Regulation of Catalytic and Non-catalytic Functions of the Drosophila Ste20 Kinase Slik by Activation Segment Phosphorylation*

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Protein kinases carry out important functions in cells by phosphorylating substrates and by means of regulated non-catalytic activities. Such non-catalytic functions have been ascribed to many kinases, including some members of the Ste20 family. The Drosophila Ste20 kinase Slik phosphorylates and activates Moesin in developing epithelial tissues to promote epithelial tissue integrity. It also functions non-catalytically to promote epithelial cell proliferation and tissue growth. We carried out a structure-function analysis to determine how these two distinct activities of Slik are controlled. We find that the conserved C-terminal coiled-coil domain of Slik, which is necessary and sufficient for apical localization of the kinase in epithelial cells, is not required for Moesin phosphorylation but is critical for the growth-promoting function of Slik. Slik is auto- and trans-phosphorylated in vivo. Phosphorylation of at least two of three conserved sites in the activation segment is required for both efficient catalytic activity and non-catalytic signaling. Slik function is thus dependent upon proper localization of the kinase via the C-terminal coiled-coil domain and activation via activation segment phosphorylation, which enhances both phosphorylation of substrates like Moesin and engagement of effectors of its non-catalytic growth-promoting activity.

Protein kinases play key roles in most cellular processes through their well known catalytic function of reversibly phosphorylating their substrates. Catalytic activity-independent functions have been ascribed to an increasing number of these proteins, leading to the emerging view of protein kinases as molecular switches (analogous to small GTPases) rather than just enzymes. The Sterile-20 (Ste20) kinases are a large group of Ser/Thr kinases with 28 members in humans divided into 2 distinct families (PAK- and GCK-like) and 10 subfamilies. Outside of the catalytic domain, which shows homology to the yeast kinase Ste20p, the 10 subfamilies show little sequence similarity to one another. Despite their structural diversity, many Ste20 kinases appear to regulate a few common cellular functions including cell proliferation and survival, cytoskeletal dynamics, and ion transport (2). Some of the Ste20 kinases are well known proteins with clearly defined physiological functions, e.g. the PAK subfamilies in cytoskeletal regulation (3), the GCK-II subfamily kinases Mst1/Mst2/Hippo in tissue growth and tumor suppression (4), and the GCK-VI subfamily kinases Osr1 and Spak in regulating ion channels (5). Many others are less well characterized. Although most of the identified functions of these kinases have been attributed to substrate phosphorylation, catalytic activity-independent functions have been proposed for some (6).

The GCK-V subfamily is composed of two kinases in mammals, Slik and Lok/Stk10. These kinases are characterized by an N-terminal Ste20-like kinase domain and a C-terminal coiled-coiled repeat-containing domain (CCD) connected by a non-conserved central linker domain (NCD) of variable length. Slik has been implicated in the regulation of a variety of cellular processes, including cell cycle progression (7), apoptosis (8, 9), and cell migration (10–12). The one fundamental function of these kinases that is evolutionarily conserved is the regulation of ezrin/radixin/moesin (ERM) family proteins. ERM proteins are important regulators of the cell cortex, acting as cross-linkers to connect the actin cytoskeleton to diverse transmembrane

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§These abbreviations used are: Ste20, Sterile-20; CCD, C-terminal coiled-coiled repeat-containing domain; NCD, non-conserved central linker domain; Slik, Ste20-like kinase; ERM, ezrin/radixin/moesin; dsRNA, double-stranded RNA; Moe.CT, C-terminal actin-binding domain of Moesin; DP, disc proper; PM, peripodial membrane; ptc, patched; ap, apopterus; nub, nubbina; P(4,5)P2, phosphatidylinositol-4,5-bisphosphate; EdU, 5-ethyl-2'-deoxyuridine; PAK, p21-activated kinase; GCK, germinal center kinase; Lok, lymphocyte-oriented kinase.
Regulation of Slik by Activation Segment Phosphorylation

proteins at the plasma membrane. Their ability to do so requires phosphorylation of a highly conserved Thr residue near the C-terminus, which disrupts autoinhibitory interactions between the N- and C-terminal domains (13). In Drosophila, phosphorylation at the critical residue (and thus activation) of Moesin was negligible in epithelial cells mutant for Slik, the single GCK-V orthologue in flies (14). Recently, depletion of Slik and/or Lok in mammalian cells was shown to strongly reduce ERM protein phosphorylation (15, 16). Mutating or depleting these GCK-V kinases in Drosophila or mammalian cells produces cellular and tissue phenotypes similar to those caused by mutating or depleting the ERM proteins themselves, including impaired epithelial tissue integrity (14, 17), disrupted organization of apical microvilli (14, 18), reduced cortical stiffness (19, 20), and misorientation of the mitotic spindle and cytokinesis defects (15, 19, 20). Taken together, these studies strongly highlight the importance of GCK-V kinase function in ERM regulation to control cell structure and epithelial organization and their potential involvement in pathological conditions where these are affected.

Drosophila slik mutants have an additional developmental phenotype that is separable from Moesin regulation. The mutant animals grow slowly, requiring approximately three times as long to reach full size in the larval stage before subsequently dying (21). Overexpression of Slik in wing imaginal discs (the epithelial precursors to the adult wing) has the opposite effect, increasing cell proliferation rates and causing overgrowth of the wing (21). Thus, as with other Ste20 kinases, including Hippo/Mst and Tao1 (22–27), Slik regulates tissue growth. There are two unusual features of Slik-driven growth. First, Slik expression had nonautonomous effects, with not only Slik-expressing cells but also surrounding cells displaying the proliferative response (21). Second, a point mutant form of the kinase that is expected to impair catalytic activity also induced the proliferative response (21). This suggests that the effect does not require catalytic activity, in line with the catalytic activity-independent allosteric functions of a number of kinases and pseudokinases (1).

Because of its involvement in both processes, Slik is well positioned to serve as one of the mechanisms for coordinating epithelial cell organization with epithelial tissue growth (28). To understand how these distinct activities of Slik may be regulated, we undertook a structure-function analysis of this kinase. Understanding how these distinct activities of Slik may be regulated light the importance of GCK-V kinase function in ERM regulation to control cell structure and epithelial organization and their potential involvement in pathological conditions where these are affected.

Experimental Procedures

Cloning and Constructs—To create the slik and moesin transgenes, PCR was used to introduce an EcoRI site immediately upstream of the initiator Met codon and a KpnI site either immediately downstream of the stop codon of full-length slik and moesin cDNAs or replacing the stop codon of moesin by PCR. For Slik, a KpnI and a NarI site were then silently introduced at codon 195 and 200 of the coding sequence, respectively. For SlikT186A, SlikT192A, and SlikT186A/T192A mutants, the first ~700 bp of the modified 5’ slik coding sequence were PCR-amplified with specific primers to introduce the mutations, and the EcoRI/KpnI-digested products encoding the N terminus of Slik were ligated together with the KpnI fragment encoding the C terminus in a modified pMT.puro3c expression vector that introduces an N-terminal Myc epitope tag and into pLlASTattB for generation of transgenic flies. For the SlikT196A mutant, an EcoRI/NarI fragment was used instead because the KpnI site is destroyed by the introduced mutation. The kinase dead slikkd mutant, in which Asp796 is mutated to Asn, has been described previously (21). The modified moesin cDNA was cloned into pMT.puro3c vectors modified to encode either an N-terminal Myc epitope tag or a C-terminal GFP tag using an EcoRI/KpnI digest. The Moesin555A and Moesin555B mutants were introduced by PCR site-directed mutagenesis. The construct for expressing the GST-MoeCT fusion protein was generated by cloning a fragment encoding the C terminus of Moesin (amino acids 311–575 of Moe-PRD) in pET-23b. For the Slik domain constructs, sequences coding for amino acids 1–321 (Slik5N), 322–988 (Slik5C), or 989–1300 (Slik5CC) of Slik were PCR-amplified with primers that introduced a 5′ EcoRI site and 3′ KpnI site. EcoRI- and KpnI-digested products were cloned into pMT.puro3c and plAST plasmids modified to encode an N-terminal Myc epitope tag. To generate the slik 5′-untranslated region (UTR) dsRNA transgene, the UTR sequence (spanning three exons between nucleotides 24,401,370 and 24,402,688 of the Drosophila chromosome 2R scaffold) was PCR-amplified from S2 cell cDNA and cloned in inverted repeat orientation in pWIZ. This same sequence, with T7 and T3 promoters introduced at either end by PCR, was used as a template for generating slik 5′-UTR dsRNA for cell culture experiments. A plasmid encoding PLC8-PH fused to GFP was generously provided by Dr. J. Brill and was re-cloned into pMT.puro3c for expression in S2 cells.

