Cloning of a Novel Phosphatidylinositol Kinase-related Kinase

CHARACTERIZATION OF THE HUMAN SMG-1 RNA SURVEILLANCE PROTEIN*

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We have cloned and characterized a new member of the phosphatidylinositol kinase (PIK)-related kinase family. This gene, which we term human SMG-1 (hSMG-1), is orthologous to Caenorhabditis elegans SMG-1, a protein that functions in nonsense-mediated mRNA decay (NMD). cDNA sequencing revealed that hSMG-1 encodes a protein of 3031 amino acids containing a conserved kinase domain, a C-terminal domain unique to the PIK-related kinases and an FKBP12-rapamycin binding-like domain similar to that found in the PIK-related kinase mTOR. Immunopurified FLAG-tagged hSMG-1 exhibits protein kinase activity as measured by autophosphorylation and phosphorylation of the generic PIK-related kinase substrate PHAS-1. hSMG-1 kinase activity is inhibited by high nanomolar concentrations of wortmannin (IC_{50} = 105 nM) but is not inhibited by a FKBP12-rapamycin complex. Mutation of conserved residues within the kinase domain of hSMG-1 abolishes both autophosphorylation and substrate phosphorylation, demonstrating that hSMG-1 exhibits intrinsic protein kinase activity. hSMG-1 phosphorylates purified hUpf1 protein, a phosphoprotein that plays a critical role in NMD, at sites that are also phosphorylated in whole cells. Based on these data, we conclude that hSMG-1 is the human orthologue to C. elegans SMG-1. Our data indicate that hSMG-1 may function in NMD by directly phosphorylating hUpf1 protein at physiologically relevant sites.

The PIK-related kinases are a subfamily of the phosphatidylinositol (PI) kinases based on homology to the core catalytic domain of phosphatidylinositol 3-kinase (PI3K) (1, 2). Regions of homology between PI kinases and the protein kinase superfamily include the ATP-binding site and the catalytic substrate-binding site (3). PIK-related kinases are distinct from PI kinases in that they are high molecular weight proteins that function as serine/threonine protein kinases, rather than lipid kinases (3). PIK-related kinases can be divided into three subgroups based on structural and functional similarities shared by certain family members. The ATM/ATR/RAD3 subgroup functions in DNA damage response pathways, and members contain regions called RAD3 homology domains (4, 5). The targets of rapamycin or TORs (Saccharomyces cerevisiae TOR1 and TOR2 and human mTOR/FRAP/RAPI1/RAPI2) were originally identified as intracellular targets of the immunophilin-immunosuppressant complex FKBP12-rapamycin (6, 7). TORs share sequence similarity within the FRB domain, which binds this complex (7). The TORs function in response to mitogenic signaling to regulate cap-dependent translation (8). Finally, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) shares no sequence similarity to other PIK-related kinases, aside from the kinase domain. DNA-PK functions in the repair of programmed DNA breaks generated by meiotic and V(D)J recombination, and those generated by genotoxic insults (9).

Recently, evidence has emerged for an essential PIK-related kinase in nonsense-mediated mRNA decay (NMD). NMD, or mRNA surveillance, is an evolutionarily conserved process by which mRNA species containing premature termination codons are preferentially degraded, thereby preventing accumulation of truncated proteins that might serve in a dominant negative or gain-of-function manner (10–18). NMD also functions to regulate the level of a number of normal mRNAs (19–22). NMD has been genetically analyzed in yeast and nematodes. Seven genes (SMG-1 to SMG-7) are involved in NMD in Caenorhabditis elegans (23). One of these genes, SMG-1 (ceSMG-1), is predicted by sequence to encode a PIK-related kinase (10). Biochemical studies of ceSMG-1 are lacking, and the protein has never been shown to have phosphotransferase activity. However, genetic evidence indicates that phosphorylation of SMG-2, an RNA helicase, is blocked in ceSMG-1 mutants (19). SMG-2 and its human homologue, human Upf1 (hUpf1), contain multiple potential PIK-related kinase (S/T)-Q and (S/T)-P phosphorylation site motifs (24). Furthermore, phosphorylation of hUpf1 is blocked by high concentrations of wortmannin (IC_{50} = 100 nM), consistent with the role of a PIK-related kinase (24). Such observations suggest that ceSMG-1 is a PIK-related kinase that may phosphorylate SMG-2. However, direct

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AY014957.

