimal region of Raf required for binding to Ras has been defined between residues 51 and 131 (11, 21-24), but whether other regions of Raf are also involved in binding to Ras is still the subject of debate. In particular, it has been claimed that the addition of residues 132-147 (or 132-149) increases the affinity of Raf (51-131) for Ras (23, 25, 26) and that the cysteine-rich region (residues 139-184) of Raf constitutes a second Ras-binding site (27).

Much of the previously reported data comparing the interactions of different Raf constructs with Ras have been obtained using nonequilibrium binding procedures involving separation steps (13, 23, 25-28), and little attempt has been made to ensure that all Raf proteins are equally active on a molar basis with respect to Ras binding. Furthermore, there is little data on the kinetics of dissociation of the Ras-Raf complex. In this study, we have concentrated on using equilibrium binding...
methods, scintillation proximity and fluorescence anisotropy assays, to compare the ability of Raf fragments to bind to Ras and to estimate the dissociation rate. An important aspect of these comparisons was to establish to what extent reported differences in apparent affinity might be due to differences in the fraction of active protein rather than in the intrinsic affinity.

**MATERIALS AND METHODS**

Cloning of Raf-(51–131) and Raf-(51–147)—DNA encoding residues 51–131 or 51–147 of c-Raf with the addition of a carboxyl-terminal Glu-Glu-Phenylmethyl sulfoxide and stop codon and incorporating BamHI and EcoRI restriction enzyme recognition sites was produced by polymerase chain reaction using Pfu polymerase (Stratagene), pGEX-Raf-(1–190) as a template and the following oligonucleotide primers: common 5′- TGA CTC GGA TCT CTT AAG ACA AGC; Raf-(51–131) 3′- CCT GCA GAA TTC TCA GAA CTC CTC GAG AATC TCT TGT; Raf-(51–147) 3′- CCT GCA GAA TTC TCA GAA CTC CTC CAG GAA CTT CGT TCC. The products were purified using Magic polymerase chain reaction PrepSpin™ (Promega), digested with BamHI and EcoRI, repurified, and ligated into digested and dephosphorylated pGEX2T vector. The sequences were confirmed using Taq terminator chemistry on an Applied Biosystems 373A automated DNA sequencer.

Expression in Escherichia coli—The pGEX 2T plasmids containing DNA encoding residues 1–190, 1–257, 51–131, or 51–147 were transformed into E. coli RR1 M15 (ATCC 35102) and, in the case of Raf-(51–147), also into BL21 DE3 (E. coli B strain lacking ompT protease). Luria-Bertani medium (50 ml) containing 200 μg of ampicillin/ml was inoculated with overnight culture at a dilution of either 1:100 (RR1 M15) or 1:20 (BL21 DE3). The cells were grown at 25°C until A500nm = 0.5 (data not shown), induction with 1 mM of 1-thio-beta-D-galactopyranoside for 4 h in the case of Raf-(51–131) and Raf-(51–147) (in BL21), overnight for Raf-(51–131) (in RR1 M15), Raf-(1–190), and Raf-(1–257), and harvested.

Purification of Raf Proteins—Raf-(1–190), Raf-(1–257), Raf-(51–131), and Raf-(51–147) were expressed in RR1 M15 as GST fusions and purified by glutathione-agarose as described previously (29, 31). Further purification of GST-Raf-(1–190) and GST-Raf-(1–257) was carried out by size exclusion chromatography using a Superose-12 gel filtration column (prep grade; 50 × 2 cm) run in 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM diethyldithrothreitol at 0.5 ml/min. Both GST-Raf-(1–257) and GST-Raf-(1–190) eluted over a relatively wide range of apparent Mr. Fractions with the least contamination by GST-fragments were pooled. In both cases the protein assay was the same for GST-Raf-(1–190) and GST-Raf-(1–257). Total concentrations of reactants present and did not take into account any differences in the percentage of active protein and also any changes in local concentration due to binding to SPA beads. The estimates of these concentrations therefore represent apparent rather than the absolute values, which might be obtained in a homogeneous system with proteins known to be 100% active. Moreover, this difference should not affect the interpretation of the experiments reported here.