Fly Strains and Reagents—Slik transgene constructs cloned in plAST-att8 plasmids were recombined into the 65B2 locus using the PhiC31 integrase system (29). UAS-slik5′-UTR-dsRNA, UAS-myc-Slik5N, UAS-myc-Slik5C, and UAS-myc-Slik5CC transgenic flies were generated by standard P-element transgenesis. UAS-Flp5C1 flies were from Dr. H. McNellis. UAS-CyclinD, UAS-Cdk4, and UAS-DmMyc flies were from Dr. B. Edgar. UAS-Dp110 flies were from Dr. S. Leevers. UAS-Eiger flies were from Dr. M. Miura. UAS-Mer5B flies were from Dr. R. Fehon. UAS-merRNAi flies (transmigrant ID 7161) were from the Vienna Drosophila Resource Centre (VDRC). All other fly stocks were from the Bloomington Drosophila Stock Centre.

The following antibody dilutions were used: guinea pig anti-Slik (21) at 1:250–1:1000 for immunostainings and 1:20000 for immunoblotting; rabbit anti-phospho-ezrin (Thr567)/radixin (Thr568)/moesin (Thr588) (Cell Signaling Technology, #3141) at 1:100 for immunostainings and 1:2000 for immunoblotting; rabbit anti-cleaved Caspase-3 (Cell Signaling Technology, #9661); mouse anti-c-Myc (Santa Cruz) at 1:1000 for immunostainings and 1:1000 for immunoblotting; rabbit anti-GFP (Towell Pines Biologs Inc.) at 1:1000.

Cell Culture—slik 5′-UTR dsRNA was synthesized in two separate in vitro transcription reactions using MEGAscript T3
and T7 kits (Ambion). RNA products were mixed in equal amounts, annealed at 95 °C for 5 min, and then slowly cooled to room temperature. All cell-based experiments were done in Drosophila S2-R+ cells grown in Schneider’s medium (Lonza) supplemented with 10% FBS (Gibco) and 50 units/ml penicillin/streptomycin (Gibco) at 25 °C. Cells were plated at 1 × 10^6/ml. For RNA interference, the appropriate dsRNA was added to the culture medium on day 0 at 1 μg/ml and left to incubate for 48 h. Plasmids were then transfected on day 2 using the X-tremeGENE HP reagent (Roche Applied Science) as described by the manufacturer. Typically, 500 ng/ml Slik plasmids and 200 ng/ml Moesin plasmids were added. On day 3, an additional dose of dsRNA was added along with 0.5 mM CuSO4 for induction. The cells were finally processed on day 5.

Immunoprecipitations, Pulldown Assays, and Western Blotting—Cells were harvested in ice-cold PBS and then lysed for 15 min at 4 °C in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40 with added 1× protease inhibitor (Roche Applied Science), 1× AEBSF (Sigma), and 1× phosphatase inhibitor (Roche Applied Science)). Lysates were centrifuged to remove cell debris (12,000 × g, 15 min, 4 °C). For immunoprecipitations, cleared lysates were incubated with the appropriate antibody for 1 h at 4 °C and then with protein A/G-agarose beads (Santa Cruz) for 1 h at 4 °C. The beads were washed in lysis buffer 3 times for 5 min and then suspended in 2× Laemmli sample buffer at 80 °C for 5 min to solubilize proteins. Proteins were separated using a standard SDS-PAGE protocol and transferred to a PVDF membrane using a semi-dry transfer apparatus. Immunoblotting was conducted according to standard procedures. For pulldown assays, cleared lysates from cells expressing either Slik^{CCD} or the PH domain of phosphatidylinositol phosphates according to the manufacturer’s protocol. S2 cells were transfected with Slik or Slik^{kd} expression vectors and induced as described above. Three days after induction, cells were washed with PBS and lysed for 15 min on ice in immunoprecipitation lysis buffer (25 mM HEPES, pH 7.2, 100 mM NaCl, 10% glycerol, 0.5% Nonidet P-40 containing 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 10 mM β-glycerophosphate, 1× PhosSTOP (Roche)). Samples were centrifuged at 14,000 rpm for 10 min at 4 °C. Rat anti-Slik antibody (21) was added to the soluble fractions, and samples were incubated for 2h at 4 °C. Protein A-agarose beads were added, and samples were incubated an additional 2 h. The beads were washed 3 times at 4 °C in 25 mM HEPES, pH 7.2, 150 mM NaCl, 0.5% Nonidet P-40 containing 5 mM EGTA, 5 mM EDTA, 10 mM β-glycerophosphate, 1 mM NaVO₄, 1× PhosSTOP, and 0.01% BSA. Beads were rinsed two times in kinase buffer (20 mM Tris, pH 7.5, 15 mM MgCl₂, 3 mM MnCl₂, 1 mM DTT, 1 mM NaVO₄, 10 mM β-glycerophosphate, 0.1 mg/ml AEBSF, 1× PhosSTOP) and then suspended in kinase buffer containing 50 μM ATP, 5 μCi [γ-³²P]ATP (for radioactive labeling), and 5 μl of GST-Moe.CT substrate. Reactions were incubated for 20 min at 30 °C, stopped by the addition of 5× sample buffer and immediate heating at 90 °C for 5 min, and separated by SDS-PAGE. Gels were incubated twice for 10 min in 20% methanol, 10% glycerol, dried, and developed by autoradiography.

Fly Crosses, EdU Labeling, and Immunostainings—Unless otherwise stated, crosses were performed at 25 °C. For some of the drivers of growth and apoptosis, crosses were performed at 18 °C. For expression of UAS-Slik transgenes in wild-type discs, Gal80^ts was used to inhibit transgene expression during embryonic stages. Parents were allowed to lay eggs at 21 °C for 2 days. One day later, offspring were transferred to 27 °C to inhibit Gal80^ts and activate GAL4-dependent transgene expression. For experiments involving rescue of dsRNA-mediated Slik depletion, crosses included a UAS-Dcr transgene and were carried out at 27 °C to maximize the slik dsRNA phenotype while minimizing the effects of transgenic Slik expression. mer dsRNA crosses were performed at 27 °C.

For immunostainings, third instar larvae were dissected in PBS to isolate the anterior halves and remove the fat, the digestive system, and salivary glands. The resulting carcasses were collected for 20 min in ice-cold PBS and then fixed using 4% paraformaldehyde in 0.2% PBS, 0.2% Tween (PBT) for 20 min. The anterior halves were then washed 3 times in 0.2% PBT followed by blocking for 1 h in BBT (0.2% BSA + 0.3% BSA). The primary antibodies diluted in BBT were added and incubated overnight at 4 °C. The next day samples were washed 3 times in 0.2% PBT and then incubated for 2h with the fluorescent secondary antibodies diluted in BBT (Invitrogen or Jackson ImmunoResearch Laboratories, Inc.). Samples were again washed 3 times in 0.2% PBT and then incubated for 10 min with 2.5 μg/ml DAPI. After a final wash in PBT, the larvae were transferred into mounting medium (90% glycerol, 10% PBS, 0.2% n-propyl gallate) and mounted. For EdU stainings, larvae were dissected in serum-free medium (EX-CELL 420, Sigma), and cleaned anterior halves were labeled for 1 h with EdU and processed using the Click-IT Edu Alexa Fluor 55 Imaging Kit (Life Technologies) according to the manufacturer’s instructions. Microscopy was performed using a LSM 700 confocal microscope (Zeiss).

