# Ras Activation of the Raf Kinase: Tyrosine Kinase Recruitment of the MAP Kinase Cascade

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ABSTRACT

A continuing focus of our work has been an effort to understand the signal transduction pathways through which insulin achieves its cellular actions. In the mid-1970s, we and others observed that insulin promoted an increase in Ser/Thr phosphorylation of a subset of cellular proteins. This finding was unanticipated, inasmuch as nearly all of the actions of insulin then known appeared to result from protein dephosphorylation. In fact, nearly 15 years elapsed before any physiologic response to insulin attributable to stimulated (Ser/Thr) phosphorylation was established. Nevertheless, based on the hypothesis that insulin-stimulated Ser/Thr phosphorylation reflected the activation of protein (Ser/Thr) kinases downstream of the insulin receptor, we sought to detect and purify these putative, insulin-responsive protein (Ser/Thr) kinases. Our effort was based on the presumption that an understanding of the mechanism for their activation would provide an entry into the biochemical reactions through which the insulin receptor activated its downstream effectors. To a degree that, in retrospect, is surprising, this goal was accomplished, much in the way originally envisioned. It is now well known that receptor tyrosine kinases (RTKs) recruit a large network of protein (Ser/Thr) kinases to execute their cellular programs. The first of these insulin-activated protein kinase networks to be fully elucidated was the Ras-Raf-mitogen-activated protein kinase (MAPK) cascade. This pathway is a central effector of cellular differentiation in development; moreover, its inappropriate and continuous activation provides a potent promitogenic force and is a very common occurrence in human cancers. Conversely, this pathway contributes minimally, if at all, to insulin's program of metabolic regulation. Nevertheless, the importance of the Ras-MAPK pathway in metazoan biology and human malignancies has impelled us to an ongoing analysis of the functions and regulation of Ras and Raf. This chapter will summarize briefly the way in which work from this and other laboratories on insulin signaling led to the discovery of the mammalian MAP kinase cascade and, in turn, to the identification of unique role of the Raf kinases in RTK activation of this protein (Ser/Thr) kinase cascade. We will then review in more detail current understanding of the biochemical mechanism through which the Ras proto-oncogene, in collaboration with the 14-3-3 protein and other protein kinases, initiates activation of the Raf kinase.

## I. Insulin and Growth Factors Promote Widespread Protein (Ser/Thr) Phosphorylation

The discovery of cyclic AMP (cAMP) in the mid-1950s provided the first paradigm for signal transduction by polypeptide hormones. In this model, the hormone interacts with its receptor at the cell surface, leading to the generation within a cell of a chemically distinct, intracellular molecule responsible for conveying all the information necessary to carry out the complete program of hormone action (Robison *et al.*, 1971). The nature of the biochemical reactions through which the "second messenger" reoriented cell function remained obscure until the discovery and elucidation of the cAMP-dependent protein kinase, protein kinase A (PKA), by Krebs and coworkers in 1968. A substantial body of evidence accumulated rapidly thereafter that demonstrated that PKA-catalyzed protein phosphorylation explains most of the actions of cAMP (Krebs, 1972). Although other cAMP effectors — such as cAMP-gated ion channels (Zagotta and Siegelbaum, 1996) and guanyl nucleotide exchangers for the small GTPase, Rap1 (DeRooij *et al.*, 1998) — have been identified more recently, PKA nevertheless remains the dominant cAMP effector.

Shortly after the discovery of cAMP, Larner and coworkers showed that insulin, like glucagon or beta-adrenergic catecholeamines, can induce stable alterations in the activity of the intracellular enzyme, glycogen synthase, which can be measured in the homogenate or after purification, if cells are disrupted under appropriate conditions (Larner, 1988). This property usually reflects the occurrence of a stable, post-translational modification (e.g., phosphorylation, dephosphorylation), suggesting a parallel between insulin and cAMP action. As the effects of insulin on the activity of enzymes such as glycogen synthase, pyruvate dehydrogenase, and hormone-sensitive triglyceride lipase are opposite to those caused by cAMP/PKA, it was anticipated that insulin would execute much of its cellular program by promoting the dephosphorylation of target proteins. This would be accomplished either by opposing cAMP generation, or by activating protein phosphatases to reverse cAMP/PKA action, or both. Direct assessment of the effect of insulin on overall protein phosphorylation in target cells readily demonstrated insulin inhibition of cAMP-directed phosphorylation, if cAMP levels were elevated by catecholamines or glucagon. Unexpectedly, however, the dominant response to insulin as the sole agonist was not dephosphorylation but rather an increase in $^{32}P$ incorporation into (Ser/Thr) residues on a variety of polypeptides, through a mechanism entirely independent of the cAMP/PKA system (Benjamin and Singer, 1975; Avruch *et al.*, 1976; Forn and Greengard, 1976). The only purpose known for agonist-stimulated Ser/Thr phosphorylation is regulation of protein target function. However, no examples of enzyme regulation by insulin-stimulated protein phosphorylation were then known. Nevertheless, reasoning that these novel, insulin-stimulated phosphorylations reflected intermedi-

ate steps in insulin signal transduction, efforts were undertaken to identify the targets and the responsible insulin-regulated protein kinases/protein phosphatases. Among the initial set of insulin-stimulated phosphoproteins identified were several metabolic enzymes (ATP-citrate lyase, Alexander *et al.*, 1979; acetyl CoA carboxylase, Witters, 1981). However, only the 40S ribosomal subunit protein S6 (Haselbacher *et al.*, 1979; Smith *et al.*, 1979) provided a substrate useful for the reliable detection of the insulin-regulated interconverting enzymes (Avruch *et al.*, 1985). Thus, once suitable extraction conditions had been defined (Novak-Hofer and Thomas, 1984), a kinase activity capable of phosphorylating 40S subunits exclusively on S6 could be reliably detected in extracts of cells treated with insulin or mitogens (Tabarini *et al.*, 1985; Erikson and Maller, 1985; Nemenoff *et al.*, 1986; Pelech *et al.*, 1986). An *in vivo* counterpart of this response is the tenfold increase in S6 kinase activity in the regenerating liver remnant that occurs within 2 hours after partial hepatectomy in the rat (Nemenoff *et al.*, 1988). Similarly, the induction of germinal vesicle breakdown in stage VI *Xenopus* oocytes by progesterone or insulin is accompanied by a dramatic increase in 40S-S6 kinase activity (Maller *et al.*, 1986).

## II. Insulin and Growth Factors Activate Multiple (Ser/Thr) Kinases: Discovery of the Mammalian MAPKinase Cascade

The *Xenopus* S6 kinase activity was first to be purified, yielding two 85- to 90-kDa kinase polypeptide isoforms (Erikson and Maller, 1985). cDNAs corresponding to these enzymes were cloned from *Xenopus* (Jones *et al.*, 1988) and murine (Alcorta *et al.*, 1989) sources and named ribosomal S6 protein kinase (Rsk) 1 and 2. *Xenopus* Rsk was shown to undergo extensive (Ser/Thr) phosphorylation *in vivo* concomitant with activation. It could be deactivated *in vitro* by treatment with protein phosphatase (Erikson and Maller, 1989). Rsk thus appeared to be activated by protein (Ser/Thr) phosphorylation, suggesting that this S6 kinase itself was the target of an insulin/mitogen-activated protein (Ser/Thr) kinase.

A second insulin-stimulated protein kinase was discovered shortly thereafter by Sturgill and coworkers. In the course of examining whether insulin activated a protein kinase activity toward the protein phosphatase modulatory protein called inhibitor-2, they observed the insulin-stimulated phosphorylation of a high Mr polypeptide contaminant, identified subsequently as the microtubule-associated protein, MAP-2 (Ray and Sturgill, 1987). Partial purification of this insulin-stimulated MAP-2 kinase from 3T3 L1 adipocytes indicated that it co-purified with a 42-kDa polypeptide, whose phosphorylation on Thr and Tyr residues was strongly stimulated by insulin (Ray and Sturgill, 1988). Remarkably, the partially purified, insulin-activated p42 MAP-2 kinase was found to phosphorylate and partially reactivate the dephosphorylated *Xenopus* Rsk (S6 kinase II), suggesting that the

MAP-2 kinase and Rsk, both insulin-activated kinases, might represent sequential elements in an insulin-regulated protein kinase cascade (Sturgill *et al.*, 1988). Independently, Ahn and Krebs (1990), fractionating protein kinases in NIH3T3 cell extracts active toward a synthetic S6 peptide, garnered evidence for the existence of an analogous mitogen-activated S6-peptide kinase and "kinase-kinase" module.

