The Minimal Fragments of c-Raf-1 and NF1 That Can Suppress v-Ha-Ras-Induced Malignant Phenotype*

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v-Ha-Ras, an oncogenic Ras mutant, causes malignant transformation of mammalian cells by recruiting c-Raf-1, a cytosolic Ser/Thr kinase, to the plasma membranes/cytoskeleton. The kinase activity of c-Raf-1 resides in the C-terminal half, which activates mitogen-activated protein (MAP) kinase kinase, while it is the N-terminal half of c-Raf-1 (Raf257, residues 1-257) that binds the Ras-GTP complex and can compete Ras GTPase-activating proteins such as NF1 for binding to Ras. However, it still remains to be clarified whether overexpression of Raf257 or its minimal Ras-binding fragment alone is sufficient to suppress Ras-induced malignancy. In this paper we demonstrate for the first time that the 81-amino acid fragment (Raf81, residues 51-131), the minimal Ras-binding fragment of Raf, indeed can suppress v-Ha-Ras-induced malignant phenotype. A further deletion of the first 6 amino acids causes 65% reduction in the Ras binding of Raf81. The resultant 75 amino acid fragment (Raf75, residues 57-131) consists of a single a-helix, five anti-parallel b-sheets and five loops. We have found that a further deletion of either the first b-sheet/loop or the last two b-sheets/loops completely abolishes Ras binding. In addition we have found that the removal of the C-terminal 35 amino acids from a Ras-binding 91-amino acid fragment of NF1 (NF91, residues 1441-1531) does not abolish its ability to suppress the Ras-induced malignancy.

The proto-oncogene product c-Raf-1 is a member of Raf (Ser/Thr kinase) family and consists of 648 amino acids (1). The normal Raf is not oncogenic, but once it is mutated, it can cause malignant transformation in mammalian cells; in particular, when it is deprived of the first 305 amino acids, it becomes highly oncogenic (2). This suggests that the N-terminal half potentially could serve as a tumor suppressor. The normal Raf is also a latent protein kinase but can be activated by several distinct mitogenic growth factors such as epidermal growth factor and platelet-derived growth factor through a chain reaction involving Grb-2, Sos and Ras-GTP complex (3). The Ras-GTP complex recruits Raf to plasma membrane-associated cytoskeleton where the activation of Raf appears to take place (4, 5). Raf in turn phosphorylates and activates a Ser/Thr/Tyr kinase called MAPK kinase kinase (MAPKKK or MEK) (3). This Raf-MEK signal cascade eventually leads to malignant transformation via phosphorylation of the elongation factor 4E at Ser53 (6), or the transcription factors c-Jun and c-Myc at their N-terminal regulatory domains (7, 8). The kinase Raf also can be activated by mutations, in particular by deletion of its N-terminal half (Raf257, residues 1-257), indicating that the Raf kinase activity resides in its C-terminal half, and that the domain Raf257 serves as an intra-molecular kinase suppressor (9).

Interestingly, Raf257 alone can suppress the transcription of specific genes activated by oncogenic mutants of Ras such as v-Ha-Ras but not by an oncogenic mutant of Raf (10), suggesting that Raf257 competes the normal full-length Raf for binding to a Ras-GTP-dependent upstream activator(s) of the kinase Raf. It was shown recently that Raf257, but not the full-length Raf, binds tightly the Ras-GTP complex (11-14), clearly indicating that (i) Raf is an immediate effector of Ras-GTP and that, (ii) the Ras binding of Raf257 is weakened or blocked by the C-terminal kinase domain of Raf. However, it still remains to identify a third protein(s) that allow the Ras-GTP complex to bind tightly the full-length Raf, and also to clarify how the C-terminal kinase domain of Raf is released from its N-terminal domain to be activated on the cytoskeleton.

It appears that Raf257 competes at least two other proteins, i.e. GAP1 and NF1, for binding to the Ras-GTP complex in vitro (11, 12). GAP1 and NF1 (neurofibromatosis) are Ras GTPase-activating proteins of 1044 and 2818 amino acids, respectively (15, 16). Mutations in the effector domain (residues 32-40) of Raf completely abolish its binding to Raf257, GAP1, and NF1 (11, 12, 17-19). We have shown previously that a series of small Ras-binding fragments of NF1 including NF91 (residues 1441-1531) can suppress v-Ha-Ras-induced malignant transformation of NIH/3T3 fibroblasts (20), suggesting that these NF1 fragments and Raf257 share the same binding domain(s) of the Ras molecule, if not exactly identical spot(s), even in vivo.