Genotypes—For the genotypes, see the following. Fig. 1, A–C, ptc-GAL4,UAS-GFP/+. Fig. 1, D–F, ptc-GAL4,UAS-GFP/+; tub-GAL80^ts/UAS-Slik. Fig. 1, G–I, ap-GAL4,UAS-GFP/slik^{EP20348}. Fig. 1, J–L, ptc-GAL4,UAS-GFP/+; tub-GAL80^ts/UAS-Slik^{kd}. Fig. 2, C and D, UAS-Dcr/+; ptc-GAL4,UAS-GFP/UAS-slik^{sUTR}dsRNA. Fig. 2E, UAS-Dcr/+; ptc-GAL4,UAS-GFP/UAS-slik^{sUTR}dsRNA;UAS-Slik^{kd}/+. Fig. 2F, UAS-Dcr/+; ptc-GAL4,UAS-GFP/UAS-slik^{sUTR}dsRNA;UAS-Slik^{kd}/+. Fig. 3, A and K, ptc-GAL4,UAS-GFP. Fig. 3B, ptc-GAL4,UAS-GFP/+; tub-GAL80^ts/UAS-Slik. Fig. 3C, ptc-GAL4,UAS-GFP/UAS-Dp110. Fig. 3D, ptc-GAL4,UAS-GFP/+;UAS-dMyc/+; Fig. 3E, ptc-GAL4,UAS-GFP/+;UAS-Ras85DV12/+. Fig. 3F, ptc-GAL4,UAS-GFP/UAS-Dcr/+. Fig. 3G, ptc-GAL4,UAS-GFP/UAS-Dcr/+.Fig. 3H, ptc-GAL4,UAS-GFP/UAS-CycD1,UAS-cdk4/+. Fig. 3I, ptc-GAL4-UAS-GFP/UAS-E2F, UAS-DP/+. Fig. 3J, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+.Fig. 3K, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+.Fig. 3L, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+.Fig. 3M, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+.Fig. 3N, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+. Fig. 3O, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+.Fig. 3P, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+.Fig. 3Q, ptc-GAL4,UAS-GFP/slik^{EP3622}.. Fig. 3R, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+. Fig. 3S, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+. Fig. 3T, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+. Fig. 3U, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+. Fig. 3V, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+. Fig. 3W, ptc-GAL4,UAS-GFP/UAS-E2F, UAS-DP/+.
Protein Digestion—Gel pieces were washed with water for 5 min and destained twice with the destaining buffer (50 mM ammonium bicarbonate, acetonitrile) for 15 min. An extra destaining buffer (55 mM iodoacetamide, 100 mM ammonium bicarbonate) for 30 min at 40 °C and then alkylated by adding the alkylating agent (LC-MS/MS) analysis.

LC-MS/MS Analysis—Before LC-MS/MS, protein digests were re-solubilized under agitation for 15 min in 21 μl of 1% acetonitrile, 1% formic acid. The LC column was a PicoFrit silica capillary column (17 cm × 75 μm inner diameter; New Objective, Woburn, MA) self-packed with C-18 reverse-phase material (Jupiter 5 μm particles, 300 Å pore size; Phenomenex, Torrance, CA) using a high pressure packing cell. This column was installed on the Easy-nLC II system (Proxeon Biosystems, Odense, Denmark) and coupled to the LTQ Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany) equipped with a Proxeon nanoelectrospray Flex ion source. The LTQ Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany) was used for precursor ion selection, and the LTQ of ThermoFisher Scientific, Bremen, Germany) was used for MS/MS fragmentation. Peptides were selected for fragmentation with collision energy of 35 eV, and the mass range was set to 400-1,800 Da. The data-dependent scan events used a maximum ion fill time of 100 ms, and target ions already selected for MS/MS were dynamically excluded for 30 s after 2 repeat counts. Nanospray and S-lens voltages were set to 1.5 kV and 50 V, respectively. The normalized collision energy was used as 27 with an activation q of 0.25 and activation time of 10 ms. Capillary temperature was 250 °C.

Protein Identification—Protein database searches were performed using Mascot 2.3 (Matrix Science). The mass tolerance for precursor ions was set to 10 ppm and that for fragment ions to 0.5 Da. The enzyme specified was trypsin, and two missed cleavages were allowed. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation, serine, threonine, and tyrosine phosphorylation were specified as variable modifications.

Results

The imaginal discs in Drosophila are a series of epithelial sacs that give rise to the adult appendages and body wall. Initially formed from invaginations of up to 50 cells in the embryonic epidermis, the discs grow rapidly during the 4 days of larval life to reach up to ~50,000 cells. The discs are divided into two continuous but morphologically distinct epithelial monolayers with their apical domains apposed and enclosing a central lumen. On one side of the lumen is the disc proper (DP) composed of pseudostratified columnar epithelial cells that will give rise to the adult appendages and body wall. Initially, each disc is a small mass of cells located at the distal end of the larva, and each disc is subdivided into a central stripe of DP cells marked by expression of Slik in a stripe of cells (based on expression of GFP) (Fig. 1A). This stripe of DP cells, as if these PM cells were highly proliferative, whereas PM cells are not (Fig. 1B). DAPI staining of nuclei reveals the tight packing of DP cells (Fig. 1C), whereas PM cells are more loosely packed (Fig. 1D). In Z-sections it was clear that this DP cell stripe is the peripodial membrane (PM), made up of large flattened squamous cells with distinctly spaced nuclei (Fig. 1E). This DP cell stripe is the peripodial membrane (PM), made up of large flattened squamous cells with distinctly spaced nuclei (Fig. 1E). This DP cell stripe is the peripodial membrane (PM), made up of large flattened squamous cells with distinctly spaced nuclei (Fig. 1E). This DP cell stripe is the peripodial membrane (PM), made up of large flattened squamous cells with distinctly spaced nuclei (Fig. 1E). This DP cell stripe is the peripodial membrane (PM), made up of large flattened squamous cells with distinctly spaced nuclei (Fig. 1E). This DP cell stripe is the peripodial membrane (PM), made up of large flattened squamous cells with distinctly spaced nuclei (Fig. 1E). 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similar dense cluster of overproliferating PM cells overlying the dorsal compartment (Fig. 1, G–I).

As we previously reported (21), the same non-autonomous effect was observed when expressing a form of Slik (Slik54d) with the critical Asp176 residue involved in binding the catalytic magnesium ion (Asp176) mutated to Asn, which is expected to disrupt catalytic activity (Fig. 1, J–L). To confirm that this pro-proliferative effect is independent of Slik kinase activity, we tested the catalytic activity of Slik54d in cells and in vitro using Moesin phosphorylation as a readout (14). Treatment of S2 cells with a dsRNA targeting the 5′-untranslated region (UTR) efficiently depleted Slik protein and led to a strong reduction of Moesin Thr556 phosphorylation in the cells (detected using a phosphospecific antiserum) (Fig. 2A). Transfection of a wild-type slik transgene lacking the 5′-UTR into slik 5′-UTR dsRNA-treated cells restored Moesin phosphorylation, whereas the comparable slik54d mutant did not (Fig. 2A). In in vitro kinase assays using [γ-32P]ATP and kinase immunoprecipitated from transfected S2 cells, wild-type Slik phosphorylated a truncated form of Moesin consisting of the C-terminal actin-binding domain (Moe.CT) (Fig. 2B). Phosphorylation of Moe.CT was abolished by mutation of the critical regulatory Thr556 (in Moesin) to Ala, confirming that Slik specifically phosphorylates this residue (Fig. 2B). In the same assay, Slik54d did not show any activity (Fig. 2B). We conclude that Slik54d does indeed lack catalytic activity.

