Regulation of Raf through Phosphorylation and N Terminus-C Terminus Interaction*

Received for publication, December 16, 2002, and in revised form, July 5, 2003
Published, JBC Papers in Press, July 14, 2003, DOI 10.1074/jbc.M212803200

Huira Chong‡ and Kun-Liang Guan§
From the Department of Biological Chemistry and the Institute of Gerontology, University of Michigan, Ann Arbor, Michigan 48109

Raf kinase is a key component in regulating the MAPK pathway. B-Raf has been reported as an oncogene and is mutated in 60% of human melanomas. The main focus of Raf regulation studies has been on phosphorylation, dephosphorylation, and scaffolding proteins; however, Raf also has its own auto-regulatory domain. Removal of the N-terminal regulatory domain, initially discovered in the viral Raf oncogene (v-Raf), results in a kinase domain with high basal activity independent of Ras activation. In this report, we show that activating phosphorylations are still required for activity of the truncated C-terminal kinase domain (called 22W). The interaction between the N-terminal regulatory domain and the C-terminal kinase domain is disrupted by activated Ras. Mutations in the Ras binding domain, cysteine-rich domain, or S259A do not affect the inhibition of 22W by the N-terminal domain. When phosphomimetic residues are substituted at the activating sites (DDED) in 22W, this results in a basal activity that is no longer inhibited by expression of the N-terminal domain, although binding to the N-terminal domain still occurs. Although the interaction between 22W/DDED and the N-terminal domain may be in a different conformation, the interaction is still disrupted by activated Ras. These data demonstrate that N-terminal domain binding to the kinase domain inhibits the activity of the kinase domain. However, this inhibition is relieved when the C-terminal kinase domain is activated by phosphorylation.

The Raf family of serine/threonine kinases is highly conserved in structure. There are three conserved regions (CR)1 in Raf kinases, CR1, CR2, and CR3 (1). CR1 contains the Ras binding domain (RBD) and the cysteine-rich domain (CRD), and both bind the activated small GTPase Ras (2, 3). CR2 is serine/threonine-rich, and CR3 is the catalytic kinase domain and contains several activating phosphorylation sites. Activated Ras binds to CR1 and recruits Raf to the plasma membrane, which is necessary for Raf activation. Phosphorylation of activating sites and dephosphorylation of inhibitory sites must be coordinated for full Raf activation. Once Raf is activated, it can phosphorylate and activate MAPK/ERK, which phosphorylates cytosolic and nuclear proteins.

Raf was first identified as a retroviral oncogene v-Raf, which has a deletion of the 5′ end of the normal cellular gene encoding CR1 and CR2 (4). This deletion correlates to an increase in transforming activity independent of Ras, which leads to the hypothesis that the N-terminal domain of Raf proteins has a regulatory function (5–9). In defining the minimal region required for cell transformation, deletion of both CR1 and CR2 but an intact kinase domain was required for high transforming activity (6, 9). Deletion of the N-terminal region renders Raf kinase activity independent of Ras.

The N-terminal domain serves the following two functions. 1) It binds to the activated form of the small GTPase Ras required for membrane recruitment. 2) It binds and suppresses the C-terminal kinase. Expression of the N terminus of c-Raf can inhibit Ras activation of the MAPK pathway in germinal vesicle breakdown (GVBD) in Xenopus oocytes by binding and sequestering activated Ras from endogenous c-Raf (10). The N-terminal half was also shown to co-immunoprecipitate and inhibit the C-terminal half of Raf. The mechanism of Raf activation may be similar to that of p21-activated kinase (PAK) (11). The Rac and Cdc42, members of the Rho family of small GTPases, directly bind the N terminus of PAK and relieve an auto-inhibited conformation held together by intramolecular interactions. The new conformation allows phosphorylation of activating sites, which are thought to help maintain an open conformation of PAK.

