The Cysteine-rich Region of Raf-1 Kinase Contains Zinc, Translocates to Liposomes, and Is Adjacent to a Segment That Binds GTP-Ras*

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Different domains of the serine/threonine kinase, Raf-1, were expressed as fusion proteins with glutathione S-transferase (GST) in Escherichia coli and purified to near homogeneity by affinity chromatography. A cysteine-rich domain of Raf-1 was found to contain 2 mol of zinc (molar basis), similar to analogous cysteine-rich domains of protein kinases. GST fusion proteins, containing the cysteine-rich domain of Raf-1, bound to liposomes in a phosphatidylycerine-dependent manner. In contrast to protein kinase C, the translocation of Raf-1 was not dependent upon diacylglycerol, phorbol ester, or calcium, nor did Raf-1 bind phorbol esters. A GST-fusion protein encoding residues 1-147 of Raf-1 bound to normal GTP-mutant GTP-B-13-Ala-GTP-ras; no binding was detected to GDP-ras. The binding of a smaller fusion protein (residues 1-130 of Raf-1) was about 10-fold weaker, inferring that a 17-amino acid sequence represents a critical binding determinant in intact Raf-1. These residues are adjacent to the amino-terminal end of, and partially extend into, the cysteine-rich domain (amino acids 139-184). A synthetic peptide corresponding to this 17-amino acid sequence blocked the interaction of Raf-1 with ras. The function of the cysteine-rich region of Raf-1 homologous to protein kinase C is to promote translocation of Raf-1 kinase to membranes and to form part of the high affinity binding site for GTP-ras.

Raf-1, a serine/threonine protein kinase, is regulated by growth factors such as insulin, epidermal growth factor, platelet-derived growth factor (1), and interleukin 2 (2) as a consequence of phosphorylation on serine/threonine (1) and tyrosine (2) residues by an unknown set of kinases. Raf-1 kinase is believed to play an essential role in cell growth as a regulator of mitogen-activated protein kinase (MAP) kinases (3) and thereby regulating the cascade leading to MAP kinase activation. The activation of MAP kinases further leads to the activation of downstream kinases including p90<sup>SR</sup> and MAP kinase-activated protein kinase 2, or phosphorylation of transcription factors such as c-myc, c-jun, p62<sup>p50</sup>, and Elk-1 (4, 5).

The small, GTP-binding protein, p21<sup>CIP</sup>, plays a critical role in many intracellular signal transduction pathways. Like other GTP-binding proteins, ras cycles between an active GTP-bound form (GTP-ras) and an inactive GDP-bound form (GDP-ras) and is linked, functionally, to several different effectors of early signal transduction events (6). Some candidate proteins known to interact with ras include the GTPase-activating protein (7), neurofibrin (8), cytotoxic T lymphocytes (9), and a novel protein histidine kinase (10). The Raf-1 kinase has only recently been identified as a direct downstream target of ras (11).

Several lines of evidence suggest that ras and Raf-1 function in the same signaling pathway and that Raf-1 lies downstream of ras. For instance, transformation by v-ras is independent of ras function (12, 13) and transformation by oncogenic ras can be suppressed by expression of a kinase-deficient ras mutant (14). Conversely, blocking ras action, either by microinjection of antibodies or through expression of a dominant negative ras mutant prevents Raf-1 and MAP kinase activation (14, 15).

Raf-1 has also been genetically assigned to act as a downstream component in ras-mediated signaling pathways during eye development of Drosophila (16) and induction of vulval differentiation in Caenorhabditis elegans (17). Finally, evidence for a direct interaction on ras-GTP with ras-1 and a MAP kinase kinase has recently been reported both in vitro and in yeast two-hybrid systems (11, 18-20). It appears that only the NH<sub>2</sub>-terminal domain of Raf-1 is required for interaction with ras.

Structurally, the Raf-1 kinase displays considerable similarity to another group of protein serine/threonine kinases, collectively known as protein kinase Cs. For example, both proteins contain an amino-terminal regulatory and a carboxyl-terminal catalytic domain. Additionally, similar cysteine-rich motifs are present within the regulatory domain of each protein. In the classical, calcium-dependent PKCs (cPKCs, 21), the two cysteine-rich zinc finger motifs are implicated in mediating the lipid-dependent activation and translocation of the kinase to the membrane (22-25). While anionic phospholipids, particularly PS, support partial activity of cPKCs in the presence of calcium, the activity is dramatically enhanced by the neutral lipid, diacylglycerol (DAG). Phorbol esters, tumor promoting agents with widespread effects in animal cells, can mimic DAG and are thought to bind to the same site(s) within the cysteine-rich regions of cPKCs. The nPKCs (PKC<sub>ε</sub>, <sub>θ</sub>, <sub>δ</sub>, like cPKCs also contain 2 cysteine-rich regions, but they lack the C2 domain and are calcium-independent. In contrast to both cPKCs and nPKCs, the aPKCs (PKC<sub>ζ</sub> and λ) contain only one cysteine-rich domain. While PKC<sub>ζ</sub> translocates to the membrane upon activation in vitro and requires PS and DAG for activity, this activity is not affected by DAG or phorbol esters (26). Structurally, Raf-1 resembles protein kinase Cs of the aPKC category; it contains...
only one cysteine-rich domain and lacks the calcium responsive element within the regulatory domain. Despite these similarities, raf-1/lipid interactions have not been characterized.