Catalytically inactive mutant forms of some kinases can dimerize with and activate wild-type kinases; for example, catalytically inactive BRAF can activate CRAF in heterodimers (30). To rule out the possibility that Slik54d promotes proliferation by activating the wild-type kinase through dimerization, we expressed it in wing discs depleted of endogenous Slik. ptc-GAL4-driven expression of a slik 5′-UTR dsRNA transgene efficiently depleted endogenous Slik (Fig. 2C) and did not affect PM cell proliferation (Fig. 2D). In this background, reintroduction of Slik54d had a similar effect as wild-type Slik in promoting non-autonomous proliferation of PM cells (Fig. 2E and F). Taken together these results confirm that the ability of Slik to drive proliferation does not require catalytic activity.

Slik Specifically Stimulates Non-autonomous Proliferation—Slik expression has pleiotropic effects in discs, accelerating cell proliferation rates and tissue growth while also increasing apoptosis (21). Altering these processes can have compensatory non-autonomous effects on tissue growth (31, 32). To see if the non-autonomous pro-proliferative effects of Slik could be an indirect consequence of its effect on growth and cell survival, we tested whether expression of other genes that affect these processes in DP cells could have a similar effect on PM morphology. Genes whose expression accelerates primarily cell growth, such as the phosphatidylinositol 3-kinase catalytic subunit Dp110 (33), Myc (34), and activated Ras85D (35) had strong cell autonomous effects in DP cells (as evidenced by an obvious increase in space between nuclei within the expression domain) but did not lead to the appearance of a cluster of PM cell nuclei as did Slik (Fig. 3, A–E). CyclinD and Cdk4, which
Regulation of Slik by Activation Segment Phosphorylation

Figure 3. Non-autonomous proliferation is a specific effect of Slik signaling. Confocal optical sections of discs expressing GFP (green) together with the indicated transgenes affecting cell growth, proliferation, and/or survival using ptc-GAL4 (unless otherwise noted). Discs were stained with DAPI (red) to reveal nuclei and in some cases with anti-activated Caspase 3 to visualize apoptotic cells. Images were taken at the level of DP (top) and PM (bottom) cells. A, GFP-expressing disc, showing normal PM morphology. B, Slik-expressing disc, showing ectopic cluster of PM cells. C–F, expression in DP cells of genes that promote cell growth (Dp110 (C), dMyc (D), Ras85D\textsuperscript{V12} (expressed with ap-GAL4), which drives expression in the dorsal disc; E), or CyclinD plus Cdk4 (F) did not alter the appearance of the PM layer, G–I, expression in DP cells of genes that promote cell proliferation (E2F + DP (G), bantam miRNA (H), or a dominant negative form of Fat (I)) did not alter the appearance of the PM layer, J–L, expression in DP cells of genes that promote apoptosis (TNF ligand Eiger (J) or Hid (K)) did not alter the appearance of the PM layer, K, control ptc-GFP disc, showing the normal low level of apoptosis and appearance of the PM layer.

Together promote cell growth in Drosophila [36], also did not noticeably alter PM morphology (Fig. 3F). Accelerating cell division rates by co-expressing the cell cycle regulator E2F and its co-factor DP [37] led to the expected autonomous reduction in DP cell size (and hence decreased nuclear spacing) without affecting PM morphology (Fig. 3G). Manipulations that coordinately accelerate cell proliferation and tissue growth, such as expression of the miRNA bantam [38] or expression of a dominant negative form of the tumor suppressor Fat (Fat\textsuperscript{Acic}) [39], induced robust overgrowth in adult wings (data not shown) but did not noticeably alter PM morphology (Fig. 3, H–J). Finally, induction of apoptosis by expressing either the tumor necrosis factor ligand Eiger (40) or the pro-apoptotic protein Hid (41), which had obvious effects on DP morphology (Eiger) and DP cell survival (Hid), did not noticeably alter PM morphology (Fig. 3, J–L). The fact that each of these regulators produced robust cell autonomous effects in DP cells without affecting PM morphology strongly suggests that the non-autonomous effect of Slik on cell proliferation is the result of Slik signaling rather than an indirect consequence of altered proliferation or apoptosis.

Slik-driven Tissue Growth, but Not Moesin Phosphorylation, Depends on Proper Localization—In immunostainings, Slik is diffusely localized throughout imaginal disc cells and is concentrated apically, where the majority of P-Moe staining is observed (21). To see which of the three domains of Slik (kinase, NCD, or CCD) (Fig. 4A) might mediate this localization, we generated and expressed transgenes encoding the domains either individually or in various combinations. A Myc-tagged full-length form of Slik recapitulated the normal localization pattern (Fig. 4, B and C). We previously reported that expression of just the kinase domain (Slik\textsuperscript{kin}) caused a redistribution of P-Moe away from the apical domain in wing disc cells (21), implying that localization of the kinase was affected in the absence of the linker and/or CCD. In fact, Slik\textsuperscript{kin} accumulated primarily in the nucleus of imaginal disc cells, as did a form of Slik lacking just the CCD (Slik\textsuperscript{ACC}) (Fig. 4, D and E). Conversely, the CCD of Slik was sufficient for apical localization (Fig. 4F). The comparable domain of mammalian Lok mediates apical microvilli localization of the kinase in cultured cells, suggesting it is a conserved localization domain (16).

Domains outside of the kinase domain of some protein kinases are responsible for their non-catalytic activities (6). For example, the regulatory domain of c-Raf alone is sufficient to mediate c-Raf-dependent inhibition of Mst2 (42). Therefore, we hypothesized that the CCD might not only localize Slik in cells but also directly mediate its growth-promoting activity. To test this, we examined the requirement for the CCD in Slik function. The CCD was dispensable for Moesin phosphorylation in S2 cells (Fig. 4G), consistent with the in vivo effects of Slik\textsuperscript{kin} expression on P-Moe re-distribution referred to above (21). In contrast, removal of the CCD abrogated the effect of Slik on proliferation (Fig. 4, H–J). Although required, the CCD alone was not sufficient to stimulate proliferation (Fig. 4K). Together these results imply that multiple regions of the protein are required for non-catalytic signaling. We conclude that the CCD is important for localizing Slik within cells, restricting Moesin phosphorylation to the appropriate apical site and positioning the protein to activate pro-proliferative signaling.

Moesin activation occurs at sites of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P\textsubscript{2}) accumulation, as its phosphorylation is dependent upon prior binding to PI(4,5)P\textsubscript{2} (43). To see if this might also be involved in membrane recruitment of Slik, we tested the ability of the CCD to bind to phosphoinositides. In pulldown assays of cell lysates from S2 cells expressing Slik\textsuperscript{ACC}, we did not detect interaction of the CCD with any phosphoinositides (Fig. 4L). Pulldown of the PI(4,5)P\textsubscript{2} binding PH domain of PLC-\delta from S2 cell lysates using PI(4,5)P\textsubscript{2}-coupled beads confirmed that the assay was working (Fig. 4L). We conclude that apical localization of Slik likely does not involve direct interaction of the CCD with membrane phosphoinositides.

