Distant N- and C-terminal Domains Are Required for Intrinsic Kinase Activity of SMG-1, a Critical Component of Nonsense-mediated mRNA Decay*

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Phosphatidylinositol 3-kinase-related kinases (PIKKs) consisting of SMG-1, ATM, ATR, DNA-PKcs, and mTOR are a family of proteins involved in the surveillance of gene expression in eukaryotic cells. They are involved in mechanisms responsible for genome stability, mRNA quality, and translation. They share a large N-terminal domain and a C-terminal FATC domain in addition to the unique serine/threonine protein kinase (PIKK) domain that is different from classical protein kinases. However, structure-function relationships of PIKKs remain unclear. Here we have focused on one of the PIKK members, SMG-1, which is involved in RNA surveillance, termed nonsense-mediated mRNA decay (NMD), to analyze the roles of conserved and SMG-1-specific sequences on the intrinsic kinase activity. Analyses of sets of point and deletion mutants of SMG-1 in a purified system and intact cells revealed that the long N-terminal region and the conserved leucine in the FATC domain were essential for SMG-1 kinase activity. However, the conserved tryptophan in the TOR-SMG-1 (TS) homology domain and the FATC domain was not. In addition, the long insertion region between PIKK and FATC domains was not essential for SMG-1 kinase activity. These results indicated an unexpected feature of SMG-1, i.e. that distantly located N- and C-terminal sequences were essential for the intrinsic kinase activity.

SMG-1 was first identified as a suppressor of morphogenetic effect on genitalia-1 in Caenorhabditis elegans. It is one of the critical components of the RNA surveillance pathway, termed nonsense-mediated mRNA decay (NMD), which is conserved from worm to human (1–4). NMD mediates rapid degradation of mRNAs bearing premature termination codons generated by genome mutations and by errors that occur during processes including transcription and splicing (5). NMD removes aberrant mRNAs containing premature termination codons from cells, thereby protecting them from accumulation of non-functional or potentially harmful truncated proteins encoded by aberrant mRNAs. In mammals, SMG-1 directly phosphorylates Upf1, a central component of NMD, at the serine/threonine-glutamine-rich ((S/T)-Q-rich) motif in the C terminus of Upf1, followed by dephosphorylation by protein phosphatase 2A (PP2A) (3, 6, 7). This phosphorylation/dephosphorylation cycle of Upf1 is essential to promoting NMD. In addition to RNA surveillance, a recent study suggested the possible involvement of SMG-1 in genome surveillance in mammalian cells (8).

SMG-1 is the newest member of the family of phosphatidylinositol 3-kinase (PI3K)-related protein kinases (PIKKs), which includes mammalian target of rapamycin (mTOR), ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) in mammalian cells. PIKKs have intrinsic serine/threonine kinase activity and are distinguished from other kinases by their unique catalytic domain (PIKK domain) similar to lipid PI3K catalytic domain, and their huge molecular weight (270–470 kDa) (9–11). SMG-1, ATM, ATR, and DNA-PKcs are also termed (S/T)-Q-directed kinases, based on their strong preference for phosphorylating serine/threonine followed by a glutamine (3, 12). PIKKs function in diverse cellular processes, including genome surveillance (ATM, ATR, and DNA-PKcs) (9, 11), mRNA surveillance (SMG-1) (1, 4), and translation control (mTOR) (10). PIKKs are also characterized by their highly conserved C-terminal region known as the FATC (FRAP, ATM, TRRAP, C-terminal) domain (13). Except for SMG-1, the FATC domain is located nearby the PIKK domain and is critical for basal kinase activities of mTOR and DNA-PKcs (14–16) or the activation of ATM by extracellular stimulation (17). Although sequences are quite different among family members, the N-terminal region of PIKKs, including that of Rad3-related; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; FRAP, FKBP-rapamycin-associated protein; TRRAP, transformation/transcription domain-associated protein; FATC, FRAP ATM TRRAP C-terminal; OCR, one-specific conserved region; WT, wild type; GST, glutathione S-transferase; BSA, bovine serum albumin; PI3K, phosphatidylinositol 3-kinase; HEK, human embryonic kidney; siRNA, small interfering RNA; CBB, Coomassie Brilliant Blue.
SMG-1, seems to have a similar structure. Computational and three-dimensional analyses predicted that N-terminal regions of DNA-PKcs, ATM, ATR, and mTOR contained many helical repeats such as HEAT (huntingtin, elongation factor 3, A subunit of PP2A, TOR1) repeat, TPR (tetratricopeptide repeat), and ARM (armadillo) repeats (18, 19).