Previous studies from our laboratory have established that the cysteine-rich domain of PKCγ is the site of Zn coordination in the protein; each cysteine-rich domain binds 2 atoms of Zn. Such regions also bind to liposomes containing PS; this association was modulated by DAG or phorbol esters (25, 27). Deletion mutagenesis studies also identified the critical residues within the cysteine-rich domain that are required in vitro for phorbol-ester binding, association to PS-enriched liposomes, and Zn coordination (24, 25). Given the similarity in the structure of this region in PKC and raf-1, we examined, in the present report, whether the cysteine-rich region of raf-1 coordinates Zn and mediates lipid interactions.

A mutant in the cysteine-rich domain of raf-1 (C168S) was recently reported to have a reduced affinity for interaction with ras (20). Another recent paper reported that the cysteine-rich region of raf-1 was not essential for binding to ras (19). Considering these observations, we set out to determine the detailed contribution of the cysteine-rich domain of raf-1 towards interactions with ras. Together, the experiments presented here address 3 major questions regarding the cysteine-rich region of raf-1, namely whether (i) it coordinates Zn, (ii) mediates lipid interactions, and (iii) is required for ras binding.

Our results indicate that the cysteine-rich domain of raf-1 is the site of coordination of 2 Zn atoms, that this region mediates association of raf-1 with liposomes enriched in PS, and that it participates in interactions with ras. A model depicting raf-1 kinase translocation to the membrane and its subsequent interaction with membrane-bound GTP-ras is shown to be an essential part of the mechanism of raf-1 kinase activation and further raises the question of whether raf-1 kinase activation and raf-ras interactions may be modulated by membrane lipids.

**Materials and Methods**

**Chemicals and Reagents**

Ninety-six well microtitre plates were obtained from Costar Corp., Cambridge, MA. Glutathione-agarose matrix (sulfur linked) was purchased from Pharmacia, Uppsala, Sweden. [α-32P]GTP and [ω-35S]ATP (both at 3000 Ci/mmol) were obtained from Du Pont NEN. All other chemicals were of the highest available commercial grade.

**Experimental Methods**

**Expression and Purification of GST-raf-1 Fusion Proteins**—Full-length raf-1 cDNA was isolated by random hexamer-primed RNA-PCR (28) from human Jurkat T cell total RNA. The PCR primers contained restriction sites for BamHI (5'-end primer) and EcoRI (3'-end primer). The RNA-PCR product was directly cloned into the BamHI-EcoRI digested pGEX-2T prokaryotic expression vector (Pharmacia LKB Biotechnology Inc.) and placed under control of the tac promoter (29). After initial propagation in DH5α cells [F' φ 80DG ZA M15 Δ(lac ZA-arg F'U169 deo R rec A1 end A1 lacI (lacZ) m15 sup E44-λ thi-1 gyr A96 rel A1; Life Technologies Inc.] the plasmid DNA containing the raf-1 insert (RafFull) was subcloned into BL-21 cells [F'ompT Rec A m6 lacI (Novagen, Madison, WI)]. For plasmids encoding shorter domains of raf-1 as a GST-fusion protein, DH5α cells were used for protein expression. Cells were grown in Luria-Bertani media fortified with ampicillin (50 μg/ml) and zinc chloride (1 mm). Transformed E. coli cells were isolated as above and used as a protein source for the following protocols previously established for PCK (24). Under these conditions, all GST-raf-1 fusion proteins were soluble. Cells were routinely harvested 1-h post-induction. While most of the smaller raf-1 constructs were successfully expressed in DH5α cells, the full-length raf-1 was exceptionally sensitive to bacterial proteases and was suitably expressed in BL-21 strain which lacks ompT and lon proteases.

The GST-fusion proteins expressed from the bacterial cells were purified on a glutathione-agarose matrix (38, 39). A spectrophotometric assay was utilized to detect the presence of GST-fusion proteins (30). Crude bacterial pellets and purified samples were analyzed by SDS-PAGE (31). Gels were either stained for protein with Coomassie Brilliant Blue or transferred to nitrocellulose (32) and probed with anti-GST or anti-raf-1 antibodies. The anti-GST antibody has been characterized elsewhere.2 Anti-raf-1 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA.

**Expression and Purification of ras Proteins**

Plasmid DNAs for normal H-ras (pAT-H*) and Ala35-mutant of H-ras (pAT-AH*) were kind gifts from Dr. Pat Casey (Duke University Medical Center) and Dr. Channing Der (University of North Carolina, Chapel Hill), respectively. The cells transformed with the ras-coding plasmid DNAs were grown and induced with isopropyl-1-thio-p-galactopyranoside following the same protocol as for the raf-1 proteins. Following expression, the cells were collected and lysed by sonication in 150 ml of buffer (20 m~ Tris-HCl, pH 7.5, 1 m~ phenylmethylsulfonyl fluoride, 50 μg/ml N-p-tosyl-l-lysine chloromethyl ketone, 50 μg/ml l-1-tosylamido-2-phenyleryl chloromethyl ketone, 2 m~ dithiothreitol, and 2 m~ EDTA). The ras protein was purified from the lysate by passing it over a DEAE column (20 ml) using a linear gradient of 0-500 m~ NaCl. The DEAE-column eluate was passed through a G-50 gel filtration column and then examined by SDS-PAGE in a gel containing 20 m~ Tris-HCl, pH 7.5, 1 m~ dithiothreitol, and 100 m~ NaCl. The purity of the preparations was typically greater than 90%.