Protein kinase cascades were first described by Krebs and coworkers, who discovered PKA as the immediate upstream activator of phosphorylase b kinase (Walsh *et al.*, 1968). Gibson's laboratory later provided evidence for the operation of a protein kinase cascade in the regulation of hydroxymethyglutaryl (HMG) CoA reductase (Ingebritsen *et al.*, 1981). However, the components of this kinase cascade, which include the AMP-activated protein kinase, were not identified at a molecular level until much later, while the AMP kinase-kinase remains unidentified. Thus, little consideration was given to the significance of protein kinase cascades, until interest was rekindled by the elucidation of the MAPK-Rsk relationship. A forceful demonstration of ubiquity of this architectural motif in signal transduction was provided by the elucidation of the molecular structure of the 44-kDa isoform of the MAP kinase. This polypeptide, named erk1 (Boulton *et al.*, 1990), is approximately 45 percent identical in the amino acid sequence of its catalytic domain to a pair of *S. cerevisiae* protein kinases, FUS3 and KSS1, that had been identified by genetic analysis as indispensable elements in the signal transduction pathway mediating the yeast response to the mating pheromones (Elion *et al.*, 1990; Courchesne *et al.*, 1989). In fact, among the genes then known to be encoding components of the pheromone response pathway, in addition to the two MAPK homologs FUS3/KSS, were three other kinases – namely, Ste20, Ste11, and Ste7. An epistatic analysis indicated that these three kinases acted in a sequential manner upstream of KSS1/FUS3 (Errede and Levin, 1993). However, no biochemical evidence was available as to whether any of these five kinases acted directly on another. The possibility remained that as-yet-unidentified gene products were interposed. Biochemical analysis of mammalian MAPK clearly showed that its activation required both Thr and Tyr phosphorylation of the MAPK polypeptide (Anderson *et al.*, 1990). Mammalian cell extracts contained a potent MAPK activator that appeared to be a protein kinase with such dual specificity (Ahn *et al.*, 1991; Gomez and Cohen, 1991). This entity was purified and molecularly cloned by several laboratories in rapid succession and proved to be a MAP kinase kinase (MAPKK, usually called MEK1) highly homologous to the yeast Ste7, the gene product immediately upstream of the yeast MAPKs, FUS3/KSS1 (Crews *et al.*, 1992; Seger *et al.*, 1992; Ashworth *et al.*, 1992; Wu *et al.*, 1993). This remarkable conservation of both the architectural design and individual structures made clear the ubiquitous and fundamental importance of the MAP kinase cascade as a signaling module.

Our primary effort at this time was focused on the elucidation of the mammalian S6 kinase. We purified this enzyme from rat liver as a 70-kDa polypeptide (Price *et al.*, 1989) and found by molecular cloning that it had a structure quite distinct from the *Xenopus*/mammalian Rsks (Banerjee *et al.*, 1990). Moreover, although the p70 S6 kinase, like Rsk, is activated through insulin-stimulated Ser/Thr phosphorylation (Price *et al.*, 1990), the p70 S6 kinase is poorly phosphorylated *in vitro* (and not at all reactivated) by the p42/44 MAPK (Price *et al.*, 1990; Mukhopadhyay *et al.*, 1992). This provided the first indication that the p70 S6 kinase and Rsk are on separate limbs of the signal transduction outflow downstream of the insulin receptor. This conclusion was strongly supported by our finding that the immunosuppressant drug rapamycin causes a potent inhibition of p70 S6kinase in intact cells, with no effect on Rsk activity (Price *et al.*, 1992). Once convinced that Rsk and p70 were on separate insulin-directed signaling pathways, we attempted to identify the components of each signaling module. We have reviewed elsewhere current information concerning the RTK-PI-3 kinase-mTOR and cell cycle-regulated pathways that impinge on p70 S6 kinase (Avruch *et al.*, in press). The remainder of this discussion will focus on the control of the MAPK/pathway by insulin and growth factors.

## III. RTKs Activate the MAPK Cascade Through Raf Kinases

One candidate RTK effector we selected for study was the cRaf-1 protein kinase. The cRaf-1 protein kinase is the cellular homolog of vRaf, one of the very few oncogenes then known to encode a protein (Ser/Thr) kinase (Rapp *et al.*, 1983; Moelling *et al.*, 1984). On inspecting the cRaf-1 amino acid sequence, a number of canonical MAPK phosphorylation sites (e.g., PXSP, XXSP) were seen to reside in a segment whose deletion had been shown to activate Raf-transforming activity, suggesting that Raf, like Rsk, might be activated by MAPK. Our efforts to examine this idea, however, were impeded by the lack of a suitable assay for Raf kinase activity. We observed that, although insulin or mitogen treatment increased the ability of immunoprecipitated cRaf1 to catalyze an autophosphorylation *in vitro*, we were not able to detect insulin/mitogen-stimulated protein kinase activity toward exogenous protein substrates previously reported (Siegel *et al.*, 1990) (primarily various histones), that could survive stringent washing of the cRaf1 immunoprecipitate. Moreover, the recombinant cRaf-1 ATP site mutant, presumably catalytically inactive, exhibited autophosphorylating and kinase activity quite similar to the recombinant wild-type cRaf-1, indicating that the bulk of these activities were due to (randomly and/or specifically) adsorbed kinases.

A fortunate clue came from the characterization of a line of v-Raf-transformed NIH 3T3 cells. We found that the endogenous p42/44 MAPKs in these cells appeared to be constitutively activated and unresponsive to further stimulation by serum or mitogens. Moreover, the activity of MAPKK in the v-Raf-trans-

formed cells was markedly greater than that in the parental NIH 3T3 cells. After partial purification, this active MAPKK could be completely deactivated by PP2A *in vitro* (Kyriakis *et al.,* 1992). The sensitivity of active MAPKK to PP2A was not unexpected, as the architecture of the *S. cerevisiae* mating pathway indicated that the yeast MAPKK Ste7 was situated downstream of another protein (Ser/Thr) kinase, Ste11 (Errede and Levin, 1993). Although the highly conserved nature of the MAPK pathway components then known suggested that a mammalian homolog of Ste11 would serve as the immediate activator (MAPKKK) upstream of MAPKK, the constitutive, phosphorylation-dependent activity of MAPKK in the v-Raf-transformed cells raised the simple possibility that the v-Raf kinase itself acted as the MAPKKK. The activation of MAPKK by Raf proved to be readily demonstrable; an aminoterminally truncated, constitutively active Raf mutant, immunoprecipitated from NIH 3T3 cells, catalyzed the phosphorylation and complete reactivation of a partially purified, PP2A-treated MAPKK (Kyriakis *et al.,* 1992). These findings were rapidly confirmed (Dent *et al.,* 1992; Howe *et al.,* 1992). Having established MAPKK as a reliable substrate for Raf kinase *in vitro,* we next showed that insulin and polypeptide growth factor treatment of a wide variety of cultured cells induced, within 2 minutes, a robust increase in the MAPKKK activity immunoprecipitated by anti-cRaf-1, with a slightly slower activation of endogenous MAPKK (Kyriakis *et al.,* 1993). These results established cRaf1 as an insulin/mitogen-activated (SerThr) kinase and defined MAPKK as one of its likely physiologic substrates. All three Raf isoforms (cRaf, B-Raf, and A-Raf) are capable of direct activation of the MAPKKs, MEK1 and MEK2. Conversely, Raf is specific for these two MAPKK isoforms and does not appear to participate in other MAPK pathways. Several MAPKKKs in addition to the Raf subfamily are now known to be capable of activating the MAPKKs, MEK1/2 *in vitro,* including cMos, MEKK1, MEKK2, MEKK3, and cCot/Tpl2 (Kyriakis and Avruch, in press). Nevertheless, Raf loss-of-function mutations invariably interrupt RTK-driven, MAPK-dependent cellular differentiation in *Drosophila* (Dickson *et al.* 1992) and *C. elegans* (Han *et al.,* 1993), indicating that Rafs are the *only* MEK 1/2-specific MAPKKKs recruited by receptor tyrosine kinases in metazoans.

## IV. Ras-GTP Recruits Raf to the Membrane to Initiate Raf Activation and Mitogenesis

We next sought to understand the mechanism by which RTKs recruit cRaf1 into an active state. Our approach was influenced most strongly by earlier work of Rapp and colleagues, who first identified the v-Raf oncogene (Rapp *et al.,* 1983) and its cellular homologs (Moelling *et al.,* 1984). The 648 amino acid (74-kDa) cRaf1 polypeptide is composed of a carboxyterminal catalytic domain (AA335 to 627) and an aminoterminal noncatalytic segment that contains a zinc

finger structure (AA139-184) homologous to those found in the PKCs (Nishizuka, 1992) (Figure 1). Deletion of the aminoterminal segment to AA303 abruptly activates the Raf-transforming activity. Most v-Raf oncogenes exhibit aminoterminal truncation, generally between AA250-300 (Stanton *et al.*, 1989; Heidecker *et al.*, 1990). The aminoterminal segment is thus an inhibitor of the catalytic domain. In addition, however, the ability of insulin and growth factors to activate cRaf1 (Kovacina *et al.*, 1990; Blackshear *et al.*, 1991) implied that the aminoterminal segment may serve as the receptor for the upstream activating signal. Strong evidence for this idea is the demonstration by Rapp and colleagues that the ability of catalytically inactive, full-length cRaf1 to inhibit RTK-induced mitogenesis (Kolch *et al.*, 1991) and transcriptional activation resides in the cRaf1 aminoterminal segment (AA 1-257). A point mutation (Cys165 Ser) in the cRaf zinc finger largely eliminates the inhibitory potency of the cRaf (1-257) segment (Bruder *et*

