Critical Contribution of Linker Proteins to Raf Kinase Activation*

Received for publication, November 1, 2001, and in revised form, December 7, 2001
Published, JBC Papers in Press, December 7, 2001, DOI 10.1074/jbc.M110498200

Anthony N. Anselmo‡, Ron Bumeister‡, Jackie M. Thomas, and Michael A. White§

From the Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

Genetic analysis of Ras signaling has unveiled the participation of non-enzymatic accessory proteins in signal transmission. These proteins, KSR, CNK, and Sur-8, can interact with multiple core components of the Ras/MAP kinase cascade and may contribute to the structural organization of this cascade. However, the precise biochemical nature of the contribution of these proteins to Ras signaling is currently unknown. Here we show directly that CNK and KSR are required for stimulus dependent Raf kinase activation. CNK is required for membrane recruitment of Raf, while KSR is likely required to couple Raf to upstream kinases. These results demonstrate that CNK and KSR are integral components of the cellular machinery mediating Raf activation.

The mechanism by which Ras activation leads to activation of downstream effectors is only beginning to be understood. In the case of Raf kinases, activation by Ras appears to involve a combination of membrane recruitment and other association-induced activity changes (1). Observations that artificially membrane-targeted variants of Raf1 are constitutively active independently of Ras in transient transfection experiments, together with observations that active Ras can recruit Raf1 to the plasma membrane, have led to the current paradigm for Ras function. That is, Ras-GTP acts as “molecular flypaper” ensnaring effector molecules at the plasma membrane where they are subsequently activated by other partially characterized membrane-associated components (2, 3). However, some recent observations are inconsistent with this model. At least in the case of Raf1, Ras association makes an important contribution to activation of Raf1 kinase activity independently of membrane recruitment (4, 5). In addition, the association of endogenous Raf1 kinases with the plasma membrane does not always correlate with the activity of the kinase or the mitogen state of the cells (6). For example, Raf1, MEK1, and ERK1/2 can be found constitutively associated with the caveolar plasma membrane of primary human fibroblasts independently of the activation state of these proteins. In addition, these components can be activated in a mitogen-dependent fashion in purified caveolae (6) strongly suggesting that the Ras/MAP kinase cascade can exist as a coherent spatially organized signal transduction machine.

Adding to the complexity, a growing number of observations have implicated non-enzymatic accessory proteins in the regulation of the Ras-Raf-MAP kinase cascade. These include molecules such as KSR1, Sur-8, CNK, and MP-1. These proteins have characteristics suggestive of roles as scaffolding and or adapter proteins (reviewed in Ref. 7). However it is still unknown whether any of these proteins directly participate in activation of the Raf/MAP kinase cascade, and, if so, whether they may function to localize kinases to sites of action, nucleate or stabilize activation complexes, enhance substrate recognition, alter kinetics of kinase activation, and/or restrict kinase specificity.

Genetic epistasis analysis of KSR, CNK, and Sur-8 alleles in Drosophila or Caenorhabditis elegans places the function of all three proteins downstream of activated Ras and upstream of or in parallel with Raf (8–12). The direct consequence of KSR, CNK, or Sur-8 alleles on the biochemical activity of the Raf/Erk cascade has not been examined. However, biochemical analysis of mammalian orthologs of these genes suggest participation of these proteins in regulation of ERK kinases (13). Sur-8 can interact with both Ras-GTP and Raf and can facilitate formation of functional Ras/Raf complexes when all three proteins are ectopically expressed, suggesting Sur-8 may function as an adapter for the Ras-GTP/Raf complex (12, 14). KSR can interact directly with MEK and will inhibit ERK activation when overexpressed (15, 16). However, low level expression of KSR can facilitate MEK and ERK activation (17). These results, coupled with the observation that overexpressed KSR can associate with Raf in a Ras-dependent manner have led to the hypothesis that KSR may function as an adapter protein to facilitate Raf-MEK and/or MEK-ERK kinase-substrate interactions (18, 19). MP1 was identified in two-hybrid screen as a MEK1-interacting protein and can simultaneously interact with both MEK1 and ERK1. Like KSR1, MP1 can inhibit or potentiate ERK activation depending upon levels of MP1 expression and may modulate the MEK-ERK interaction (20).

The stoichiometry of scaffolds relative to the components they can assemble is likely to be strictly regulated. A surfeit of scaffold may disperse the very components that must function together to mediate a signal transduction cascade (21, 22). Therefore ectopic expression analysis is less than ideal for characterization of potential scaffold/adapter proteins and may be partially responsible for paradoxical or apparently contradictory observations of protein function (13, 22, 23). For this reason, we sought a biochemically tractable model system in which to examine the consequences of loss-of-function of scaffolding proteins on regulation of the MAP kinase cascade.