Two different mechanisms have given rise to oncogenic forms of Raf kinases. v-Raf has a deletion of the entire 5′ end of the cellular gene that encodes for the N-terminal regulatory domain, v-Raf has high transforming activity because of a loss of the auto-inhibitory domain. Recently, mutations in B-Raf were identified in 60% of human melanomas and identified B-Raf as an oncogene (12, 13). A common oncogenic mutation of B-Raf has a Val to Glu mutation in the activation loop of the kinase domain and results in high kinase activity. This mutation, V599E, lies between two conserved phosphorylation sites, Thr-598 and Ser-601, in the activation loop of B-Raf, previously identified by our lab (14, 15). We have shown that acidic residue substitutions of the phosphorylation residues in the activation loop elevates B-Raf kinase activity. Our studies on Raf regulation provides a molecular mechanism of how B-Raf is activated by oncogenic mutations associated with human cancers. Hence, relief of auto-inhibition and phosphomimetic mutation are two mechanisms that convert Raf to oncogenes.

We sought to investigate the role of the N-terminal domain...
in Raf regulation and the effects of phosphorylation on the auto-inhibition induced by the N terminus. We observed that the activation loop sites are still necessary for kinase activity even when the auto-inhibitory effects of the N terminus are relieved. Phosphomimetic substitutions of the four activation sites with acid residues renders the kinase domain resistant to inhibition imposed by the N-terminal domain, although the interaction with the N-terminal domain is not disrupted. Furthermore, this interaction is still disrupted by activated Ras. Our results suggest a complex mechanism of Raf regulation by phosphorylation and N terminus-C terminus interactions.

EXPERIMENTAL PROCEDURES

**Plasmid Constructs**—FLAG-tagged full-length c-Raf was generously provided by Dr. Kevin Pumiglia. All c-Raf mutations were described previously and made by PCR mutagenesis. Truncated c-Raf constructs were created by PCR with flanking BamHI/EcoRI sites, cloned into pH5-myc or pCDNA3 vectors. C-terminal deletions were 5'-tagged with Myc, and N-terminal deletions were 3'-tagged with FLAG. Clones were verified by DNA sequencing.

**Cell Culture and Transfection**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. All cells were transfected in serum-free media using LipofectAMINE (Invitrogen) following the manufacturer’s instructions.

**In Vitro Kinase Assay**—For c-Raf kinase assays, HEK293 cells grown in 6-well plates were transfected with 200 ng of the c-Raf constructs mentioned above with or without 10 ng of K-RasV12. 24 h after transfection, some cells were treated with 50 ng/ml of EGF for 3 min. Cells were washed in ice-cold phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 5 mM EDTA, 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotons). After centrifugation, FLAG-tagged kinase was immunoprecipitated with anti-M2 antibody (Sigma). Immunocomplexes were collected by incubation with protein-G-Sepharose beads and washed with RIPA buffer, and a final wash was done in HEPES buffer. Immunoprecipitated c-Raf was then incubated with 0.1 μg of GST-MEK2 in 20 μl of kinase buffer (10 mM HEPES, pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol, 50 μM ATP) for 20 min at 30°C. 14 μl of supernatant from the reaction was transferred to a new tube and incubated for 10 min at 30°C with 6 μl of kinase buffer containing 0.1 μg of GST-ERK. 10 μl of kinase buffer containing 4 μg of GST-ERK-1 and 5 μCi of [γ-32P]ATP (ICN) was added to the reaction and incubated for an additional 20 min at 30°C. Reactions were stopped upon the addition of SDS-sample buffer and boiled for 5 min. Reactions were resolved by SDS-PAGE and visualized by autoradiography. Recombinant GST-MEK1, GST-ERK1, and GST-ELK-1 were expressed in bacteria and purified by glutathione-agarose affinity chromatography.

For ERK kinase assays, cells were co-transfected with 100 ng of HA-ERK and 50 ng of wild type, or mutant e-Raf constructs or 10 ng of K-RasV12. HA-tagged ERK kinase was immunoprecipitated using anti-HA antibody (Covance) and protein-G-Sepharose beads. Immunocomplexes were incubated in kinase buffer containing 4 μg of GST-ERK-1 and 5 μCi of [γ-32P]ATP for 20 min at 30°C. Reactions were stopped and resolved as mentioned above.