To determine how closely the NF1-binding and Raf-binding sites (if they differ) on the same Ras molecule are situated in three dimensions, and to further identify the key Ras-binding residues of Raf by x-ray crystallography, NMR analysis, or site-directed mutagenesis, it is important to produce the smallest Ras-binding fragment of Raf. We have demonstrated previously that a 78-amino acid fragment of NF1 (NF78, residues 1441-1518) is the minimal GTPase-activating peptide (20). Furthermore, we have shown that a Ras-binding 91-amino acid fragment of NF1 (NF91, residues 1441-1531) can suppress v-Ha-Ras-induced malignant transformation of NIH/3T3 fibroblasts (20). Although, so far nobody has demonstrated that Ras-binding fragments of Raf can suppress v-Ha-Ras-induced malignant transformation. In this paper, using a series of N- and C-terminal deletion mutants of Raf257, we demonstrate that its 81-amino acid frag-

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ment Raf1 (residues 51-131) has the highest affinity for the Ras-GTP complex and its overexpression indeed can suppress v-Ha-Ras-induced malignant phenotype, i.e., anchorage-independent cell growth. In addition we demonstrate that the smaller NF1 fragments such as NF78 and NF56 (residues 1441-1496) still can suppress v-Ha-Ras-induced malignancy, although their affinity for Ras is extremely low in vitro, suggesting that a third protein may be involved in stabilizing the Ras-NF1 interaction in vido.

EXPERIMENTAL PROCEDURES
Preparation of Recombinant c-Raf-1 and NF1 Mutants and Ras GTPases—The normal v-Ha-Ras and oncogenic v-Ha-Ras GTPases were purified from Escherichia coli according to the procedure of Gibbs et al. (25). To produce a series of human c-Raf-1 and NF1 fragments as GST (glutathione S-transferase) fusion proteins in the bacteria, we have subcloned the corresponding polymerase chain reaction DNA fragments into the bacterial expression vector pGEX-2TH (21). Each GST fusion protein was then affinity-purified from the bacterial extract as described previously (20, 21, 22, 24).

In Vitro Assay for the Binding of Ras-GTP Complex to c-Raf-1 or NF1 Mutants—The purified v-Ha-Ras GTPase (25 pg) was incubated with [γ-32P]GTP (37 °C for 15 min in 1 ml of a GDP-GTP exchange buffer containing 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 3 mM ATP, 1 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin. To stop the GDP-GTP exchange, 15 mM MgCl2 and 20 μM ZnCl2 were then added to the reaction mixture. For the binding assay, an aliquot of the [γ-32P]GTP-v-Ha-Ras complex (100 μl) was then incubated at 25 °C for 15 min with GST fusion proteins containing c-Raf-1 or NF1 mutants that were immobilized on GSH-agarose beads. The mixture was transferred onto a glass microfiber filter (Whatman GF/C), and the filter was washed four times with 1 ml of a binding buffer (cold) that was identical to the GDP-GTP exchange buffer except that 2.5 mM EDTA was replaced by 10 mM MgCl2 and 20 μM ZnCl2. The radioactivity of [γ-32P]GTP-v-Ha-Ras complex left on the filter was measured in a scintillation liquid. Since the oncogenic v-Ha-Ras GTPase is never activated by either NF1 or Ras, the GAP activity of NF1 or Raf fragments (if any) does not interfere with this binding assay.

Construction of the Plasmids Expressing Raf1, NF78, or NF56 in Mammalian Cells—An EcoRI DNA fragment of 0.3 kilobases, containing at the 5' end a Kozak sequence (GCC GCC ACC ATG) followed by a termination codon (TGA) and HindIII linker (AAGCTT), was prepared by polymerase chain reaction (PCR) and subcloned into the retroviral vector pMV7, which encode the human NF1 residues 1441-1518 (NF78) and residues 1441-1496 (NF56), respectively, were also prepared in a similar manner.

Assay for Colony-Forming Ability in Soft Agar of v-Ha-Ras-transformed Cells Expressing Raf1, NF78, or NF56—v-Ha-Ras-transformed NIH3T3 cells (21) were transfected with 1 μg of the plasmids Raf81/ pMV7, NF78/pMV7, NF56/pMV7, or pMV7 alone (as a control) as complexes with liposomes (35 pg) as described previously (23). Colony-forming ability of the parental clone and each transfectant was examined in soft agar by incubating 1000 cells/plate at 37 °C for 2 weeks under the standard culture conditions (21). The colonies were stained with crystal violet and counted.