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The abbreviations used are: PIK, phosphatidylinositol kinase; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; SMG, suppressor with morphogenetic effect on genitalia; hSMG-1, human SMG-1; Upf, up frameshift; NMD, nonsense-mediated mRNA decay; TOR, targets of rapamycin; DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase; RACE, rapid amplification of cDNA ends; kb, kilobase pairs; PCR, polymerase chain reaction; EST, expressed sequence tag; KD, kinase-deficient; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; dH2O, distilled H2O; LIP, lambda-interacting protein.
phosphorylation of SMG-2 or hUpf1 protein by ceSMG-1 or an SMG-1-like kinase in mammalian cells has never been demonstrated.

In the present report, we describe the cloning and characterization of the human orthologue to ceSMG-1, which we term human SMG-1 (hSMG-1). hSMG-1 encodes a novel PIK-related kinase with significant homology to the TORs and ceSMG-1. Biochemical characterization of transiently expressed FLAG-tagged hSMG-1 protein indicates that it is a protein kinase that exhibits both autophosphorylation and substrate-specific phosphorylation. FLAG-hSMG-1 directly phosphorylates hUpf1 protein at sites phosphorylated in whole cells, indicating that hSMG-1 is a physiologically relevant hUpf1 protein kinase.

### Experimental Procedures

hSMG-1 cDNA Cloning and Plasmid Construction—*5′-RACE (rapid amplification of cDNA ends) was performed using K562 (human chronic myelogenous leukemia) Marathon Ready cDNA (CLONTECH Laboratories, Inc.) and gene-specific primers toward the 5′-end of KIAA0421 (NCBI gi: 2887416; accession number AB007881), a partial cDNA with significant sequence similarity to PIK-related kinases, to generate an additional 1000 base pairs of cDNA sequence. The remaining hSMG-1 cDNA sequence was obtained from a long pre-stretch 5′-STRETCH (A-gt10) cDNA library (CLONTECH Laboratories, Inc.) by PCR. The full-length cDNA sequence, derived from 12 overlapping clones, was verified by alignment of four independent full-length clones. Comparison of the sequence to the GenBank™ EST data base identified KIAA0220 (NCBI gi: 1504021; accession number D68974), which corresponds to amino acids 95–647 of the full-length hSMG-1 cDNA. The hSMG-1 coding sequence can be accessed via GenBank™ accession number AY014957 (NCBI gi: 372334).

FLAG-tagged, full-length hSMG-1 cDNA was generated by PCR amplification of three overlapping hSMG-1 fragments corresponding to nucleotides 1–2580 (fragment A), nucleotides 2531–6540 (fragment B), and nucleotides 6481–9096 (fragment C). The initiating methionine codon was mutated to glycine (ATG to GTG) to generate a 5′-FLAG-tagged cDNA. The A, B, and C fragments were cloned into pCR-XL-TOPO (Invitrogen), and FLAG-tagged, full-length cDNA (pCMV-FLAG-hSMG-1) was assembled by directional cloning into the pCMV-TAG2B (Stratagene) and then subcloned into pcDNA (Promega). Clones were verified by sequencing.

Three kinase-deficient (KD) hSMG-1 cDNAs were generated by site-directed mutagenesis of nucleotide 5114 (GAC to GCC), or of beta-actin 5171 (GAT to GAG or GAT to GCT) within motif I (KD1) and motif II (KD2 and -3) of the catalytic domain. All hSMG-1 clones DNA were confirmed by sequencing. Sequence alignments were performed using the Vector NTI Suite II program (InforMax Inc.).