**Co-immunoprecipitation Analysis**—HEK293 cells grown in 6-well plates were transfected with 200 ng of empty vector, wild type, or mutant e-Raf 22W constructs with or without 350 ng of c-Raf N-terminal wild type or mutant constructs. 24 h after transfection, cells were lysed in NuPAGE F-40 buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Nonidet P-40, 50 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotons) and immunoprecipitated with anti-M2 antibody (Sigma) or anti-Myc antibody (Covance) and protein-G-Sepharose beads. Immunocomplexes were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The presence of the N terminus of c-Raf was detected with anti-Myc (9E10) antibody, and the C terminus of c-Raf was detected with anti-FLAG antibody. Anti-HA (Covance), anti-Src (Santa Cruz Biotechnology), anti-PY20 (Transduction Laboratories), anti-phospho-Raf (Cell Signaling), and anti-phospho-ERK (BioSource) antibodies were also used in this study.

RESULTS

**Both Relief of Auto-inhibition and Phosphorylation of Activating Sites Contribute to c-Raf Activation**—The maintenance of the auto-inhibited state is facilitated by a pair of 14-3-3-binding sites in c-Raf, Ser-259 in the N terminus, and Ser-621 in the C terminus (16–20). Ser-259 is a conserved inhibitory site and phosphorylated by Akt (21, 22) and protein kinase A (23), and mutation of this site to alanine results in high basal activity. To determine whether the removal of an inhibitory signal was sufficient to activate Raf or whether other phosphorylation events must occur, we made alanine or phenylalanine mutations of the four activating phosphorylation sites, S338A, Y341F, and T491A/S494A (24), and we assessed these mutants for their Raf kinase activity (Fig. 1A). The constructs were transfected into HEK293 cells, immunoprecipitated, and subjected to a Raf in vitro kinase assay. Wild type c-Raf has low basal activity that is increased when Ser-259 is mutated to alanine. Mutation of the activating...
phosphorylation sites to alanine decreases the high basal activity of S259A. However, phosphomimetic substitution of these activation sites (S259A/DDED) further increases the basal activity of S259A. These data suggest that removal of the inhibitory phosphorylation site in full-length c-Raf is not sufficient for Raf activation, and activating phosphorylation sites are still required.

The Ser-239 mutants have an intact RBD, which can still bind activated Ras at the plasma membrane, and CRD, which is thought to mediate the N terminus-C terminus interaction (10). To determine whether the relief of auto-inhibition mediated by the N-terminal domain was sufficient for Raf activation, we deleted the first 304 amino acids of c-Raf, which consisted of CR1 and CR2, and we assessed the kinase activity of the C-terminal domain construct (305–648, called 22W).

Consistent with the high transforming activity (24), 22W has high basal kinase activity (Fig. 1B) compared with full-length c-Raf. When the activating phosphorylation sites are mutated to alanine or phenylalanine, the high basal activity is suppressed (Fig. 1C). When these sites are substituted by phosphomimetic residues, the high basal activity of 22W is slightly increased. Hence, removal of an inhibitory signal or auto-inhibitory domain still requires activating phosphorylation sites to activate Raf kinase.

**The N Terminal of c-Raf Is Able to Inhibit ERK Activation by the C-terminal Kinase Domain of Raf—Expression of the N-terminal domain of Raf, consisting of two Ras-binding sites, was shown to inhibit GVBD induced by activated Ras or the catalytic domain of c-Raf in Xenopus oocytes (10). These observations indicate that the N-terminal domain of Raf may inhibit the function of the C-terminal kinase domain. To test if Raf-(1–305) was able to inhibit ERK activation, we co-transfected HEK293 cells with ERK with or without the N-terminal domain (amino acids 1–305) of c-Raf and stimulated with EGF or RasV12 (Fig. 2A). ERK activity was induced by EGF and RasV12; however, the co-expression of Raf-(1–305) inhibited the activation of ERK. This N terminally mediated inhibition is presumably through binding of activated Ras (endogenous Ras in the case of EGF stimulation) and sequestering Ras from components of the MAPK pathway such as endogenous Raf.