In addition to common conserved features in PIKKs, SMG-1 has unique sequences. The TOR SMG-1 homology (TS) domain, which we renamed from FRBH (FKBP-rapamycin binding homology) domain of mTOR (supplemental Fig. IA) (3). However, there is no evidence suggesting that SMG-1 interacts with the FKBP-rapamycin complex (2, 3). Furthermore, the N-terminal region of SMG-1 shows unique conserved sequences (one-specific conserved region, OCR) among species (OCR1–OCR3) (supplemental Fig. IB). In addition, human SMG-1 has a quite large insertion sequence between the PIKK domain and the FATC domain (supplemental Fig. IA) (1, 3, 20). Although these features suggest involvement of these unique sequences in the activity of SMG-1, structure-function relationships of SMG-1 remain completely unknown.

In this study, we investigated the structure–activity relationships of human SMG-1. For this purpose, we constructed a variety of point and deletion mutants and evaluated their kinase activities both in vitro and in vivo. Our analysis revealed that the FATC domain was important for SMG-1 kinase activity, and the essential amino acid residue for SMG-1 kinase activity was different from that of mTOR, but another conserved amino acid was required. Surprisingly, the N terminus of SMG-1, which is far from the catalytic domain, strongly affected SMG-1 kinase activity. On the other hand, the long insertion region between the PIKK domain and the FATC domain was not critical for SMG-1 kinase activity. Taken together, this first comprehensive analysis of relationships of kinase activity and structural features of SMG-1 suggested that SMG-1 activity was provided through both the N-terminal region and FATC domains.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The SR vector based on FLAG-SMG-1 wild type (WT) has been described previously (7). FLAG-SMG-1 D2331A encoding a kinase-dead mutant of SMG-1 was created by subcloning His-SMG-1 D2331A (3) digested with Apal/Sall into FLAG-SMG WT digested with Apal/Xhol. For FLAG-SMG-1 L3646A and W3653A, BgIII/BamHI fragments encoding the C-terminal region of SMG-1 were subcloned into pBluescript, and point mutations were inserted by site-directed mutagenesis using the QuikChange kit following the manufacturer’s instructions (Stratagene). FLAG-SMG-1 WT was digested with Xhol/Nhel to remove the FATC domain, and the Xhol/Nhel fragment of subcloned plasmids containing the mutation was inserted into it. For FLAG-SMG-1 W1962F, Kpnl/EcoRI fragment encoding the TS domain of SMG-1 was subcloned into pBluescript, and point mutations were inserted by site-directed mutagenesis, as described above. FLAG-SMG-1 WT was digested with EcoRV, and the EcoRV fragment of the subcloned plasmid containing the mutation was inserted into it. For FLAG-SMG-1-(Δ1–152), -(Δ1–617), -(Δ1–800), -(Δ1–998), -(Δ1–1377), -(Δ1–1478), -(Δ1–1708), -(Δ1–1859), and -(Δ1–2066): Smal/Xhol (Δ1–152), Spel/Xhol (Δ1–617), Eco122I/Xhol (Δ1–800), HindIII/Xhol (Δ1–998), Apal/Xhol (Δ1–1377), EcoRV/Xhol (Δ1–1478), Kpnl/Xhol (Δ1–1708), or Dral/Xhol (Δ1–152) fragments from FLAG-SMG-1 WT were inserted into SR-FLAG vectors. For Δ631–649, Δ631–679, Δ631–791, and Δ746–791: the SpeI fragment encoding the OCR1 of SMG-1 was subcloned into pBluescript, and PCR-based deletion mutants were constructed using the primers for Δ631–649 (5’-TAGCGCCACATCCTATTA-3’ and 5’-GCTGTTCTACCTTCGGCATT-3’); Δ631–679 (5’-TAGCGCCACATCCTATTA-3’ and 5’-TTTATCTCTAGACCTAGTT-3’); Δ631–791 (5’-TAGAGATGTGTCGATTGTCG-3’); and Δ746–791 (5’-TAGAGATGTGTCGATTGTCG-3’). The SpeI fragment of subcloned and deleted plasmids was inserted into FLAG-SMG-1 WT digested with SpeI. For Δ994–999, the SpeI fragment encoding OCR2 was subcloned into pBluescript and digested by EcoT22I and self-ligated. Afterward, the BstXI fragment of deleted subcloned plasmids was inserted into SR-FLAG-SMG-1 WT digested with BstXI. For Δ912–967 and Δ912–1014, the SpeI/HindIII fragment encoding OCR2 was subcloned into pBluescript, and PCR-based deletion mutants were constructed using the primers for Δ912–967 (5’-AGATGCTGTCTTTGGCAG-3’ and 5’-AGATGCTGTCTTTGGCAG-3’); Δ912–1014 (5’-AGATGCTGTCTTTGGCAG-3’ and 5’-AATGCAGAAAATCCAGGAC-3’). Thereafter, the BstXI fragment of deleted subcloned plasmids was inserted into SR-FLAG-SMG-1 WT digested with BstXI. For Δ1375–1477, FLAG-SMG-1 WT was digested by Apal/HindIII, blunt-ended, and self-ligated, and for Δ1675–1708, FLAG-SMG-1 WT was digested by Eco52I and EcoRV, and also blunt-ended, and self-ligated. For Δ2771–3254, Δ2771–3359, Δ2513–3490, and Δ2513–3579, the BgIII/BamHI fragment encoding the C-terminal and 3’UTR regions of SMG-1 were subcloned into pBluescript, and PCR-based deletion mutants were constructed using the primers for Δ2771–3254 (5’-GGTACAAAGGGCGAAGAAATA-3’ and 5’-GGTACAAAGGGCGAAGAAATA-3’); Δ2771–3359 (5’-GGTACAAAGGGCGAAGAAATA-3’ and 5’-ACTGGTGCTTGAACATCCTATT-3’); Δ2513–3579 (5’-TATTTCCTCACGATGTTTGGCCCC-3’ and 5’-ACCCTGGAAACTGAAAC-3’); Δ2513–3579 (5’-AGATGCTGTCTTTGGCAG-3’). Thereafter, the EcoRI/Xhol fragments of the subcloned and deletion plasmid were inserted into FLAG-SMG-1 WT EcoRI/Xhol-digested plasmid.