Northern Blot Analysis—Total cellular RNA from human HL60, K562, and HEK-293 cells was extracted using TRIzol® reagent (Life Technologies, Inc.) supplemented with 10% bovine calf serum (K562 cells) or 10% fetal calf serum (HL60 cells), 100 µg/ml penicillin, and 100 IU/ml streptomycin as described previously (25). Human embryonic kidney 293 (HEK-293) cells were maintained in DMEM medium (Life Technologies, Inc.), 10% bovine calf serum, 100 µg/ml streptomycin, and 100 IU/ml penicillin. Subconfluent HEK-293 cells were transiently transfected using LipofectAMINE Plus (Life Technologies, Inc.) with ~1–3 µg of DNA/plate and harvested for analysis 48 h after transfection.

hSMG-1 Immunopurification and Protein Kinase Assay—FLAG-WT- or -KD-hSMG-1 was immunopurified from transiently transfected HEK-293 cells using anti-FLAG M2® conjugated agarose beads (Sigma). Brieﬂy, cells were rinsed once with ice-cold 1× PBS, scraped, and transferred into microcentrifuge tubes, and lysed in 200 µl of lysis buffer (50 mM β-glycero-phosphate, 10 mM potassium phosphate, 50 mM NaF, 1 mM EDTA, and 1 mM EGTA, pH 7.4) supplemented with 1 mM DTT, 10 µg/ml each of leupeptin, pepstatin, aprotinin, 1 mM phenylmethyl sulfonfyl fluoride, 2.5 mM MgCl2, 0.1 mM DTT, and 0.1% Tween 20. Cells were homogenized using a 1.5-ml pestle followed by microcentrifugation at 12,000 rpm for 20 min at 4 °C. Immunopurification was performed by incubating 800–1000 µg of clonal lysate with 30 µl of anti-FLAG M2®-conjugated agarose beads for 2 h at 4 °C with gentle rotation. Beads were washed twice with 1 ml of each of the following buffers: lysis buffer supplemented with 1 mM DTT and 0.1% Tween 20, 500 µl NaCl, 1 mM DTT and 0.1% Tween 20, wash buffer (50 mM Tris, 1 mM EDTA, and 1 mM EGTA), and kinase buffer (10 mM HEPES, 50 mM NaCl, 0.1 mM EDTA, and 1 mM EGTA). hSMG-1 was immunopurified from HEK-293 cells transiently transfected with 1 µg of pcDNA-hUpf1 DNA (26) for 48 h and used as substrate for immunopurified FLAG-hSMG-1 kinase assay as described below.

Protein kinase assays were performed in kinase buffer containing 100 µM ATP, 10 mM MnCl2, 1 µg of recombinant PHAS-1 (Calbiochem) or immunopurified FLAG-hUpf1 protein, and 5 µCi of [γ-32P]ATP (PerkinElmer Life Sciences) in a final volume of 20 µl. Reactions were performed at 30 °C and terminated after 30 min by the addition of 20 µl of 2× SDS-PAGE sample buffer followed by boiling for 5 min. Samples were analyzed in 4–20% acrylamide Tris-HEC (Bio-Rad) or NuPAGE 3–8% acrylamide Tris acetate gels (Invitrogen) and transferred to nitrocellulose. Radioactivity was detected using an InstantImager (Packard) and autoradiography on X-omat film (Eastman Kodak Co.). For wortmannin treatment, immunopurified FLAG-hSMG-1 was incubated with the indicated amount of wortmannin or solvent (Me2SO) in a final volume of 50 µl at room temperature for 1 h, then washed twice with kinase buffer, and the kinase assay performed as indicated above. Immunoblot analysis of FLAG-tagged proteins was performed with 0.1 µl ice-cold KCl, excised, recovered by electroloration, and used to immunize rabbits for polyclonal antibody production.

