K-Ras Lys-42 is crucial for its signaling, cell migration, and invasion

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Ras proteins participate in multiple signal cascades, regulating crucial cellular processes, including cell survival, proliferation, and differentiation. We have previously reported that Ras proteins are modified by sumoylation and that Lys-42 plays an important role in mediating the modification. In the current study, we further investigated the role of Lys-42 in regulating cellular activities of K-Ras. Inducible expression of K-RasV12 led to the activation of downstream components, including c-RAF, MEK1, and extracellular signal–regulated kinases (ERKs), whereas expression of K-RasV12/R42 mutant compromised the activation of the RAF/MEK/ERK signaling axis. Expression of K-RasV12/R42 also led to reduced phosphorylation of several other protein kinases, including c-Jun N-terminal kinase (JNK), Chk2, and focal adhesion kinase (FAK). Significantly, K-RasV12/R42 expression inhibited cellular migration and invasion in vitro in multiple cell lines, including transformed pancreatic cells. Given that K-Ras plays a crucial role in mediating oncogenesis in the pancreas, we treated transformed pancreatic cells of both BxPC-3 and MiaPaCa-2 with 2-D08, a small ubiquitin-like modifier (SUMO) E2 inhibitor. Treatment with the compound inhibited cell migration in a concentration-dependent manner, which was correlated with a reduced level of K-Ras sumoylation. Moreover, 2-D08 suppressed expression of ZEB1 (a mesenchymal cell marker) with concomitant induction of ZO-1 (an epithelial cell marker). Combined, our studies strongly suggest that posttranslational modification(s), including sumoylation mediated by Lys-42, plays a crucial role in K-Ras activities in vivo.

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2 The abbreviations used are: MEK, mitogen-activated protein kinase/extracellular signal–regulated kinase; ERK, extracellular signal–regulated kinase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; Dox, doxycycline; PI3K, phosphatidylinositol 3-kinase; SUMO, small ubiquitin-like modifier; EMT, epithelial and mesenchymal transition.
Function of K-Ras Lys-42

We have previously observed that Lys-42 mediates sumoylation of Ras proteins, which appears to be important for their activity (20). Lys-42 is located between switch I (amino acids 32–38) and II (amino acids 59–67) domains that mediate the interaction with its regulators and effectors (Fig. 1A). Thus, we speculate that Lys-42 mutation would have an impact on Ras functions in vivo. To further determine whether Lys-42 was crucial for promoting Ras activation and its downstream signaling, K-RasV12 and K-RasV12/R42 expression was subjected to induction by doxycycline (Dox). We observed that inducible expression of K-RasV12 was strongly associated with the activation of downstream components, including c-Raf, MEK, and ERK, and that the activation of downstream signaling components was K-Ras concentration–dependent (Fig. 1B). On the other hand, expression of K-RasV12/R42 greatly suppressed the activation of c-Raf, MEK1, and ERKs, albeit its expression was comparable with that of K-RasV12 after the addition of Dox, strongly supporting the notion that Lys-42 is crucial for the activation of Ras as well as its major signaling cascade of RAF/MEK/ERK. Because Ras is known to interact with PI3K (21), we measured the activation kinetics of AKT after induced expression of K-RasV12 and K-RasV12/R42. Intriguingly, AKT activation was not affected in cells expressing K-RasV12/R42 as compared with those expressing oncogenic K-RasV12.

To determine the mechanism(s) by which Lys-42 mediates K-Ras signaling, we determined whether it may be involved in regulating protein-protein interaction. HEK293 cells were transfected with plasmids expressing FLAG-tagged K-RasV12 or K-RasV12/R42 for 24 h. We also co-transfected the cells with or without SENP1 expression plasmid (for an isopeptidase specific for removing SUMO moiety from protein substrates). Equal amounts of cell lysates from various transfectants were immunoprecipitated with the anti-FLAG antibody. FLAG immunoprecipitates, along with cell lysate inputs, were blotted with antibodies to c-Raf, MEK, and ERK. c-Raf, MEK, and ERK phosphorylation was reduced in cells expressing K-RasV12, suggesting that K-Ras sumoylation may also play a role in mediating Ras signaling.