Recently, it has been demonstrated that the Drosophila Schneider L2 cell line (S2) responds to insulin by activation of endogenous ERK-A (ERK2 ortholog) through activation of the canonical receptor-coupled Ras-Raf kinase cascade, just as has been previously characterized in mammalian cells. Together

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* This work was supported by Grant CA71443 from the National Cancer Institute and Grant 1-1414 from the Welch Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed: Dept. of Cell Biology, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390. Tel.: 214-648-2861; Fax: 214-648-8694; E-mail: michael.white@utsouthwestern.edu.
1 The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular-regulated kinase; MEK, MAP kinase/ERK kinase; ds, double-stranded; RIP, radioligand precipitation buffer; PMA, phorbol 12-myristate 13-acetate.
with the observation that S2 cells are extremely amenable to double-stranded RNA-mediated interference of gene expression (RNAi), thus allowing analysis of loss-of-function phenotypes, these cells become an ideal model system in which to characterize the contribution of accessory proteins to the regulation of the Ras/MAP kinase cascade (24).

To directly assess the contribution of putative scaffold/adaptor proteins to regulation of the Ras/MAP kinase cascade, we examined the consequences of knocking down the expression of KSR1, CNK, Sur-8, and MP1 on stimulus-dependent activation of this cascade. We show here that both CNK and KSR are required for activation of ERK in response to insulin and phorbol ester. We demonstrate that the molecular level at which both CNK and KSR1 impact this cascade is directly at the Raf kinase level.

**EXPERIMENTAL PROCEDURES**

*Materials—Double-stranded DNA (dsDNA) was prepared and used according to Clemens et al. (24). Schneider L2 (S2) cells were cultured in *Drosophila* serum-free media (Invitrogen) supplemented with 16.5 mM l-glutamine (Invitrogen) and 50 μg/ml gentamicin (Sigma). Total RNA was prepared using the High Pure RNA Isolation Kit (Roche Molecular Diagnostics). Reverse transcriptase-PCR was performed using Superscript First Strand Synthesis system for reverse transcriptase-PCR (Invitrogen). Antibodies against phospho-ERK and total ERK were purchased from Sigma (M5670, M8159). Anti-phospho-Akt antibody was from Cell Signaling (9271). Antibody against *Drosophila* total Akt was a generous gift from Brian Hemmings. Draf and Dras antibodies were generous gifts from Deborah Morrison and Helmut Kramer, respectively. Antibodies against MEK1/2 and phospho-MEK1/2 were from Cell Signaling (9122) and Sigma (no. 127-67).**

**Raf Kinase Assay—** S2 cells were stimulated with 10 μg/ml human recombinant insulin for 0, 5, 10 min and immediately lysed in a modified RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 5 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate). After rotation for 20 min at 4 °C, the cell lysates were cleared by centrifugation at 17,000 × g for 15 min. From the cleared lysates, D-Raf was immunoprecipitated with 2A of a polyclonal rabbit anti-D-Raf antibody. The immunoprecipitates were then washed three times in RIPA buffer (150 mM NaCl), two times in a high salt RIPA buffer (500 mM NaCl), and finally two times in 25 mM HEPES + 10 mM MgCl₂. To the α of washed immunoprecipitates were added 30α of kinase reaction buffer (25 mM HEPES, pH 7.4, 10 mM MgCl₂, 83 μM ATP, and 0.5 μg of recombinant His-MEK1ΔN1(k97M)). After incubation for 30 min at 30 °C, MEK1 phosphorylation was assayed using an antibody that specifically recognizes Ser(P)-217/221.

Cell Fractionation—S2 cells were resuspended in homogenization buffer (20 mM Tris, pH 7.5, 0.25 M sucrose, 5 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 20 mM NaF) and incubated for 15 min on ice. The cells were then subject to nitrogen cavitation at 600 psi. Upon release of pressure, the disrupted cells were centrifuged at 17,000 × g for 5 min. The supernatant was then centrifuged for 1 h at 100,000 × g. P100 designates the pellet material, whereas S100 designates the soluble supernatant. 1% of the total S100 and 10% of the total P100 fractions from each sample were loaded for Western analysis. Signal intensities from anti-Raf immunoblots were quantitated using an Alpha Inotech digital imaging system together with the Flouro-Chem software package (Scimetrics).

**RESULTS AND DISCUSSION**

To assess the contribution of putative scaffolding/adaptor proteins to ERK activation, S2 cells were treated with dsRNAs targeted to KSR-1, CNK, the *Drosophila* ortholog of Sur-8 (GenBank™ accession AE003717), and the *Drosophila* ortholog of MP1 (CG5110 accession AAF53620). As reported by Clemens et al. (24), addition of double-stranded RNA to the culture media resulted in a robust and specific reduction in transcript levels for the targeted genes (Fig. 1). Targeting Sur-8 or MP1 had no detectable effect on activation of ERK (not shown), however, we found that both CNK and KSR are required for activation of ERK in response to insulin (Fig. 2A). Akt activation was unaffected by loss of KSR or CNK, demonstrating that insulin signaling was not generally inhibited (Fig. 2B). As expected, dsRNA directed against Ras also blocked ERK activation (Fig. 2A). In contrast to KSR and CNK, downregulation of Ras partially reduces activation of AKT by insulin.
Fig. 2B). This is consistent with published observations suggesting a contribution of Ras to activation of phosphatidylinositol 3-kinase (25, 26).