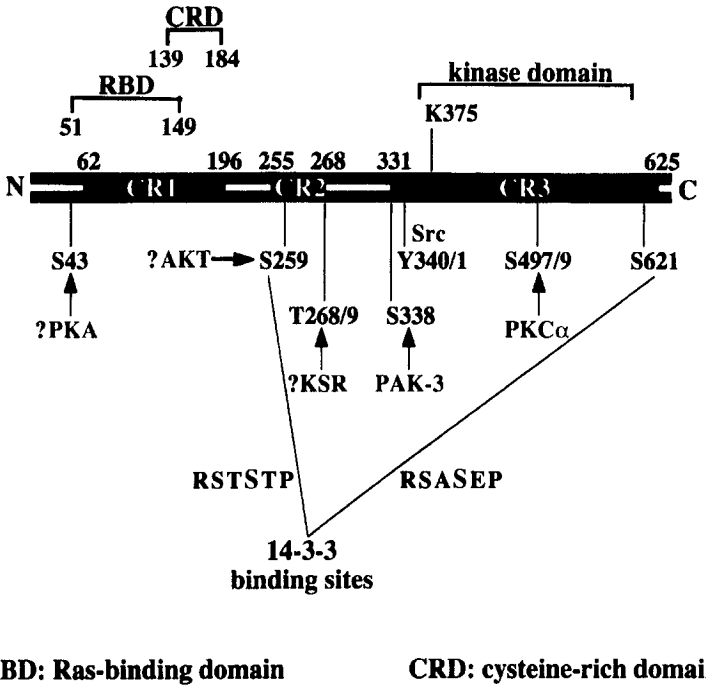


FIG. 1. cRaf1: Domain structure and relevant, known phosphorylation sites. CR = conserved regions in c-, A-, and B-Raf.

*al.*, 1992). As to the nature of this upstream activating signal, the cRaf1zinc finger binds zinc (two moles) and phosphatidylserine (Ghosh *et al.*, 1994) but does not bind diacylycerol (DAG). The structural homology between the Raf- and PKC-zinc fingers, however, suggested that Raf activation might be initiated by the binding of some other signal-dependent membrane lipid. Nevertheless, to cover the possibility that the upstream activator required the participation of, or was itself a cellular polypeptide, we employed the yeast two-hybrid expression system to inquire whether the Raf-1 aminoterminal segment (AA1-257) interacted with cellular polypeptides in a manner that was dependent on an intact zinc finger. One of the first cDNAs found to interact with the Raf aminoterminus in this manner encoded the small GTPase, Rap-1b.

Rap1 had been discovered in 1988 independently by three groups (Kawata *et al.*, 1988; Kitayama *et al.*, 1989; Pizon *et al.*, 1988), one of which isolated Rap 1 through selection of cDNAs capable of causing reversion of the transformed phenotype induced by v-Ras (Kitayama *et al.*, 1989). Rap1 is 50 percent identical to Ras in overall amino acid sequence; however, the two small GTPases share complete identity in the region corresponding to the Ras effector domain, amino acids 32-40 (Figure 2). This segment had been identified through a comprehensive examination of the effects of site-specific mutation on the transforming activity of the GTPase-deficient, constitutively active V12 Ras. Mutations in Ras amino acids 32-40 were found to severely inhibit V12 Ras-transforming activity without

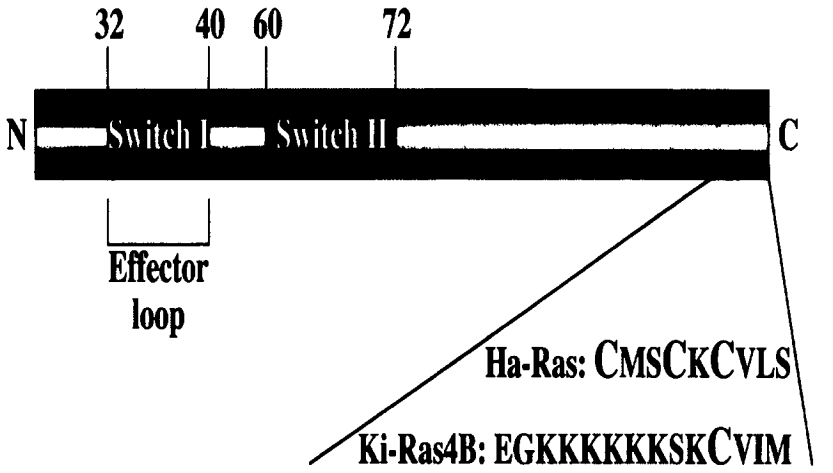


FIG. 2. Ras: Domains relevant to activity. Switch = segments whose configuration is altered by guanyl nucleotides. The cysteine residues subject to prenylation (CAAX) or palmitoylation (Ha-Ras only) are enlarged.

affecting Ras cellular localization or the ability of cRas to bind, hydrolyze, or exchange guanosine triphosphate (GTP) (Marshall, 1993). The ability of mutations in this segment to abrogate transformation without altering the biochemical activities of Ras known to be critical to transforming activity led to the proposal that the structure of this segment was important to the ability of Ras to recruit its mitogenic "effectors." This view was reinforced when comparison of the crystal structure of Ras liganded with either guanosine diphosphate (GDP) or GTP showed that the loop encoded by Ras AA31-39 (i.e., the "effector" loop identified by mitogenesis) was one of only two segments that differed in configuration (switch 1 and 2) between inactive and active Ras (Figure 2). The inhibitory action of Rap1 on Ras-induced transformation could be rationalized with this hypothesis if their identical "effector" loops enabled Rap 1 to interact with Ras "effectors" but in a manner that was nonproductive for the activation of mitogenesis (Marshall, 1993). Our finding that the proto-oncogene Raf interacted directly with the anti-oncogene Rap was consistent with this formulation and implied that the Raf activator was likely to be Ras rather than Rap. In fact, expression of cRaf1 with V12 Ras gave strong activation of Raf kinase activity, whereas V12 Rap1 was without effect. Moreover, we readily demonstrated that Ras interacted strongly with Raf 1-257 in the yeast expression system (Zhang *et al.*, 1993). To determine whether this interaction was truly direct, baculoviral-recombinant, full-length V12 Ha-Ras polypeptide was purified from Sf9 cells and examined for its ability to bind directly to a purified, immobilized prokaryotic recombinant GST-Raf (1-257) fusion protein. Ras charged with GTPγS bound avidly to GST-Raf, whereas Ras charged with GDPβS was only slightly retained; GST bound neither form of Ras. Mutation of the Raf zinc finger (GST-Raf 1-257 C168S) reduced the binding of V12 Ras GTPγS and abolished binding to V12 Ras GDPβS. Fully processed, membrane-bound V12 Ha-Ras and cytoplasmic (farnesylated but incompletely palmitoylated) V12 Ha-Ras polypeptides behaved in a manner similar to each other, binding preferentially to wild-type Raf, as compared to the Raf C168S mutant. Prokaryotic (unprocessed) recombinant Ras liganded with GTP also bound to GST Raf (1-257) but bound equally well to GST Raf (1-257, C168S). Thus, the initial binding studies established that Ras bound to the aminoterminal regulatory domain of Raf in a GTP-dependent manner. Ras carboxyterminal processing, although not required for high-affinity, GTP-dependent Ras-Raf interaction *in vitro*, did appear to influence the interaction. Reciprocally, an intact Raf zinc finger, while not necessary for the GTP-dependent Ras-Raf binding *in vitro,* somehow influenced avidity, at least for processed Ras. An indirect estimate of the affinity of this interaction was enabled by the ability of GST-Raf (1-257) to inhibit ($IC_{50}$, 0.13 μM) the stimulation of Ras-GTPase activity caused by the addition of p120 Ras-GAP (Zhang *et al.*, 1993).

The direct, specific binding of the aminoterminal regulatory domain of the cRaf1 proto-oncogene to GTP-liganded, "active" Ras established Raf as a strong candidate to serve as a mitogenic effector of Ras. Because the interaction of such an effector with Ras would be expected to occur through the Ras "effector" loop, we examined a panel of Ras effector loop mutants that had been characterized quantitatively for their transforming ability relative to V12 Ras, measured as focus formation in NIH 3T3 cells. The ability of V12 Ras and the various effector Ras loop mutants to bind to Raf (1-257), as assessed quantitatively in the yeast two-hybrid system, paralleled almost completely the relative transforming efficiency of these Ras variants. This close correlation provided additional strong evidence in support of Raf as a direct effector of Ras's mitogenic program (Zhang *et al.,* 1993). Contemporaneously, several other laboratories provided evidence for an association of Ras-GTP and Raf, either in the two-hybrid system, or *in vitro* using purified polypeptides, or both (reviewed in Avruch *et al.,* 1994).