Lys-42 affects several signaling pathways mediated by K-Ras

Given that Ras proteins are crucial for numerous biological functions, including cell proliferation, migration, and stress responses, we next examined whether K-RasV12/R42 expression affected signaling pathways other than the RAF/MEK/ERK signaling axis. Through the analysis of a kinase array, we observed that K-RasV12/R42 expression down-regulated phosphorylation/activity of a few well-studied protein kinases (Fig. 2A and B) and Figs. S1 and S2). For example, p38 and JNK phosphorylation was reduced in cells expressing K-RasV12/R42, suggesting that K-Ras sumoylation may also play a role in mediating stress responses. Moreover, K-RasV12/R42 also compromised phosphorylation of Chk2 and FAK, two protein kinases involved in DNA damage responses and cell adhesion, respectively.

K-RasV12/R42 suppresses cell migration and invasion

K-Ras oncogenic mutations (e.g., Val-12) occur early in carcinogenesis of major human malignancies, which is known to promote cell migration and invasion of cancer cells (18, 19). Because Lys-42 mutation greatly compromised Ras signaling, we measured whether expression of K-RasV12/R42 would affect cell migration promoted by the oncogenic counterpart in a conventional wound-healing assay. We observed that NIH3T3 cells transfected with FLAG-K-RasV12 displayed rapid closing of the wound gap due to active cell migration compared with that of
cells transfected with FLAG-K-RasV12/R42 or vector alone (Fig. 3, A and B), indicating that Lys-42 mutation significantly compromised its ability to promote cell migration. Expression of transfected K-Ras was efficient (Fig. 3C). To confirm that sumoylation plays a role in cell migration, we ectopically expressed K-RasV12 or K-RasV12/R42 in MCF7 cells. We observed that expression of K-RasV12/R42 mutant also suppressed MCF7 cell migration promoted by K-RasV12 (Fig. 3D and E). This suppression was associated with down-regulation of the MAPK signaling pathway coupled with reduced expression of Snail and Claudin-1 (Fig. 3F), two gene products involved in cell migration and epithelial and mesenchymal transition (EMT) (22). Expression of the K-RasV12/R42 mutant also somewhat affected cell proliferation, which did not seem to be associated with increased cell death or cell cycle arrest (Figs. S3 and S4).

Given the crucial role of K-Ras in tumor development in pancreas (23), we then performed wound-healing experiments using a pancreatic cell line (MiaPaCa-2). We transfected MiaPaCa-2 cells with K-Ras and various mutant constructs. We observed that expression of WT K-Ras significantly stimulated cell migration, which was further promoted by Val-12 mutation (Fig. 4, A and B). In contrast, expression of K-RasR42 mutant suppressed cell migration, and the effect was more pronounced in cells expressing K-RasV12/R42, strongly suggestive of a role of Lys-42 in mediating K-Ras biological and pathological activities.

We further examined whether Arg-42 affected the ability of K-Ras to promote cell invasion. Tet293 cells stably transfected with K-RasV12 or K-RasV12/R42 expression plasmid, along with parental cells, were subjected to a transwell migration assay. We observed that compared with the parental control cells, significantly increased invasiveness was observed in Tet293-K-RasV12 cells and that Tet293-K-RasV12/R42 cells exhibited greatly reduced transwell migration similar to that of parental cells (Fig. 5A and B). Moreover, the addition of Dox to Tet293-K-RasV12 cells, but not Tet293-K-RasV12/R42 cells, induced expression of Snail (Fig. 5C), a gene product involved in epithelial–mesenchymal transition (24). Consistent with these observation, transfection of K-RasV12 expression plasmid, but not K-RasV12/R42 or vector, greatly promoted transwell migration of NIH3T3 cells (Fig. 6A and B). Combined, these observations strongly suggest that Lys-42 plays an essential part in mediating oncogenic activities of K-Ras.

