

Endophilin Regulates JNK Activation through Its Interaction with the Germinal Center Kinase-like Kinase*

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The endophilin family of proteins function in clathrin-mediated endocytosis. Here, we have identified and cloned the rat germinal center kinase-like kinase (rGLK), a member of the GCK (germinal center kinase) family of c-Jun N-terminal kinase (JNK) activating enzymes, as a novel endophilin I-binding partner. The interaction occurs both *in vitro* and in cells and is mediated by the Src homology 3 domain of endophilin I and a region of rGLK containing the endophilin consensus-binding sequence PPRPPPPR. Overlay analysis of rat brain extracts demonstrates that endophilin I is a major Src homology 3 domain-binding partner for rGLK. Overexpression of full-length endophilin I activates rGLK-mediated JNK activation, whereas N- and C-terminal fragments of endophilin I block JNK activation. Thus, endophilin I appears to have a novel function in JNK activation.

Src homology 3 (SH3)¹ domains are protein modules that bind to specific proline-rich sequences (1). SH3 domains were identified originally in proteins of signal transduction cascades, and they have also been found to mediate protein-protein interactions important for regulation of endocytosis (2). Recent studies have revealed biochemical and functional interactions between protein components of the regulatory machinery for endocytosis and those of signal transduction pathways (3). For example, both intersectin and amphiphysin II are SH3 domain-containing proteins that function in endocytosis and also bind to the Ras-activating enzyme mammalian son-of-sevenless

(mSos) (4, 5). Overexpression of the SH3 domains of intersectin blocks epidermal growth factor-mediated activation of Ras (5) and the extracellular signal-regulated kinase (Erk) MAP kinases (6, 7) demonstrating the functional significance of the intersectin-mSos interaction.

Another SH3 domain-containing protein that functions in clathrin-mediated endocytosis is endophilin (8–10). Three isoforms of endophilin have been identified: endophilin I, which is brain-specific; endophilin II, which is ubiquitously distributed; and endophilin III, which is expressed in brain and testis (8–13). Overexpression of the C-terminal SH3 domain of endophilin I-permeabilized mammalian cells (14) or in lamprey synapses (15, 16) blocks clathrin-coated vesicle formation. At its N terminus, endophilin I encodes a lysophosphatidic acid acyltransferase activity that has been suggested to regulate membrane curvature and hence clathrin-coated vesicle formation (17).

Through its SH3 domain, endophilin I has been reported to interact with a variety of partners including the endocytic proteins synaptojanin 1, dynamin 1, and the amphiphysins (8–10, 18), as well as the β 1-adrenergic receptor (19) and specific metalloprotease disintegrins (20). To better understand these and other interactions mediated by endophilin I, we screened a rat brain expression library with the endophilin I SH3 domain. We identified synaptojanin 1 and dynamin 1, and surprisingly, we also identified and cloned a rat homologue of the human germinal center kinase-like kinase (hGLK). hGLK was identified originally based on its homology to members of the germinal center kinase (GCK) family of protein kinases (21) and is a group I GCK (22). Group I GCKs are mitogen-activated protein kinase kinase kinase kinases (MAP4Ks) that function upstream of c-Jun N-terminal kinase (JNK) in a variety of cell types (22). Group I GCKs are composed of an N-terminal kinase domain and a C-terminal regulatory domain with multiple SH3 domain consensus-binding sites (22). The mechanisms by which events at the cell surface are linked to activation of GLK and other GCKs remain poorly understood. Moreover, the identity of SH3 domain-containing binding partners for GLK and the potential role for such proteins in regulation of GLK function have not been addressed.

The identification of rat GLK (rGLK) as an endophilin I-binding partner suggests a role for endophilin in the regulation of GLK function. To explore this role, we first confirmed the rGLK-endophilin I interaction in multiple systems *in vitro* and by co-immunoprecipitation analysis. Overlay assays of rat brain extracts revealed endophilin I as a major SH3 domain-binding partner for rGLK. Moreover, endophilin I was found to regulate GLK-mediated JNK activation. These data implicate endophilin I in the activation of JNK and provide evidence of a link between the endocytic machinery and the JNK signaling pathway.

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¹ The abbreviations used are: SH3, Src homology 3; Erk, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; GCK, germinal center kinase; GLK, germinal center kinase-like kinase; h, human; r, rat; GST, glutathione S-transferase; PCR, polymerase chain reaction; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence tag.

EXPERIMENTAL PROCEDURES

cDNA Expression Library Screen—An oligo(dT)-primed λ -ZAP II rat brain cDNA expression library, size-selected for clones greater than 4 kilobase pairs (Ref. 23; gift from Dr. Terry Snutch, University of British Columbia), was plated at 20,000 plaque-forming units/150-mm plate. Protein expression was induced using isopropyl- β -D-thiogalactopyranoside-soaked nitrocellulose filters that were then screened by overlay assays (24) using a GST fusion protein encoding the SH3 domain of endophilin I (9). Positive plaques were purified by two additional rounds of screening, and the cDNAs were isolated and identified by DNA sequence analysis. The longest clone encoding rGLK was completely sequenced on both strands.

Antibodies—Polyclonal antibodies against amphiphysin I/II (25) and endophilin I (1903) (9) were described previously. Monoclonal anti-FLAG M2 and anti-phospho-JNK (Thr¹⁸³-Tyr¹⁸⁵) antibodies were purchased from Sigma and New England Biolabs, respectively.