The identification of Raf as a Ras effector, the concurrent discovery of the role of Grb2 and son of sevenless (SOS) in coupling RTKs to Ras GTP charging, and the earlier discovery of Raf as the conduit for RTK signals into the MAPK pathway together enabled the first description of an RTK signaling pathway from ligand binding to an ultimate intracellular target (e.g., transcription factors such as ternary complex factor (TCF) are regulated directly by MAPK-catalyzed phosphorylation). The ubiquity of the Ras-MAPK pathway in cellular differentiation during development attests to the biologic importance of this pathway. Moreover, the Ras-Raf interaction established in concrete terms a paradigm for the biochemical action of a small GTPase. This has proven applicable to the entire superfamily (i.e., GTP induced reconfiguration of a binding surface on the G protein, enabling a high-affinity interaction with target proteins, which are thereby recruited to the G protein, resulting in either target activation and/or target apposition to critical substrates). The central role of protein kinases as effectors of the small GTPases has been repeatedly demonstrated with the subsequent discovery of the p21-activated kinases (PAKs) and (probably) mixed-lineage kinases (MLKs) as effectors of the Rac1 and Cdc 42 GTPases, the rho-activated kinases (ROKs) and protein-kinase C-related kinases (PRKs) as effectors of the Rho A GTPases, germinal center (GC) kinase as a probable Rab8 effector, and so forth. Finally, mutant active forms of Ras are present in an estimated 30 percent of human cancers (Bos, 1989). Considerable evidence indicates that that transforming activity of all tyrosine kinase oncogenes and most nonnuclear oncogenes requires activation of endogenous Ras. Thus, the biochemical mechanism of Ras signaling is crucial to the understanding of oncogenesis and to the development of rationally targeted anticancer therapies

In collaboration with Mark Marshall, we undertook a more-refined analysis of the sites of interaction between Raf and Ras-GTP. Using the competitive inhibition of p120 Ras-GAP, a Raf fragment (AA 51-149) was found to bind

prokaryotic recombinant Ras-GTP with an affinity tenfold higher ($IC_{50}$,12 nM) than the full Raf aminoterminus (AA1-257). Further deletion, especially from the aminoterminus, reduced affinity for Ras-GTP significantly (Chuang *et al.*, 1994). Subsequent mutagenesis of this Raf segment by substitution of single or multiple amino acids revealed especially strong inhibitory effects of substituting A for the basic residues R67, K84, K87, and especially R89, as well as L86 (Barnard *et al.*, 1995). R89 had been identified independently as a loss-of-function mutation in *Drosophila* Raf (Fabian *et al.*, 1994). Conversely, substitution of acidic residues D33, E37, D38 on the Ras effector loop by uncharged amino acids reduced affinity for Raf by 20- to 50-fold or more. This analysis suggested that the GTP-dependent binding between Raf and Ras involved multiple basic residues in the Raf aminoterminal segment, interacting with acidic and/or hydrophilic residues on the Ras effector loop. The subsequent visualization of a co-crystal between prokaryotic recombinant Rap1 GTP and the Raf fragment AA 51-131 strongly reinforced this view (Nassar *et al.*, 1995).

## V. Once at the Membrane, the Raf Zinc Finger Binds to a Second Site on Ras

The relatively clearcut picture of the structural basis of the high-affinity, GTP-dependent binding of Raf to Ras did not actually clarify the question of how the Ras-Raf interaction functions to promote Raf activation. In fact, discrepancies between the requirements for Raf-Ras binding as presented above and those for Raf activation became evident as these data accumulated. Thus, several mutations in Ras just outside of the effector loop (e.g., Ras N26 H27 to GI; Ras V45E) result in dramatic inhibition of Ras-transforming activity, with essentially no inhibition of the binding *in vitro* of Ras-GTP to Raf (1-257) (Chuang *et al.*, 1994; Barnard *et al.*, 1995). The discrepancy we found most striking was that the Raf fragment (51-149) showing the highest affinity for prokaryotic recombinant Ras-GTP in a direct, *in vitro* binding assay (Chuang *et al.*, 1994) lacked nearly all of the zinc finger structure. However, an intact Raf zinc finger appeared to be important to the Ras-Raf interaction, as assessed both in the very-sensitive yeast two-hybrid system and *in vitro* using baculoviral, recombinant Ras polypeptides (Zhang *et al.*, 1993). In addition, the zinc finger was clearly important to Ras-dependent Raf activation. Mutation of the Raf zinc finger (C168S), although causing a small increase in Raf transactivating function in serum-deprived cells, completely abolished the ability of V12 Ras to further augment Raf activity (Bruder *et al.*, 1992).

If the Raf zinc finger is not critical for the high-affinity, GTP-dependent binding of Raf to Ras, why does zinc finger mutation disturb Raf activation so severely? Two mutually exclusive explanations for these apparently conflicting data seemed possible. First, the Raf Cys-rich domain could make an important direct contribution to Raf activation and/or the Ras-Raf interaction that was poorly

reflected in the *in vitro* binding assay because of unappreciated technical issues. Second, the zinc finger mutation might simply cause a propagated disturbance in the structure of the contiguous Ras-GTP-binding domain, thereby interfering with Ras binding *in vivo*. Attempting to distinguish these alternatives, we sought to avoid the loss-of-function phenotype engendered by the point mutations by replacing the Raf zinc finger with the homologous, diacylglycerol (DAG)-binding zinc finger structure from PKCγ (Luo *et al.*, 1997). We then examined whether the newly introduced PKC zinc finger was itself functionally intact and could effectively substitute for the endogenous Raf zinc finger. The results were quite clearcut: the Raf/PKCγ fusion proved to be a fully activatable protein kinase, which had acquired the ability to bind active phorbol ester (TPA) *in vivo* and *in vitro*. The Raf1/PKCγ fusion was activated by TPA in intact cells but not *in vitro*, indicating that TPA activated Raf1/PKCγ by recruiting the fusion protein to the membrane, rather than by an allosteric mechanism. Conversely, in sharp contrast to wild-type Raf 1, the Raf/PKCγ fusion, like the Raf (C165, 168S) zinc finger mutant, showed virtually no activation in response to epidermal growth factor (EGF). Thus, the presence of a functional, DAG-binding zinc finger in place of the native Raf zinc finger did not interfere with the ability of the catalytic domain to be activated; however, it did not enable Ras-dependent activation of the Raf kinase domain any better than a mutant Raf zinc finger. Seeking an explanation for this outcome, we compared the association of wild-type and variant Raf polypeptides with V12 Ras. Surprisingly, in spite of earlier studies pointing to the dispensability of the zinc finger for high-affinity Ras-Raf binding *in vitro*, we found that both the Raf (C165, 168S) and Raf/PKCγ polypeptides were markedly impaired in their ability to associate with V12 Ras during coexpression in COS cells and *in vitro*, to an extent comparable to that caused by mutation of the critical basic residues (K84 ALK87 to A) in Raf's primary Ras-binding domain. This indicated that the Raf zinc finger contributed in a significant way to the creation of a stable Ras-Raf interaction. The difference between prokaryotic (unprocessed) recombinant Ras and mammalian recombinant Ras in their ability to bind wild-type Raf vs. Raf with mutant or variant zinc fingers proved to be attributable to Ras prenylation. Abolition of Ras farnesylation by conversion of Ras C 186 to S does not impair high-affinity, GTP-dependent binding of Ras to Raf *in vitro*. However, it does abolish the ability of mammalian recombinant Ras-GTP to distinguish between wild-type Raf and Raf (C165,168 S) zinc finger mutant, as was observed for prokaryotic (unprocessed) recombinant Ras. Thus, the Raf zinc finger binds to an Ras epitope, distinct from the effector loop, that is fully expressed only on prenylated Ras (Luo *et al.*, 1997).