Affinity-purified antibody was generated using purified GST-hSMG-1 resolved by SDS-PAGE in 10% acrylamide preparative gels, transferred to nitrocellulose, and visualized by staining with fast green. Blotting was performed on nitrocellulose or PVDF membranes in 100 mM carbonate buffer containing 1% bovine serum albumin in PBS containing 0.1% Tween 20 (1% BSA/PBST), and then incubated with a 1:1000 dilution of affinity-purified hSMG-1 antibody in 1% BSA/PBST for 1 h at room temperature. The nitrocellulose was washed twice for 10 min in PBST, once in PBS alone, and antibody eluted into a minimal volume of 0.5 M acetic acid at pH 2.5 for 1 h. The eluted antibody was immediately neutralized by addition of 3 volumes of 1% PBS/PBS and dialyzed three times for 30 min against ice-cold PBS. Purified antibody was concentrated using a Centricon-10 (Amicon Inc.) and stored at −20 °C in PBS containing 10% glycerol.

Immunoblot analysis of hSMG-1 was performed using a 1:10 dilution of affinity-purified antibody in 1% BSA/PBST. Total cell lysates from HEK-293 cells were separated in a 7.5% acrylamide Tris-HEC gel (Bio-Rad), transferred to nitrocellulose and blocked in 5% nonfat dry milk/PBST for 1 h prior to incubation with affinity-purified hSMG-1 antibody for 2 h. Membrane was washed three times in PBST, incubated in a 1:10,000 dilution of goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) in 5% milk/PBST for 1 h, and washed twice in PBST and once in PBS. Chemiluminescent detection of antigen-antibody complexes was performed using X-Omat® West Sensitive X-ray Film (Kodak).
Cloning of a Novel PIK-related Kinase Orthologous to C. elegans SMG-1

RESULTS AND DISCUSSION

Cloning of hSMG-1 cDNA—In a search for sequences with homology to ceSMG-1, we used the GenBank™ data base of ESTs to identify a partial cDNA, termed KIAA0421 (NCBI gi: 2887416; accession number AB007881) with homology to PIK-related kinases (30). Both 5'-RACE and PCR screening were used to clone the full-length hSMG-1 cDNA as described under “Experimental Procedures.” The starting methionine codon was identified using two criteria: 1) the presence of upstream in-frame stop codons in three independent clones and 2) a Kozak consensus context. The open reading frame is encoded by 9096 base pairs and predicts a protein of 3031 amino acids with a predicted molecular mass of 79.7 kDa. The full nucleotide and amino acid sequence of hSMG-1 can be accessed through GenBank™ accession number AY014957 (NCBI gi: 372334). Interestingly, a fourth independent clone was identified using two criteria: 1) the presence of upstream homology to ceSMG-1 in three major regions: an N-terminal 200-amino acid region of 44% similarity, a 1000-amino acid region in the middle of the coding sequence of 46% similarity that includes the FRB-like and kinase domain (Fig. 1B), and a C-terminal 88-amino acid region of 61% similarity encompassing the FATC domain (Fig. 1A). Two additional regions of homology between ceSMG-1 and hSMG-1 (termed SMG-1 homology Domains 1 and 2; SD1 and SD2) have not previously been described in other PIK-related kinases and may constitute unique functional domains characteristic of ceSMG-1 and hSMG-1.

hSMG-1 Is Widely Expressed as a High Molecular Weight RNA and Protein—To assess hSMG-1 expression, RNA blot hybridization was performed using total RNA from K562, HL60, and HEK-293 cells (Fig. 2, A–C). hSMG-1 is expressed as an RNA of ~11.6 kb in HL60 and K562 cells as determined using a probe spanning nucleotides 5937–6890 (probe 1) of hSMG-1 (Fig. 2A). This probe overlaps with the first 150 nucleotides of the reported LIP sequence, suggesting that the reported ~7.5-kb LIP mRNA (33) is not expressed in these cells. To confirm this observation, RNA blot hybridization was performed using a LIP-specific probe (probe 2, nucleotides 6741–7812) and a hSMG-1-specific probe (probe 3, nucleotides 3521–4654). Like probe 1, these two probes detect an 11.6-kb RNA in HL60, K562, and HEK-293 cells, but no smaller RNA(s) corresponding to LIP (Fig. 2, B and C). RNA blot hybridization of cancer cell lines and human tissues using probe 1 (Fig. 2, D and E) detected hSMG-1 RNA in each of the cancer cell lines with relatively low levels in lung carcinoma (A459) and melanoma (G361) cell lines. hSMG-1 RNA was also detected in the