Generation of GST Fusion Protein Constructs—GST fusion proteins encoding full-length endophilin I or its isolated SH3 domain were described previously (9). GST fusion protein constructs of rGLK encoding various regions of the regulatory domain were generated using an rGLK cDNA clone as a template in PCR reactions. The reactions were performed with the forward primer 5'-GCGGGATCCCCCTGACGAGGTCTTTG (nucleotides 826–843) and the following reverse primers: GST-GLK-(276–541), 5'-GCGCCCGGGTCAGGCACAGTGGATTTC-AAG (nucleotides 1605–1623); GST-GLK-(276–498), 5'-GCGCCCGGGT-CAGTTTCGTGCCTCTCTGCTC (nucleotides 1477–1494); GST-GLK-(276–445), 5'-GCGCCCGGGTCACCTGATGAGGGACATC (nucleotides 1319–1335); GST-GLK-(276–406), 5'-GCGCCCGGGTCACAAGG-TTGAATGTTTAGAGTC (nucleotides 1198–1218). The resulting PCR products were subcloned into the corresponding *Bam*HI and *Sma*I sites of pGEX-2T (Amersham Pharmacia Biotech), and the resulting GST fusion proteins were expressed and purified using standard procedures.

Generation of Constructs for Mammalian Expression—Mammalian expression constructs for full-length endophilin I and endophilin I lacking the SH3 domain (delta SH3) were generated by digesting the corresponding GST fusion protein constructs (9) with *Bam*HI and *Eco*RI with the resulting inserts subcloned into the corresponding sites of pcDNA3 (Invitrogen). An N-terminal green fluorescent protein (GFP)-tagged mammalian expression construct for the SH3 domain of endophilin I was generated by PCR using the full-length cDNA (11) as a template, with the forward primer 5'-GCGAGATCTCTCAGCCAA-GAAGGGAATATC and the reverse primer 5'-GCGGAATTTCAAT-GGGGAGAGCAACCAG. The resulting PCR product was digested with *Bgl*II and *Eco*RI and subcloned into the corresponding sites of pEGFP-C2 (CLONTECH). To generate a construct encoding rGLK with an N-terminal FLAG tag, PCR was performed with the forward primer 5'-GCGAAGCTTGCCACCATTGACTACAAAGACGATGACGACAAA-CCGCAGGAGGACTTCG (nucleotides 34–49), which includes an initiation ATG codon within the context of a Kozak consensus sequence (26) and a FLAG epitope (DYKDDDDK), and the reverse primer 5'-GCGG-GATCCTCACTGTGTGACAAAGGGATG (nucleotides 808–825). The resulting PCR product was digested with *Hind*III and *Kpn*I and subcloned into the same sites at the 5'-end of the longest rGLK library clone in pBluescript. The resulting FLAG-tagged rGLK construct was then digested with *Hind*III and *Bam*HI sites, and the insert was subcloned into the same sites in pcDNA3. FLAG-tagged GCK (27) and JNK constructs were generous gifts from Dr. John Kyriakis (Massachusetts General Hospital) and Dr. Nathalie Lamarche-Vane (McGill University), respectively.

Binding Assays—For cultured cells, 10-cm² dishes of transfected HEK-293T cells were washed in phosphate-buffered saline (20 mM NaH₂PO₄, 0.9% NaCl, pH 7.4) and scraped into buffer A (20 mM HEPES-OH, pH 7.4, 150 mM NaCl, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μ g/ml aprotinin, and 0.5 μ g/ml leupeptin). The cells were sonicated and passed through a 25-gauge needle. Triton X-100 was added to a final concentration of 1%, and following incubation for 20 min at 4 °C the samples were centrifuged at 75,000 rpm in a Beckman TLA 100.1 rotor. Aliquots of the soluble supernatant were diluted to 2 mg of protein/ml in buffer A with 1% Triton X-100, and 1-ml samples were incubated overnight at 4 °C with ~25 μ g of GST fusion proteins prebound to glutathione-Sepharose. The bead samples were subsequently washed in buffer A with 1% Triton X-100, eluted with SDS-PAGE sample buffer, and prepared for Western blot analysis. For brain extracts, adult rat brains were homogenized in buffer B (20 mM HEPES-OH, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μ g/ml aprotinin, and 0.5 μ g/ml leupeptin) and centrifuged at 750 \times g_{max} for 15 min. The supernatant was

centrifuged at 45,000 rpm in a Sorval T-865 rotor for 1 h, and the soluble supernatant was diluted to 2 mg/ml in buffer B. Triton X-100 was added to a final concentration of 1%, and 1-ml aliquots were incubated o/n at 4 °C with ~25 μ g of GST fusion proteins prebound to glutathione-Sepharose. The beads were subsequently washed three times in 1 ml of buffer B with 1% Triton X-100, eluted with SDS-PAGE sample buffer, and prepared for Western blot analysis. Overlay assays were performed as described (24).

Immunoprecipitation Assays—Extracts from HEK-293T cells transfected with various cDNA constructs were prepared as described above and were precleared with either protein A-Sepharose or protein G-agarose beads. The precleared supernatants were then incubated with pre-immune serum or anti-endophilin I antibody (1903) coupled to protein A-Sepharose beads or with anti-FLAG antibody coupled to protein G-agarose beads. After 5 h at 4 °C, the beads were washed extensively in buffer A with 1% Triton X-100 and prepared for Western blotting analysis.

JNK Assays—HEK-293T cells were co-transfected with FLAG-tagged JNK and a variety of constructs as indicated in the figure legends. 48 h post-transfection, the medium was removed and the cells were scraped and boiled in sample buffer. The samples were separated on SDS-PAGE and used for Western blot analysis.

RESULTS

Identification and Cloning of Rat Germinal Center Kinase-like Kinase as an Endophilin I-binding Protein—To better understand the complete range of endophilin I functions, we sought to identify its full complement of SH3 domain-binding partners. We thus screened a rat brain expression library with a GST fusion protein encoding the SH3 domain of endophilin I. From a total of approximately 600,000 clones screened, 18 encoded potential endophilin I-binding proteins (data not shown). As expected, the majority of the clones (eight) encoded for synaptojanin 1. Only one clone was found to correspond to dynamin 1. Interestingly, three independent, non-amplified isolates were found to encode for a rat homologue of the human serine/threonine protein kinase, hGLK (21). It is particularly striking that more independent clones of rGLK were isolated than for dynamin I, which is an abundant brain protein and is a well established endophilin I-binding partner (9, 10). This result is consistent with a high affinity interaction between endophilin I and rGLK.