**GST-raf-1 Constructs**

**PCR Mutants**—Clones of the different domains of raf-1 were obtained by PCR amplification of the corresponding regions of the full-length raf-1 DNA template cloned into pGEX-2T vector. Following PCR, the amplified fragment was ligated directionally into the BamHI-EcoRI site on digested pGEX-2T plasmid vector. In all cases, PCR primers were optimally designed based on a computer-assisted algorithm (AMPLIFY, University of Wisconsin, Madison, WI).Built-in BamHI and EcoRI restriction sites were introduced into the 5'- and 3'-primer sequences, respectively, to facilitate directional cloning. The following raf-1 deletion constructs were obtained by PCR cloning: RafN1 (residues 1-130 of raf-1); RafC1 (residues 128-196 of raf-1); RafC1 (residues 273-648 of raf-1). The primer sequence employed in these studies are as follows (for details, see Table I): 5'-ACGGATCCCGACATACAGGG-3' (5DP1); 5'-AAGCAGGAT-TCGTTGCTGCTTAGAAGG-3' (5DP2); 5'-TACCTGGAATCTCTCCTACGAAGACAGGCAGCCT-3' (5DP3); 5'-CCGGATCCCTGAGTATTCTCGGATCAT-3' (5DP4); 5'-ATAGAATCTCGAACTAAGAGGG-3' (5DP5); 5'-TTTGGATCCAGTAGCCCCAACAATG-3' (5DP6); 5'-TTGGATCCAGTAGCCCCAACAATG-3' (5DP7); 5'-TTGGATCCAGTAGCCCCAACAATG-3' (5DP8). The entire DNA sequence of RafCys and the sequences of the splice junctions of all other PCR generated constructs were verified by dideoxy sequencing (33) using Sequenase II T7 DNA polymerase and sequencing reagent kits according to the manufacturer's directions (U. S. Biochemical Corp.).

**Deletion Mutants**—A subset of raf-1 mutants were obtained by deletion mutagenesis. Using a full-length GST-raf-1 construct in pGEX-2T as a template, different regions of raf-1 were removed by digestion with suitable restriction endonucleases following by religation of the digested plasmid. The followingraf-1 fragments were obtained by employing this approach: RafN2 (residues 1-147 of raf-1); RafN3 (residues 1-330 of raf-1). Allraf-1 constructs employed are summarized in Table I.

It is to be noted that since in all cases raf-1 domains were expressed as fusion proteins to GST, the actual regions of raf-1 that start or end was determined from the secondary amino acid sequence of the GST fusion protein reconstructions. The initial methionine being supplied by the GST. For the sake of convenience, the GST-raf-1 fusion proteins have been identified as from amino acid residues 1-ω, where ω is the terminal residue.

**Liposome Association of GST-Cys**

Experiments to determine the liposome association of the different GST-fusion proteins were based on previously established protocols (34, 35) and performed as described elsewhere (24, 25).

**Zinc Measurements**

Zinc was quantitated by atomic absorption spectroscopy as previously described (27, 36). All zinc absorbance spectra were calculated based upon such zinc measurements and protein quantitation by

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the Amido Black dye binding assay (37), assuming a molecular mass of 74 kDa for intact raf-1, 27 kDa for GST, and an atomic mass of 65 for zinc. The observed standard error of stoichiometries calculated for several determinations of samples separated from the adventitious zinc by gel filtration reflected the error of the measurements involved and in the vicinity of 10% of the averaged values.

**Assays for Measuring raf-ras Interactions**

**Direct Binding Assay**—Fifty picomoles of H-ras protein was loaded with [γ-32P]GTP or [β-35S]GDP by preincubation for 15 min at 30 °C in buffer A (20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 0.3% Triton X-100). GST-raf fusion protein was then added (usually 20 pmol) in a final volume of 50 µl and allowed to interact for 1 h at 4 °C. A slurry of glutathione-agarose resin (100 µl) was added to the reaction and allowed to bind for 30 min at room temperature with occasional mixing. The tubes were then microcentrifuged for 10 min and the supernatant discarded. The resin was washed three times with phosphate-buffered saline, 0.3% Triton X-100 buffer. The washed resin was directly subjected to liquid scintillation counting.