The first direct demonstration of a second binding site between Ras and Raf was provided by Hu *et al.* (1995). This group showed that a recombinant Raf fragment (AA 132-206) encompassing the Cys-rich zinc finger bound directly to

Ras *in vitro*. In contrast to the GTP-dependent, high-affinity (nM) binding of the Raf aminoterminal segment (e.g., AA 48-148) to Ras, the binding of the Raf zinc finger to Ras exhibits a much-lower affinity that is entirely GTP independent and abolished by the Raf (C168S) mutation. Our estimates indicate that Raf (130-220) has a binding affinity for prenylated Ras in the low micromolar range (Figure 3). Campbell and coworkers (Williams *et al.*, 2000), using a sensitive fluoresence assay, demonstrated that the enzymatic farnesylation of prokaryotic recombinant Ras *in vitro* greatly increases its affinity for the Ras zinc finger (to a Kd near 20-30 μM). This low-affinity, GTP-independent binding becomes physiologically relevant only through the proximity induced by the primary, GTP-dependent Ras-Raf interaction. Hu and colleagues (1995) further showed that the Ras mutations N26G and V45E, which greatly impair V12 Ras-dependent Raf activation without significantly interfering with the GTP-dependent binding of Ras to Raf *in vitro*, greatly inhibit the GTP-independent binding of Ras to the Raf zinc finger

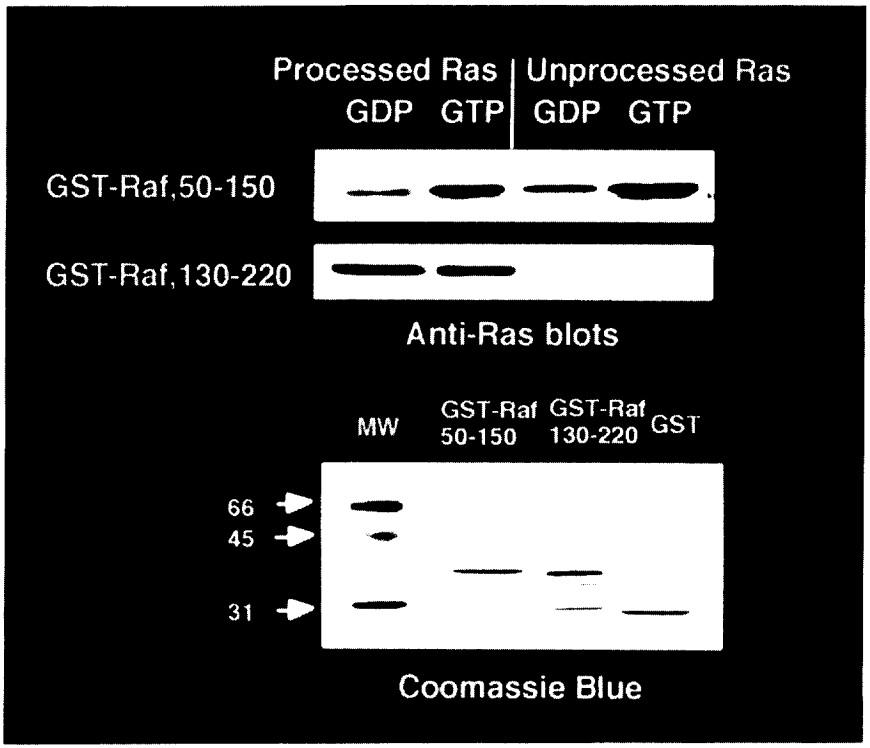


FIG. 3. Binding *in vitro* of processed (prenylated) or unprocessed Ras to two overlapping domains in the cRaf1 aminoterminal segment.

domain (AA132-206). How Ras prenylation reconfigures this epitope (sometimes called the Ras "activation" domain) to increase its affinity for the Raf zinc finger remains to be established.

## VI. The Interaction of the Raf Zinc Finger with Ras Is Critical for Raf Activation

The idea that Ras was dispensable once Raf had been recruited to the membrane was inferred from the observation that fusion of the carboxyterminal membrane targeting sequence from Ki-Ras (Figure 2) onto cRaf1 to create Raf CAAX enables Raf to promote transformation of NIH 3T3 cells in a manner resistant to inhibition by N17 Ras (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Subsequent work has shown this interpretation to be incorrect; the resistance of Raf CAAX to N17Ras only indicates that the GTP-dependent function of Ras has been bypassed. A continued interaction between Ras and Raf is necessary to enable Raf activation. The Raf zinc finger is critical for this function.

An extensive, surface-scanning mutagenesis (Daub *et al.*, 1998) of the Raf zinc finger region identified S177, T182, and M183 as well as L149 and F151 (Williams *et al.*, 2000) as residues critical to Ras-Raf binding energy. Mutation of Raf K144, R164, and L160 has little affect on Ras binding but interferes strongly with Raf activation, even in the context of Raf CAAX. An elegant, although indirect, demonstration of the importance of the second site, GTP-independent Ras-Raf interaction to Raf activation, was provided by the Ras E37G effector loop mutant, which is unable to bind Raf in a GTP-dependent manner. White and colleagues (1995) used the yeast two-hybrid system to select a Raf mutant (S257L) that is capable of binding to Ras E37G. The Raf (Ser257 Leu) mutation does not restore high-affinity, GTP-dependent binding to Ras E37G but causes a slightly higher level of basal kinase activity that is further augmented by both wild-type Ras and Ras E37G (Jaitner *et al.*, 1997). Raf (S257L) CAAX, like wild-type Raf CAAX, is transforming. Coexpression with Ras E37G substantially increases Raf (S257L) CAAX-dependent focus formation, kinase activity, and MAPK activation. However, Ras E37G has no effect on wild-type Raf CAAX (Mineo *et al.*, 1997). The Raf S257L mutation thus facilitates the second site Ras-Raf interaction that is crucial to Raf activation.

How does the zinc finger participate in Raf activation? The ability of Raf microinjected into *Xenopus* oocytes to activate germinal vesicle breakdown (GVBD) provides an exquisitely sensitive assay for Raf activity. Raf wild-type and the Raf (R89L) mutant do not activate GVBD. However, various mutations in the zinc finger — C165, 168S, F163I, P181L, as well as R143E or W and K144E — enable significant GVBD, even in the Raf (R89L) background (Cutler and Morrison, 1997; Cutler *et al.*, 1998; Winkler *et al.*, 1998). These results suggest that the Raf zinc finger, in addition to its ability to bind Ras, is the element

in the Raf aminoterminal domain that participates directly in the inhibition of the Raf catalytic domain. The binding of Ras to the Raf zinc finger may therefore displace the zinc finger from the catalytic domain and promote the disinhibition of the catalytic domain. Support for this view is provided by the ability of Raf 1-330 to inhibit the ability of the catalytically active Raf kinase domain (Raf 306-648) to cause GVBD. Raf 1-330 (R89L) also inhibits but the Raf zinc finger mutants indicated above as allowing GVBD in full-length Raf abolish the ability of Raf 1-330 to inhibit Raf 306-648 (Cutler *et al.*, 1998). Reciprocally, introduction of Y340/341D mutations into Raf 306-648 abrogates inhibition by Raf 1-330, suggesting that the zinc finger inhibits catalytic function by an interaction near Y340 and 341 (i.e., the aminoterminal region of the catalytic domain) (Cutler *et al.*, 1998; Roy *et al.*, 1997).

Interestingly, Rap 1, which binds both cRaf1 and B-Raf in a GTP-dependent manner, is incapable of activating cRaf1 *in vivo* but activates B-Raf both *in* vivo and on direct addition *in vitro* (Ohtsuka *et al.*, 1996). This response to Rap1-GTP depends on the respective Raf zinc finger structures. Swapping the zinc fingers between B-Raf and cRaf1 swaps the susceptibility to activation by Rap1 (Okada *et al.*, 1999). The cRaf zinc fingers actually bind Rap1 more tightly than Ras (Hu *et al.*, 1997). Rap1 inhibition of Ras-dependent Raf activation therefore may occur through Rap1 sequestion of the Raf zinc finger rather than through the more-aminoterminal, GTP-dependent interaction sites. The differential affinity of Ras and Rap 1 for the cRaf zinc finger is attributable to residue 31, which is K in Rap 1 and E in Ras; Ras E31K behaves like Rap 1. Conversely, the B-Raf zinc finger binds comparably to both small GTPases. A B-Raf zinc finger mutant (K252E M278T) with selectively enhanced binding to Rap 1 is no longer activated by Rap1 but continues to be activated by Ras (Okada *et al.*, 1999). This behavior is consistent with the idea that the Raf zinc finger-Ras interaction must be strong enough to enable displacement of the zinc finger from the catalytic domain. An excessively strong interaction between the small GTPase and the Raf zinc finger, however, will block activation.

## VII. Conversion of cRaf-1 to a Stably Active State Requires Raf Phosphorylation

The discovery of the Ras/Raf interaction provided an instant insight into both the function of Ras and the regulation of Raf. The subsequent, more-torturous elaboration of the complexities of this interaction, partially described above, uncovered the dual role of Ras: first, to recruit Raf to the membrane through a GTP-dependent interaction between the Ras effector loop and Raf 51-149 (the Ras-binding domain, RBD) and then to initiate the disinhibition of the catalytic domain by a GTP-independent interaction between a second site on Ras with the Raf zinc finger. Altogether, however, these steps do not fully account for the

mechanism of Ras-dependent cRaf1 activation *in vivo*. Ras-dependent activation *in vivo* converts Raf to a stably activated form in the cytosol, no longer bound to Ras. In our hands, addition of purified, fully processed Ras failed to alter the activity of purified, baculoviral recombinant cRaf1 (Zhang *et al.*, 1993). Stokoe and McCormick (1997) subsequently reported evidence for such direct activation. However, the extent of activation appeared to be very slight, compared to that achieved *in vivo*, and also appeared to require the continued presence of Ras-GTP. We infer that this reflects an intermediate step in the conversion of Raf to a stably activated, Ras-free form, a view supported by other work (Mizutani *et al.*, 1998). As indicated earlier, such stable activation most often reflects the introduction of a post-translational modification, usually phosphorylation. In fact, cRaf1 is extensively phosphorylated *in vivo* prior to activation and its phosphorylation increases several-fold after RTK activation. Moreover, coexpression of cRaf1 with a variety of protein (Ser/Thr) kinases (e.g., PKCα, Kolch *et al.*, 1993; PKCζ, van Dijk *et al.*, 1997) or tyrosine kinases (e.g., Src, Fabian *et al.*, 1993; Marais *et al.*, 1995; Jelinek *et al.*, 1996; Mason *et al.*, 1999; Xia *et al.*, 1999) or JAK2 (Xia *et al.*, 1996), either in mammalian or Sf9 cells, results in the phosphorylation and activation of cRaf1. Reciprocally, dephosphorylation of active cRaf1 with protein (Ser/Thr) phosphatases, alone or together with protein tyrosine phosphatases (Dent *et al.*, 1995) as well as the dual-specificity phosphatase Cdc25A (Mason *et al.*, 1999), have all been reported to deactivate cRaf1. Nevertheless, the manner in which altered phosphorylation promotes the Ras-dependent activation of cRaf1 remains elusive.