Regulation of Slik Catalytic Activity by Phosphorylation—Both in wing discs and in S2 cells, Slik is a phosphoprotein, as evidenced by an increase in mobility in SDS-PAGE gels after treatment of lysates with \lambda- phosphatase (Fig. 5A). At least some of this is likely attributable to autophosphorylation, as Slik is capable of phosphorylating itself in vitro kinase assays (Fig. 5B). To see if Slik may also be a substrate of other kinases, we compared the phosphorylation status of transfected Slik and Slik\textsuperscript{kd} in S2 cells depleted of endogenous Slik. As expected, transfected Slik shifted in response to \lambda- phosphatase treatment (Fig. 5C). Interestingly, we observed a very similar shift of Slik\textsuperscript{kd}
in response to λ-phosphatase treatment (Fig. 5C). This suggests that Slik is also phosphorylated and potentially regulated by other kinases.

We used a LC-MS/MS approach to map phosphorylated residues in Slik. We carried out this analysis on Slik protein isolated from transfected S2 cells and on endogenous Slik isolated from third instar larvae. All 19 of the phosphosites we identified (Table 1) mapped to either the kinase domain or NCD, with 15 of them observed in both S2 cells and embryos (Fig. 5D). Two clusters of phosphorylation sites, one in the activation segment (Thr186, Thr192, Thr196) and one just beyond the C-terminal clusters of phosphorylation sites, one in the activation segment inositides was detected. As expected, PLC

Phosphorylation at one or more residues from both the small (N-) and large (C-) lobes, properly aligned. This is generally required for efficient catalysis (for review, see Refs. 45 and 46). Ste20 kinases are typical in this respect, with activation segment phosphorylation being crucial for the activity of several (47–50).

Phosphorylation at three Ser/Thr residues in the activation segments of Slk and Lok has been observed numerous times (≥48 occurrences at each site for the two proteins combined) in independent LC-MS/MS analyses (44). Phosphorylation at two of these sites, Thr186 and Ser189, of human Slk (corresponding to Thr186 and Thr189 in Slk), was observed in a crystal structure of the kinase domain of this protein (51), and mutational analysis indicated that both phospho-residues are important for activation of its catalytic activity toward an exogenous substrate (52). Mutation of the third site, Thr193 (corresponding to Thr196 in Slik), impaired catalytic activity of the isolated kinase domain of Slk (52). Consistent with this, we found that mutating both Thr186 and Thr192 in Slk to Ala (SlkT186A/T192A) strongly impaired its ability to rescue Moesin phosphorylation in endogenous Slk-depleted cells (Fig. 6A). Phosphorylation at either site was sufficient for at least some activity, as both single mutants either fully (SlkT186A) or partially (SlkT192A) rescued Moesin phosphorylation (Fig. 6A). We observed the same trend in in vitro kinase assays (Fig. 6B). In contrast, mutation of the single site Thr196 to Ala (SlkT196A) strongly impaired catalytic activity both in cells and in vitro (Fig. 6A and B).

To carry out similar tests in vivo, we generated transgenic flies for expressing the wild-type and mutant forms of Slik. All
Regulation of Slik by Activation Segment Phosphorylation

**FIGURE 5.** Slik is a phosphoprotein in vivo. Western blot of lysates from S2 cells or third-instar wing discs treated without (−) or with (+) λ-phosphatase, probed with anti-Slik antibody, reveals that Slik is a phosphoprotein in cells and in tissues. **A**, in vitro kinase assay using Slik or Slik kd immunoprecipitated from S2 cells. **B**, Western blot of lysates from S2 cells treated with empty vector (−) or with (+) λ phosphatase. **C**, summary of phosphorylation site mapping by LC-MS/MS. Sites identified in Slik isolated from S2 cells, sites in Slik from wing discs, and sites in green were identified in both. Sites identified more than five times in the Phosphosite database for mammalian Slik are indicated below.

transgenes were recombined into the same site in the genome using the φC31-based integration system (29) to ensure equal mRNA expression. We used nud-GAL4 to drive expression of the slik 5’-UTR RNAi transgene, which efficiently depleted endogenous Slik protein and caused a strong reduction of Moesin phosphorylation specifically throughout the wing pouch of developing wing discs, as compared with controls (Fig. 6, C and D). Co-expression of wild-type Slik increased Moesin phosphorylation to well above endogenous levels (Fig. 6E). Co-expression of either Slik T186A/T192A or Slik T196A partially rescued Moesin phosphorylation but only to about endogenous levels (Fig. 6, F and G), confirming that both mutants have impaired activity. Taken together, our results suggest that phosphorylation of Slik at Thr196 and either Thr186 or Thr192 is required for efficient phosphorylation of its substrates. We were unable to detect any difference in the subcellular distribution of the activation segment mutants versus wild-type Slik (Fig. 6, E–G, insets), suggesting that apical localization occurs independently of activation.

The Non-catalytic Function of Slik in Growth Control Is Dependent upon Kinase Activation—Because Slik can promote tissue growth independently of substrate phosphorylation, it remained an open question whether this activity is regulated in any way. To test whether activation loop phosphorylation could play a role in this regard, we used the rescue assay to assess the ability of activation loop mutants of Slik to drive non-autonomous proliferation. Expression of wild-type Slik or Slik D176N with ptc-GAL4 caused fully penetrant lethality, with many animals dying at pupal stage. In contrast, Slik T186A/T192A and Slik T196A expression was much better tolerated, with some or all animals surviving to adult stage, respectively (data not shown). Interestingly, whereas expression of wild-type Slik or Slik D176N in endogenous Slik-depleted DP cells triggered the expected non-autonomous proliferation effect (Fig. 2, E and F), Slik T186A/T192A did so much more weakly and Slik T196A not at all (Fig. 7, A and B). Thus, although it does not require catalytic activity per se, the ability of Slik to induce non-autonomous proliferation in discs does require that the kinase be “activated” by activation segment phosphorylation, providing a level of regulation.

Slik has been linked to regulation of the tumor suppressor protein Mer/NF2 which, like Moesin, is a FERM domain-containing protein. Based on work in both flies and mammals, Mer/NF2 mediates its growth-suppressive effects by activating the Hippo (Hpo)/Mst pathway (53–56). Like Moesin, Mer/NF2 exists in active and inactive conformations, with the transition from active to inactive states driven by phosphorylation at a critical residue (Ser518 in human NF2) (57). Manipulating Slik activity altered Mer phosphorylation, which led to the suggestion that Slik promotes growth by phosphorylating and inhibiting Mer (58). This is hard to reconcile with the observation that Slik promotes tissue growth independently of its catalytic activity. Furthermore, the residue in Mer/NF2 corresponding to the critical Thr in Moesin that is phosphorylated by Slik (Thr576 in human NF2) does not appear to be involved in regulating its activity (59).

To see if inhibition of Mer by Slik could be sufficient to explain its effects on growth, we compared the phenotypic effects of expressing Slik or inhibiting Mer in discs. ap-GAL4-driven expression of Slik in dorsal DP cells induced robust non-autonomous proliferation of PM cells directly overlying the DP dorsal compartment (Fig. 8A). In contrast, under the same conditions neither expression of a dominant negative form of Mer (Mer A586V) (60) nor mer dsRNA had nonautonomous effects on PM cell proliferation (Fig. 8, B and C), although both induced robust overgrowth of the dorsal wing surface that caused the wings to curve downward (Fig. 8, D and E, and not shown). Taken together, our results suggest that Mer is not the main target of Slik in regulating tissue growth.