**ELISA Format Assay**—Pured ras protein (20–50 pmol) was loaded onto the wells of a 96-well titer plate and allowed to bind overnight at 4 °C in the presence of 50 µM GTP-S. The remaining binding sites were blocked with 0.1% bovine serum albumin (EIA grade, Sigma) or 0.5% gelatin, in phosphate-buffered saline. GST-raf fusion protein was added and incubated with ras at 37 °C for 1 h, in the presence of 50 µM GTP-S. The plate was washed in phosphate-buffered saline, 0.1% Tween 20 and reacted with excess primary antibody, goat anti-ras polyclonal antiserum (1:4000 dilution) for 1 h at room temperature, in the presence of 50 µM GTP-S. Excess primary antibody was washed off the plate which was subsequently incubated with sheep anti-goat IgG-alkaline phosphatase conjugate (Sigma) at 1:2000 dilution, in the presence of 50 µM GTP-S. p-Nitrophenyl phosphate was used as chromogen. The resulting absorbances were quantitated at 405 nm on an ELISA plate reader (Bio-Rad). The intensity of the absorbance was directly related to the amount of GST-ras protein bound. By using the GST-ras proteins in a serial dilution, a set of binding curves were plotted by plotting the absorbance against GST-ras protein concentration. A quantitative measurement of the relative binding affinities of the different ras domains was made by determining, from the binding curves, the concentration of proteins required for half-maximal binding to ras according to the equation: \( y = c + m0/(m0 + n2) \), where \( y \) is the observed absorbance at 405 nm, \( c \) is the background binding, \( m0 \) is the concentration of the ras protein used, and \( n2 \) is the absorbance at maximum binding, and \( m2 \) is the protein concentration at half-maximal binding.

**RESULTS AND DISCUSSION**

**Expression of GSTraf Domain Fusion Proteins**—Human raf-1 GST fusion proteins were expressed in *E. coli* using the pGEX-2T expression vector. The details of the expression protocol employed are described under "Materials and Methods." raf-1 GST fusion proteins were expressed in E. coli using the pGEX-2T expression vector. The details of the expression protocol employed are described under "Materials and Methods." Following this procedure, about 3–4 mg of pure GST-fusion protein was obtained from a 250-ml induced E. coli culture. The level of purity for most of the fusions, assessed by SDS-PAGE followed by either staining with Coomassie Brilliant Blue or immunoblotting with an anti-raf-1 antibody (Fig. 1). The level of purity for most of the fusions, assessed by scanning densitometry of the gels, was typically greater than 90%; all preparations employed exceeded the 75% purity obtained for the full-length GST-raf-1 fusion. The presence of two independent zinc binding sites in the cysteine-rich region of raf-1 is inferred, with each involving 3 conserved cysteines and 1 conserved histidine residue of the cysteine-rich domain of protein kinase C contains two atoms of Zn2+ binding. The cysteine-rich domain of protein kinase C contains two atoms of Zn2+ binding. The cysteine-rich domain of protein kinase C contains two atoms of Zn2+ binding.

**Analysis of Zinc Content**—The cysteine-rich domain of raf-1 kinase shows significant homology to the cysteine-rich domain of protein kinase C. Both proteins contain the consensus motif His-X12-Cys-X2-Cys-X4-X2-Cys-X2-Cys-X2-His-X2-Cys-X2-Cys which may form a suitable site(s) for Zn2+ binding. The cysteine-rich domain of protein kinase C contains two atoms of zinc bound to two independent coordination spheres each consisting of 3 cysteines and 1 histidine present in the domain (24, 25).

Table I summarizes all raf-1 constructs employed in these studies and shows the zinc content of 5 different raf-1 fusion proteins (RafN2, RafN3, RafCys, RafC1, and RafFull). A short NH2-terminal fragment, RafN2 (aa 1-147) did not contain zinc. RafCys (aa 128-196) contained, on an average, 1.9 mol of zinc. RafN3 (aa 1-64) contained 0.22 mol of zinc. RafC1 (273-648) contained 1.85 mol of zinc. RafN2 (aa 1147) contained 0.23 mol of zinc. RafFull (full-length) contained 2.90 mol of zinc. GST contained 0.01 mol of zinc.

**Table I**

| Name     | Residues | Method obtained | Zn stoichiometry |
|----------|----------|-----------------|------------------|
| RafN0    | 1–64     | PCR-5DP1-3DP1*  | ND*              |
| RafN1    | 1–130    | PCR-5DP1-3DP2   | ND               |
| RafCys   | 128–196  | PCR-5DP2-3DP3   | 0.23 ± 0.02 (n = 2) |
| Ram3    | 1–330    | PCR-S1-3DP1     | 1.85 ± 0.3 (n = 2) |
| RafN3    | 1–330    | Del. Mut*       | 2.11 ± 0.25 (n = 6) |
| RafC1    | 273–648  | PCR-5DP3-3DP4   | 1.09 ± 0.14 (n = 6) |
| RafFull  | 1–648    | PCR-5DP1-3DP4   | 2.90 ± 0.30 (n = 6) |
| GST      |          |                 | 0.01 ± 0.01 (n = 6) |

* ND, not determined.

**Full-length raf-1 (RafFull)** contained 3 mol of zinc/mol of protein; 2 mol of zinc were coordinated in the NH2-terminal domain, RafN3 (aa 1–330). The carboxyl-terminal of raf-1 (RafC1, aa 273–648) contained the other mole of zinc. The exact site of coordination of the COOH-terminal zinc was not further investigated.

Analysis of these results indicates that the cysteine-rich regions of PKC and raf-1 contained similar amounts of zinc. The presence of two independent zinc binding sites in the cysteine-rich region of raf-1 is inferred, with each involving 3 conserved cysteines and 1 conserved histidine residue of the 50-amino acid C2H2 consensus motif for this region, similar to the coordination model discussed for PKC (25).