The longest rGLK clone isolated was sequenced on both strands to generate a coding sequence that aligned to hGLK, starting at amino acid 3 of its published sequence (21), and appeared to be complete at the C-terminal end. To obtain the extreme 5'-end, we searched the data base of expressed sequence tags (dbEST) with a sequence from near the 5'-end of our isolated clones. Four overlapping rat ESTs were identified leading to a complete rGLK coding sequence containing 11 additional amino acids (Fig. 1A). These amino acids, which extend the rGLK sequence 8 amino acids beyond the predicted start of hGLK, were homologous to other members of the GCK family (data not shown). The differences in the extreme N-terminal end of hGLK and rGLK may represent a species difference. However, multiple human EST clones were identified, which when aligned with the 5'-end of hGLK could define an N-terminal sequence identical to that for rGLK (data not shown).

Protein alignments revealed that the N-terminal kinase domain of rGLK is 99 and 72% identical to hGLK and hGCK, respectively (Fig. 1B). The C-terminal regulatory domain of rGLK, which includes a proline-rich region, is 95% identical with hGLK and 44% identical with hGCK (Fig. 1B).

rGLK Binds the SH3 Domain of Endophilin I via an Endophilin Consensus-binding Sequence—To confirm the endophilin I-rGLK interaction, we transfected HEK-293T cells with a construct encoding rGLK containing an N-terminal FLAG tag (FLAG-rGLK). The expressed protein bound strongly to a GST-endophilin I SH3 domain fusion protein (GST-SH3)

A

ATGACCACGGGGCTTCGACCTGTCGCCCGGAACCCGAGGAGGACTTCGAGCTGATCCAAACGCATAGGACGCGGCACCTACGCGGACGCTCTACAAGGCACGGAATGTCAACACTGGAGAA 120
 MNPGGFDFLSRRNPQEDFELIQIRIGSGTYGDVYKARNVNTGE
 TTAGCCGAATTAAGTGATAAAATGGAACAGGAGAGACTTCGCGGTGTACAGCAAGAAATCATTATGATGAAAGACTGCAACATGCAACATCGTTGCTTATTTGGAAGTTAT 240
 LAAAIKVIKLEPGEDFAVVQQEIIIMMKDKKHANIVAYFGSSY
 CTCAGGCAGATAAATTTGGATTGATGGAATTTTGGAGGTGGCTCTTACAGGACATCTATCATGTAACGGCCCTTTCAGAGCTGCAGATTGCATACGTTAGCAGGGAACA 360
 LRRDCKLWICMEFCGGGSLQDIYHVVTGPLSEELQIAYVVSRET
 CTCAGGGATTGATTACCTTCACAGTAAGGAAAAATGCAGAGATATAAGGGCGCTAACATCCTACTAACGGATAACGGTCATGTGAAATTAGCTGATTTGGAGTTTCCGCACAG 480
 LQGLYYLHSGKMKHRRDIKGANILLTDNGHVKLADFVGVSAAQ
 ATAACAGTACAATGCCAACGGAAGTCCTTATGTTGATACCCATATTTGGATGGCCAGAAAGTCGCGGTGTGAGCGGAAAGGAGGTACAATCAGCTGTGTGACCTCTGGGCTGTG 600
 ITATIAKRRKSFIGTPYWMMAPEVAAVERKGGYNQLCDLWAV
 GGCATCACTGCATCGAGCTGGCGAGCTCCAGCACCATGTTGACCTTACCCAATGAGGCGATTGTTCTAATGACAAAAGCAATTTCCAGCTCTCTAAATTAAGATAGTTG 720
 GITATIELEALQPPMFDLHPMRALFLMTKSNFQPPPKLKDKL
 AAGTGTCAAATAGTTTCCATCATTTTGTGAAGATGGCTCTTACAAAAATCAAAAAAAGACCCACTGCAGAAAAGCTGCTACAGCATCCCTTTGTACACAGCCTCTGACGAGGTCT 840
 KWSNSFHHFVVKMAALTKNPKKRPTAEKLLQHPPFVTQPLTR
 TTGGCAATCGAGTTGTCGACAGGTGAATATCCAGATATCCACATACCAGACTTTGACGATGATACCCAGAGCCTCTTGTGCTGTACACATAGAAATCTTCACTAGTAGG 960
 LAIEELLDKVNNPFDHSTYHDFDDDDPEPLVAVPHRIPSTSR
 AATGTGAGAGAAAGACACGCTCAGAGATAAACTTTGGTCAGGTAAAATTTGACCGCCCTTAAGGAAGGACAGAGCGCATGTAACCTGATCTACAATAGAGTACGGGCAA 1080
 NVREEKTRSEINFQKFDPLRKEETEPHHELDLQLEYGQ
 GGACACAAAGTAATATCTTTTAGGTGGAACAGAGTCTTCTGAAGTCTGTTGAAGAGGAGCTGCACAGCAGGACACGTGGCACACTTAGAAGATGACGAAGGGGACGATGACGAC 1200
 GHQSLGNGKSLKSVLEELHQRGHVAHLEDDGDDDD
 TCTAAACATTCAACCTTGAAGCAAGTCCACCTCTCTGCCACCAAGCTTAATCCATCTCCATACCCAGGATACACATCTTCTGAGGACAGTAATCAAGGCACATCAAGAGA 1320
 SKHSTLKLAKVPPPLPPPKPKKSIPIQDTHSSSEDSNQGTIKR
 TGTCCCTCATCAGGAGCCAGCAAGCCATCCACGTTCTCTCTAGACACACCCCGGCTACCCCGCAGAAAGCTGCTGTTTATGCAATGGGTGAGCTCTCTCAGCTCAAT 1440
 CPSSGSPAKPSHVPPRPPLPLPQKPAVLGNVGSFPQLN
 GGTGAACGGGAGCGGTGCTGCATCAGCAGAGAGTGAGCAGAGAGGACGAACCTCTCCGAAAAGAGAAAGGAGCTGCCAAGCAATAGTAATGGCCTTCCCAACACCTAA 1560
 GERDGSVHQQQSEQRGTNLSRKEKKDVPKPISNGLPPTPK
 GTGCATATGGGAGCATTTTTCAAGGTTTTTAATGGATGTCCTTGAATCCATCTGTCACATCTGGATAAATCCGGACACGAGAGACAGTACTTGTATTTGGCGCTGAAGAG 1680
 VHMGAACFSPKVFNGCPLKIHCATSWINPDTRDQYLIIFGAE
 GGCATTTATACCTTAACTCAATGAACCTTCAATGAACATCAATGAACAGCTGTTCCCGCAGGTGTACGTGGTTGTATGTAATGAACAACCTGCTTACTGTCCGTATCCGCAAGCC 1800
 GIIYTLNLNELEHETSMELQFPFRRCTWLYVMNNCLLSVSGKA
 TCTCAGCTTTATCCCACAATTTACCAGGGCTTTTCGATTATGCAAGCAGATGCAAGATTACCTGTAGCCATCCAGCACACAGCTCCCGGATCGAATCTGCCAGGAATTTGCT 1920
 SQLYSHNLPGLFDYARQMQLPVAIPAHHKLDPDRILPRKFA
 GTATCAGCAAGATTCCTGAAACCAAGTGGTGTGAGAGTGTGCGTGTGAGAAATCCTTACACGGCCATAAGTACCTGTGCGGAGCACTTACAGTACGATGTTCTATTAGATGG 2040
 VSAKIPETIKWCQKCCVVRNPNPYTGHKYLCGALQTSIVLLEW
 GTTGAGCAATCAGAGATTATGTAAATGAACATAGAGTTTCAATGCCGTGTGCGCTCCGATGTTTCGAGATGCTGGTGTGCGAGAGCAGGAGTACCTCTGGTCTGTGTGGC 2160
 VEPMQKFMLEIKHIEFPMPCLRMFEMLVVPEQEYPLVVCV
 GTCAGTAGAGGGGAGACTTCAACCAAGTGGTGTGAGACGTCATCCAAATTTACCTCGTGTGTCAGAGTGCAGATACCCGAGACAAACGTCACCATGTGACCCAG 2280
 VSRGRDFNQVVRFEETVNPNTSSSWFTESDTPQTNTVTHVTQ
 CTGAGAGAGACCATCTCGTGTGCTGGATTGTTGTATAAAATAGTGAATCTCCAAGGAAGATTGAATCCAGCAGAAAATATCTTCAGAGCTCACCTTCGATTTCCAGATAGAA 2400
 LERDITILVCLDCCKIKIVNLQGRRLKSSRLSSSELTFFDFQIE
 TCCATAGTTGTCTACAGACAGTGTGTAGCTTTCTGGAACACGGGATGCAAGGTAGAAGTTTATAGTCTACAGAGGTAAACAAGAAATTCAGATAACAAGAATCTTCAGACTT 2520
 SIVCLGQDSSLVLAFWKHHGMQGRSPFRSNEVTQEISDNTIRIPRL
 CTTGGATCTGACAGGTTGTGGTTTGGAAAGTAGACCACTGACAAACCAACAGCAATATAGCAATTTATACATCTTGGCGGGCCAGAAAACAGCTACTGA 2622
 LGSDRVVVLLESRRPTDNPNTANSNLYILAGHENS Y.