**RESULTS**

Interaction of Ras with Raf-(1–257) and Raf-(1–190)—Raf-(1–257) and Raf-(1–190) were expressed as GST fusions and purified by chromatography on glutathione-agarose. In both

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cases, the glutathione eluate contained, in addition to full-length protein, many degradation products (Fig. 1, tracks 1 and 3), the majority of which were immunoreactive with anti-GST (data not shown). The intact proteins were further purified by size-exclusion chromatography. After this step, the GST-Raf-(1–257) was about 90% pure (Fig. 1, track 2), whereas the GST-Raf-(1–190) eluted as a broad peak and hence the preparation still contained many contaminating proteins (Fig. 1, track 4). The ability of these Raf fusion proteins to bind to Ras was initially monitored using ELISA, similar to that used by Ghosh et al. (25). When Ras was bound to the wells and the plate subsequently incubated with an excess of GST-Raf, a clear Ras-dependent binding signal was observed with both GST-Raf-(1–190) and GST-Raf-(1–257) (Fig. 2). However, when GST-Raf-(1–190) was bound to the plate, which was then incubated with excess Ras, no significant Ras-dependent signal was obtained (Fig. 2).

Binding of Ras to Raf has frequently been monitored by using glutathione-agarose to precipitate a GST-Raf/Ras complex (affinity precipitation). Both GST-Raf-(1–190) and GST-Raf-(1–257) fusion proteins were able to precipitate Ras-GTP. However, the amount of Ras precipitated was markedly substoichiometric in that using microgram quantities of GST-Raf, the bound Ras could not be detected by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining but only by immunodetection on Western blots (data not shown). It was possible that the inability to detect binding of Ras to GST-Raf-(1–190) when the latter was prebound to the plate and the low stoichiometry seen in the affinity precipitation assay might have been because both are nonequilibrium binding assays involving separation steps. Therefore we attempted to measure Ras/Raf binding using a scintillation proximity assay. A SPA procedure, employing protein A fluoromicrospheres coated with anti-GST, has recently been used to measure the binding of GST-NF1–334 to Ras complexed with [3H]GTP under equilibrium conditions (29). However, when this assay was performed using 0.03 μM Ras[3H]GTP with either GST-Raf-(1–257) or GST-Raf-(1–190), the signal obtained was at least 40-fold lower than that with the same molar concentration of GST-NF1–334 (data not shown).

Interaction of Raf-(51–131) with Ras Monitored by SPA—The data presented above suggested that the primary reason for the weak signal in the SPA, the low stoichiometry of affinity precipitation, and the dependence of the ELISA signal upon the order of addition was not the nonequilibrium nature of the assays. An alternative explanation was that the Raf fusion proteins were much less than 100% active with respect to binding to Ras. The extensive degradation of GST-Raf-(1–190) or GST-Raf-(1–257) when expressed in E. coli (Fig. 1, tracks 1–4) suggested that these regions are not stable domains, in which case a large proportion of the expressed proteins might be incorrectly folded and hence inactive. We therefore examined the properties of a fragment of Raf comprising residues 51–131, which represents the minimal Raf binding region (11, 21–24).

As observed by others, GST-Raf-(51–131) expressed at high levels in E. coli as a soluble protein, which showed little degradation. The fusion protein was virtually pure after glutathione elution and could also be cleaved with thrombin to produce pure Raf-(51–131) (Fig. 1, tracks 7 and 8). The proteins were demonstrated to be intact by their reactivity with the YL1/2 antibody (which recognizes the Glu-Glu-Phe epitope placed at the carboxyl terminus of the construct) and by electrospray mass spectrometry (data not shown).

GST-Raf-(51–131) was added to protein A SPA beads, anti-GST, and Ha-RasLeu-61[3H]GTP in a scintillation proximity assay. In contrast with the longer Raf constructs described above, a signal similar to that with GST-NF1–334 was produced. As has been reported with GST-NF1–334 (29), the signal increased with increasing concentrations of GST-Raf until a maximum was reached, and it then decreased (Fig. 3a). These results are consistent with the hypothesis that with increasing concentration of GST-Raf, the signal increases as more Ras-GST-Raf complex is formed and then decreases due to competition between free and Ras-bound GST-Raf for binding to the limiting amount of anti-GST antibody. The specificity of the signal was confirmed in that it was dependent upon the presence of both anti-GST and GST-Raf and was abolished by the neutralizing anti-Ras antibody, Y13–259 (Fig. 3b).