**Translocation of Raf-1 Kinase to Phospholipid Vesicles**—Previous results (24) have established a role for the cysteine-rich domain of PKCγ in its translocation to liposomes containing PS, in vitro. This translocation was shown to be strongly enhanced by the presence of DAG or PDBu and was essentially independent of calcium. In an analogous manner, the cysteine-rich domain of raf-1 kinase may be responsible for its translocation to the cell membrane to interact with its upstream target, membrane-anchored ras. Therefore, we tested whether
raf-1 would associate with liposomes containing PS.

Fig. 2A summarizes results from experiments quantitating the association of different raf-1 fusion proteins with PS-liposomes containing PS concentrations which gave the highest degree of association for GST-PKCγ fusion proteins (24, 25). Apparent association, seen in the absence of lipids, is referred to as background. This probably reflects nonspecific binding of the fusion proteins with the tubes or their aggregation. The extent of these interactions were found to vary considerably depending upon the nature of the fusion protein (see also Refs. 24 and 25). The difference between association measured in the presence of DAG. The association of raf-1 cysteine-rich domain (lanes 1–5) or presence (lanes 6–10) of 10 mol % sn-1,2-dioctanoylglycerol. The concentration of PS in mol % were as follows; lanes 1 and 6, 77; 2 and 7, 50; 3 and 8, 25; 4 and 9, 10; 5 and 10, 0. PC was the other component in the liposomes.

The only mutants of raf-1 that showed significant association with liposomes contained the cysteine-rich domain, either wholly or in part (RafN3 and RafN2, respectively). The low level of liposomal association observed with full-length raf-1 may reflect steric constraints imposed on the cysteine-rich domain by additional elements present within raf-1. These observations support the hypothesis that raf-1 kinase may translocate from the cytosol to the cell membrane (analogous to PKC, Ref. 40) through regions within the cysteine-rich domain and that translocation could be a regulated process.

In Fig. 2B, we further investigated the dependence of raf-1 liposomal association on phospholipid composition and the presence of DAG. The association of raf-1 cysteine-rich domain increased with an increase in the concentration of PS in the liposome from 10 to 77 mol % (lanes 1–5). However, neither the presence of diacylglycerol (lanes 6–10) nor phorbol ester (data not shown) enhanced association significantly. Additionally, no binding to phorbol ester ([^3H]PDBu) by the cysteine-rich region of raf-1 was ever detected (data not shown). The translocation of full-length PKCy was enhanced by calcium, full-length raf-1 was insensitive to the presence of calcium (data not shown).

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These results define important functional distinctions between PKC and raf-1. Translocation of cytoplasmic proteins to the cell membrane, for the purpose of signal transduction, may be regulated by second messengers generated in response to the extracellular signal (such as DAG and Ca\(^{2+}\) for PKC). Enhanced translocation of PKC to liposomes in the presence of DAG and Ca\(^{2+}\) in vitro corroborates the observation in vivo where upon cell stimulation PKC translocates to the membrane (40). The finding that both DAG and PDBu fail to enhance liposome association of raf-1 kinase suggests that raf-1 and PKC respond to distinct signaling pathways. Thus, despite the fact that translocation was mediated in both cases by similar structural domains, this selectivity in response to second messengers (DAG, Ca\(^{2+}\)) and pharmacophores (PDBu) could provide a critical means of distinguishing between signal transduction pathways.

**Differential Binding of GST-Raf Proteins to H-ras**—The observation that the association with PS-liposomes at PS concentrations mimicking the inner leaflet of the plasma-membrane tricon P-30 or P-100 (depending upon the molecular masses of ras. Glutathione was removed from the purified protein fraction mimicking the inner leaflet of the plasma-membrane and the assay depended critically on two factors: (i) the absence of glutathione in the affinity purified GST-raf protein constructs; and (ii) the inhibition of the intrinsic GTPase activity of ras, Mg\(^{2+}\) ions were omitted from all incubation buffers. The raf-ras interaction was studied at 4 °C; assays performed at 37 °C reduced the interaction about 5-fold (data not shown).

Fig. 3A (white bars) depicts the results of the binding of different raf-1 fusion proteins to normal ras in the “direct binding assay.” The NH\(_2\)-terminal construct RafN2 (aa 1-147) bound maximally to H-ras. Similar binding was observed for RafN3 (aa 1-330, data not shown). The full-length raf-1 fusion protein (RafFull), and a slightly smaller NH\(_2\)-terminal fragment (RafN1; aa 1-130) bound less strongly to ras (4-7-fold decrease). No binding beyond background was observed for the COOH-terminal fragment (RafC1; aa 273-648) or a shorter NH\(_2\)-terminal fragment (RafN0; aa 1-64, data not shown).

The observed maximal binding of the NH\(_2\)-terminal constructs of raf-1 agrees with reported observations (20). The differences in binding observed between RafN1 and RafN2 suggest that a major ras binding determinant is located in a stretch of amino acid residues 130-147 in human c-raf-1. This is also a region that flanks and partially extends into the NH\(_2\)-terminal of the cysteine-rich domain of raf-1 (amino acids 139-184). The weaker binding observed with full-length ras, compared to the NH\(_2\)-terminal fragment, indicates that the binding determinant in the intact protein may not normally be fully accessible for interaction with ras. This site may be exposed as part of an activation mechanism of raf-1 in response to other lipid or protein cofactors or phosphorylation.