**SUMO inhibitor blocks cell migration in a K-Ras–dependent manner**

Ras proteins were the first and remain the best-studied oncogene products. However, to date, no compounds that target Ras have been approved for cancer treatments in the clinic. Given that sumoylation plays a crucial role in activating Ras, we next determined whether 2-D08, a SUMO E2 inhibitor (25), would suppress tumor cell migration in a manner that depends on K-Ras activation. To this end, we selected MiaPaCa-2 and BxPA-3 cells for our studies, as MiaPaCa-2 cells harbor mutated K-RasV12, whereas BxPC-3 cells contain WT K-Ras. Wound-healing assays revealed that MiaPaCa-2 cells closed the wound gap much faster than BxPC-3 cells did, suggesting that expression of oncogenic K-Ras enhances cell migration (Fig. 7A and B). Significantly, SUMO E2 inhibitor, 2-D08, inhibited MiaPaCa-2 cell migration in a concentration-dependent manner, whereas it had little effect on BxPC-3 cells.
Moreover, 2-D08 treatment suppressed expression of ZEB1, a gene product positively associated with cell migration and EMT (Fig. 7D). In contrast, it promoted expression of ZO-1, a gene product whose expression is negatively associated with cell proliferation and migration. Immunoblotting revealed that K-Ras sumoylation levels were lower in BxPC-3 cells than those in MiaPaCa-2 cells (Fig. 7D). These results are thus consistent.
with our early observation that cells expressing oncogenic Ras contain more sumoylated counterpart (20). These results also explain why MiaPaCa-2 cells are sensitive to treatment with 2-D08.

Based on our studies, we propose the following model to explain Lys-42 in mediating K-Ras biochemical and biological functions (Fig. 8). K-Ras is modified by sumoylation, which in turn regulates its physical interaction with downstream component c-Raf via the Ras-binding domain (RBD). Other domain structure(s) of Raf, such as the SUMO-interacting motif, may recognize the SUMO moiety, positively promoting the interaction between Ras and Raf. Lys-42 mutation significantly compromises its sumoylation and thus the physical interaction with Raf. Desumoylated K-Ras has a significantly reduced ability to activate downstream components owing to compromised interaction with c-Raf, leading to reduced biological/pathological functions, including cell migration and invasion.

Figure 7. SUMO inhibitor blocks migration of pancreatic cancer cells with K-RasV12 mutation. A, both BxPC-3 (without K-Ras mutation) and MiaPaCa-2 (with K-RasV12 mutation) cells were employed for wound-healing assays. Cells with open scratches were treated with various concentrations of 2-D08 for 18 h. Representative images of cells before (CNTL) and after recovery in the presence of 2-D08 for 18 h are shown. B, cell migration of various treatments, as shown in A, was quantified. Data are summarized from three independent experiments. C, pancreatic cell lines as indicated were immunoprecipitated with the Ras antibody (Pan-Ras) or IgG as control. Immunoprecipitates, along with lysate controls, were blotted with antibodies to SUMO2/3 or to K-Ras. D, MiaPaCa-2 cells treated with or without 2-D08 were lysed, and equal amounts of cell lysates were blotted with antibodies to N-cadherin (N-Cad), ZEB1, ZO-1, PARP-1, and β-actin, respectively. Molecular weight markers are also indicated.

Discussion

Posttranslational modifications play an essential role in regulating the subcellular localization, stability, and/or activity of numerous cellular proteins. Ras proteins are modified by almost all known posttranslational modifications (9, 26). Lysine residues in proteins are major targets for posttranslational modifications, including acetylation, methylation, ubiquitination, and sumoylation. In fact, extensive research in the past has demonstrated that many lysine residues in Ras proteins are modified (12, 14). We recently demonstrated that Lys-42 of Ras proteins is important for sumoylation, a previously undocumented posttranslational modification for the oncogene product (20). In the current study, we have further analyzed the functional consequence of K-RasR42C in mediating its biologic and oncogenic functions. We have demonstrated that inducible expression of K-RasV12/R42C mutant inhibits the activation of the RAF/MEK/ERK signaling axis. Moreover, expression of this mutant suppresses cell migration and invasion in multiple cell lines.