In defining the minimal region required for transformation, a range of deletions was identified that resulted in high transforming activity (6, 24). Two c-Raf deletion constructs, 325–648 and 305–648 (22W), were used to stimulate ERK phosphorylation at the TEY motif required for activity in the absence of EGF or RasV12 stimulation (Fig. 2B). The N terminus (1–305) was also able to inhibit ERK activity induced by the C terminus of c-Raf. The mode of inhibition in this case is assumed to be through the N terminus-C terminus interactions because the activity of these truncated c-Raf constructs is independent of Ras stimulation. We observed a doublet of Raf-(305–648) (Fig. 2B) likely because of phosphorylation. Similarly, slower migrating bands were also detected for Raf-(325–648). Interestingly, the slower migrating bands of the kinase domain (both Raf-(305–648) and Raf-(325–648)) were suppressed by N-terminal co-expression. These data suggest that the N-terminal domain inhibits phosphorylation of the kinase domain and provides a possible mechanism of inhibition.

**The Interaction between the N Terminus and C Terminus Is Not Disrupted by Phosphomimetic Mutations in the C Terminus and Requires the Full N-terminal Domain**—The N-terminal regulatory region of c-Raf is able to immunoprecipitate with the C-terminal catalytic region, and this interaction is thought to correlate with the inhibitory effect seen when the N terminus is co-expressed with the C terminus. To investigate the mechanism of inhibition, we tested whether the phosphomimetic substitutions of the four activating sites, Ser-338, Tyr-341, Thr-491, and Ser-494, could block the interaction between the N terminus and C terminus of c-Raf. S338D/Y341D (DD), T491E/S494D (ED), S338D/Y341D/T491E/S494D (DDED), and T491A/S494A (AA) substitutions were made on 22W and co-transfected with Raf-(1–305). The 22W constructs were immunoprecipitated, and the presence of the N terminus in the immunocomplexes was assessed by Western blot (Fig. 3A). The N terminus was able to co-immunoprecipitate with the 22W regardless of the mutation made. Acidic residue substitutions at these activation sites increased the basal activity of 22W (Fig. 1C). Although these substitutions may not fully mimic phosphorylation, these data suggest that the degree of activation of the C terminus does not alter the ability of the C terminus to associate with the N terminus.

The inhibition of GVBD by the N terminus was also compromised when the CRD was mutated (10). It was suggested that
this region was the domain that mediated the interaction with the C terminus. By using N-terminal fragments in co-immunoprecipitation assays, we wanted to define the N-terminal region of interaction. The N-terminal fragments used spanned the CRD but deleted either the CR2 (fragment 1–190) or the RBD (fragment 130–305). These N-terminal fragments were co-transfected with 22W and immunoprecipitated, and the presence of 22W was assessed by Western blot analysis (Fig. 3B). 22W was unable to co-immunoprecipitate with any of the fragments and was found in a complex only when expressed with the entire N terminal Raf-(1–305); however, it is possible these fragments may be unstable or misfolded. To address this, we made a CRD mutation of the conserved cysteines (C165S/C168S) (25). The interaction with the CRD N-terminal mutant and 22W was still detectable by Western blot analysis (Fig. 3C).

**Ras Regulates the Interaction between the N Terminus and C Terminus**—The first step in Raf activation is Ras binding to Raf through the Ras binding domain (RBD) and the cysteine-rich domain (CRD). Although Ras binding has been part of the model of Raf activation, the main role of Ras binding has been in facilitating the membrane recruitment of Raf. We tested if activated Ras also aids in the dissociation of the N terminus and C terminus. Cells were transfected with 22W and the N terminus in the presence or absence of RasV12 or Src. When RasV12 was co-expressed, the interaction between Raf-(1–305) and 22W was disrupted, and Raf-(1–305) was no longer able to co-immunoprecipitate with 22W (Fig. 4A). This disruption was not seen when cells were co-transfected with increasing amounts of Src. These data suggest that it is Ras that disrupts the interaction between the N and C termini of c-Raf and that activating kinases such as Src are not involved at this step.