**Specificity of raf-ras Interactions**—In order to validate the authenticity of the interaction of the different GST-raf-1 fusion proteins with ras, we investigated the binding interaction as a function of ras structure, ras concentration, and ras-nucleotide (GTP/GDP) conformation. The direct binding assay was used in all experiments.

Fig. 3A (hatched bars) shows the results of the binding of different raf-1 domains to a mutant form of ras (Ala\(^{35}\)ras). The mutant ras did not interact with any of the GST-raf fusion proteins at all. Thr\(^{35} \rightarrow \) Ala comprises an effector domain mutant of ras which disrupts the “effector loop” required for the interaction of ras with mammalian GTPase-activating protein and Saccharomyces cerevisiae adenyl cyclase (18). The observation that none of the GST-raf-1 fusion proteins bound to Ala\(^{35}\)ras indicates that the different subdomains of raf-1 as well as full-length raf-1 binds within the “effector domain” of ras.

Fig. 3B shows the binding profile of different raf-1 fusion proteins as a function of ras concentration. When the [\(^{32}\)P]GTP concentration was kept unchanged at 50 nM, and the ras concentration varied from 0 to 200 nM, the ras-raf binding saturated at around 50 nM ras for both RafFull (full-length raf-1) as well as RafN2 (aa 1-147). Background binding was observed with either ras alone or a carboxyl-terminal fragment of Raf-1 (RafC1; aa 273-648). Fig. 4 shows the binding profile of RafN2 as a function of the guanyl nucleotide (GTP plus GDP) was kept constant in all the assays, but the relative concentration of each was varied from
0 to 100%. All the tubes contained the same amount of radioactivity. Our results indicate that the binding of RafN2 to ras progressively declined as the ratio of the GDP-bound ras increased in the assay. GTP-bound ras is the physiologically "active" state of ras, and GDP-ras is the "inactive" or "resting" conformation of ras (6). Recent evidence suggests that the binding of ras to ras-1 in cells is strongly dependent on the nature of the guanyl nucleotide bound to ras (43). The clear preference of RafN2 for binding to GTP-ras over GDP-ras suggests that the peptide is most likely mimicking the actual binding of full-length ras-1 to ras.

Interaction of ras and ras Assessed by ELISA Format Assay—

All the experiments described so far were carried out by the direct binding assay where the interaction of ras-1 domains with ras occurred in solution. In order to further assess the binding of ras-1 mutants to ras, we developed an "ELISA based assay" (see "Experimental Methods"). The success of this assay depended on the availability of a high-titer anti-GST polyclonal antiserum. This high-titer antibody, coupled to an alkaline phosphatase-based detection system, enabled us to quantitate the ras-ras interactions at a high signal-to-noise ratio.

Fig. 5 shows the data from a typical binding experiment in the ELISA format assay. To prevent significant hydrolysis of GTP during the time course of the assay, the ras was loaded with a nonhydrolyzable GTP analog, GTPyS. Binding curves were generated by serially varying the concentration of the test proteins (different ras domains) while maintaining the ras in

![Graph](attachment://Graph.png)

**Fig. 4. Dependence of rasN2-ras interaction on GTP/GDP ratio.** RafN2 (400 nm) and ras (1 pm) were incubated in the presence of different ratios of [α-32P]GTP and β-35S-GDP, maintaining the same total nucleotide concentration and the same amount of radioactivity in the assays. The data shown is a mean of assays performed in triplicate.

![Graph](attachment://Graph.png)

**Fig. 5. Binding of different GST-ras-1 fusion proteins to H-ras in the ELISA format assay.** Details of the assay have been described under "Experimental Methods." Absorbance data was plotted as a function of the test protein concentration and fitted by a curve-fitting algorithm. The data shown is the mean of three independent assays done in triplicate.

Table II shows a quantitative measurement of the relative binding affinities of the different ras domains. The quantitation was made by determining, from the binding curves in Fig. 5, the concentration of proteins required for half-maximal binding to ras (see "Experimental Methods"). A scale of relative binding affinities was constructed by normalizing half-maximal binding values of all fusion proteins to that of RafN2 which was arbitrarily set at 1. It is clear that RafN2 (aa 1-147) and RafN3 (aa 1-330) bound with the highest affinity to ras. Full-length ras bound with about a 4-fold decrease in affinity, whereas the cysteine-rich domain (RafCys; residues 128-196) bound with a 5.5-fold lower affinity. The binding of RafN1 (aa 1-130) was weaker (~10-fold) than that of RafN2 or RafN3. The very high numbers associated with RafN0 (aa 1-64) or RafC1 (aa 273-684) indicated an insignificant binding to ras (63- and 25-fold less, respectively).

While a possible reason for the weaker binding of full-length ras-1 to ras has been explained before, the apparent weaker binding of the cysteine-rich domain of ras-1 (RafCys) may be due to steric restraints arising from the presence of a large GST protein immediately adjacent to the relatively small peptide coding for the ras-1 cysteine-rich domain. The results obtained in the ELISA format assay are in close agreement with the results of the direct binding assay. Thus both RafN2 and RafN3 bound with the highest affinity to ras, with RafN1, RafCys, and RasFull binding less tightly. Constructs that did not bind in the direct binding assay, RafN0 and RafC1, exhibited very high values for half-maximal binding, indicating weak interaction.