Discussion

The *Drosophila* Ste20 kinase Slik is one of a growing number of kinases with both catalytic activity-dependent and -independent functions (6). The non-catalytic functions of kinases are mediated in different ways, including scaffolding of protein...
Regulation of Slik by Activation Segment Phosphorylation

TABLE 1
Summary of Slik phosphopeptides identified by LC-MS/MS

| Phosphosite  | Peptide sequence | m/z   | Charge | Error | Mascot score |
|--------------|------------------|-------|--------|-------|--------------|
| Thr3        | LYNNIKMDyTPDAPFEMVQEGLDGAFKG | 1076.4779 | +3 | 3 | 43 |
| Thr46, Thr192 | NKKHTMQKHypTIGTPYWMAPELVCLCEFT | 936.41748 | +4 | 5 | 31 |
| Thr192, Thr196 | NKHTMQKHDPYIGTPYWMAPELVCLCEFT | 936.416500 | +4 | 4 | 29 |
| Ser212     | NSALQLDLDpLSLGQSDIDLpPTPTSLR | 1169.56800 | +3 | <1 | 84 |
| Ser212     | NSALQLDLDSSLpLSLGQSDIDLpPTPTSLR | 1169.56555 | +3 | 1 | 85 |
| Thr212     | NSALQLDLDSSLQpSDIDLpPTPTSLR | 1169.565824 | +3 | <1 | 87 |
| Thr212     | ATPyTPDPPHTKDDNAAAQppHHTK | 579.067020 | +5 | 1 | 25 |
| Thr31      | VPAPAlPSQQTPQPPpVQpppTPPApQTAAVLPQK | 1177.604860 | +3 | <1 | 30 |
| Ser212     | GKAPpPSpSLGpLpAAPpASpQTPSP | 666.838620 | +4 | 2 | 49 |
| Ser349     | PAASpDSQpSPK | 584.748050 | +2 | 1 | 41 |
| Ser349     | STTHpHEpSSTDpFpSLSLDgLGTpTppHK | 801.343260 | +4 | 1 | 64 |
| Thr341     | STTHpHEpSSTDpFpSLSLDgLGTpTppHK | 801.343510 | +4 | 1 | 66 |
| Ser341     | STTHpHSEPSTDpFpSLSLDgLGTpTppHK | 821.338260 | +4 | 5 | 62 |
| Thr341     | pSDpSSLpLENSGpS | 707.796810 | +2 | 1 | 72 |
| Ser341     | FKpQKpSPpDSLpLENSGpS | 771.008970 | +3 | <1 | 37 |
| Ser341     | SPpDSLpLENSGpS | 707.795590 | +2 | <1 | 82 |

FIGURE 6. Silk catalytic activity is regulated by activation segment phosphorylation. A, “in-cell” assay of Silk catalytic activity. B, in vitro kinase assay of Silk activity using the C-terminal activation binding domain of Moesin fused to GST (GST-Moe.CT) as a substrate. Silk186A was fully active, and the activity of Silk192A, Silk186A,192A, and Silk196A was strongly impaired. C, confocal images of wing discs in which transgenes were expressed throughout the wing pouch using the nub-GAL4 driver and immunostained with antibodies against Silk (green) and phospho-Moessin (red). Silk and phospho-Moessin are normally ubiquitously throughout the discs, as seen in control discs with nub-GAL4-driven expression of GFP (blue) (C). nub-GAL4-driven expression of a dsRNA transgene targeting the 5′-UTR of silk efficiently depleted Silk protein and led to a strong reduction in Moesin phosphorylation (E). Although they retained some activity, Silk186A,192A and Silk196A were substantially less active (F and G). Insets in E–G show magnified views of Silk staining at a fold in the disc, where strong Silk accumulation in the apical region of cells on either side of the fold, optically sectioned longitudinally (arrowheads), is clearly visible for all three transgenic Silk variants.

complexes, allosteric regulation of other proteins, and competition for binding partners. We were interested to know whether the regulatory mechanisms that control Silk catalytic activity, which is required for phosphorylation of the substrates Moesin and thus epithelial tissue integrity in vivo, also control its non-catalytic growth-promoting function. Our results indicate that activation segment phosphorylation, which typically stabilizes kinases in their active conformation, activates the catalytic activity of Silk and is also important for its non-catalytic function. In contrast, localization of the kinase via its conserved CCD is not required for catalytic activity but is essential for Silk to drive cell proliferation.

Analysis of the crystal structures of many protein kinase domains in the phosphorylated active and non-phosphorylated inactive states has revealed how activation segment phosphorylation affects kinase conformation and thus catalytic activity (45, 46). In the structures of protein kinase A (PKA) and others, the primary activation segment phosphate group (on Thr196 in PKA) stabilizes the active conformation by making electrostatic contacts with several conserved motifs in the kinase core. Some kinases also contain a secondary phosphorylation site, whose contacts with several conserved motifs in the kinase core. Some kinases also contain a secondary phosphorylation site, whose main function is less clear. Of the three activation segment residues we identified, Thr192 in Silk aligns most closely with Thr196 in PKA and is conserved (Ser or Thr) in all Ste20 kinases. In most of these proteins, this residue is the primary regulatory phosphorylation site in the activation loop (2). For some, this residue is autophosphorylated (e.g. Pak2 (61), hMink (62), Silk

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Regulation of Slik by Activation Segment Phosphorylation

A–C, confocal images of the PM layer of wing discs expressing Slik (A), a dominant negative form of Mer (Mer8888), (B), or a mer dsRNA transgene (C) in the DP layer using ap-GAL4, labeled with EdU. Only Slik induced non-autonomous proliferation of PM cells. D and E, pictures of adult flies in which GFP (as a control) (D) or Mer8888 (E) was expressed using ap-GAL4. Mer8888 expression in the dorsal wing disc caused robust overgrowth of the dorsal surface of the wing blade, as evidenced by the severe downward curvature.

FIGURE 8. Merlin is not the main target of Slik in regulating tissue growth. (51)). For others, such as Osr1 and Spak (50), Hippo (27), Pak1 (49), and Hpk1 (48), the site can also be trans-phosphorylated, providing a point of intersection with other signaling pathways. The residue corresponding to Thr196 is less well conserved, being present in Slk, Lok, and Hippo/Mst kinases but not in the PAK kinases or Osr1 and Spak. In those kinases where it is present, this site appears to function as a secondary phosphorylation site (2, 46). Our results with Slik fit this model, as mutation of Thr192 alone had more of an effect on catalytic activity than Thr186. Phosphorylation of at least one of these residues is important for efficient catalysis, as the double mutant had low activity. Both are likely autophosphorylated, as phosphate groups were observed at the equivalent sites in bacterially expressed recombinant Slk and Lok kinase domains (51). However, our genetic analyses suggest that at least one of these sites is likely also trans-phosphorylated (see below).

In addition to these two well defined sites, Slik activity was strongly impaired by mutation of Thr196. This residue is situated at a hinge point near the start of the “p + 1” loop region of the activation segment, so-called because several residues in this region make contact with the p + 1 residue in the substrate. The equivalent residue in other kinases also makes contact with conserved residues in the catalytic loop (46), meaning that modification of this residue could influence both the active structure and interaction with substrates. This Thr residue is invariant in all Ste20 kinases (2), and phosphorylation at this site appears to be fairly common (based on analysis of the Phosphosite MS/MS database (44)), especially for certain kinases (e.g. Slk and Lok, Spak and Osr1, and Ysk/Stk25). In several Ste20 kinases, including Slk, Spak, and Mst1, Ala substitution at this site impairs substrate phosphorylation (52, 63). Thus the role of phosphorylation at this site in regulating kinase function is conserved both between at least some Ste20 kinase family members and between species. The mechanism remains to be determined.

Although there are few cases where the involvement of activation segment phosphorylation in regulating catalytic activity-independent activities of kinases has been assessed, there is evidence that it can either be required (as in activation of topo-IIα and Parp-1 by ERKs (64, 65)) or can cause a switch from catalytic activity-independent to -dependent signaling (for yeast Kss1 (66)). We found that the catalytic activity-independent growth promoting function of Slik shows a similar dependence on activation loop phosphorylation as catalytic activity; Thr190 and either Thr186 or Thr192 need to be phosphorylated for an efficient proliferative response. Although Slik can auto-phosphorylate, it is likely also trans-phosphorylated by one or more other kinases, as the wild-type and catalytically inactive proteins were phosphorylated to a similar extent in endogenous Slik-depleted cells. Interestingly, the catalytically inactive Slikkd mutant protein cannot autophosphorylate but is capable of promoting cell proliferation even when expressed in cells depleted of endogenous Slik. This suggests that the activation segment of Slik can be phosphorylated by another kinase to activate non-catalytic pro-proliferative signaling.