14-3-3 binds phosphorylated residues in the N terminus and C terminus of c-Raf to facilitate the maintenance of an inactive c-Raf conformation as well as affecting the subcellular localization of c-Raf to non-activable complexes (16–20). Ras binding to the RBD may displace 14-3-3 from phosphorylated Ser-259 revealing the second Ras-binding site in the CRD (1). To determine whether Ras binding disrupts the N terminus-22W interaction directly or indirectly by displacing 14-3-3 from the N terminus, we used a N-terminal mutant, Raf-(1–305/S259A) in co-immunoprecipitation assays. The alanine mutation destroys the Ser-259 phosphorylation site and subsequently the 14-3-3-binding site. This S259A mutant was still able to co-immunoprecipitate with 22W, and these interactions were also disrupted by RasV12 (Fig. 3A and 4B). Although

---

**Fig. 3. N terminus and C terminus of c-Raf interact.** A, cells were co-transfected with the N-terminal domain of c-Raf (Myc-(1–305)) and 22W (kinase domain, amino acids 305–648) containing various activating phosphorylation site mutants as indicated. 22W was immunoprecipitated with anti-FLAG, and the presence of the N-terminal domain was assessed by anti-Myc Western blot (top panel). B, 22W was co-transfected with various N-terminal domain truncations as indicated. The N-terminal domain was immunoprecipitated (IP) with anti-Myc antibody and the presence of 22W was detected by anti-FLAG Western blot (WB) (top panel). C, 22W was co-transfected with CRD (C165S/C168S) or S259A N-terminal domain (1–305) mutant. The N-terminal domain was immunoprecipitated with anti-Myc antibody, and the presence of 22W was detected by anti-FLAG Western blot (top panel).
14-3-3 may facilitate or stabilize the interaction, the N-terminal 14-3-3-binding site, Ser-259, is not required for the N terminus to interact with the C terminus. However, the role of 14-3-3 in the regulation of the N terminus-C terminus conformation may not be ruled out. Endogenous 14-3-3 is still able to co-immunoprecipitate with the N-terminal domain mutant S259A (Fig. 4C), supporting the presence of other 14-3-3-binding sites (26).

The Phosphomimetic Mutation, 22W/DDED, Is Resistant to Inhibition by the N-terminal Domain—Mutations in the C terminus and the N terminus have no detectable negative affect on their interaction with each other. CRD and Ser-259 mutations are still able to interact with 22W. Activating substitutions in 22W that elevate the kinase activity also retain their interaction with the N terminus. We next sought to investigate if this interaction resulted in inhibition of activity. Cells were co-transfected with wild type 22W, N-terminal mutants, and ERK. ERK was immunoprecipitated and assayed for kinase activity (Fig. 5A). 22W was able to activate ERK and was inhibited when the wild type Raf(1–305) was co-expressed. The RBD, CRD, and S259A N-terminal mutants were also able to inhibit ERK activation by 22W. Inhibition of 22W activity by the CRD mutant is contrary to the GVBD assays; however, this disparity may be due to differences in the assays used. 22W/DD and 22W/DDED both have high basal kinase activity (Figs. 1C and 5C). When 22W/DD and 22W/DDED were co-transfected with Raf(1–305) and ERK, Raf(1–305) was able to inhibit 22W/DDED-activated ERK phosphorylation on the TEY motif (Fig. 5C). However, 22W/DD activity was mostly resistant to inhibition by Raf(1–305). These data suggest that phosphorylation of Ser-338/Tyr-341 may play an important role to relieve the inhibitory effect by the N-terminal domain.

The N terminus was able to inhibit 22W but not when the activation sites were substituted by phosphomimetic residues. We then asked if the N terminus would be able to inhibit an activated full-length c-Raf kinase where the activation sites were not mutated. Mutation of Ser-259 to alanine in full-length c-Raf results in an elevated basal activity by eliminating an inhibitory phosphorylation site (Fig. 1A) (22). This mutant also eliminates a 14-3-3-binding site that is thought to facilitate an auto-inhibited state. c-Raf/S259A was co-transfected with HA-ERK with or without N-terminal constructs. The wild type N terminus can provide a 14-3-3-binding site through its Ser-259. c-Raf/S259A strongly activates ERK activity, as does 22W and 22W/DDED (Fig. 5D). However, where both wild type (1–305) and S259A (1–305/S259A) constructs of the N terminus can inhibit 22W activity, it cannot inhibit ERK activation by S259A-c-Raf. Our results suggest the full-length c-Raf/S259A mutant is resistant to inhibition by the N-terminal domain, and although 14-3-3 may contribute, the interaction between the N terminus and C terminus of c-Raf is not dependent on 14-3-3 binding to phosphorylated Ser-259.