**Cell Culture and Transfection**—HEK293T cells and HeLa TetOff (Clontech) cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics and were incubated at 37 °C with 5% CO2. For the in vitro kinase assay experiments, HEK293T cells (2 × 106 cells for the in vitro kinase assay and 1 × 106 cells for the kinase amount-dependent in vitro kinase assay) were transfected with the indicated FLAG-SMG-1 mutant plasmids using Polyfect (Qiagen) or FuGENE 6 (Roche Applied Science), and cells were collected after 36–48 h.

Depletion of endogenous proteins by RNA interference was carried out by using siRNAs as described by Usuki et al. (21),
except the transfection reagents. The following target sequences were used: non-silencing (5'-AAUUCUCCGAAC-GUGUCAGU-3') and SMG-1 3' UTR (5'-GGAAAGAUUU-GUGCAUUCATT-3'). Non-silencing siRNAs that were guaranteed to show no gene silencing activity were commercially prepared by Qiagen. HeLa TetOff cells were transfected with small interfering (siRNA) duplexes (as above) at a final concentration of 70 nM using RNAiFect (Qiagen). At 36 h post-transfection, the cells were trypsinized and reseeded in 12-well plates. After a further 24 h, the cells were transfected with the same siRNAs together with 1 μg of the indicated FLAG-SMG-1 constructs using Lipofectamine 2000 (Invitrogen). The cells were harvested 48 h after the second transfection.

**Protein Detection and Quantification**—Samples were separated by 5.5–12% SDS-PAGE. Western blotting was performed using the indicated antibodies with a standard ECL system (GE Biosciences). Anti-SMG-1, Upf1, phospho-Upf1 (3B8), SMG-7 antibodies were generated as done previously (3, 6), and the anti-FLAG M2 antibody was purchased from Sigma. Coomassie Brilliant Blue (CBB) staining was performed according to standard procedures. To measure the amounts of immunopurified mutant proteins from ~150 to 430 kDa, silver staining was used, because Western blotting efficiency was largely different for each molecular weight of mutants (supplemental Fig. 2). Silver staining was performed using the Silver Quest silver staining kit (Invitrogen) following the manufacturer’s protocols. Signals were scanned by LAS 3000 (Fuji film) and quantified by MultiGauge (Fuji film). Phosphoproteins were detected by autoradiography using BAS 2500 (Fuji film), and signals were quantified as described above.

**In Vitro Kinase Assay**—FLAG-SMG-1 proteins were immunopurified from transiently transfected HEK293T cells. Cells were lysed in lysis buffer F (20 mM Tris–HCl at pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 20 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM NaF, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 100 mM okadaic acid (Calbiochem), and protease inhibitor mixture (Sigma)), and FLAG-SMG-1 proteins were collected on anti-FLAG affinity gels (Sigma). Immunoprecipitates were washed four times in lysis buffer F and twice in kinase reaction buffer (10 mM Hepes–KOH at pH 7.5, 50 mM β-glycerophosphate, 50 mM NaCl, 1 mM dithiothreitol, and 10 mM MnCl2). Kinase reactions were performed for 15 min at 30 °C in 50-μL kinase reaction mixtures (kinase reaction buffer containing 5 μg of substrate, 5 μM ATP, and 5 μCi of [γ-32P]ATP). As substrate, GST or GST fusion peptides containing Upf1 serine 1078 (3) were used. To measure the kinase amount shown in Fig. 1, immunopurified FLAG-tagged mutants were eluted twice with 50 μl of kinase reaction buffer containing 1 mg/ml FLAG peptide for 30 min at 4 °C. The eluted proteins and BSA (as control) were separated by 7% SDS-PAGE and visualized by silver staining. Amounts of eluted proteins were calculated from a BSA standard curve, and eluted proteins were used in an in vitro kinase assay, as described above.