Profiling a kinase array reveals that expression of the K-RasV12/R42C mutant leads to changes in phosphorylation of protein kinases that mediate several distinct downstream pathways. Notably, we have observed that the phosphorylation level of FAK, a kinase functioning as a biosensor or integrator to control cell motility, is also significantly reduced in cells expressing K-RasV12/R42C. This is consistent with our subsequent study showing that expression of K-RasV12/R42C leads to a weakened ability of cell migration and invasion.

One intriguing observation is that there were no significant changes of physical interaction between K-RasV12/R42C and c-Raf compared with K-RasV12 and c-Raf; however, when SENP1 was expressed, the interaction between K-RasV12/R42C and c-Raf was greatly reduced (Fig. 1C). There are two possibilities to explain these results. 1) Lys-42 regulates K-Ras sumoylation but is not the residue accepting the SUMO moiety. In other words, the Arg-42 mutant reduces, but does not abolish, K-Ras sumoylation. Thus, further inhibition of K-Ras sumoylation by 2-D08 synergizes with Arg-42 mutation, leading to reduced physical interaction with c-Raf as well as compromised downstream sig-
naling. 2) An alternative explanation is that sumoylation of another component(s) upstream of Ras is required for its full activation and efficient interaction with c-Raf. Supporting this, it has been reported that Grb2 is modified by sumoylation, which positively regulates ERK signaling through promoting its interaction with Sos1.

K-Ras is the most frequently mutated oncogene product in human malignancies. K-Ras mutations are frequently associated with tumor development, poor prognosis, and therapeutic resistances. Ras proteins have been generally considered druggable targets, given their enzymatic activities and roles as key components of signaling pathways for cell survival, proliferation, transformation, and differentiation (1, 9, 27). Despite extensive research in the past, no pharmacological agents are available for malignancies derived partly or primarily from K-Ras mutations. One aspect of K-Ras biology that has not been extensively studied is the role of lysine residues in mediating its oncogenic/pathologic activities because of their involvement in several types of posttranslational modifications. Our observations that demonstrate the importance of lysine residues in oncogenic functions open up new avenues for identifying druggable targets, including those essential for participating in ubiquitination, sumoylation, acetylation, and methylation. As a proof-of-principle, we show that SUMO E2 inhibitor 2-D08 is capable of blocking cell migration that is at least in part promoted by K-RasV12 mutation.

We realize that Lys-42 lies in close proximity to the so-called switch domain and that a mutation at Lys-42 could in theory cause a conformation change, leading to compromised interactions with upstream regulators and/or downstream effectors. On the other hand, available evidence suggests that the substitution of Lys with Arg at residue 42 does not appear to perturb its conformation: 1) K-RasV12/R42 regulates AKT signaling/activation in a manner similar to that of K-RasV12 (Fig. 1B); 2) K-RasV12/R42 interacts with Ral.GDS, Raf, and PI3K in the same manner as that of WT Ras or RasV12 mutant regardless of GTP loading (21). However, when the overall sumoylation status in the cells is compromised due to expression of SENP1, K-RasV12/R42 displays weakened interaction with c-Raf compared with K-RasV12 (Fig. 1C), supporting the notion that sumoylation plays an essential role in Ras downstream signaling.

Activated K-Ras functions to drive oncogenesis in major human malignancies. The K-RasV12 mutant is thought to be constitutively locked in the active, GTP-bound state, which makes it very challenging to inhibit its oncogenic activity through traditional drug design approaches. One approach is to disrupt signaling between K-Ras protein and its downstream effector(s) via a small molecular compound, thus blocking its interaction with Sos1, interaction with Sos1.