The N-terminal Regulatory Domain Blocks Phosphorylation—The interaction between the N-terminal domain and the
Fig. 5. 22W-DDED is resistant to inhibition by the N-terminal domain. A, HA-ERK was co-transfected with 22W with Myc-1–305-wt, −R89L, −CRD, or −S259A constructs. HA-ERK was immunoprecipitated (IP) and assayed for kinase activity shown in the top right panel. The phosphorylation of ERK on the TEY motif was also assessed in the top left panel. B, ERK kinase activity was assessed as in A except 22W-DDED.
was co-transfected. C, HA-ERK and 22W/DD or 22W/ED were co-transfected with or without Myc-(1–305). The top panel shows ERK phosphorylation on the TEY motif. D, HA-ERK was co-transfected with 22W, 22W-DDED, or c-Raf-S259A and Myc-(1–305) or Myc-(1–305)/S259A, as indicated. HA-ERK was assayed for kinase activity and is shown in the top panel. WB, Western blot.

REGULATION OF RAF

C-terminal domain does not necessarily correlate with inhibition of Raf activity. We hypothesized that this interaction may inhibit phosphorylation of activating sites. Wild type c-Raf and 22W were co-transfected with or without RasV12, immunoprecipitated, and the phosphorylation of Thr-491 was assessed by Western blot using a phospho-specific antibody (14, 15). Ras increases the phosphorylation of wild type c-Raf at the activation loop phosphorylation sites (Fig. 6A) (14, 15). The phosphorylation state of this site is higher in the 22W construct than the wild type full-length Raf and is not further increased by RasV12. When 22W is co-transfected with Raf(1–305), the phosphorylation of Thr-491 is blocked (Fig. 6B). These data further support a role for the N-terminal domain in inhibiting the phosphorylation of the C-terminal kinase domain.

Discussion

We have demonstrated that activating phosphomimetic substitutions on the C-terminal domain of c-Raf are resistant to inhibition imposed by the auto-inhibitory N-terminal domain. The wild type C-terminal domain has high basal activity, which is suppressed by alanine mutations of the activating phosphorylation sites Ser-338, Tyr-341, Thr-491, and Ser-494. Phosphomimetic substitutions of the activating phosphorylation sites (DDED) in the C-terminal domain result in a slightly higher basal kinase activity in vitro and is able to activate ERK in vivo. Removing the inhibitory signal on Ser-259 is also able to induce high basal activity of the full-length c-Raf kinase. Phosphorylation on Ser-259 creates a 14-3-3-binding site and is thought to maintain c-Raf in an auto-inhibited conformation as well as mediate subcellular localization (27). Mutation of Ser-259 to alanine results in high basal activity that is suppressed by alanine mutations of the phosphorylation activating sites and increased when these sites are substituted by acidic residues. Dephosphorylation of the inhibitory site, Ser-259, and phosphorylation of the activation sites, Ser-338, Tyr-341, Thr-491, and Ser-494, must be coordinated for full c-Raf kinase activity. These data also suggest that relief of auto-inhibition is still reliant on activating phosphorylation mechanisms.

The inhibition of the kinase domain by the N-terminal domain is through direct interaction. From previous studies, it was hypothesized that this interaction was through the CRD of the N-terminal domain, and phosphomimetic substitution on an activation site outside of the activation loop interferes with this interaction (10). This model of activation is similar to the model of PAK activation (11). The N terminus of PAK binds the C terminus and blocks the kinase cleft. The small GTPases Rac or Cdc42 bind the N-terminal domain and open the conformation allowing phosphorylation of sites near the kinase domain that maintain this open conformation. The wild type N-terminal domain of c-Raf is able to co-immunoprecipitate with the C-terminal domain and inhibit its activity. The N-terminal domain is also able to co-immunoprecipitate with 22W/DDED; however, this interaction does not result in the inhibition of 22W/DDED activity. These observations suggest that phosphomimetic substitutions do not interfere with the interaction between the N terminus and the C terminus of c-Raf and that these activating mutations are resistant to the inhibitory effect imposed by the N terminus.