sites within the catalytic domain (Fig. 1C). In addition, hSMG-1 contains a short C-terminal region at amino acids 3001–3031 (termed FATC for FRAP, ATM, TRRAP at C-terminus), found in the majority of the PIK-related kinases (Fig. 1A) (31). Among the well characterized PIK-related kinases, hSMG-1 exhibits the highest sequence homology to the TORs, which extends beyond the kinase domain to the FRB (FKBP12-rapamycin binding) domain (Fig. 1, A and B). Mutation analysis of the FRB domain of mTOR has defined it as the site of binding for rapamycin, a selective inhibitor of TOR (8, 32). Several residues within this domain are also required for mTOR kinase activity (8). Sequence alignment indicates that hSMG-1 lacks a critical serine residue that is required for binding of the FKBP12-rapamycin complex to mTOR (Fig. 1B) (32). However, the FRB domain of hSMG-1 retains a conserved tryptophan residue, which when mutated in mTOR abolishes kinase activity (Fig. 1B) (8). Conservation of this tryptophan residue suggests that it is important for hSMG-1 kinase activity.

The overall structure of hSMG-1 differs from that of other PIK-related kinases, since the kinase domain is not located at the extreme C terminus (Fig. 1A). Rather, a large region is located between the kinase domain and the FATC region that is about 100% identical to LIP (lambda-interacting protein). LIP was identified by yeast two-hybrid screening as a protein that interacts with the zinc finger domain of PKC/α (33). LIP cDNA was reported to be 2142 base pairs, coding for a protein of 713 amino acids with a predicted molecular mass of 79.7 kDa (NCBI gi: 5542015; accession number U32581) (33). The observation that LIP forms part of hSMG-1 raised the possibility that LIP is a splicing variant of hSMG-1. However, our Northern and immunoblot analyses argue against this hypothesis (see below).

hSMG-1 Shares Sequence Homology to ceSMG-1, a C. elegans Protein Required for Nonsense-mediated mRNA Decay—In addition to homology to mTOR, hSMG-1 exhibits significant homology to ceSMG-1 in three major regions: an N-terminal ~200-amino acid region of 44% similarity, a 1000-amino acid region in the middle of the coding sequence of 46% similarity that includes the FRB-like and kinase domain, and a C-terminal ~88-amino acid region of 61% similarity encompassing the FATC domain (Fig. 1A). Two additional regions of homology between ceSMG-1 and hSMG-1 (termed SMG-1 homology Domains 1 and 2; SD1 and SD2) have not previously been described in other PIK-related kinases and may constitute unique functional domains characteristic of ceSMG-1 and hSMG-1.

hSMG-1 contains several highly conserved motifs found in all PIK-related kinases, including a conserved ATP-binding site (Lys1525), and motif I (D1705-XXXVX1710) and motif II (D1724XX)
The majority of human tissues at varying levels. Therefore, hSMG-1 RNA is widely expressed in multiple tissues and cell lines.

Immunoblot analysis using an affinity-purified hSMG-1 antibody detected a single immunoreactive band in HEK-293 cells with an apparent molecular mass consistent with the predicted molecular mass of 340 kDa. Importantly, no lower molecular mass bands of ~80 kDa consistent with LIP (33) were detected. The antigen used to produce our hSMG-1 antibody consisted of the C-terminal region of the reported LIP cDNA. Therefore, it is likely that our antibody would detect LIP if it were expressed in these cells. We have also failed to detect LIP by immunoblot analysis in K562 and HL60 cells (data not shown).

hSMG-1 Is a Bona Fide PIK-related Kinase—To assess hSMG-1 protein kinase activity, full-length wild-type (WT) and kinase-deficient (KD1) hSMG-1 were expressed as FLAG-tagged proteins in HEK-293 cells. Cultures were transfected with empty vector (pCI-neo), pCI-FLAG-WT-hSMG-1, or pCI-FLAG-KD1-hSMG-1 and FLAG-tagged protein immunopurified using anti-FLAG M2® beads. FLAG-hSMG-1 immunoprecipitates were assayed for autophosphorylation and phosphorylation of recombinant PHAS-1 in the presence of Mn$^{2+}$ and $[^{32}]$P]ATP (Fig. 3B). PHAS-1 was chosen as substrate because it contains multiple (S/T)-Q and (S/T)-P phosphorylation motifs that serve as general phospho-acceptor sites for many PIK.