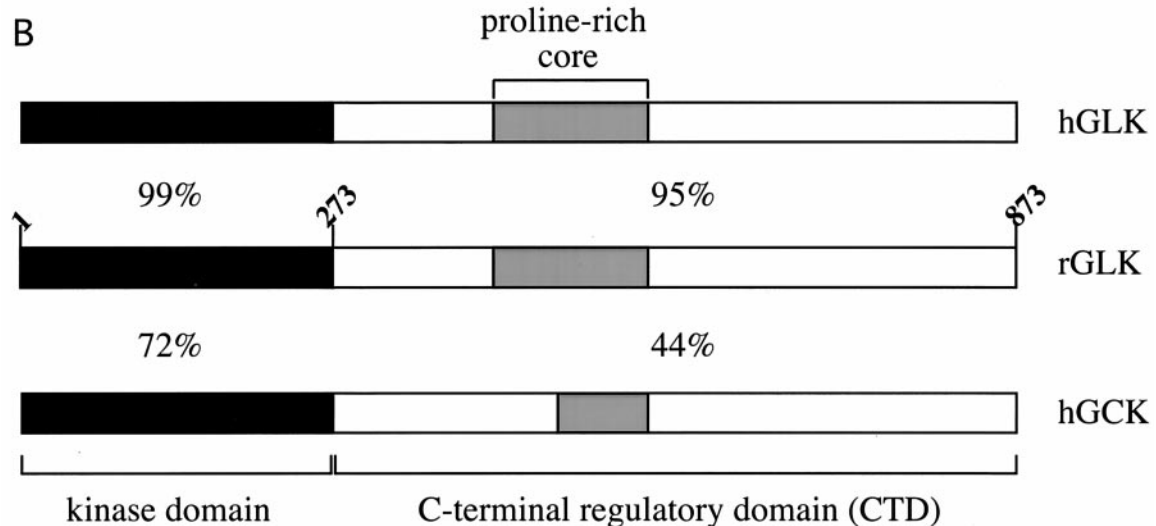


FIG. 1. Sequence and structure of rGLK. A, the nucleic acid and complete coding sequence of rGLK is shown. The N-terminal sequence, determined from four overlapping rat EST clones, is underlined. The boxed amino acids represent consensus SH3 domain-binding sites. The nucleic acid position numbers are indicated on the right. B, rGLK is shown aligned to hGLK and hGCK. The N-terminal kinase domain and C-terminal regulatory domain (including the proline-rich core) are indicated. The amino acid position numbers of rGLK that define each domain are indicated along the top of the protein. The percent identity between the various domains is indicated.