Reverse Transcriptase PCR Analysis of Raf81 and v-Ha-Ras Expression—Total RNAs from each transfected clone were isolated by the method of Chomczynski and Sacchi (22). 5 μg of each RNA preparation was then used as a template to synthesize cDNA by avian myeloblastosis virus reverse transcriptase (Promega) and a Ribonuclease cDNA synthesis kit (Promega). The same aliquots of all cDNA preparations were used as templates for PCR to amplify selectively human Raf81 and v-Ha-Ras DNA sequences using the corresponding pairs of sense and antisense primers as described previously (21, 23). Under these conditions, the amount of PCR products is linearly proportional to the initial amount of cDNA used as template and is far below the saturation point. Standard titration curves were drawn based on the incorporation of [α-32P]GTP into the PCR bands, using both cloned v-Ha-Ras and c-Raf-1 DNAs at various concentrations (0.1–10 pg) as PCR templates (data not shown).

RESULTS AND DISCUSSION
Ras-binding Domain of c-Raf-1—The fragment Rafl57, consisting of the first 257 amino acids of human c-Raf-1, has been shown previously to be sufficient to bind to the Ras-GTP complex directly in vitro (11, 12). Furthermore, using a two-hybrid system in yeast, Voetjek and colleagues (26) have provided indirect evidence suggesting, if not proving, that either the first 50 amino acids of c-Raf-1 or any residues downstream of position 132 in c-Raf-1 are not required for its Ras binding. To further these findings and identify the smallest Ras-binding domain of the Raf, we have affinity-purified a series of N- and C-terminal deletion mutants of the Raf as GST fusion proteins from E. coli (see Fig. 1A) and examined in vitro the direct binding of the v-Ha-Ras-[γ-32P]GTP complex to these Raf-GST fusion proteins, which are linked to GSH-agarose beads (see Fig. 1B). In this assay GST alone does not bind to the v-Ha-Ras-GTP complex. First we have confirmed that the 81-amino acid fragment
TABLE I

Anchorage-independent growth of Raf81 transfectants derived from v-Ha-Ras transformants

| Clones | Large | Medium | Small | Total | Suppression |
|--------|-------|--------|-------|-------|-------------|
| Vector alone | 126   | 100    | 134   | 360   | 0%          |
| Raf81 transfectants: | | | | | |
| Clone 15 | 0     | 2      | 5     | 7     | 98%         |
| Clone 10 | 9     | 8      | 33    | 50    | 88%         |
| Clone 37 | 8     | 11     | 87    | 106   | 71%         |
| Clone 47 | 19    | 24     | 120   | 163   | 55%         |
| Clone 14 | 32    | 22     | 58    | 112   | 69%         |
| Average | 14    | 13     | 61    | 88    | 76%         |

1000 cells of each transfectant were plated in soft agar, and after 2 weeks the number of colonies formed in each dish was counted. The size of colony: Large, more than 100 cells; Medium, around 30 cells; Small, less than 10 cells/colony. Each presented value was the average of the data from three independent experiments, and the standard deviation in each case was less than 5%.

of c-Raf-1 called Raf81 (residues 51–131) binds Ras tightly. It is of interest to note that the apparent affinity of Raf81 for Ras is significantly higher than that of Raf257. These observations were confirmed recently by Marshall and colleagues (27). The removal of the first 6 amino acids from Raf81 reduces significantly, but does not abolish, its binding to Ras (Fig. 1B). The resultant 75-amino acid fragment called Raf75 has been shown to consist of a single helix (residues 79–89), five anti-parallel β-sheets and five loops (28): β-sheets I (57–61), II (67–71), III (96–101), IV (110–112), and V (125–130). We found that the deletion of either the first β-sheet/loop or the last two β-sheets/loops abolishes completely the Ras binding; neither Raf66 (residues 66–131), Raf52 (residues 51–102), nor Raf37 (residues 66–102) binds to Ras (Fig. 1B). These observations indicate that (i) so far Raf75 (residues 57–131) is the smallest fragment that still binds Ras, although its affinity for Ras appears 3-fold lower than that of Raf81, and that (ii) some residues in both β-sheets I and IV-V are absolutely essential for its binding to Ras. We are currently identifying the key Ras-binding residues of Raf75 by further systematic deletion/point mutations. So far Leu6' in the first loop and two basic residues in the helix (Lys34 and Arg35) were found to be absolutely essential for Ras binding.