Rate of Dissociation of Raf from Ras—The SPA procedure allowed us to estimate the rate of dissociation of Raf from the Ras/Raf complex. GST-Raf was displaced from binding to Ras[3H]GTP by the addition of either 1.7 μM NF1–334 or 1.0 μM unlabeled Ras to an equilibrated Rast/GST-Raf/SPA bead mixture, and the loss of SPA signal was monitored. The effect of either addition was an 80% decrease in signal within 3 min with little further decrease over the next 50 min. Therefore the rate constant for the dissociation of GST-Raf from Ras at 22°C must be greater than 0.5 min⁻¹. The results were the same.
using a SPA in which Ha-Ras-Leu-61[\textsuperscript{3H}]GTP was replaced by K-RasVal\textsuperscript{12}-mant-GTP.

In order to establish that the rapid dissociation rates observed in the SPA were not an artifact of the assay procedure, an alternative method of monitoring the dissociation of the Ras-Raf complex was used. The fluorescence anisotropy signal given by Ha-RasLeu-61 being mant-GTP has been shown to increase when binding to p120-GAP or to NF1–334 occurs (34). A similar increase in anisotropy was seen after the addition of GST-Raf-(51–131) to a solution of K-RasVal\textsuperscript{12}-mant-GTP (Fig. 4) or Ha-RasLeu-61-mant-GTP (data not shown). Subsequent addition of an excess of unlabeled Ha-RasLeu-61-GTP reduced the signal to that of Ras-mant-GTP alone within the time taken to make the addition (<0.5 min) at 25 °C, suggesting a rate constant for dissociation of >1 min\textsuperscript{-1} (Fig. 4). As expected, after further addition of excess GST-Raf-(51–131), the fluorescence anisotropy signal increased again to the level before addition of unlabeled Ras (data not shown).

Comparison of Raf-(51–131) with Raf-(51–147)—Residues 132–147 have been implicated as increasing the affinity of binding of Raf to Ras. Since the measurements on which these conclusions were based were performed under nonequilibrium conditions, and no data were presented to show that the proteins were intact and fully active, we devised experiments to compare rigorously the affinity of a Raf fragment encoding residues 51–147 with that encoding Raf residues 51–131 described above.

Raf-(51–147) was expressed as a GST fusion again with the added C-terminal tripeptide sequence, Glu-Glu-Phe. This produced soluble protein, which was apparently homogeneous by SDS-polyacrylamide gel electrophoresis. It reacted with anti-GST, but it showed a very low reactivity with the YL1/2 antibody compared with the GST-Raf-(51–131) (data not shown), suggesting degradation at the carboxyl terminus. Electrospray mass spectrometry revealed several species; the major one (M, 36874) corresponded to loss of residues 144–147 and would have been produced by proteolytic cleavage between the paired basic residues Arg-143 and Lys-144. This degradation was largely prevented by expression in the ompT-deficient E. coli strain BL21 (31). Electrospray mass spectrometry showed that a high percentage of the protein purified from this strain both as a GST-fusion and as a thrombin-cleaved fragment was now of the calculated M, for the intact protein. GST-Raf-(51–147) gave a significant signal in the SPA (Fig. 5). In order to compare the affinity of Ras for Raf-(51–131) and Raf-(51–147), it was necessary to ascertain whether both Raf proteins were similarly active. The titration of Ras[\textsuperscript{3H}]GTP into a mixture of protein A SPA beads, anti-GST, and a fixed amount of protein active in binding Ras. There was very little difference in the maximum SPA signal between GST-NF1–334 and GST-Raf-(51–131), but the same concentration of GST-Raf-(51–147) gave 50% of this signal (Fig. 5). At that concentration of Ras required to saturate GST-Raf-(51–131) binding, GST-Raf-(1–257) and GST-Raf-(1–190) gave about 20 and 2% of this signal, respectively, consistent with the hypothesis suggested above that these two proteins contain high proportions of inactive protein. The apparent K\textsubscript{D} for the Ras binding interaction estimated from titrations with increasing Ras concentrations using the model described under “Materials and Methods” was found to be similar for GST-Raf-(51–147) and GST-Raf-(51–131) (Table I). When this calculation was repeated making the assumption that the GST-Raf-(51–147) was only 50% active, the apparent K\textsubscript{D} was only increased by 6%, which is within the experimental error.