To detect phosphorylated substrate, the reaction products were separated by 12% polyacrylamide gels, and the gels were stained with CBB. The gels were then dried, phosphoproteins were detected by autoradiography, and signals were quantified as described above. To measure the amount of SMG-1 proteins, the same reaction products were separated by 5.5% polyacrylamide gels, and Western blotting (Figs. 2 and 4B) or silver staining (Figs. 1, 3, and 4A) was performed to detect SMG-1 proteins. After that, the membranes or stained gels were subjected to autoradiography to measure autophosphorylation. The kinase activity or the autophosphorylation levels of SMG-1 proteins in Figs. 2–4 was corrected by phosphorylated signals of substrates or SMG-1 proteins themselves divided by the number of moles of SMG-1 proteins measured as described above. Statistical analysis was performed by using Student’s t test.

**RESULTS**

The FATC Domain Is Essential for SMG-1 Kinase Activity—To evaluate the intrinsic kinase activity of SMG-1, we immunopurified recombinant SMG-1 from HEK293T cells transfected with a SMG-1 expression construct, and in vitro kinase activity was evaluated using GST–Upf1 WT (1072–1085) (3) as a substrate. Previous reports showed that SMG-1 associated with other NMD-related proteins such as Upf1 and SMG-7 (3, 7), but immunopurified recombinant SMG-1 did not show any signs of contaminations in silver staining (Fig. 1B, lanes 6–10) and Western blotting (data not shown), indicating the high purity of the enzyme. As shown in Fig. 1, C and D, substrate phosphorylation depended on the amount of FLAG-SMG-1 WT. Furthermore, the fact that FLAG-SMG-1 D2331A, a kinase inactive point mutant in the PIKK catalytic domain (3), did not have any detectable kinase activity supported the validity of the assay. Similar results were also obtained when phosphate incorporation into SMG-1 was evaluated (data not shown). To simplify the assay system, we also performed a kinase assay without elution of FLAG-SMG-1 proteins (see “Experimental Procedures”) (Fig. 2).

In PIKKs, several point mutations in the region other than the PIKK catalytic domain affect their kinase activity (14–16,22). Therefore, we first tested point mutants in which conserved amino acids between SMG-1 and PIKKs were changed using the above kinase assay system. We constructed the W1962F point mutant in which tryptophan at residue 1962 in the TS domain was replaced with phenylalanine. The corresponding residue in mTOR (Trp-2027) was critical for kinase activity (supplemental Fig. 1A) (22); however, FLAG-SMG-1 W1962F retained kinase activity and autophosphorylation, and kinase activity was ~50% compared with FLAG-SMG-1 WT (Fig. 1, C and D, and Fig. 2). This suggested that tryptophan 1962 in the TS domain was not critical for SMG-1 kinase activity, which was different from the results for mTOR.

The next set of point mutants that we used were point mutants (L3646A and W3653F) in the FATC domain, in which conserved leucine or tryptophan between FATC domains in PIKKs were substituted with alanine or phenylalanine essential for the basal/regulatory kinase activity of mTOR (16) (supplemental Fig. 1A). The L3646A mutant showed greatly reduced kinase activity (7.9% compared with WT). On the other hand, the W3653F mutant retained significant activity (50% compared with WT) (Fig. 1, C and D, and Fig. 2B, black bar). Importantly, similar results were obtained with a different substrate (GST-p5311–30) (data not shown), and with SMG-1

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autophosphorylation (Fig. 2, white bar.) These results suggested that mutation at the conserved FATC domain strongly affected SMG-1 activity and that the critical amino acid for kinase activity in SMG-1 was different from those of mTOR and ATM. Because data for kinase activity with or without the elution step were almost similar to each other (Figs. 1D and 2B), we thereafter performed a kinase assay without an elution step.