but not to GST alone (Fig. 2A, upper panels). To further demonstrate the interaction specificity, we assessed the binding of FLAG-tagged GCK (27) (generously provided by Dr. John Kyriakis). No binding of FLAG-GCK to the SH3 domain of endophi-

lin I was detected (Fig. 2A, upper panels). We next performed the converse pull-down experiments. Full-length endophilin I expressed in HEK-293T cells bound specifically to a GST fusion protein encoding the proline-rich core of rGLK (GST-GLK-

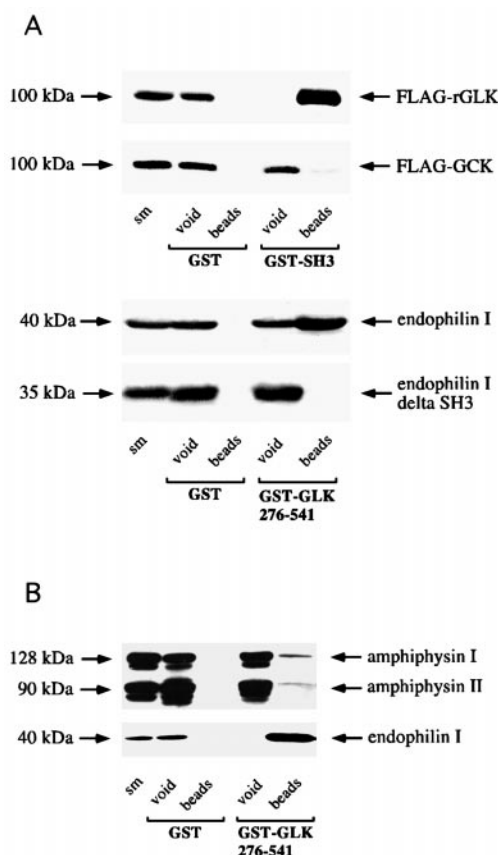


FIG. 2. Characterization of endophilin I-rGLK interactions. A, Triton X-100 soluble extracts were prepared from HEK-293T cells transfected with FLAG-rGLK or FLAG-GCK (upper panels) or with full-length endophilin I or endophilin I lacking the SH3 domain (endophilin I delta SH3, lower panels). The extracts were incubated with GST or with GST fusion proteins of either the SH3 domain of endophilin I (GST-SH3) or a portion of the proline-rich regulatory domain of rGLK (GST-GLK₂₇₆₋₅₄₁) prebound to glutathione-Sepharose. Proteins specifically bound to the beads were processed for Western blots along with an aliquot of the soluble extract (starting material (sm)) and an equal amount of the unbound material (void). The panels show immunoblots with anti-FLAG (upper panels) or anti-endophilin I (lower panels). B, GST or GST-GLK(276–541) conjugated to glutathione-Sepharose beads were incubated with soluble extracts from rat brain. Proteins specifically bound to the beads along with equal aliquots of the soluble brain extract (starting material (sm)) and unbound material (void) were processed for Western blots with an antibody recognizing amphiphysin I and II (upper panel) or endophilin I (lower panel).

(276–541)) (Fig. 2A, lower panels). In contrast, an endophilin I construct lacking the SH3 domain (endophilin I delta SH3) did not bind to the same rGLK fusion protein (Fig. 2A, lower panels). The endophilin I-rGLK interaction was also demonstrated from brain tissue. GST-GLK(276–541) strongly affinity-selected endophilin I from brain extracts, whereas amphiphysin I and II, two major SH3 domain-containing proteins in brain (25), bound only weakly to the fusion protein (Fig. 2B). The binding of amphiphysin I and II, although weak, is specific as neither protein nor endophilin I bound to GST alone (Fig. 2B).

The proline-rich core of rGLK contains multiple consensus SH3 domain-binding sites. To begin to identify the proline-rich motif(s) responsible for endophilin I binding, we generated GST fusion protein constructs deleting increasing amounts of the C terminus of the rGLK regulatory domain (Fig. 3A). Whereas constructs consisting of amino acids 276–541 (GST-GLK(276–541)) and 276–498 of rGLK (GST-GLK(276–498)) bound strongly to endophilin I from brain extracts, a construct encoding amino acids 276–445 (GST-GLK(276–445)) showed weak binding, and a construct encoding amino acids 276–406 (GST-

GLK(276–406)) failed to bind (Fig. 3B). The major endophilin I-binding site is thus located between amino acids 445 and 498 of rGLK with a second, weaker site between amino acids 405 and 446. Within the region of rGLK from amino acids 445–498 is the sequence PPRPPPPR (Fig. 3C), which closely conforms to the previously described SH3 domain consensus-binding sequence for the endophilins (28). This sequence is thus the likely endophilin I-binding site in rGLK.