The interactions between Raf fragments and Ras were further monitored by assessing the ability of Raf or NF1–334 (as thrombin-cleaved proteins) to compete with GST-Raf or GST-NF1–334 for binding to Ras[\textsuperscript{3H}]GTP in the SPA (Fig. 6 and Table I). NF1–334 abolished the binding of Ras to GST-NF1–334, GST-Raf-(51–131), or GST-Raf-(51–147). The potency of inhibition was identical in all three assays (I\textsubscript{50} = 50 nM) (Table
Similarly, Raf-(51–131) and Raf-(51–147) abolished the signal from SPAs using Ras binding to GST-Raf-(51–131), GST-Raf-(51–147), or GST-NF1–334, with similar potencies (Table II). Thus, again there was no evidence of any significant difference in Ras binding affinity between the two Raf fragments.

**DISCUSSION**

Our initial studies used proteins containing residues 1–257 and 1–190 of Raf, both of which contain the minimal Ras-binding region (residues 51–131). Using ELISA and affinity precipitation techniques, these longer fragments appear to be unable to bind Ras stoichiometrically. Both techniques involve separation steps and therefore do not measure equilibrium binding but the amount of binding detected in a scintillation proximity assay under equilibrium conditions was also much lower than expected for the amount of Raf proteins added. Significantly, the amount of binding detected by ELISA was much higher when excess Raf was allowed to bind to Ras attached to the plate than when excess Ras was allowed to bind to Raf attached to the plate. These data suggested that the low stoichiometry was not due to the nonequilibrium nature of the binding procedures and that the Raf-(1–257) and Raf-(1–190) proteins were far from being fully active with respect to binding Ras. This was confirmed using the Raf-(51–131) protein, which gave the same amount of Ras binding as an equivalent molar concentration of NF1–334 when measured under the SPA equilibrium conditions, compared with the 5- and 50-fold lower maximum values obtained using GST-Raf-(1–257) and GST-Raf-(1–190), respectively. It was notable that the longer Raf fragments were extensively degraded in *E. coli*, whereas the Raf-(51–131) was stable. Probably the longer fragments were incorrectly folded and hence both sensitive to proteolytic degradation and also much less active with respect to Ras binding.

We have examined for the first time the kinetics of dissociation of Ras (either Leu-61 or Val-12 mutants) from Raf. Experiments were performed in which Ras was displaced from the Ras/Raf complex and the amount of remaining complex determined using both SPA and fluorescence anisotropy procedures. The rate of dissociation was very fast, and the dissociation rate constant was greater than 1 min⁻¹. In view of these high dissociation rates, nonequilibrium binding procedures, such as have been frequently used in studies of the interaction of Ras with...
with Raf, will not give true measures of affinity. Given such a high dissociation rate, it is surprising that Ras binding could be observed by either affinity precipitation or ELISA procedures, both of which involve extensive washing. The explanation for this is probably a combination of two factors. First an avidity effect of the local concentration of protein bound to either the beads or the plate (cf. Ref. 35) and second the detection procedures employed (either radioactivity or antibody), which are so sensitive that grossly substoichiometric binding can be detected. Indeed where it is possible to calculate data from the literature, it would appear that the binding was substoichiometric (12, 36).