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**Fig. 1. Sequence of human SMG-1 and alignment to PIK-related kinase family members.** A, a schematic diagram showing homology between hSMG-1, cSMG-1, and mTOR. The FRB domain (FRB), kinase domain (Kinase Domain), and the C-terminal FATC region (FATC) are indicated. In addition, two regions of homology between hSMG-1 and cSMG-1, SD1 and SD2, are indicated. Numbers represent percent identity and similarity to hSMG-1, respectively. B, alignment of the FRB-like domain of hSMG-1 with cSMG-1 and mTOR. Shading indicates identical and similar amino acids. The critical tryptophan residue required for mTOR kinase activity is indicated (box labeled Kinase Activity). Also indicated is a Ser residue important for rapamycin binding to mTOR (box labeled RAPFKBP12 site). C, alignment of the kinase domains of hSMG-1, cSMG-1, and mTOR. Shading indicates identical and similar amino acids. Indicated is the conserved Lys within the ATP binding domain (box labeled Motif I), and conserved motif I and motif II sequences (box labeled Motif I and Motif II, respectively). Amino acid mutations introduced in kinase-deficient hSMG-1 variants (KD1#, KD2#, and KD3#) are indicated with arrows.

**Fig. 2. Expression of hSMG-1 RNA.** Total RNA from HL60 (A–C), K562 (A–C), and HEK-293 (B and C) cells was hybridized with hSMG-1-specific probes corresponding to nucleotides 5937–6890 (probe 1) (A), nucleotides 6741–7812 (probe 2) (B), or nucleotides 3521–4654 (probe 3) (C) within the hSMG-1 cDNA. RNA hybridization of the Human Cancer Cell Line MTN® Blot (D) and Human 12-Lane MTN® Blot (E) (CLONTECH Laboratories, Inc.) using probe 1. Hybridization to human β-actin probe is shown as a control for RNA loading (β-actin).
hSMG-1 is expressed in HEK-293 cells and exhibits intrinsic protein kinase activity. A, endogenous hSMG-1 protein expression was determined in HEK-293 cell lysates by immunoblot analysis using affinity-purified hSMG-1 antibody. A single band is detected with an apparent molecular mass consistent with the predicted molecular mass of 340 kDa. B, HEK-293 cells were transfected with empty vector (pcDNA3) (–), pcDNA-FLAG-WT-hSMG-1 (WT), or pcDNA-FLAG-KD1-hSMG-1 (KD) and immunopurified FLAG-hSMG-1 was assayed for autophosphorylation and phosphorylation of PHAS-1. Immunoblot analysis using FLAG antibody (anti-FLAG) demonstrates the presence of equivalent amounts of FLAG-WT-hSMG-1 and FLAG-KD-hSMG-1. C, HEK-293 cells were transfected with empty vector (pcDNA3) (–), pcDNA-FLAG-WT-hSMG-1 (WT), or pcDNA-KD-hSMG-1 (KD) and immunopurified hSMG-1 was incubated with the indicated concentration of wortmannin (WM (nM)) or diluent (0.1% MeSO) (–) for 1 h prior to assay for autophosphorylation ([32P]hSMG-1) and phosphorylation of PHAS-1 ([32P]PHAS-1). Immunoblot analysis using FLAG antibody (anti-FLAG) indicates that equivalent amounts of FLAG-WT-hSMG-1 and FLAG-KD-hSMG-1 were analyzed. D, the data presented in C are plotted as the percent PHAS-1 phosphorylation versus wortmannin concentration. The IC50 of hSMG-1 for wortmannin is 105 nM using PHAS-1 as substrate. E, top panel, HEK-293 cells were transfected with pcDNA3 (-), pcDNA-FLAG-WT-hSMG-1 (WT), or pcDNA-FLAG-KD-hSMG-1 (KD). FLAG-tagged proteins were immunopurified and incubated with rapamycin alone (R), rapamycin-FKBP12 complex (R-F), FK506 alone (FK), or FK506-FKBP12 complex (FK-F) for 1 h prior to assay for autophosphorylation and phosphorylation of PHAS-1. Immunoblot analysis using FLAG antibody (anti-FLAG) demonstrates the presence of FLAG-hSMG-1 in the reactions. Bottom panel, mTOR was immunopurified from HEK-293 cells using mAb1 antibody and incubated with either rapamycin alone (R) or rapamycin-FKBP12 complex (R-F) for 1 h prior to assay for protein kinase activity using PHAS-1 as substrate ([32P]PHAS-1). Immunoblot analysis using anti-FLAG antibody demonstrates the presence of mTOR in the reactions (anti-FLAG).
hSMG-1 Phosphorylates hUpf1 Protein at Sites Phosphorylated in Whole Cells—hUpf1, the human orthologue to C. elegans SMG-2 and the S. cerevisiae Upf1 protein, is an ATP-dependent helicase required for NMD in mammalian cells (10, 26, 41, 42). hUpf1, like SMG-2, is a phosphoprotein whose phosphorylation is inhibited by high concentrations of wortmannin (IC_{50} = 100 nM) (24). These observations, and the fact that hUpf1 is rich in (S/T)-Q and (S/T)-P motifs that serve as phosphorylation sites for PIK-related kinases, suggest that a PIK-related kinase may mediate hUpf1 protein phosphorylation (24). This hypothesis is supported by the observation that phosphorylated SMG-2 is not detected in a ceSMG-1 mutant (25). PIK-related kinases are capable of mediating phosphorylation of hUpf1, as hUpf1 protein is an ATP-dependent helicase required for NMD in mammalian cells (10, 26). These biochemical and genetic data suggest that hSMG-1 may be a physiologic hUpf1 protein kinase.