**Competition of rasN2-ras Interaction by Peptides—**Results from the direct binding assay and the ELISA format assay indicated a strong interaction of RafN2 (residues 1-147) with ras while a similar interaction with RafN1 (1-130) was considerably weaker. This implies that the region of ras-1 between amino acids 130 and 147 may constitute a significant binding determinant for ras. We tested the ability of a synthetic peptide P1 (containing amino acids 131-147 of human ras-1) to inhibit the interaction of RafN2 with ras.

The results of the experiment are shown in Fig. 6. An ELISA format based assay with RafN2 (starting at 5 pmol) and ras (maintained at 50 pmol) generated a typical binding curve in the absence or presence of competitor peptide P1, or in the

| Protein (pmol) | OD (405 nm) |
|--------------|-------------|
| 0            | 0.1         |
| 5            | 0.15        |
| 10           | 0.2         |
| 15           | 0.25        |
| 20           | 0.3         |
| 25           | 0.35        |

- **raf-N3 (1-330)**
- **raf-N2 (1-147)**
- **raf-Full (1-648)**
- **raf-Cys (128-196)**
- **raf-N1 (1-130)**
- **raf-C1 (188-648)**
- **raf-N0 (1-64)**
- **GST**
TABLE II
Measurement of relative binding affinities of differentraf-1 constructs toH-ras in the ELISA format assay

| GST-raf construct (amino acids) | Relative concentrations for half-maximal binding | Half-maximal binding |
|---------------------------------|-----------------------------------------------|-------------------|
| RafN0:1-64                      | 63.0                                          | 391 ± 263         |
| RafN1:1-130                     | 9.4                                           | 57.6 ± 0.36       |
| RafN2:1-147                     | 1.0                                           | 6.2 ± 0.5         |
| RafN3:1-330                     | 1.2                                           | 7.2 ± 0.8         |
| RafFull:1-648                   | 3.7                                           | 22.9 ± 3.2        |
| RafCys:128-196                  | 5.5                                           | 34.4 ± 5.2        |
| RafC1:273-648                   | 25.0                                          | 153 ± 46          |

Fig. 6. Competition of RafN2-ras interaction by peptides. Fifty picomoles of H-ras was loaded with GTPγS and immobilized on 96-well titer plates. Following blocking with 0.1% bovine serum albumin in phosphate-buffered saline, ras was incubated with either buffer, 500 pmol of peptide P1 or P2 for 30 min at 4 °C in a volume of 100 μl. RafN2 was then added to the wells in 2-fold serial dilutions, beginning at 5 pmol. The remainder of the assay was followed as described under "Experimental Methods."

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REFERENCES
1. Iwai, Y., Iwaki, M., Nagao, M., Kodawski, T., Takaku, F., and Kasuga, M. (1993) J. Biol. Chem. 268, 7033-7039
2. Turner, B. C., Tonks, N. K., Rapp, U. R., and Reed, J. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5544-5548
3. Davis, R. J. (1993) J. Biol. Chem. 268, 14553-14566
4. Howe, I. R., Leveers, S. J., Gomez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1996) Cell 71, 335-342
5. Marais, R., Wynne, J., and Freisem, R. (1993) Cell 73, 381-394
6. Kaziro, Y., Iizuka, Y., Kosaka, T., Nakafuku, M., and Satoh, T. (1993) Annu. Rev. Biochem. 60, 349-460
7. Polakis, P., and McCormick, F. (1993) J. Biol. Chem. 268, 9157-9160
8. Schaper, G. L., Ciment, G., Stocker, K. M., and Bailer, I. (1995) Mol. Chem. Neuropharmacol. 18, 267-279
9. Skipper, J., and Strauss, H. J. (1993) J. Exp. Med. 177, 1493-1498
10. Motojima, K., and Goto, S. (1993) FEBS Lett. 319, 75-79
11. Moodie, S. A., Willsumen, B. M., Weber, J. M., and Wolfman, A. (1993) Science 260, 1658-1661
12. Feig, L. A., and Cooper, G. M. (1988) Mol. Cell. Biol. 8, 2325-2342
13. Smith, S. R., DeGutis, S. J., and Stacey, D. W. (1988) Nature 320, 540-543
14. Kolch, W., Heidecker, G., Lloyd, F., and Rapp, U. (1991) Nature 349, 426-428
15. Robbins, D. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6924-6928
16. Dickson, B., Spranger, F., Morrison, D., and Hafen, E. (1992) Nature 360, 665-668
17. Han, M., Golden, A., Han, Y., and Stenberg, P. W. (1993) Nature 366, 133-140