The Long Insertion Region between the PIKK Domain and the FATC Domain Is Not Essential for SMG-1 Kinase Activity—Human SMG-1 contains a unique extraordinarily large (1182 amino acids) insertion region between the PIKK domain and the FATC domain (Fig. 1A and supplemental Fig. 1A) in contrast to the other PIKKs. Distant FATC domain was essential for its kinase activity as shown above. However, the role of the insertion region remained unknown. Next, we developed deletion mutants FLAG-SMG-1- (ΔH90042771–3254), - (ΔH90042771–3359), - (ΔH90042513–3490), and - (ΔH90042513–3579) lacking different lengths (amino acids 484–1067) of insertion regions. Although ~40–90% of insertion regions were deleted in these mutants, all retained their kinase activity (Fig. 3, black bar, and supplemental Fig. 2A), and activities were ~32–52% of WT. However, there was no correlation between the remaining length of the insertion region and kinase activity. We also measured kinase activity for a different substrate, GST-p5311–30, and kinase activities of all mutants for GST-p53 11–30 were similar to that for GST-Upf11072–1085 (data not shown).

Interestingly, the autophosphorylation level of SMG-1 (ΔH90042513–3490) was ~4-fold higher than that of WT (Fig. 3, white bar, and supplemental Fig. 2A). These results indicated that the insertion region unique to human SMG-1 was not essential but was involved in kinase activity.

N-terminal SMG-1 Conserved Regions Are Necessary for Kinase Activity—The N-terminal half of SMG-1 does not seem to contain any distinct domains but is predicted to contain many helical repeats (supplemental Fig. 1B). To evaluate sequences in the N-terminal region of SMG-1 required for kinase activity, we first developed N-terminal deletion mutants FLAG-SMG-1- (Δ1–152), - (Δ1–617), - (Δ1–800), - (Δ1–998), - (Δ1–1375), - (Δ1–1475), - (Δ1–1707), - (Δ1–1858), and - (Δ1–2067) and measured their kinase activities, as...
Intriguingly, all of the mutants (except for FLAG-SMG-1-(H90041–152)) showed a great reduction in kinase activity. Only FLAG-SMG-1-(H90041–152) retained 60% kinase activity compared with WT. Similar experiments using a different substrate (GST-p5311–30) gave very similar results to those obtained for GST-Upf11072–1085 (data not shown). Furthermore, mutants (except for FLAG-SMG-1-(H90041–617)) showed no significant autophosphorylation (0.02–0.3%) (see “Discussion”) (Fig. 4A, white bar, and supplemental Fig. 2B). These results indicated that the end of the N-terminal region of SMG-1 containing OCR1 was extremely important for kinase activity.

To further investigate the region essential for kinase activity within the N-terminal region of SMG-1, we constructed a set of internal deletion mutants: FLAG-SMG-1-(H9004631–649), -(H9004631–679), -(H9004631–791), -(H9004746–791), -(H9004912–967), -(H9004912–1014), -(H90041375–1477), and -(H90041675–1708). These constructs were expressed in HEK293T cells, but expression of FLAG-SMG-1-(H90041631–649), -(H9004631–679), -(H9004912–967), and -(H90041675–1708) tended to be lower than that of WT (data not shown). Unexpectedly, only FLAG-SMG-1-(H90041675–1708) retained significant kinase activity similar to WT, and other mutants showed greatly reduced activity (Fig. 4B, black bar).

FLAG-SMG-1-(H9004631–649), with only 19 deleted amino acids in OCR1, and FLAG-SMG-1-(H9004912–999), with only 6 amino acids in OCR2, showed kinase activity of ~8 and 13% of WT, respectively. Similar results were obtained for autophosphorylations (Fig. 4B, white bar). Taken together, these results indicated that the N-terminal half of SMG-1 was generally essential for kinase activity.

In Vivo Kinase Activity of SMG-1 Mutants—We examined whether the sequence required for intrinsic kinase activity of SMG-1, as described above, was required for phosphorylation of Upf1 in vivo. For this purpose, we established an in vivo assay system to evaluate phosphorylation of endogenous Upf1 by exogenous FLAG-SMG-1 protein in HeLa TetOff cells. We cotransfected siRNAs targeted to the 3’UTR of SMG-1 (21) and FLAG-SMG-1 WT, which do not have SMG-1 3’UTR, into HeLa TetOff cells. As shown in Fig. 5, knock down of endogenous SMG-1 resulted in suppression of Upf1 phosphorylation to 32% of that in control cells transfected with control siRNA (Fig. 5, lanes 1 and 2). Importantly, cotransfection of siRNAs and FLAG-SMG-1 WT restored Upf1 phosphorylation at 70%, whereas coexpression of kinase-dead mutant FLAG-SMG-1 D2331A did not (Fig. 5, lanes 3 and 4), indicating that this system could be used to evaluate kinase activity of exogenous SMG-1 against endogenous Upf1 in vivo. Thus, we used this assay system to
evaluate the activities of some characteristic SMG-1 mutants, i.e. FLAG-SMG-1-(Δ1–617), -(Δ631–649), -(Δ746–791), -(Δ912–1014), -(Δ1375–1477), and -(Δ1675–1708). Consistent with in vitro kinase activity assays, the FLAG-SMG-1-(Δ1675–1708) mutant that retained kinase activity in vitro did rescue Upf1 phosphorylation (Fig. 5, lanes 9). On the other hand, FLAG-SMG-1-(Δ1–617), -(Δ631–649), -(Δ746–791), -(Δ912–1014), and -(Δ1375–1477), which have little or no kinase activity in vitro, did not restore sufficient Upf1 phosphorylation in vivo (Fig. 5, lanes 5–8 and 10). These results showed that SMG-1 mutants that retained kinase activity in vitro corresponded to SMG-1 mutants that kept kinase activity in vivo.