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Experimental procedures

Cell culture and transfection

HEK293T (kidney carcinoma), MCF7 (mammary carcinoma), NIH3T3 (fibroblast), MiaPaCa-2, and BxPC-3 cell lines obtained from the American Type Culture Collection were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (100 μg/ml penicillin and 50 μg/ml streptomycin sulfate; Invitrogen) at 37 °C under 5% CO2. Transfection of individual cell lines was achieved with either LF2000 (Invitrogen) or Fugene HD (Roche Diagnostics) following the manufacturers’ protocol. Transfection efficiency was estimated to be between 80 and 100% in all cases through co-transfecting a GFP-expressing plasmid (data not shown).

Plasmids

HA-tagged SUMO3 and UBC9 expression plasmids were obtained from Addgene. K-Ras and its mutant cDNAs were obtained using the QuikChange site-directed mutagenesis kit (Agilent Technologies). All mutations were confirmed by DNA sequencing.

Protein extracts and immunoblotting

Total cell lysates were prepared in a buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% IGEPAL, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with a mixture of protease and phosphatase inhibitors. HEK293T Tet-on cells were engineered to express K-RasV12 or K-RasV12/R42 mutants in the presence of doxycycline. At various times after the addition of doxycycline, cells were collected and lysed in the same buffer as above. Protein concentrations were measured using the bicinchoninic acid protein assay reagent kit (Pierce). Equal amounts of protein lysates from various samples were used for SDS-PAGE analysis followed by immunoblotting. Antibodies to HA, FLAG, phospho-c-Raf, Raf1, Ral.GDS, phospho-MEK, MEK, phospho-ERK42/44, ERK1/2, Snail, Claudin-1, N-Cad, ZEB1, ZO-1, PARP-1, and actin were purchased from Cell Signaling Technology. Specific signals on immunoblots (polyvinylidene difluoride) were visualized using enhanced chemiluminescence (Super-Signal, Pierce).

Immunoprecipitation

Cells were lysed in TBSN buffer (20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na2VO4, and 20 mM β-glycerol phosphate). The cell lysates were clarified by centrifugation at 15,000 × g for 20 min at 4 °C. Cleared lysates (1 mg) were added to FLAG M2 agarose (Sigma) followed by incubation in the TBSN buffer for 1 h at 4 °C. After incubation, proteins bound to each resin were washed extensively with the binding buffer, eluted in the SDS-PAGE sample buffer, and analyzed by SDS-PAGE.

Wound-healing assay

NIH3T3 fibroblast cells were transiently transfected with plasmid vector alone or pcDNA-K-RasV12 or pcDNA-K-
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RasV12/R42 for 24 h before they were used for the wound-healing assay. Transfected cells were serum-starved for 18 h and then wounded by manual scraping with a pipette tip. Plates were washed several times to remove nonadherent cells, and cells were cultured in the same medium containing 1% fetal bovine serum. Assessment of cell migration into the wounded area was performed under a light microscope after an additional 18 h of incubation.

**Phosphokinase array**

For phosphokinase array analysis, cellular extracts (500 mg) were incubated with the Phospho-Kinase Array Kit (Proteome Profiler; R&D Systems, Abingdon, UK) following the manufacturer’s instructions. Densitometry values were estimated by the ImageJ software and were expressed as arbitrary units. Average signal of the pair of duplicate spots, representing each phosphorylated kinase protein, was calculated after subtraction of background values (pixel density) from negative control spots and normalization to average values from positive control spots.

**Cell invasion assays**

HEK293T and NIH3T3 cells were transiently transfected with pcDNA plasmid vector (control), pcDNA-K-RasV12, or pcDNA-K-RasV12/R42. Twenty-four h posttransfection, cells (4 × 10^4 cells/well) were seeded onto transwell inserts and incubated at 37°C for 12 h. Nonmigrated cells were removed from the upper face of the transwell insert using a cotton swab. Cells that migrated through membranes were stained. Cell density in each treatment was recorded under a light microscope.

**Statistical analysis**

Each experiment was performed at least three times. The data were plotted as the mean ± S.D. Student’s *t* test was used for all comparisons. A *p* value of <0.05 was considered statistically significant.

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