We also tested if mutation of the CRD or Ser-259 14-3-3-binding site affected the interaction or function of the N-terminal domain in inhibiting the C-terminal domain. Both mutations were able to bind the wild type C-terminal domain. Hence, the CRD and Ser-259 are not critical for the interaction between the N and C termini, although they may contribute to the interaction. These N-terminal mutants were also able to inhibit the kinase activity of the C-terminal domain but not of the DDED mutant. This further supports that the DDED mutation is resistant to inhibition by the N terminus and that N terminus-C terminus interactions do not necessarily result in inhibition.

Another role for the N terminus of c-Raf is to bind Ras, which leads to the recruitment of Raf to the plasma membrane (28, 29). This first step is essential to the activation of Raf. We observed that Ras disrupts the interaction between the N terminus and the C terminus. Ras is required not only for the recruitment c-Raf to the membrane but also to relieve the auto-inhibited conformation. Furthermore, although the interaction between the N terminus and 22W/DDED may be
an alternate conformation, this interaction is also disrupted by Ras.

Our observations may further explain why the mutated B-RAFT gene found in 60% of human melanomas has high transforming activity (12) and is not inhibited by the presence of the N-terminal domain in the full-length kinase. The mutation V599E inserts a negative charge between the two conserved activation loop phosphorylation sites, Thr-598 and Ser-601, which are necessary for B-Raf activity (15). This single Val to Glu mutation is sufficient to mimic phosphorylation of both activation loop sites and induce activity. The B-Raf-V599E activity would be unaffected by interactions between its N terminus and C terminus. Similarly, phosphomimetic substitutions on full-length c-Raf result in high basal activity as they are impervious to inhibition imposed by N-terminal interactions with the C terminus.

We have further defined two roles for the N-terminal domain in the regulation of c-Raf activity. One role is to keep c-Raf in an auto-inhibited conformation making phosphorylation sites inaccessible to their kinases when cells are unstimulated. The second role is to bind Ras and mediate the recruitment of Raf to the plasma membrane. Data from this report and previous studies suggest the following model for Raf regulation. Raf is maintained in an auto-inhibited state through N-terminal-C-terminal interactions and facilitated by 14-3-3 binding to sites located in both domains. Upon Ras activation, GDP is exchanged for GTP and Ras is able to bind and recruit Raf to the membrane. Dephosphorylation on Ser-259 releases 14-3-3; however, N- and C-terminal domains are still able to interact. The loss of Ser-259 phosphorylation and Ras binding may occur concomitantly. The N-terminal and C-terminal domain interactions are disrupted by GTP-bound Ras, and the activating phosphorylation sites are unmasked and available to be phosphorylated by activating kinases. Once the activating sites are phosphorylated, the kinase is active and resistant to inhibition by the N-terminal domain. Therefore, activated Raf maintains high kinase activity even when dissociated from membrane-bound Ras. Consistent with this model is our observation that 22W/DDED binds the N-terminal domain but is not inhibited by the interaction. The main inhibitory function of the N-terminal domain is likely to prevent the phosphorylation and activation of the C-terminal kinase domain. Co-immunoprecipitation of the N-terminal domain with the C-terminal domain shows less phosphorylation on the activation loop site Thr-491. Furthermore, it has been reported that the interaction between PAK, the Ser-338 kinase, and Raf is enhanced by the deletion of the N-terminal regulatory domain of Raf (30). The relief of auto-inhibition as well as dephosphorylation of inhibitory sites and phosphorylation of activating sites must occur to reach full Raf activation.

Acknowledgments—We thank members of the Guan Lab for technical assistance and critical review of the manuscript.