A very definite conclusion is that phosphorylation of one or more residues on the cRaf1 activation loop, the most-common mechanism for phosphorylation-dependent kinase activation, is not contributory to Ras-dependent Raf activation (Barnard *et al.*, 1998). Much attention has been devoted to the role of phosphorylation at Y340/341, inasmuch as conversion of these residues to A greatly impedes cRaf1 activation. However, conversion to D (which is the amino acid found at these two sites in B-Raf) results in increased basal activity (Fabian *et al.*, 1993; Marais *et al.*, 1995; Jelinek *et al.*, 1996; Mason *et al.*, 1999). This site is phosphorylated *in vivo* in response to overexpression of Src, in a manner augmented by V12Ras. We and others, however, have been unable to detect significant cRaf1 tyrosine phosphorylation during activation by insulin, EGF, or platelet-derived growth factor (PDGF). Thus, while phosphorylation of Y340/41 is a plausible and attractive mechanism for activation, especially in view of the role of this region as the probable site of negative regulation of Raf kinase activation by the Raf zinc finger, the evidence that Y340/41 phosphorylation participates in RTK-initiated, Ras-dependent activation of cRaf is unpersuasive.

A more-compelling body of evidence points to an important role for cRaf Ser338 phosphorylation in facilitating Raf activation. As first shown by Marshall

and coworkers, conversion of S338/339 to A blocks Raf activation by V12 Ras or vSrc and abrogates the transforming activity of Raf CAAX. Conversion of S338/339 to D is not itself activating but restores responsiveness to V12Ras and vSrc as well as the transforming activity of Raf CAAX (Diaz *et al.*, 1997). These workers subsequently identified PAK3 as a kinase capable of phosphorylating *in vitro* a peptide whose sequence is corresponds to that surrounding cRaf1 Ser338/339 (King *et al.*, 1998). Substantial evidence was presented for the participation of PAK3 in S338 phosphorylation *in vivo*, acting downstream of Cdc42 and/or Rac1, which can be recruited either by V12Ras or by RTKs, through the recruitment and activation of PI-3 kinase (Sun *et al.*, 2000). PAK3 may not be the only kinase active on the Raf S338 site; the identity of the S338 kinase is likely to vary, depending on the initiating stimulus (e.g., tumor necrosis factor receptors (TNFRs) vs. RTKs). Moreover, it is plausible that some stimuli (e.g., hematopoeitic cytokine receptors) use Y341 phosphorylation rather than S338 phosphorylation for this "priming" or facilitative function. Nevertheless, phosphorylation at S338, while necessary, is not sufficient to activate Raf *in vivo* or *in vitro*.

## VIII. Raf Binds Chaperones and 14-3-3 Proteins

Our inability to activate cRaf1 by direct addition *in vitro* of purified, fully processed Ras-GTP and Mg-ATP led us to the view that additional components are necessary to complete the process of Raf activation. We therefore again used two-hybrid expression cloning to seek proteins that interacted with cRaf1 and immediately recovered cDNAs encoding the 14-3-3ζ polypeptides (Luo *et al.*, 1995). Concurrently, purification of baculoviral recombinant cRaf1 yielded a complex of proteins consisting of the 74-kDa Raf polypeptide, heat shock protein (hsp) 90, hsp 50 (also known as cdc37), and the 14-3-3 proteins (Luo *et al.*, 1995). The association of these heat shock proteins with other protein kinases, particularly Src, had been previously described (Pratt, 1998). While these chaperones are undoubtedly critical for ensuring the proper conformation of the kinase, we considered it unlikely that they participated actively in the regulation of kinase activity. We therefore examined the role of the 14-3-3 proteins in Raf regulation. The 14-3-3s are a family of 28- to 30-kDa polypeptides known to assemble as homo- or heterodimers and repeatedly rediscovered as binding partners for a diverse array of polypeptides (Aitken, 1996). As first shown by Muslin *et al.* (1996), the 14-3-3s bind to motifs that contain phosphoserine (and probably phosphothreonine) residues, situated in a specific sequence. This context was defined as RSXS(P)XP or RXXXS(P)XP using synthetic peptides (Yaffe *et al.*, 1997). However, it is clear that a considerably broader specificity is accommodated. This tolerance is explained in part by the dimeric nature of the 14-3-3 assembly. The stable binding of a peptide containing a single phosphoserine motif

to 14-3-3 requires a high-affinity (nM) interaction. A polypeptide with multiple phosphoserines, even though these are encompassed in lower-affinity motifs, may achieve stable association with 14-3-3 due to the approximation of a low-affinity motif enabled by association of the first phosphoserine site with a 14-3-3 half dimer.

We carried out a deletion analysis of the 245 amino acid 14-3-3ζ polypeptide. This analysis demonstrated that the 14-3-3 dimerization interface involved an extensive part of the aminoterminal half of the 14-3-3ζ polypeptide, whereas the Raf binding function resided primarily in the carboxyterminal half of the 14-3-3 polypeptide (Luo *et al.*, 1995). Structures of 14-3-3ζ crystals confirmed this arrangement, revealing each 14-3-3ζ polypeptide as an L-shaped set of antiparallel helices, with the N-terminal four helices involved in dimerization and the carboxyterminal helices free, with helices 3, 5, 7, and 9 forming the inner walls of a cavity within the dimer that accommodates its binding partners (Liu *et al.*, 1995). Modeling the phosphoserine peptide into the 14-3-3 structure indicates that the two facing binding sites within the cavity are arranged in an antiparallel fashion. The most-striking result of our analysis was the finding that, although cRaf1 bound quite well *in vivo* to a GST-14-3-3 (139-245) fusion protein, the cRaf 1 polypeptide recovered with this monomeric form of 14-3-3 is completely devoid of catalytic activity, either before or after EGF stimulation (Luo *et al.*, 1995). This strongly suggested that a dimeric 14-3-3 was critical to the process of Raf activation.

## IX. Raf Dimerization Promotes Raf Activation

The ability of monomeric GST 14-3-3 to bind cRaf1 suggested the possibility that the native 14-3-3 dimer might enable cRaf1 to be dimerized, either with itself or with another kinase that could catalyze Raf phosphorylation and activation. Coexpression of variously epitope-tagged Raf polypeptides demonstrated that a small fraction of recombinant Raf is recovered as homodimers and that the abundance of these homodimers is unaffected by EGF stimulation (Luo *et al.*, 1996). We next inquired whether forced dimerization of Raf polypeptides affected Raf activity or susceptibility to activation. A set of three tandem FKBP-12 polypeptides was fused to the Raf aminoterminus, each conferring a single binding site for the macrolide drug FK506 or its dimeric congener, FK1012. Addition of FK1012 to cells expressing FK506-binding protein (FKBP)-Raf promoted the oligomerization of FKBP-Raf in a manner that was inhibited competitively by the monomeric FK506. The oligomerization of Myc-Raf was unaffected by FK1012. Similarly, FK1012 produced a time- and concentration-dependent activation of FKBP-Raf but not wild-type Raf that was about half the magnitude seen with EGF. Strikingly, however, FK1012 and EGF together produced a synergistic

activation of FKBP-Raf. Activation of FKBP-Raf by both EGF and FK1012 was inhibited by coexpression with N17 Ras and abolished completely by mutation in either FKBP-Raf Ras-binding domain (RBD) (K84 ALK-A84AA) or zinc finger (C165,168S) (Luo *et al.*, 1996). Thus, Raf dimerization strongly promotes Ras-dependent activation *in vivo*. Whether Raf dimerization is a necessary precondition for Raf activation *in vivo* and whether dimerization is regulated are not yet known. Nevertheless, it is striking that Inouye *et al.* (2000) recently observed that the ability of Ras-GTP to partially activate cRaf1 *in vitro* is entirely dependent on Ras dimerization. Ras dimerization apparently occurs spontaneously when Ras is inserted into a phospholipid membrane *in vitro* and perhaps within caveoli *in vivo*. Ras and Raf dimerizations appear to be significant elements in the Raf activation process in a manner somewhat analogous to the ligand-induced dimerization of the RTKs themselves. In contrast to the RTKs, however, it is unclear whether Raf dimerization is initiated by dimeric Ras, as Raf homodimers are recovered from cells free of Ras. Nevertheless, further analysis of the structural basis for Raf homodimerization revealed that the Raf (1-257) aminoterminal fragment can homodimerize, whereas the constitutively active carboxyterminal Raf fragment known as BXB-Raf (1-25/306-648) does not. Inasmuch as BXB Raf binds 14-3-3 avidly, while Raf (1-257) binds Ras but exhibits no binding to 14-3-3 in the two-hybrid system, it seems clear that Raf homodimerization is not mediated by 14-3-3. However, a role for Ras in initiating Raf dimerization remains tenable (J. Avruch *et al.*, unpublished observations).