The N-terminal End of SMG-1 Is Required for the Interaction with SMG-7.—To evaluate the role of the N-terminal region of SMG-1, we next examined the interaction between SMG-1 and its known partner proteins Upf1, Upf2, and SMG-7 (7). Upf1 can independently and directly interact with both the N-terminal half (amino acids 1–2223) and C-terminal half (amino acids 2068–3657) of SMG-1, whereas Upf2 can interact directly with the N-terminal region (amino acids 1–617) and C-terminal region (amino acids 1523–2513) of SMG-1, whereas Upf2 can interact directly with both the N-terminal and C-terminal regions of SMG-1 (Fig. 3). These results showed that SMG-1 mutants that retained kinase activity in vitro corresponded to SMG-1 mutants that kept kinase activity in vivo.

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FIGURE 3. The insertion region between the PIKK and FATC domains of SMG-1 is not critical for kinase activity. Kinase activities of the FLAG-SMG-1 insertion region-deleted mutants. The left panel is a schematic structure of deletion mutants. The right panel represents the kinase activity levels of deletion mutants from an in vitro kinase assay, as described in the legend to Fig. 2. Recombinant SMG-1 levels were determined by silver staining, and substrates were detected by autoradiography followed by CBB staining. The black bars indicate the kinase activity of SMG-1 proteins for GST-Upf11072–1085, and the white bars indicate autophosphorylation levels of SMG-1 proteins. Values represent mean ± S.D. from three independent trials. aa, amino acid(s).

We next examined the interaction between SMG-1 and SMG-7. As for SMG-7, we measured the interaction of overexpressed SMG-1 mutants and endogenous SMG-7, as we have an excellent antibody to SMG-7 (6). Interestingly, the interaction with SMG-7 was reduced in FLAG-SMG-1-(Δ1–152), and further reduction of the interaction was observed in FLAG-SMG-1-(Δ1–617); however, other mutants (-(Δ912–1014), -(Δ1675–1708), and -(Δ2513–3580) did not change the affinity for SMG-7 (Fig. 6). This result indicated that the N-terminal end of SMG-1 is required for the interaction with SMG-7.

DISCUSSION

Analyses of kinase activity of SMG-1 mutants in vitro and in vivo revealed the specific structural features of SMG-1 and the common conserved features for PIKKs.