Anti-Ras Action of Raf81 in Vivo—Since the Raf257 has been shown to suppress the v-Ha-Ras-induced transcription of specific genes in NIH/3T3 cells (10), and among the Raf fragments Raf81 shows the highest affinity for Ras, its potential anti-Ras action was also examined by overexpressing Raf81 in the v-Ha-Ras-transformed NIH/3T3 cells under the control of a strong LTR promoter of the retroviral vector pMV7. As shown in Table I, the Raf81-expressing cells exhibit a much lower colony-forming ability than the Ras-transformed parental cells in a soft agar, indicating that Raf81 also effectively interacts with v-Ha-Ras in vivo. These Raf81-transfected cells express both v-Ha-Ras and Raf81 as revealed by a semiquantitative reverse transcriptase PCR analysis (see Fig. 2A). In an attempt to establish the correlation between the in vitro Ras binding and the in vivo anti-Ras action of the Raf fragments, we are currently examining the anti-oncogenicity of Raf75 and several other Raf fragments, which are smaller than Raf81 but larger than the non-Ras-binding fragment Raf37.

Anti-Ras Action of NF78 and NF56 in Vivo—We have shown previously (20) that (i) the NF1 fragments of 91 and 78 amino acids (NF91, residues 1441–1531 and NF78, residues 1441–1518) can still activate normal Ras GTPases, but the NF56 (residues 1441–1496) does not, and that (ii) NF91 can suppress strongly v-Ha-Ras-induced malignant transformation. In an attempt to identify further the minimal NF1 fragment capable of suppressing the oncogenicity of v-Ha-Ras, we have examined the possible anti-Ras action of both the GTPase activating NF78 and the non-GTPase activating NF56 by overexpressing these NF1 fragments in the v-Ha-Ras-transformed cells. As shown in Table II, out of 20 randomly picked transfectants (G418-resistant cells), 7 NF56 transfectants (35%), and 12 NF78 transfectants (60%) showed the soft agar colony forming ability less than 10% of that of the parental Ras transfectants that were transfected with the vector alone. These observations suggest that both NF78 and NF56 are still anti-oncogenic as Raf81 and NF91 (20). Further Northern blot analysis has confirmed that the reduction of anchorage-independent growth of v-Ha-Ras transfectants by either NF56 or NF78 transfections is due not to any loss of v-Ha-Ras gene expression, but to overexpression of the NF1 fragments (see Fig. 2B).

Ras Binding Activity of NF78 and NF56—Under the in vitro conditions where both Raf81 and Raf75 form a stable complex with v-Ha-Ras, v-Ha-Ras[32P]GTP, no stable complex formation of either NF78 or NF56 was detected with the Ras-GTP (see Fig. 1B). Since NF78 was shown previously to activate normal Ras GTPases (20), it is clear that NF78 binds Ras, but its affinity for Ras must be much lower than that of Raf81 or Raf75 in vitro. Under different assay conditions, however, NF56 still can

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bind a Ras-GTP complex (c-Ha-Ras-\(\gamma\)-S)GTF) in vitro. Despite the great difference in the affinity for Ras between NF78/NF56 and Raf81 in vitro, their anti-oncogenicity (anti-Ras action in vitro) appears to be quite similar. These observations suggest that a third protein is involved in the stabilization of the Ras-GTP complex with these NF1 fragments in vitro. Since the microtubule-binding domain and GTPase-activating/Ras-binding domain of NF1 overlap each other (29,30), it is possible that the third protein may be microtubule. Alternatively, either Ras or the NF1 fragments are posttranslationally modified, perhaps phosphorylated, to form a tight complex. We are currently identifying the protein(s) in NIH3T3 cell extracts that are involved in the stabilization of the NF78-v-Ha-Ras complex in vitro.

It is of interest to note that the two distinct Ras-GTP-binding fragments, i.e. NF78 (residues 1441–1518) and Raf75 (residues 57–131), of a similar molecular size appear to share little sequence homology, if any. This might suggest that the NF78-binding spot probably differs from the Raf75-binding spot even if the two fragments appear to bind rather competitively the same general area (or domain) of the Ras molecule. X-ray crystallographic analysis of both NF78-v-Ha-Ras-GTP and Raf75-v-Ha-Ras-GTP complexes in three dimensions is now under way to identify the key Ras residues as well as the residues of Raf75 and NF78 that are responsible for their unique interactions. This would provide valuable information not only for our better understanding of the Raf-Ras or NF1-Ras interaction, but also for further designing a highly specific anti-Ras chemical drug, which would be potentially useful for the treatments of Ras-associated cancers, which represent about 30% of total human carcinomas, notably more than 90% of pancreas carcinomas and 50% of colon carcinomas (31).

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### Addendum
After the submission of this manuscript, two reports appeared which confirm our observation that Raf81 is the minimal Ras-binding fragment of c-Raf-1 (27, 28). The latter report (28) further shows an NMR solution structure of this Raf fragment.

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