## X. Binding of Raf to Dimeric 14-3-3 Is Necessary for the Initiation and Maintenance of Kinase Activity

Previous work had provided evidence for the binding of 14-3-3 to the Raf zinc finger (Michaud *et al.*, 1995; Clark *et al.*, 1997) as well as to the phosphoserines at 259 and 621 (Muslin *et al.*, 1996; Michaud *et al.*, 1995; Rommel *et al.*, 1996). We mutated S259 and S621 to A and examined the effects on the interaction of Raf with 14-3-3 in the two-hybrid assay and on the activation of Raf kinase in COS cells in response to EGF (Tzivion *et al.*, 1998). Removal of either S259 or S621 singly did not eliminate interaction with 14-3-3. However, the double mutation S259/621A eliminated interaction with 14-3-3 completely without affecting the interaction of Raf with Ras or MEK. Thus, each Raf polypeptide contains two phosphoserine-based 14-3-3-binding sides. The S259A (single) mutant exhibits a modest increase in basal kinase activity and two- to three-fold greater activation in response to EGF than is seen with wild-type Raf. Thus, the S259 phosphorylation is inhibitory and contributes to the maintenance of unstimulated Raf in a low-activity state. Conversely, the S621A mutant (and the S259/621A double mutant) is completely inactive. To ascertain whether the inactive state of the S621A mutant is due to the loss of 14-3-3 binding from that site,

we displaced 14-3-3 from the recombinant Raf *in vitro* by addition of a synthetic phosphopeptide whose sequence corresponds to the motif surrounding cRaf1 S621. The displacement of 14-3-3 from Raf resulted in the complete deactivation of the Raf kinase, both wild type and Raf (S259A). This deactivation is not due to interference with Raf oligomerization, which was unaltered by displacement of 14-3-3. Moreover, the activity of FKBP-Raf induced by the dimeric ligand FK1012 was inhibited by displacement of 14-3-3. Thus, the binding of 14-3-3 to Raf is necessary both for initiation and maintenance of the active state. The deactivation of Raf caused by displacement of 14-3-3 can be reversed simply by addition *in vitro* of prokaryotic, recombinant 14-3-3. However, Raf must have been previously activated *in vivo* to be (re)activated by 14-3-3 *in vitro*. The ability of 14-3-3 to reactivate Raf *in vitro* is dependent on Raf serine phosphorylation, as treatment of Raf with protein phosphatase 1γ after 14-3-3 displacement prevents reactivation by 14-3-3. Moreover, reactivation *in vitro*, just like activation *in vivo*, depends on the integrity of the 14-3-3 dimer. We constructed a mutant, monomeric 14-3-3 polypeptide by introducing a series of mutations into the dimer interface. Monomeric, full-length 14-3-3 bound Raf avidly *in vivo* but, as with the monomeric GST 14-3-3 (139-245), the Raf bound to monomeric, full-length 14-3-3 was devoid of activity. Moreover, in contrast to wild-type, dimeric 14-3-3, prokaryotic, recombinant monomeric 14-3-3 is completely unable to support the reactivation of wild-type Raf or Raf (S259A) *in vitro* after displacement of endogenous 14-3-3. Thus, Raf (S259A), which contains only one known 14-3-3-binding motif (at S621) nevertheless requires a dimeric 14-3-3 to sustain activity *in vivo* and to restore activity *in vitro*. The explanation for this requirement is not known. Our working hypothesis is simply that a 14-3-3 dimer is required because a second, yet to be identified, phospho (Ser/Thr)-dependent 14-3-3 binding site exists on active cRaf1 (S259A). In fact, we believe that the introduction of this phosphorylation underlies the stable activation of Raf (i.e., the active state requires the simultaneous binding of both S621P and this putative new phosphorylation site on a single Raf polypeptide by a single 14-3-3 dimer) (Tzivion *et al.*, 1998 ). We recently have identified a set of novel phosphorylation sites in the Raf catalytic domain and are examining the role of those sites in the initiation and maintenance of Raf activation.

## XI. Mechanism of Ras-dependent cRaf1 Activation

Our current hypothesis (Figure 4) for the mechanism of RTK-induced cRaf activation is the following:

1) cRaf1 resides in the cytosol in an inactive state, bound in a complex with hsp90, hsp50, and the 14-3-3 protein. The configuration of inactive Raf involves the occlusion of the catalytic domain by the Raf zinc finger, which obstructs

access to the ATP-binding site. The inactive configuration is stabilized by the binding of a 14-3-3 dimer to Ptd Ser259 and Ptd Ser621, which acts like a clothespin, folding the Raf polypeptide into a closed configuration. Mutation of the zinc finger or the S259A mutation each relaxes slightly the inhibited configuration. A portion of Raf polypeptides exist as dimers, an interaction mediated, directly or indirectly, through a segment in the aminoterminal (1-257) region, yet to be fully defined.

2) Ligand activation of RTK promotes Ras-GTP charging, which creates a high-affinity binding site for cRaf1 residues (51-149), the primary Ras-binding domain. This enables recruitment of a Raf dimer to Ras-GTP dimer at the membrane.

3) Once bound to the Ras effector loop at the membrane, Raf then interacts through its zinc finger with a second epitope on Ras that includes Ras N26, H27, and E45. Its configuration is unaffected by GTP-GDP but is fully developed only on prenylated Ras.

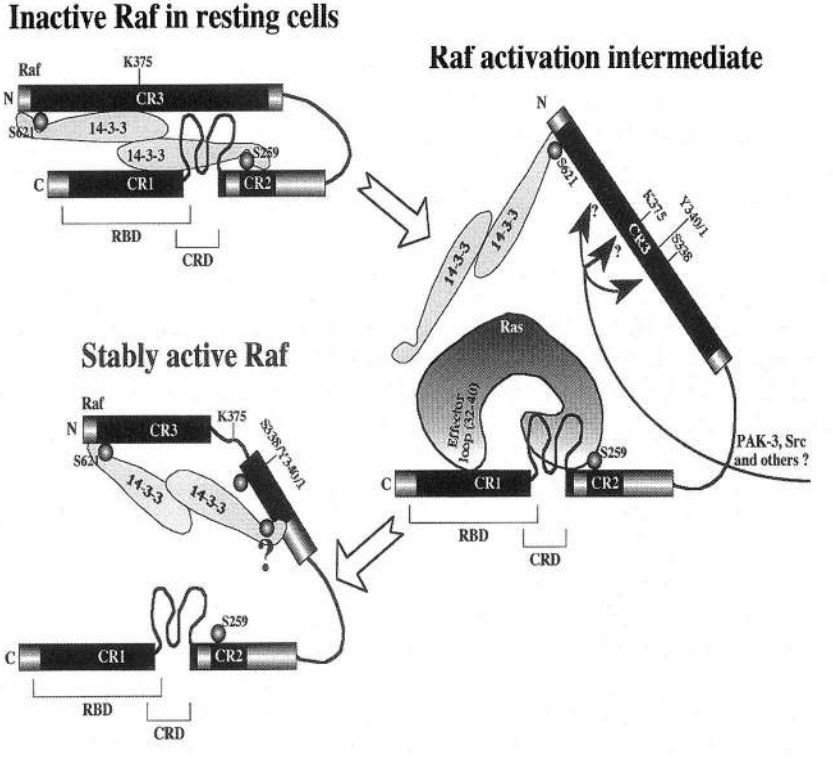


FIG. 4. A model for Ras-dependent cRaf1 activation.

4) Once at the membrane, the Raf catalytic domain also may bind to phosphatidic acid (PA) through a segment between cRaf1 residues 389-423 (Ghosh *et al.,* 1996). Although PA does not alter Raf activity *per se,* it may contribute to the activation process *in vivo* (Ghosh *et al.,* 1996; Rizzo *et al.*, 2000).

5) The second site Ras-Raf interaction, perhaps aided by phosphatidylserine binding to the zinc finger, promotes the displacement of the 14-3-3 half-dimer from the P-Ser259 site (Rommel *et al.,* 1996). This enables a partial activation of Raf kinase, which remains dependent on the continued presence of Ras. Ras binding to the Raf zinc finger also interferes with the interaction of the zinc finger with the region around Raf S338-Y341, further relaxing the inhibited configuration.