We identified 16 phosphorylation sites outside of the activation segment of Slik in our MS/MS analyses. The majority of these were in the non-conserved central domain, and their relevance is unclear. One cluster of sites just beyond the C-terminal limit of the kinase domain (Ser340, Ser342, Ser345, and Ser354) appears to be conserved in spacing, if not in actual primary sequence, with phosphorylation sites in mammalian Slk and Lok. Interestingly, two of the corresponding sites in mammalian Slk (Ser347 and Ser348) mediate negative regulation by casein kinase II in response to v-Src activity (67), suggesting the possibility that these may be conserved trans-regulatory phosphorylation sites.

Consistent with studies of mammalian Slk (16), we found that the coiled-coil-containing CCD of Slik mediates apical localization of the protein in epithelial cells of the imaginal disc. Although a model involving membrane recruitment of Slik by PI(4,5)P2 would have fit well with ERM protein biology, we did not detect any interaction of the CCD with phosphoinositol lipids. Interestingly, the CCD also appears to be involved in regulated membrane recruitment of Slk in non-polarized mouse embryonic fibroblasts through interactions with the LIM domain transcription cofactors Ldb1 and Ldb2 (68). Although we found that it determines where in cells Moesin gets activated, the CCD is not required for phosphorylation per se. In fact, previous work suggests that the Slk CCD acts as an autoinhibitory domain (68), something that we have not tested.
Regulation of Slik by Activation Segment Phosphorylation

In contrast, the CCD is absolutely required for activation of pro-proliferative signaling. Although non-catalytic activities of many kinases are mediated by parts of the protein outside of the kinase domain, the CCD alone is not capable of stimulating proliferation. Our data are more consistent with a model in which localization via the CCD instead brings Slik into the vicinity of other proteins required for signaling, and activation loop phosphorylation places Slik in a conformation that enables it to interact with these proteins to initiate the signal.

We do not know how Slik promotes cell proliferation in the wing disc. Although Slik in some way influences the phosphorylation of Mer, the importance of that for the Slik-driven growth phenotypes is not clear for two reasons. First, Slik-driven growth does not require catalytic activity. Second, although it causes the expected tissue overgrowth response, direct inhibition of Mer activity in wing discs does not induce non-autonomous proliferation, which is a characteristic effect of Slik. Thus, although Mer regulation may contribute to the growth effects of Slik, it does not appear to be the main target. The direct target of Slik in growth signaling remains to be identified.

Author Contributions—V. P. was involved in the design of and performed and analyzed experiments shown in Figs. 1, 2, 5, 6, and 7. A. N. and N. D. designed, performed, and analyzed experiments shown in Fig. 8. F. S. performed and analyzed experiments shown in Figs. 4G, 5A, and 5D. A. P. performed and analyzed the experiment shown in Fig. 2B. D. M. was involved in the design of experiments in Figs. 2 and 5. K. O. performed and analyzed experiments shown in Fig. 4L. D. R. H. conceived of the study, was involved in the design of all experiments, and performed and analyzed experiments shown in Figs. 1, 3, 4, and 6.

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References
1. Shaw, A. S., Kornev, A. P., Hu, J., Ahuja, L. G., and Taylor, S. S. (2014) Kinases and pseudokinases: lessons from RAF. Mol. Cell. Biol. 34, 1538–1546
2. Delpere, E. (2009) The mammalian family of sterile 20p-like protein kinases. Pflugers Arch. 458, 953–967
3. Kumar, R., Gurusuraj, A. E., and Barnes, C. J. (2006) p21-activated kinases in cancer. Nat. Rev. Cancer 6, 459–471
4. Avruch, J., Zhou, D., Fitamant, J., Bardeesy, N., Mou, F., and Barrufet, L. R. (2012) Protein kinases of the Hippo pathway: regulation and substrates. Semin. Cell Dev. Biol. 23, 770–784
5. Gagnon, K. B., and Delpere, E. (2012) Molecular physiology of SPAK and OSMR: two Ste20-related protein kinases regulating ion transport. Physiol. Rev. 92, 1577–1617
6. Rauch, J., Volinsky, N., Romano, D., and Kolch, W. (2011) The secret life of kinases: functions beyond catalysis. Cell Commun. Signal. 9, 23
7. O’Reilly, P. G., Wagner, S., Franks, D. J., Cailliau, K., Browaeys, E., Dissous, C., and Sabourin, L. A. (2005) The Ste20-like kinase SLK is required for cell cycle progression through G2. J. Biol. Chem. 280, 42383–42390
8. Hao, W., Takano, T., Guillemette, J., Papillon, J., Ren, G., and Cybulsky, A. V. (2006) Induction of apoptosis by the Ste20-like kinase SLK, a germinal center kinase that activates apoptosis signal-regulating kinase and p38. J. Biol. Chem. 281, 3075–3084
9. Sabourin, L. A., and Rudnicki, M. A. (1999) Induction of apoptosis by SLK, a Ste20-related kinase. Oncogene 18, 7566–7575
10. Burakov, A. V., Zhapparova, O. N., Kovalenko, O. V., Zinovkina, L. A., Potekhina, E. S., Shanina, N. A., Weiss, D. G., Kuznetsov, S. A., and Nadezhina, E. S. (2008) Ste20-related protein kinase LOSK (SLK) controls microtubule radial array in interphase. Mol. Biol. Cell 19, 1952–1961
11. Quizi, I. L., Baron, K., Al-Zahrani, K. N., O’Reilly, P., Sriman, R. K., Conway, J., Laurin, A. A., and Sabourin, L. A. (2013) SLK-mediated phosphorylation of paxillin is required for focal adhesion turnover and cell migration. Oncogene 32, 4666–4663
12. Wagner, S., Flood, T. A., O’Reilly, P., Hume, K., and Sabourin, L. A. (2002) Association of the Ste20-like kinase (SLK) with the microtubule: role in Rac1-mediated regulation of actin dynamics during cell adhesion and spreading. J. Biol. Chem. 277, 37685–37692
13. Fehon, R. G., McClatchey, A. I., and Bretscher, A. (2010) Organizing the cell cortex: the role of ERM proteins. Nat. Rev. Mol. Cell Biol. 11, 276–287
14. Hipfner, D. R., Keller, N., and Cohen, S. M. (2004) Slik Sterile-20 kinase regulates Moesin activity to promote epithelial integrity during tissue growth. Genes Dev. 18, 2234–2248
15. Machicoane, M., de Frutos, C. A., Fink, J., Rocancourt, M., Lombardi, Y., Garel, S., Piel, M., and Echard, A. (2014) SLK-dependent activation of ERM controls LGN-NuMA localization and spindle orientation. J. Cell Biol. 205, 791–799
16. Viswanatha, R., Ohouo, P. Y., Smolka, M. B., and Bretscher, A. (2012) Local phosphocrosslinking mediated by LOK/SLK restricts ezrin function to the apical aspect of epithelial cells. J. Cell Biol. 199, 969–984
17. Speck, O., Hughes, S. C., Noren, N. K., Kulikauksas, R. M., and Fehon, R. G. (2003) Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. Nature 421, 83–87
18. Karagiosis, S., and Ready, D. F. (2004) Moesin contributes an essential structural role in Drosophila photoreceptor morphogenesis. Development 131, 725–732
19. Carreno, S., Kouranti, I., Glusman, E. S., Fuller, M. T., Echard, A., and Payre, F. (2008) Moesin and its activating kinase Slik are required for cortical stability and microtubule organization in mitotic cells. J. Cell Biol. 180, 739–746
20. Kunda, P., Pelling, A. E., Liu, T., and Baum, B. (2008) Moesin controls cortical rigidity, cell rounding, and spindle morphogenesis during mitosis. Curr. Biol. 18, 91–101
21. Carreno, S., Kouranti, I., Glusman, E. S., Fuller, M. T., Echard, A., and Payre, F. (2008) Moesin and its activating kinase Slik are required for cortical stability and microtubule organization in mitotic cells. J. Cell Biol. 180, 739–746
22. Harvey, K. F., Pfleger, C. M., and Harrian, I. K. (2003) The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell 114, 457–467
23. Pantalacci, S., Tapon, N., and Léopold, P. (2003) The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat. Cell Biol. 5, 921–927
24. Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C., and Halder, G. (2003) Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. Nat. Cell Biol. 5, 914–920
25. Wu, S., Huang, J., Dong, J., and Pan, D. (2003) Hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salivador and warts. Cell 114, 445–456
26. Poon, C. L., Lin, J. I., Zhang, X., and Harvey, K. F. (2011) The sterile 20-like kinase Tao-1 controls tissue growth by regulating the Salvador-Warts-Hippo pathway. Dev. Cell 21, 896–906
27. Boggiano, J. C., Vanderzalm, P. J., and Fehon, R. G. (2011) Tao-1 phosphorylates Hippo/MSI kinases to regulate the Hippo-Salvador-Warts tumor suppressor pathway. Dev. Cell 21, 888–895
28. Teppas, U. (2012) The apical polarity protein network in Drosophila epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. Annu. Rev. Cell Dev. Biol. 28, 655–685
29. Bischof, J., Maeda, R. K., Hediger, M., Karch, F., and Basler, K. (2007) An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. U.S.A. 104, 3312–3317
Regulation of Slik by Activation Segment Phosphorylation