Genetic analysis of the developmental phenotypes induced upon hyperactivation of ERK suggests that KSR and CNK both act at the level of Raf or in parallel with Raf (8, 11). However, the observation that KSR can interact with MEK and ERK suggests that this protein may function as a linker to potentiate the MEK/ERK kinase-substrate interaction (16, 27, 28). As with ERK, we found insulin activation of MEK, as displayed with an anti-Ser(P)-217/221 MEK antibody, was inhibited by KSR or CNK dsRNAs (data not shown). To examine Raf activity, anti-dRAF immunoprecipitates were mixed with recombinant kinase-dead human MEK1 in vitro kinase reactions. As shown in Fig. 3, both CNK and KSR are required for insulin activation of Raf kinase activity.

The observation that Ras, but not CNK or KSR, contributes to activation of AKT in response to insulin suggests that insulin activation of Raf is not affected by down-regulation of CNK or KSR. This places the activity of KSR and CNK squarely at the level of Raf activation. Overexpressed CNK is enriched at sites of cell/cell contact potentially via PH domain-mediated interaction with phosphatidylinositol phosphates. CNK can also associate with Raf when overexpressed in cells (11). These observations hint that CNK may contribute to plasma membrane compartmentalization of Raf. Multiple studies suggest Raf must be targeted to the plasma membrane prior to activation (13). Consistent with observations in mammalian cell culture systems, we find Raf protein both in the membrane particulate fraction (P100) and the soluble fraction (S100) of mechanically disrupted S2 cells. Down-regulation of CNK resulted in a dramatic reduction of Raf protein in the P100 fraction independently of insulin stimulation (Fig. 4). Surprisingly, the presence of Raf in the P100 fraction is more dependent upon CNK than Ras. This result strongly suggests that the contribution of CNK to Raf activation is at least in part through appropriate compartmentalization of Raf proteins to the site of activation. In contrast, down-regulation of KSR had no detectable effect on Raf compartmentalization (data not shown).

To further elaborate a general requirement of KSR and CNK
to mediate Raf activation, we screened for additional ERK stimuli in S2 cells. We found that ERK is activated in response to 1 μM phorbol 12-myristate 13-acetate (PMA). A body of literature suggests that PMA activation of ERK is mediated by PKC and Raf independently of Ras (29–32). The majority of these studies employed dominant inhibitory Ras variants to exclude a role for Ras activity. However, recent studies using neutralizing Ras antibodies demonstrate that Ras is required for PMA activation of ERK (33, 34). Consistent with these later studies, we found that down-regulation of Ras blocks PMA activation of ERK (Fig. 5). Similarly, CNK and KSR are required for PMA-induced activation of ERK (Fig. 5).

In summary, we have provided the first direct biochemical evidence that CNK and KSR are integral components of the cellular machinery required for Raf activation. Transfected epitope-tagged CNK, and a related mammalian protein MAGUIN (MAGUK-interacting protein), can both interact with Raf in cells suggesting that native CNK and Raf form complexes (11, 35). Both transfected CNK and MAGUIN partially localize to plasma membrane compartments (11, 35). These observations lead to the hypothesis that CNK may participate in regulating compartmentalization of Raf in cells. However, expression of MAGUIN was not sufficient to recruit Raf1 to the plasma membrane (35). Therefore, there have been no direct observations supporting this hypothesis. Here, we have directly shown that inhibition of native CNK expression blocks native Raf activation, and prevents compartmentalization of Raf at the plasma membrane, suggesting the biochemical contribution of CNK to Raf activation is at least partially due to facilitation of appropriate cellular localization.

KSR contains a putative serine/threonine kinase domain (8). One group has reported that immunoprecipitated KSR can phosphorylate Raf in vitro, which is compelling evidence for a biochemical relationship between Raf and KSR (36). On the other hand, others find no evidence for an intrinsic kinase activity of KSR and contest the classification of this protein as a kinase (23, 37, 38). Several reports do show that CNK immunoprecipitates from cells are tightly associated with kinases that can phosphorylate KSR itself, but these CNK-associated kinases reportedly do not utilize Raf1 as a substrate in vitro (23, 38). Our direct biochemical observation of the requirement of KSR for Raf activation is consistent with either the possibility that KSR is indeed a Raf kinase, or that it is a linker protein required to couple Raf to the upstream kinases that are responsible for activating phosphorylation events on Raf. Unlike CNK, KSR does not appear to be required to compartmentalize Raf at the plasma membrane (data not shown). Studies are currently underway to employ the system described here to identify critical kinases responsible for Raf activation.

Acknowledgments—We thank Deborah Morrison, Brian Hemmings, and Helmut Kramer for generous gifts of antibodies. We thank Bing Xu and Melanie Cobb for help and advice with Raf kinase assays.

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