6) PAK3-catalyzed phosphorylation of S338 impedes the reassociation of the Raf zinc finger with this segment, limiting autoinhibition.

7) cRaf1 undergoes phosphorylation at an unidentified site in the catalytic domain, distinct from the S338-Y340 region, which creates a new binding site for the 14-3-3 half-dimer just displaced from Ptd Ser259. The binding of 14-3-3 to this putative site stabilizes an open, active configuration of cRaf1, enabling its release from Ras-GTP into the cytosol in a stably active state. Deactivation is initiated by protein (Ser/Thr) phosphatase action.

Steps 1-6 in this model are well supported by available data; step 7 is speculative and under active investigation. Other models, which differ in significant details regarding the mechanism of activation, have been proposed (Roy *et al.,* 1998; Thorson *et al.,* 1998; McPherson *et al.,* 1999).

## XII. Ras Effectors Other Than Raf

Although the Raf kinases are the most-securely established, direct mitogenic effectors of Ras, other Ras effectors crucial to mitogenesis have emerged (Shields *et al.,* 2000). The existence of such elements was predictable from the early observation that although both v-Raf and v-Ras are able to transform fully NIH 3T3 cells and other cells of fibroblastic origin, most human Ras oncogenes are found in cancers of epithelial origin (e.g., pancreas, colon, nonsmall cell lung cancers). In such cell backgrounds, v-Ras is itself transforming but v-Raf is not. Inhibitors of MAPK activation uniformly inhibit the growth of Ras-transformed epithelial cell lines in cell culture. This indicates that Ras activation of the Raf-MAPK pathway is necessary to transformation; however, mitogenic pathway(s) in addition to Raf-MAPK clearly are required. Moreover, a variety of Ras effector loop mutations have been shown to attenuate or eliminate Ras-transforming activity while retaining the ability to elicit components of the vRas biologic response and to complement each other to enable mitogenesis (White *et al.,* 1995; Joneson *et al.,* 1996). These mutants have been very useful in parsing the outflows from

V12 Ras (Miller *et al.*, 1998). At this time, two families of Ras-regulated signaling molecules in addition to the Rafs have been clearly identified.

Rodriquez-Viciana *et al.* (1994) first demonstrated that constitutively active Ras can promote the accumulation of 3′OH phosphorylated Ptd Ins lipids. Subsequent to the molecular cloning of the p110 catalytic subunits of the type 1a PI-3 kinases, this group showed that a region near the p110 aminoterminus binds directly to Ras-GTP in preference to Ras-GDP. A point mutation (K227E) that abrogates this interaction increases basal p110 activity (about four-fold) but abolishes the response to V12 Ras (Rodriquez-Viciana *et al.*, 1996). Moreover, the ability of V12 Ras to cause membrane ruffling, a Rac-regulated response, is abolished by the PI-3 kinase inhibitor wortmannin (Rodriquez-Viciana *et al.*, 1997). Thus, type 1a PI-3 kinases appear to be among the mitogenic effectors recruited by V12 Ras. The caveat, however, relates to the role of Ras in the activation of PI-3 kinase in response to RTK activation. Unlike Raf, whose recruitment to the membrane and subsequent activation in response to RTKs is entirely dependent on its nM affinity for and recruitment by Ras-GTP, the affinity of Ras-GTP for p110 is substantially lower than for Raf (Rodriquez-Viciana *et al.*, 1994,1996). In response to RTK activation, type 1a PI-3 kinases are not recruited to the membrane through their low-affinity interaction with Ras-GTP but rather by the avidity of the SH2 domains of the p85/55 adaptor subunits of the type1A PI-3 kinases for the tyrosine-phosphorylated RTKs and docking protein (e.g., insulin receptor substrate (IRS), FGF receptor substrate (FRS)). Thus, in the context of ligand activation of the RTK, the impact of Ras-GTP on PI-3 kinase activity is secondary and collateral, serving to augment the activation engendered by direct recruitment of the p85/p110 PI-3 kinase heterodimer by RTK and/or docking proteins. Conversely, the idea that the constitutively active V12 Ras oncogene is able to promote a direct activation of PI-3 kinase, sufficient to recruit some Ptd Ins 3,4,5 $P_3$ ($PIP_3$) downstream effectors in achieving cellular transformation, is quite plausible. Such effectors include the PKB/cAkt protein kinases and members of the Rho subfamily of GTPases (e.g., Rac1), which are activated by $PIP_3$-induced recruitment of Dbl-family guanylnucleotide exchange factors (Shields *et al.*, 2000).

A second, well-established group of Ras-GTP binding partners is a family of guanynucleotide exchange proteins (GNEFs) for the RalA GTPases (Shields *et al.*, 2000). These GNEFs — including Ral-GDS, RGL, and Rlf — were identified as candidate Ras effectors through two-hybrid screens. Each is capable of binding directly to Ras GTP, in preference to Ras GDP *in vitro*. Dominant inhibitors of RalA can interfere with Ras-induced mitogenesis. However, the role of RalA in Ras signaling and the specific biochemical effectors of the RalA GTPase are poorly understood. Ral GDS also binds to Rap1 GTP *in vitro*, with an affinity that apparently is higher than for Ras-GTP. This situation is reversed from cRaf1,

which clearly prefers Ras GTP over Rap 1 GTP. Thus, the question of whether the Ral GDS are effectors for Ras, Rap1, or both is currently unresolved. This exemplifies as well the current uncertainty over the actual biologic functions of the Rap1 GTPases (Zwartkruis and Bos, 1999). Based on work in *Drosophila,* it appears probable that Rap1 has functions entirely distinct from its ability to interfere with Ras signaling (Li *et al.,* 1997; Asha *et al.,* 1999). The latter phenotype, in fact, may occur only when Rap1 is overexpressed and may not reflect a physiologic function of Rap1 (Zwartkruis *et al.,* 1998). Whereas Rap1 opposes Ras-induced activation of cRaf1, both Ras and Rap1 are capable of promoting by direct addition the activation *in vitro* of B Raf (Yamamori *et al.,* 1995; Ohtsuka *et al.,* 1996), the isoform predominant in neural cells *in vivo* (Vossler *et al.,* 1997). Interestingly, constitutively active Rap1 is transforming in some cell backgrounds (e.g., Swiss 3T3 cells, but not NIH 3T3 cells) (Altshuler and Rheiro-Neto, 1998), possibly as a function of its ability to support the activation of B-Raf.

Our own efforts to identify additional Ras effectors have primarily employed two-hybrid screens using constitutively active forms of Ras and Rap1. Although we never encountered p110 PI3 kinase, we have recovered cDNAs corresponding to all three Ral-GDS isoforms from several cDNA libraries. In addition, we have retrieved an array of noncatalytic proteins, some of which (e.g., AF6) have been characterized as candidate Ras-GTP effectors by others. We are presently attempting to elucidate the biologic function of NORE1, a 46-kDa noncatalytic polypeptide that binds to Ras *in vivo* in response to EGF (Vavvas, 1998). We presume NORE 1 to be an adaptor protein and have recently found it to be constitutively associated with a protein kinase of the Ste20 subfamily. The significance of this association to the biologic programs controlled by NORE1 acting downstream of Ras remains to be defined.

## XIII. Conclusion

The Ras-Raf-MAPK pathway is arguably the most-extensively characterized and thoroughly studied signal transduction pathway in metazoans, certainly over the last decade. This scrutiny reflects the central role of this pathway in development, cellular differentiation, and, especially, mitogenesis. Continuous overactivity of this pathway is necessary to the growth of a substantial fraction of human malignancies. The pathway components therefore are attractive targets for antiproliferative therapies. This chapter has focused selectively on Ras and cRaf1, components that we have investigated directly. The understanding of the interaction between these elements and their operation during the process of Raf activation has expanded greatly but remains incomplete. Moreover, the outflows from Ras other than Raf, and the targets of Raf other than the MAPPKs (MEK 1 and 2), are areas where significant discoveries remain to be accomplished. Finally, we have not discussed a number of elements that appear to be important modulators

of the activity of either Ras or Raf or of the interaction of Ras and Raf with each other. Such molecules include the Ras-GAPs, p120 and NF1/2, and Sprouty (Casci *et al.,* 1999), an inhibitory element acting on Ras by an unknown mechanism. By contrast, SUR8 (Steward *et al.,* 1999), connector enhancer of kinase suppressor of Ras (CNK) (Therrien *et al.*, 1999), and kinase suppressor of Ras (KSR) (Li *et al.,* 2000) are positive modulators of Ras-Raf signaling that interact directly with Ras, Raf, or both. Other modulators are Raf kinase inhibitor protein (RKIP), a putative Raf inhibitor (Yeung *et al.,* 1999), and Bag-1, a Bcl2-like, putative Raf activator (Wang *et al.,* 1996). Understanding the mechanism of action and biologic role of these novel elements will keep this field vibrant for many years to come.

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