First, we found that a single point mutation (L3646A) in the FATC domain strongly affected SMG-1 kinase activity (Fig. 1, C and D, and Fig. 2), suggesting that the FATC domain probably locates close to the catalytic domain spatially. Furthermore, the fact that the large insertion region between the PIKK domain and the FATC domain was not critical for SMG-1 kinase activity in vitro (Fig. 3) may indicate that the large insertion region is not critical for the orientation between the PIKK and the FATC domains. This is consistent with the fact that lengths of insertion regions of SMG-1 are not conserved among species (supplemental Fig. 1A) (1, 3, 20). The substrate-induced conformational change between the PIKK and FATC domains was also suggested for the case of DNA-PKcs based on the electron microscopy observations (23, 24). Other reports show that the FATC domain is necessary for basal kinase activity in mTOR and DNA-PKcs, although not in ATM (15–17). A drastic effect of the single amino acid mutation in the FATC domain of SMG-1 on an intrinsic kinase activity of SMG-1 suggests that the role of the FATC domain for SMG-1 kinase activity is similar to those for mTOR and DNA-PKcs. Among the conserved amino acids in the FATC domains of PIKKs, Leu-3646 was essential for SMG-1 kinase activity, but Trp-3653 was not (Figs. 1 and 2). The L3646A mutation of SMG-1 might disrupt the folding of the FATC domain itself, because the corresponding leucine in yeast TOR1 (Leu-2459) is required to make the correct folding of the FATC domain together with other hydrophobic and aromatic residues (25). The essential role of corresponding leucine in ATM (Leu-3045) for its ionized radiation-inducing activation (17) suggests that the possible contribution of Leu-3646 resides in the regulation of SMG-1 kinase activity by extracellular stimulation, such as ionized radiation (8), besides basal kinase activity. Another conserved amino acid
FIGURE 4. The N-terminal region of SMG-1 is essential for kinase activity. A, kinase activities of FLAG-SMG-1 sequential N-terminal deletion mutants. The left panel is a schematic structure of deletion mutants. The right panel represents the kinase activity levels of deletion mutants from an in vitro kinase assay, as described in the legend to Fig. 2. Recombinant SMG-1 levels were determined by silver staining, and substrates were detected by autoradiography followed by CBB staining. Values represent mean ± S.D. from three independent trials. B, kinase activities of FLAG-SMG-1 mutants that lack short amino acid sequences in the N-terminal region. The left panel is a schematic structure of deletion mutants, and the right panel represents the kinase activity levels of deletion mutants from an in vitro kinase assay, as described above. Recombinant SMG-1–1 levels were determined by immunoblotting, and substrates were detected as described above. The black bars indicate the kinase activity of SMG-1 proteins for GST-Upf11072–1085, and the white bars indicate autophosphorylation levels of SMG-1 proteins. Values represent mean ± S.D. from three independent trials. aa, amino acid(s).
(Trp-1962) in the TS domain weakly affected SMG-1 kinase activity (Fig. 2), whereas the corresponding amino acid in mTOR, i.e., Trp-2027, was critical for kinase activity (22). There is no evidence that SMG-1 interacts with FKBP12-rapamycin-binding complex, which interacts with mTOR in the TS domain; however, the TS domain might regulate SMG-1 kinase activity similar to mTOR by binding to other unknown proteins or lipids.

Second, we showed that N-terminal sequences were required for the kinase activity of SMG-1. The Δ1–617 mutant, which lacks most of OCR1, showed significantly reduced kinase activity compared with WT (Fig. 4A). Although the Δ1–617 mutant, which contains all sequences of the isoform as reported by Dunn et al. and Brumbaugh et al. (amino acids 626–3657; 340 kDa) (2, 8), showed detectable kinase activity, its autophosphorylation activity was only 4.5% of WT and was normalized against total Upf1 and graphed. Values represent mean ± S.D. from three independent trials.

There is no evidence that SMG-1 interacts with FKBP12-rapamycin-binding complex, which interacts with mTOR in the TS domain; however, the TS domain might regulate SMG-1 kinase activity similar to mTOR by binding to other unknown proteins or lipids.

Detailed truncation and internal deletion analyses of the N-terminal region of SMG-1 revealed that the whole N-terminal region was required for SMG-1 kinase activity. N-terminal halves of PIKKs comprise tandem helical repeats (supplemental Fig. 1B) (18, 19), and these helical repeats mediate interactions with distinct binding partners (26–28). However, there is little evidence whether this region is involved in the intrinsic kinase activity of PIKKs. N-terminal truncation analyses of mTOR and ATM showed that deletion of >1000 amino acids far from the catalytic domain, containing tandem helical repeats, did not seriously affect kinase activity (22, 29). However, in SMG-1, a small deletion in the N-terminal region strongly affected kinase activity in vitro and in vivo (Fig. 4). Because immunopurified SMG-1 proteins used for the kinase assay showed no signs of contamination by other proteins (Fig. 1B), the cause of inactivation observed for many of the SMG-1 mutants did not seem to involve failure in association with unknown accessory proteins. Therefore, we suggest that the N-terminal region of SMG-1 is required for maintaining the proper conformation of the catalytic PIKK domain. Note that all of the mutants tested, except for Δ1–152 and Δ1–617, could associate with known direct partner proteins Upf1, Upf2, and SMG-7, supporting the idea that they retain the ability to interact with these proteins.

Three-dimensional analyses of DNA-PKcs also revealed that the N-terminal regions of DNA-PKcs were located near the

**FIGURE 5.** In vivo Upf1 phosphorylation activity of SMG-1 mutants. A, HeLa TetOff cells transfected with siRNA against SMG-1 3’UTR together with the indicated FLAG-SMG-1 mutants were separated by 6% SDS-PAGE, and FLAG-SMG-1 mutants, endogenous Upf1, and phosphorylated Upf1 were detected by Western blotting. Black and white arrowheads indicate endogenous SMG-1 isoforms of 430 and 400 kDa, respectively. Asterisks indicate exogenous FLAG-SMG-1 proteins. B, amount of phosphorylated Upf1 was normalized against total Upf1 and graphed. Values represent mean ± S.D. from three independent trials.