One of the main aims of this work was to compare the affinity of Ras binding to the two different length Raf fragments, residues 51–131 and 51–147. In view of the high dissociation rates and the substoichiometric binding observed with the longer fragments, it was considered particularly important to use an equilibrium binding method (the scintillation proximity assay) and to establish that the proteins being compared were intact, pure, and had comparable biological activities. Initially both proteins were expressed in E. coli RR1LM15. However, although both purified proteins appeared homogeneous by SDS electrophoresis, the 51–147 protein was proteolytically degraded such that residues 144–147 and the added Glu-Glu-Phe epitope were missing. Full-length protein was obtained using the ompT protease-deficient E. coli strain BL21. The sensitivity to proteases suggests that the region around residue 144 is not highly structured, whereas Raf (51–131) has a well-defined structure (24, 37). The 51–147 protein gave a maximal signal in the SPA about half that of the 51–131 protein, suggesting that not all of the protein was functionally active. The affinity of interaction between Ras and Raf was measured both directly through a titration in which the Ras concentration was varied and indirectly by measuring the ability of NF1–334 or Raf to prevent binding of Ras to GST-Raf. The affinity of interaction between Ras and NF1–334 was nearly identical to that between Ras and Raf (51–131), and the latter was similar to that previously reported (13, 21–23). We detected no significant differences in affinity for Ras between Raf (51–131) or Raf (51–147) using any of these procedures, even when corrections were made for the activity of the GST-fusion proteins assessed by the maximal signal obtained in the SPA. Hence, in contradiction of the conclusions of Ghosh et al. (25, 26) and Chuang et al. (23), we find no evidence that residues 132–147 form a critical determinant in the Ras/Raf interaction. We do not know definitively why there is a discrepancy in our conclusions, but the following factors would be sufficient to explain it. First, non-equilibrium binding procedures involving separation steps were used to estimate affinities, and the apparent affinities would therefore reflect dissociation rates rather than absolute affinities. Second, the integrity of the proteins was not established. Third, the stoichiometry of interaction was not reported, and hence the proteins may not have been fully or equally active, resulting in measurement of apparent rather than true affinities. In our opinion, the latter is likely to be particularly significant. In support of this, longer GST-Raf constructs gave a weak maximal SPA signal (Fig. 5), and Raf (51–147), when expressed as a biotinylated transcarboxylase (Pinpoint) fusion, gave no SPA signal.  

Using the SPA, we showed that the thrombin-cleaved Raf fragments abolished the signal given by GST-NF1–334 binding to Ras (Fig. 6a) and that NF1–334 abolishes the signal given by GST-Raf binding to Ras (Figs. 6, b and c). Other groups have shown that Raf inhibits the stimulation of Ras GTP hydrolysis by p120GAP and neurofibromin (12, 13, 22, 23), but as this inhibition appeared to be less than 100%, it was unclear whether the proteins were fully competitive. Thus, this is the first demonstration that Raf and NF1–334 block the physical interaction between Ras and NF1–334 and Ras and Raf, respectively, and we conclude that Raf and NF1–334 cannot be bound simultaneously to Ras.

There are conflicting inferences in the literature about the dissociation rate for the Ras/Raf interaction. Since Ras/Raf binding is readily observed using nonequilibrium techniques such as affinity precipitation or ELISA, there has been a tacit assumption that the dissociation rate must be relatively slow so that a stable complex is formed. In contrast, Zhang et al. (13) suggest that their inability to co-immunoprecipitate Ras and full-length Raf from cell lysates may be due to the transient nature of the interaction. Our own experiments, which are the first to directly measure the dissociation kinetics, clearly demonstrate that the Ras binding domain of Raf dissociates relatively rapidly from the nucleotide binding domain of Ras-GTP (Fig. 4). Similar results were obtained both with Val-12 and Leu-61 Ras variants, consistent with the report that the affinities of interaction of Raf with Val-12 and Leu-61 Ras, and also normal Ras, are similar (21). Based on the assumption that the dissociation rate of Raf from Ras is very slow, it has been postulated that the Ras/Raf signal is terminated by the intrinsic GTP hydrolysis rate (21, 24). Although the signal might indeed be at least in part, terminated by the intrinsic GTPase, as discussed in Refs. 3 and 30, the data presented here suggest a rapid equilibration between Ras and Raf (and also between Ras and neurofibromin and Ras and p120-GAP) in the cell, which would allow GTPase activating proteins to down-regulate signaling through Raf. Further work on the kinetics of dissociation of full-length Raf from processed Ras-GTP attached to the plasma membrane might help to obtain a better understanding of the system in the cell.

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Equilibrium and Kinetic Measurements Reveal Rapidly Reversible Binding of Ras to Raf

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