Endophilin I Is a Major rGLK-binding Protein—Members of the GCK family have been reported to interact with a number of different SH3 domain-containing proteins (22). To determine whether rGLK has multiple SH3 domain-binding partners, we performed overlay assays of brain extracts with the GST-rGLK fusion protein constructs described above. Remarkably, GST-GLK(276–541) reacted with a single 40-kDa band that perfectly co-migrated with endophilin I as determined by Western blot with an endophilin I antibody (Fig. 4A). GST-GLK(276–498), which like GST-GLK(276–541) interacts with endophilin I in pull-down assays, also bound to the 40-kDa band. In contrast, GST-GLK(276–445) and GST-GLK(276–406), which bind to endophilin I in pull-down assays, either very weakly or not at all, do not recognize the 40-kDa band (Fig. 4A). A similar specificity was seen with overlay assays of recombinant endophilin I (data not shown). These data thus suggest that endophilin I is the major rGLK-binding protein in the brain. Previously, we performed overlays with the SH3 domain of endophilin I that revealed dynamin I and synaptojanin 1 as the major endophilin I-binding partners in the brain (9). However, dynamin I and rGLK both migrate on SDS-PAGE at ~100 kDa, so it is possible that the 100-kDa band detected in these experiments represented a mixture of dynamin I and rGLK. To further explore this issue, we performed overlays with GST or GST-endophilin I on brain extracts run on gels side-by-side with immunoprecipitated FLAG-rGLK and FLAG-GCK. Endophilin I reacted strongly with bands in the brain extract at 100 and 145 kDa, identified previously as dynamin and synaptojanin, respectively (9). Endophilin I also reacted with FLAG-rGLK, which migrated almost perfectly with dynamin, whereas no reactivity was seen with FLAG-GCK (Fig. 4B). A Western blot with an anti-FLAG antibody confirmed the presence of FLAG-rGLK and FLAG-GCK in the immunoprecipitated samples (Fig. 4B). Together, these results suggest a strong and highly specific interaction of endophilin I with rGLK.

Co-immunoprecipitation of rGLK and Endophilin I—To demonstrate the interaction in cells, lysates from HEK-293T cells co-transfected with FLAG-rGLK and full-length endophilin I were subjected to immunoprecipitation analysis with an anti-endophilin I antibody (1903) and an anti-FLAG antibody. Immunoprecipitation of either endophilin I or FLAG-rGLK led to the co-immunoprecipitation of the respective binding partner (Fig. 5A). In control experiments, no co-immunoprecipitation was seen when cells were co-transfected with FLAG-rGLK and an endophilin I construct lacking the SH3 domain (endophilin I delta SH3) (Fig. 5A).

Endophilin I Regulates GLK-mediated JNK Activation—It was previously reported that overexpression of hGLK leads to JNK activation in transfected HEK-293 cells (21). We thus used this system to determine whether endophilin I functions in GLK-mediated JNK activation. Specifically, we monitored JNK activation using an anti-phospho-JNK antibody following transfection of GLK and different endophilin I constructs. Consistent with Diener *et al.* (21), we found that overexpression of rGLK is sufficient to activate JNK (Fig. 5B). Interestingly, overexpression of full-length, untagged endophilin I along with rGLK causes an increase in JNK activation *versus* expression of GLK alone (Fig. 5B). Quantitative analysis of eight inde-