well documented protein domains such as the src homology 2 (SH2) and 3 (SH3) domains. In the present report, we have investigated the structural and functional properties likely to be associated with the cysteine-rich domains of human raf-1 kinase. Our present data indicates that the raf-1 cysteine-rich domain contains an arrangement of sulfur and nitrogen ligands suitable for the coordination of 2 mol of zinc analogous to that reported for PKC (25); the probable coordination geometry for zinc for PKC was illustrated in Ref. 25. Besides the 2 zinc coordination sites formed by the Cys3-His units, the cysteine-rich domain also contains an arrangement of hydrophobic and positively charged residues which may define critical binding sites for anionic phospholipids, such as PS. We have experimentally determined that the cysteine-rich region of raf-1 does indeed associate with liposomes containing PS and may thus function to anchor raf-1 to the cell membrane. However, in contrast to PKC, translocation of raf-1 to liposomes in vitro was not enhanced by DAG, phorbol ester, or calcium, and may reflect the differential responsiveness of PKC and raf-1 to different signaling systems. Additionally, while the cysteine-rich region was implicated in binding zinc and translocating to PS liposomes, a region in raf-1, adjacent to and extending partially into the cysteine-rich domain provided a critical determinant for binding to GTP-ras. The cysteine-rich region of raf-1 may, thus, play a multifunctional role; it is conceivably involved in the targeting of raf-1 to the inner surface of the plasma membrane as well as binding to activated, membrane-bound ras (49). A model summarizing such putative interactions is shown in Scheme I. Both lipid-protein and protein-protein interactions may play a critical role in the activation of the raf-1 kinase which in turn phosphorylates and activates MAP kinase kinase kinases and leads to MAP kinase activation. The likely formation of the ras-raf-1 complex in the cell membrane could serve as the scaffolding for the formation of a multisubunit signaling complex in the membrane since MAP kinase kinase is known to be tightly associated with the raf-1 kinase (50). The presence of these proteins at the membrane places them in an environment where their activities could be modulated by lipids. This leads to the speculation that "mitogenic lipids" could exert their effects by regulating the association of subunits present in the complex or the activities of the subunits. Whether or not the cysteine-rich domain serves a common functional role in other proteins in mediating protein-lipid interactions and other signaling functions remains to be elucidated.
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18. Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6213-6217
19. Vojtek, A. B., Hohenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205-214
20. Zhang, X., Settleman, J., Kyriakis, J. M., Takeuchi-Suzuki, E., Eledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) Nature 364, 309-313
21. Nishizuka, Y. (1992) Science 258, 607-614
22. Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4863-4867
23. Burns, D. J., and Bell, R. M. (1991) J. Biol. Chem. 266, 18330-18338
24. Quest, A. F. G., Bardes, S. G., and Bell, R. M. (1994) J. Biol. Chem. 269, 2563-2570
25. Quest, A. F. G., Bardes, S. G., and Bell, R. M. (1994) J. Biol. Chem. 269, 2961-2970
26. Schwartz, J. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8310-8313
27. Quest, A. F. G., Bloomenthal, J., Bardes, S. G., and Bell, R. M. (1992) J. Biol. Chem. 267, 10193-10197
28. Wang, A. M., Doyle, M. V., and Mark, D. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9717-9721
29. Smith, D. B., and Johnson, R. S. (1988) Gene (Amst.) 76, 31-40
30. Habig, W. H., and Jakoby, W. B. (1981) Methods Enzymol. 77, 398-405
31. Laemmli, U. K. (1970) Nature 227, 680-685
32. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354
33. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
34. Boni, L. T., and Rand, R. R. (1985) J. Biol. Chem. 260, 10619-10625
35. Sossin, W. S., and Schwartz, J. H. (1992) J. Neurosci. 12, 1165-1168
36. Quest, A. F. G., Bardes, S. G., Bloomenthal, J., Berchardt, B., and Bell, R. M. (1993) Methods Neurosci. 13, 138-153
37. Schaffner, W., and Weissmann, C. (1973) Anal. Biochem. 56, 502-514
38. Simons, P. C., and Vandendael, D. L. (1977) Anal. Biochem. 85, 334-341
39. Mannervik, B., and Guthenberg, C. (1981) Methods Enzymol. 77, 231-235
40. Kraft, A. S., and Anderson, W. B. (1983) Nature 301, 821-823
41. White, D. A. (1973) in Form and Function of Phospholipids: The Phospholipid Composition of Mammalian Tissues (Ansell, G. B., Hawthorne, J. N., and Dawson, R. M. C., eds) pp. 441-478, Elsevier, Amsterdam
42. Hannun, Y. A., and Bell, R. M. (1996) J. Biol. Chem. 261, 9341-9347
43. Koide, H., Satoh, T., Nakafuku, M., and Kaziro, Y. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5683-5686
44. Rino, J., and Gierras, L. M. (1992) Annu. Rev. Biochem. 61, 387-418
45. Ren, R., Mayer, B. J., Cochet, P., and Baltimore, D. (1993) Science 259, 1157-1161
46. Katzav, S., Martin-Zanca, D., and Barbacid, M. (1989) EMBO J. 8, 2283-2290
47. Hall, C., Monfries, C., Smith, P., Lim, H. H., Korona, R. A., Ahmed, S., Vanniasingham, V. M., Leung, T., and Lim, L. (1990) J. Mol. Biol. 211, 11-16
48. Casey, P., Solaki, P., Der, C. J., and Buss, J. E. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8323-8327
49. Crews, C. M., and Erickson, R. L. (1993) Cell 74, 215-217