30. Heidorn, S. J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomn, N., Hussain, J., Reis-Filho, J. S., Springer, C. I., Pritchard, C., and Marais, R. (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. Cell 140, 209–221

31. Johnston, L. A. (2009) Competitive interactions between cells: death, growth, and geography. Science 324, 1679–1682

32. Ryoo, H. D., Goren, T., and Steller, H. (2004) Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. Dev. Cell 7, 491–501

33. Leevers, S. J., Weinkove, D., MacDougall, L. K., Hafen, E., and Waterfield, M. D. (1996) The evolution of the eukaryotic protein kinases as dynamic molecular switches. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 351, 2267–2270

34. Fievet, B. T., Gautreau, A., Roy, C., Del Maestro, L., Mangeat, P., Louvard, D., Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Matakatsu, H., and Blair, S. S. (2006) Separating the adhesive and signaling functions of the Fat and Dachsous protocadherins. EMBO J. 25, 3551–3560

35. Igaki, T., guest editor. (2004) Regulation of Slik by Activation Segment Phosphorylation. J. Biol. Chem. 280, 2364–2383

36. King, C. C., Gardiner, E. M., Zenke, F. T., Bohl, B. P., Newton, A. C., Hemmings, B. A., and Bokoch, G. M. (2000) p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PKD1). J. Biol. Chem. 275, 41201–41209

37. Vitari, A. C., Deak, M., Morrice, N. A., and Alessi, D. R. (2005) The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. Biochem. J. 391, 17–24

38. Pike, A. C., Rellos, P., Niesen, F. H., Turnbull, A., Oliver, A. W., Parker, S. A., Turk, B. E., Pearl, L. H., and Knapp, S. (2008) Activation segment dimerization: a mechanism for kinase autophosphorylation of non-consensus sites. EMBO J. 27, 704–714

39. Luhovy, A. Y., Jaber, A., Papillon, J., Guillemette, J., and Cybulsky, A. V. (2012) Regulation of the Ste20-like kinase, SLK: involvement of activation segment phosphorylation. J. Biol. Chem. 287, 5446–5458

40. Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H., and Halder, G. (2006) The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat. Cell Biol. 8, 27–36

41. Zhang, N., Bai, H., David, K. D., Dong, J., Zheng, Y., Cai, J., Giovanni, M., Liu, P., Anders, R. A., and Pan, D. (2010) The Merlin/NF2 tumour suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Dev. Cell 19, 27–38

42. Yu, J., Zheng, Y., Dong, J., Klusza, S., Deng, W. M., and Pan, D. (2010) Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. Dev. Cell 18, 288–299

43. Yin, F., Yu, J., Zheng, Y., Chen, Q., Zhang, N., and Pan, D. (2013) Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. Cell 154, 1342–1355

44. Rong, R., Surace, E. I., Haidke, C. A., Gutmann, D. H., and Ye, K. (2004) Serine 518 phosphorylation modulates merlin intramolecular association and binding to critical effectors important for NF2 growth suppression. Oncogene 23, 8447–8454

45. Hughes, S. C., and Feon, R. G. (2006) Phosphorylation and activity of the tumor suppressor Merlin and the ERM protein Moesin are coordinately regulated by the Slik kinase. J. Cell Biol. 175, 305–313

46. Surace, E. I., Haidke, C. A., and Gutmann, D. H. (2004) Effect of merlin phosphorylation on neurofibromatosis 2 (NF2) gene function. Oncogene 23, 580–587

47. Lajeuennesse, D. R., McCartney, B. M., and Feon, R. G. (1998) Structural analysis of Drosophila merlin reveals functional domains important for growth control and subcellular localization. J. Cell Biol. 141, 1589–1599

48. Zhan, N., Huang, Z., Jakobi, R., Tuazon, P. T., Alnemri, E. S., Litwack, G., and Traugh, J. A. (1998) Cleavage and activation of p21-activated protein kinase y-PAK by CPP32 (caspase 3): effects of autophosphorylation on activity. J. Biol. Chem. 273, 28733–28739

49. Lim, J., Lennard, A., Sheppard, P. W., and Kellie, S. (2003) Identification of residues which regulate activity of the STE20-related kinase hMINK. Biochem. Biophys. Res. Commun. 300, 694–698

50. Gagnon, K. B., England, R., and Delpierre, E. (2006) Characterization of SPAK and OSR1, regulatory kinases of the Na–K–2Cl cotransporter. Mol. Biol. Cell. 17, 689–698

51. Shapiro, P. S., Whalen, A. M., Tolwinski, N. S., Wilsbacher, J., Froelich-Ammon, S. I., Garcia, M., Osberoff, N., and Ahn, N. G. (1999) Extracellular signal-regulated kinase activates topoisomerase I alpha through a mechanism independent of phosphorylation. Mol. Cell. Biol. 19, 3551–3560

52. Cohen-Armon, M., Visochek, L., Rzozensal, D., Kalal, A., Geistrikk, I., Klein, R., Bendetz-Nexer, S., Yao, Z., and Seger, P. (2007) DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation. Mol. Cell 25, 297–308

53. Madhani, H. D., Styles, C. A., and Fink, G. R. (1997) MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. Cell 91, 673–684

54. Chaar, Z., O’Reilly, P., Gelman, I., and Sabourin, L. A. (2006) v-Src-dependent down-regulation of the Ste20-like kinase SLK by casein kinase II. J. Biol. Chem. 281, 28193–28199

55. Storkbeck, C. J., Wagner, S., O’Reilly, P., McKay, M., Parks, R. J., Westphal, H., and Sabourin, L. A. (2009) The Ldb1 and Ldb2 transcriptional cofactors interact with the Ste20-like kinase SLK and regulate cell migration. Mol. Biol. Cell 20, 4174–4182