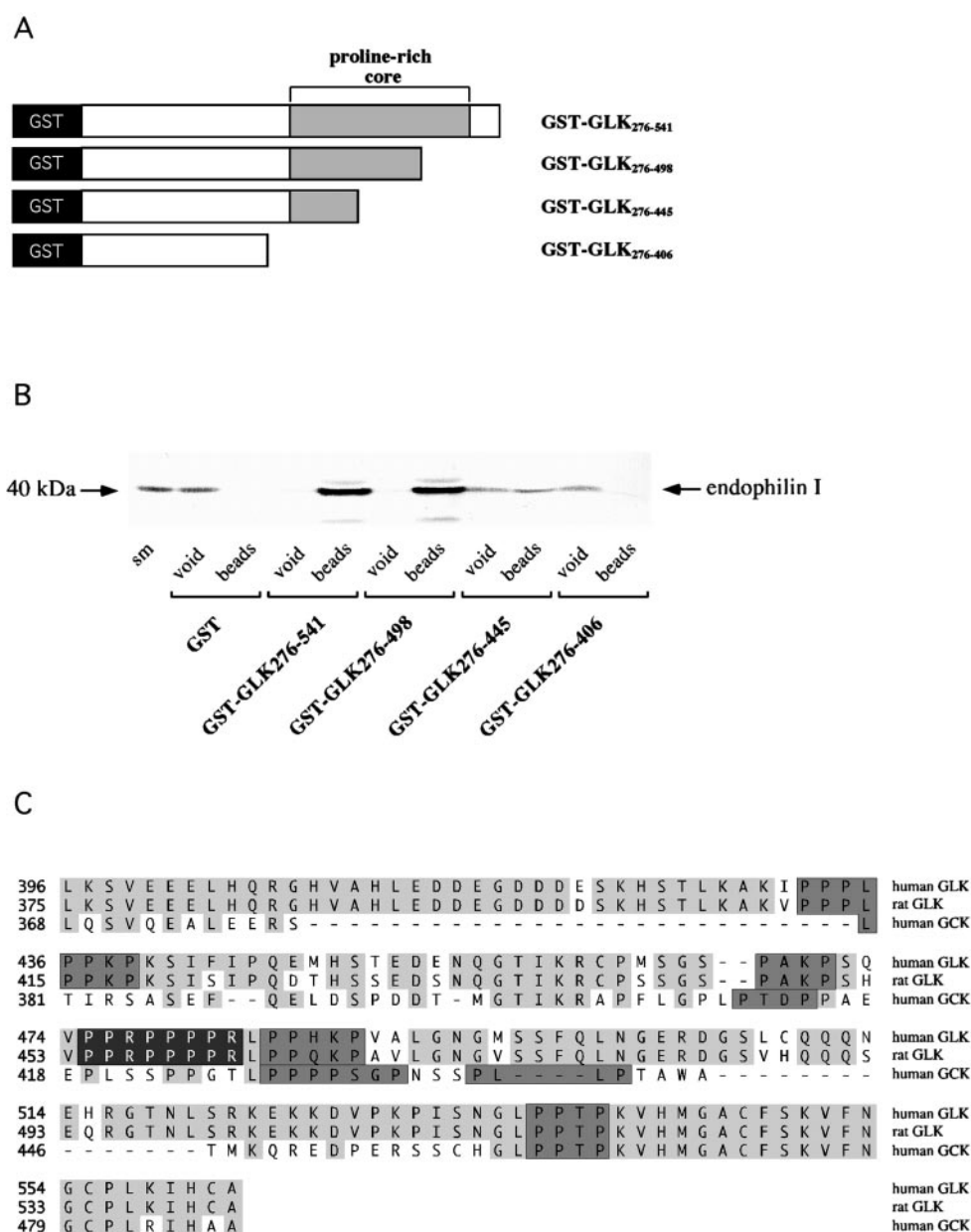


FIG. 3. Identification of an endophilin-binding site in rGLK. *A*, domain model of GST fusion protein constructs of the proline-rich core of rGLK. *B*, soluble extracts prepared from rat brain were incubated with the rGLK fusion proteins conjugated to glutathione-Sepharose. Proteins specifically bound to the beads along with aliquots of the soluble brain extract (starting material (*sm*)) and equal amounts of the unbound material (*void*) were processed for Western blot with an antibody against endophilin I. *C*, amino acid sequence alignment of a portion of the regulatory domains including the proline-rich cores, of hGLK, rGLK, and hGCK. Homologous residues are *lightly shaded*, SH3 domain-consensus binding sites are *more darkly shaded*, and the likely endophilin I-binding site is the *most darkly shaded*.

pendent experiments revealed a statistically significant ($p < 0.05$; two-tailed t test) 2.01-fold stimulation. Further, overexpression of a GFP-tagged form of the SH3 domain of endophilin I or of the untagged N terminus of endophilin I lacking the SH3 domain (delta SH3) completely blocked GLK-mediated JNK activation (Fig. 5*B*). Given that the SH3 domain of endophilin I was expressed with a GFP tag, we sought to further demonstrate the specificity of this construct to block rGLK-mediated JNK activation. Thus, JNK activation was measured following overexpression of rGLK with the GFP-SH3 domain construct or with GFP alone. Whereas the GFP-endophilin I SH3 domain blocked rGLK-mediated JNK activation, GFP alone had no effect (Fig. 5*C*). Together, these data demonstrate that endophilin I can function to regulate GLK-mediated JNK activation.

DISCUSSION

Previous studies have established a role for endophilin I in clathrin-mediated endocytosis (14–16). Our data, demonstrating a functional role for endophilin I in rGLK-mediated JNK activation, suggest an additional and perhaps complementary role for endophilin I in signaling via the JNK pathway. Several recent studies have demonstrated that proteins that function in endocytosis can also function in the regulation of cell signaling. For example, both amphiphysin II and intersectin demonstrate SH3 domain-dependent interactions with the Ras-activating enzyme mSos (4, 5), and overexpression of the SH3 domains of intersectin blocks epidermal growth factor-mediated activation of Erk1/2 (6, 7). Moreover, β -arrestin 1, a clathrin-binding protein that functions to target activated G protein-coupled receptors to clathrin-coated pits, also functions as a

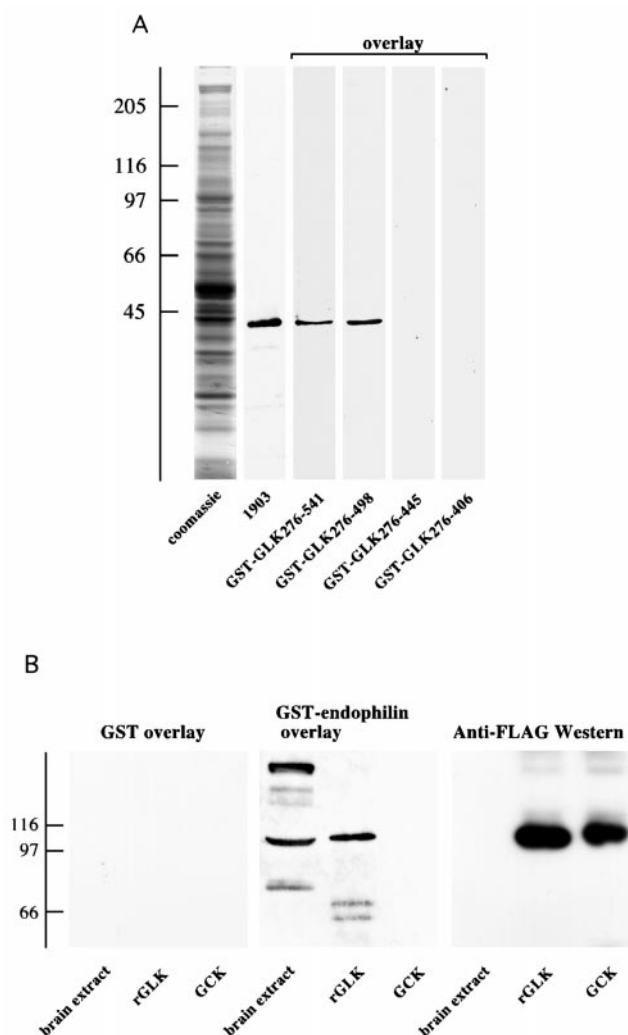


FIG. 4. rGLK overlay assays. A, overlay assays using the GST-rGLK fusion protein constructs shown in Fig. 3A were performed on strips of rat brain post-nuclear supernatants on nitrocellulose. Coomassie Blue staining reveals the complement of proteins, and an endophilin I antibody (1903) was used to indicate the migratory position of rat brain endophilin I. B, overlays using GST alone or GST-endophilin I were performed on an aliquot of rat brain extract as well as on FLAG-rGLK and FLAG-GCK immunoprecipitated from transfected cells. An anti-FLAG Western blot reveals the presence of rGLK and GCK on the blots.

scaffolding protein for Erk1/2 and its upstream activating kinase, Raf-1 (29). In fact, agonists of the G_{α_q} -coupled proteinase-activated receptor 2 stimulate the formation of a complex containing receptor, β -arrestin 1, Raf-1, and activated Erk1/2 on endocytic vesicles (29). Thus, proteins that function in endocytosis can also regulate the Erk MAP kinases.