REFERENCES
1. Morrison, D. K., and Cutler, R. E. (1997) Curr. Opin. Cell Biol. 9, 174–179
2. Mott, H. R., Carpenter, J. W., Zhong, S., Ghosh, S., Bell, R. M., and Campbell, S. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8312–8317
3. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1995) Cell 74, 205–214
4. Rapp, U. R., Goldeborough, M. D., Mark, G. E., Bonner, T. I., Grinfeld, J., Reynolds, F. H., Jr., and Stephenson, J. R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4218–4222
5. Bonner, T. I., Kerby, S. B., Sutrame, P., Gunnell, M. A., Mark, G., and Rapp, U. R. (1985) Mol. Cell. Biol. 5, 1400–1407
6. Heidecker, G., Hulsehle, M., Cleveland, J. L., Kolch, W., Beck, T. W., Lloyd, P., Pawson, T., and Rapp, U. R. (1990) Mol. Cell. Biol. 10, 2503–2512
7. Moelling, K., Heimann, B., Beimling, P., Rapp, U. R., and Sander, T. (1984) Nature 312, 558–561
8. Shimuzu, K., Nakatsuy, Y., Sekiguchi, M., Hatamura, K., Tanaka, K., Terada, M., and Sugimura, T. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5641–5645
9. Stanton, V. P., Jr., and Cooper, G. M. (1987) Mol. Cell. Biol. 7, 1171–1179
10. Cutler, R. E., Jr., Stephens, R. M., Saracino, M. R., and Morrison, D. K. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9214–9219
11. Lei, M., Lu, W., Meng, W., Parrini, M. C., Eck, M. J., Mayer, B. J., and Harrison, S. C. (2000) Cell 102, 387–397
12. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffenden, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewir, E., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, L., Stevens, C., Watt, S., Hosper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargraves, D., Pritchard-Jones, K., Maitland, N., Chenex-Trench, G., Riggins, G. J., Bignell, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002) Nature 417, 949–954
13. Rajagopalan, H., Bardelli, A., Lengauer, C., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. (2002) Nature 418, 934
14. Chong, H., Lee, J., and Guan, K. L. (2001) EMBO J. 20, 3716–3727
15. Zhang, B. H., and Guan, K. L. (2000) EMBO J. 19, 5429–5439
16. Dent, P., Jelinek, T., Morrison, D. K., Weber, M. J., and Sturgill, T. W. (1995) Science 268, 1902–1908
17. Jasmot, M., and Hancock, J. F. P. (1991) Oncogene 20, 3949–3958
18. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) Cell 84, 889–897
19. Trivison, G., Luo, Z., and Avruch, J. (1998) Nature 394, 88–92
20. Yip-Schneider, M. T., Miao, W., Lin, A., Barnard, D. S., Trivison, G., and Marshall, M. S. (2000) Biochem. J. 351, 151–159
21. Zhang, B. H., Tang, E. D., Zhu, T., Greenberg, M. E., Vojtek, A. B., and Guan, K. L. (1991) J. Biol. Chem. 266, 31620–31629
22. Zimmermann, S., and Moelling, K. (1999) Science 286, 1741–1744
23. Dhillon, A. S., Pollock, C., Steen, H., Shaw, P. E., Mischak, H., and Kolch, W. (2002) Mol. Cell. Biol. 22, 3237–3246
24. Stanton, V. P., Jr., Nichols, D. W., Laudano, A. P., and Cooper, G. M. (1989) Mol. Cell. Biol. 9, 629–647
25. Michaud, N. R., Fabian, J. R., Mathes, K. D., and Morrison, D. K. (1995) Mol. Cell. Biol. 15, 3390–3397
26. Clark, G. J., Drugan, J. K., Rossman, K. L., Carpenter, J. W., Rogers-Graham, K., Fu, H., Der, C. J., and Campbell, S. L. (1997) J. Biol. Chem. 272, 20990–20993
27. Dhillon, A. S., Meikle, S., Yazici, Z., Eulitz, M., and Kolch, W. (2002) Mol. Cell. Biol. 22, 3237–3246
28. Baron, T. I., Kerby, S. B., Sutrame, P., Gunnell, M. A., Mark, G., and Rapp, U. R. (1985) Mol. Cell. Biol. 5, 1400–1407
29. Shih, S. F., Yamamoto, K., Maitani, M., and Kolch, W. (1998) Mol. Cell. Biol. 18, 64–71
30. Marais, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) EMBO J. 14, 3136–3145
31. Miseo, C., Anderson, R. G., and White, M. A. (1997) J. Biol. Chem. 272, 10345–10348
32. Zang, M., Hayne, C., and Luo, Z. (2002) J. Biol. Chem. 277, 4395–4405
Regulation of Raf through Phosphorylation and N Terminus-C Terminus Interaction
Huira Chong and Kun-Liang Guan

J. Biol. Chem. 2003, 278:36269-36276.
doi: 10.1074/jbc.M212803200 originally published online July 14, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212803200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 19 of which can be accessed free at
http://www.jbc.org/content/278/38/36269.full.html#ref-list-1