**FIGURE 6.** Interaction of FLAG-SMG-1 mutants with SMG-7. HEK293T cells expressing the indicated FLAG-SMG-1 mutants were lysed with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.4% Nonidet-P40, 200 μg/ml RNase A, 100 mM okadaic acid, and protease inhibitors and were immunoprecipitated (IP) with anti-FLAG antibody. After the immunoprecipitates were separated by 7% SDS-PAGE, FLAG-SMG-1 mutants and endogenous SMG-7 (short isoform and long isoform) were detected by Western blotting using anti-FLAG and anti-SMG-7 antibodies.
C-terminal region containing the PIKK domain and the FATC domain (23, 24, 30), and this conformation changed in response to binding to the DNA and Ku subunit. In addition, the x-ray crystal structure of the catalytic subunit of the class IB γ isoform of PI3K (PI3Kγ), whose catalytic domain is similar to PIKK domain, revealed that the helical domain akin to the HEAT repeat provides a core scaffold, the surface of which interacts with other domains including catalytic domain (31, 32). In this case, such a folding manner makes an N-terminal Ras-binding domain, which is distantly positioned from the catalytic domain in the amino acid sequence position, a close contact to the catalytic domain, enabling a Ras-induced PI3K activation. A mutation of the helical domain of the class Ia δ isoform of PI3K (PI3Kδ), the structure of which is similar to PI3Kγ, affects the kinase activity of PI3Kδ (33). Taken together with our biochemical study and the three-dimensional analyses of DNA-PKcs and PI3K, we suggest that the N-terminal region of SMG-1 would locate near the catalytic domain, and such specific conformation might be required for an intrinsic kinase activity of SMG-1. The catalytic domains of most protein kinases transit between an active closed state and an inactive open state, which are formed by the N- and C-terminal lobes of the catalytic domain (34–36). We suggest that the N-terminal region of SMG-1 would modulate the folding structure of the catalytic domain. Our findings that the N-terminal region is required for SMG-7 and presumably for Upf1 bindings support the notion that the large N-terminal domain forms a large scaffold for other accessory proteins and regulates the catalytic activity of SMG-1. SMG-7 is required for recruiting the SMG-5-SMG-7-PP2A complex to the phosphorylated Upf1 (6). Our recent study has also revealed that SMG-7 can interact with SMG-1-Upf1 complex in the absence of the phosphorylation of Upf1 (7). Thus, it is possible that SMG-7 can have dual activities, recruitment of PP2A phosphatase complex to phosphorylated Upf1, and suppression of SMG-1 catalytic activity through the interaction with the N-terminal domain.

A recent study (37) reports that the N-terminal region of mTOR is required for multimerization in response to nutrients. We also observed multimerization of SMG-1 via both the N- and C-terminal regions (supplemental Fig. 4, A and C). However, this multimerization became undetectable in the presence of 1% Triton X-100 (supplemental Fig. 4B), a similar condition for our immunopurification and kinase assays. Thus, multimerization may not be related to SMG-1 kinase activity as far as our assay conditions were concerned, although we could not exclude the possibility that multimerization might regulate SMG-1 kinase activity or affinity to other proteins in vivo. Although autophosphorylations of ATM and DNA-PKcs have been reported to be involved in regulation of their activities (9, 38, 39), a role of autophosphorylation of SMG-1 observed in this study still remains unclear, because accelerated autophosphorylation of Δ2513–3490 did not significantly affect the kinase activity.

We found that all mutants tested for Upf1 phosphorylation in vitro showed kinase activity in vitro (Fig. 5). We could not compare the kinase activity between in vivo and in vitro accurately, because the amount of SMG-1 mutants in Fig. 5 could not be measured accurately due to the difference of molecular weight, although purified SMG-1 proteins could be measured in an in vitro kinase assay (Figs. 1–4). However, the mutants, which possessed kinase activity in vitro, retained the ability to phosphorylate Upf1 more than the kinase-inactive mutant did. We failed to obtain SMG-1 mutants with intrinsic kinase activity but without interaction with other essential components. For example, Upf2 binds to the C-terminal region of SMG-1 (7), and our previous study established that in vivo phosphorylation of Upf1 required interaction between SMG-1 and Upf2 (7). However, all mutants retained interaction with Upf2 (supplemental Fig. 3C).

In conclusion, our extensive mutational analysis of SMG-1 revealed a novel aspect into nature of the basal kinase activity of SMG-1, the newest member of the PIKK family. Characterization of SMG-1 kinase activity will provide important clues for understanding the regulation of SMG-1 kinase activity and mRNA surveillance.

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