Recent studies have also suggested a function for endocytic proteins in regulation of the JNK MAP kinases. For example, following stimulation with ligand, the angiotensin II type 1A receptor is targeted to the endocytic pathway through interactions with β -arrestin 2 (30). Interestingly, β -arrestin 2 also binds to JNK3, and activation of the angiotensin II type 1A receptor leads to the formation of a receptor- β -arrestin 2-JNK3 complex on endocytic vesicles (30). The formation of this complex is necessary for ligand-induced JNK3 activation (30). Further, Fish *et al.* (31) determined that overexpression of wild-type dynamin II, but not a GTPase defective mutant of the protein, activates the transcription factor p53 and induces apoptosis. These data suggest a possible role for dynamin in JNK activation as JNKs are potent effectors of apoptosis (32). Moreover, dynamin 1 binds directly to mixed lineage kinase 2, an

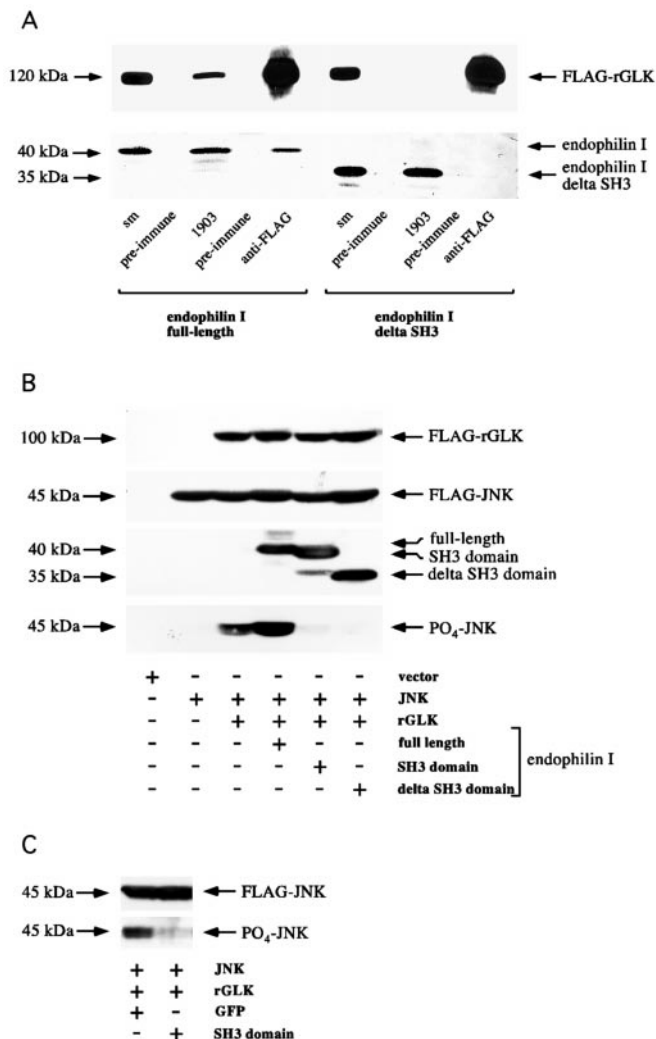


FIG. 5. Endophilin I regulates rGLK-mediated JNK activation. A, Triton X-100-soluble extracts of HEK-293T cells, co-transfected with FLAG-rGLK and either full-length endophilin I or endophilin I lacking the SH3 domain (*endophilin I delta SH3*), were used for immunoprecipitation analysis using anti-FLAG, anti-endophilin I (1903), or pre-immune 1903 (*pre-immune*). Proteins specifically bound to the beads were processed for Western blots with anti-FLAG (*upper panel*) or anti-endophilin I (*lower panel*) antibodies. B, HEK-293T cells were transfected with control plasmid, FLAG-JNK, FLAG-rGLK, full-length endophilin I, endophilin I lacking the SH3 domain, or GFP-SH3 domain of endophilin I either alone or in various combinations as indicated. 48 h following transfections, the cells were scrapped and processed for Western blots with anti-FLAG, anti-endophilin, or anti- PO_4 -JNK antibodies as indicated. C, HEK-293T cells were transfected with FLAG-JNK, FLAG-rGLK, GFP, or GFP-SH3 domain of endophilin I as indicated. 48 h after transfections, the cells were scrapped and processed for Western blots with anti-FLAG, anti-endophilin, or anti- PO_4 -JNK antibodies as indicated. For all of the blots, the migratory positions of the proteins are indicated on the left.

upstream activator of JNK (33). Thus, distinct protein components of the endocytic regulatory machinery appear to function in the activation of MAP kinase signaling pathways including those involving JNKs. The data presented here, demonstrating a functional interaction between endophilin I and rGLK, further support the importance of such a link.

A clue to the mechanism by which endophilin I may regulate hGLK-mediated JNK activation has come from the study of Diener *et al.* (21) who determined that deletion of the regulatory domain of hGLK, including the SH3 domain-binding sites, resulted in a kinase that had full catalytic activity but that was significantly impaired in its ability to activate JNK. These data are consistent with a model in which the regulatory domain

targets rGLK for the interactions necessary for JNK activation. Thus, endophilin I may function as an adaptor protein to target rGLK to an upstream activator or to a downstream effector functioning in JNK activation. In fact, other GCKs appear to undergo specific targeting events. For example, the group 1 GCK family member HPK1 is activated following its recruitment to the epidermal growth factor receptor via interactions with the SH3 domain-containing adaptor protein Grb2 (34). GCK itself is targeted to the Golgi complex via its interactions with the membrane-trafficking protein Rab8 (35). Regardless of its precise functional role, the data presented here unveil a novel interaction between endophilin and rGLK and strongly suggest a role for endophilin I in regulation of JNK activity.

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