To directly test this hypothesis, immunopurified FLAG-hUpf1 protein was used as a substrate for immunopurified FLAG-WT-hSMG-1 or FLAG-KD-hSMG-1 (Fig. 4 A). FLAG-WT-hSMG-1 directly phosphorylates hUpf1 protein in vitro, whereas FLAG-KD-hSMG-1 is incapable of doing so. To determine whether hSMG-1 phosphorylates physiologically relevant sites on hUpf1, the sites of hSMG-1-mediated phosphorylation were compared with those phosphorylated in whole cells. HEK-293 cells were transiently transfected with pCI-FLAG-hUpf1 and incubated with [32P]orthophosphate for 12 h, and 32P-labeled FLAG-hUpf1 was immunopurified. Comparative two-dimensional tryptic phosphopeptide mapping (Fig. 4 B) demonstrates that hUpf1 phosphorylated by hSMG-1 contains two major phosphopeptides (Fig. 4 B, in vitro, labeled 1 and 2). A similar phosphopeptide pattern consisting of two major phosphopeptides was observed with hUpf1 phosphorylated in whole cells (Fig. 4 B, whole cells, labeled 1 and 2). Analysis of a mixture of these digests revealed that phosphorylated 1 and 2 are identical. Thus, hSMG-1 phosphorylates hUpf1 at the same two major sites phosphorylated in whole cells.

We conclude that hSMG-1 is a physiologically relevant hUPF1 kinase. The physiologic role of hSMG-1-mediated phosphorylation of hUpf1 is unknown. One possible role is to regulate hUpf1 function in NMD. We have been unable to generate direct evidence of a critical role for hSMG-1 in NMD, likely due to the low levels of KD-hSMG-1 expression that we can achieve (26). Further studies aim to directly assess the importance of hSMG-1 and hSMG-1-mediated phosphorylation of hUpf1 protein in NMD in human cells.
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