Differences in the Regulation of K-Ras and H-Ras Isoforms by Monoubiquitination[*]

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Background: Ras proteins are critical regulators of cellular growth and are differentially modified by ubiquitination.
Results: Chemical ubiquitination and immunoprecipitation assays demonstrate that monoubiquitination causes sustained H-Ras activation in the absence of oncogenic mutations.
Conclusion: The mechanism by which H-Ras is activated by monoubiquitination is both isoform-specific and site-specific.
Significance: Monoubiquitination adds a new level of regulation and complexity to isoform-specific Ras signaling.

Ras GTPases are signaling switches that control critical cellular processes including gene expression, differentiation, and apoptosis. The major Ras isoforms (K, H, and N) contain a conserved core GTPase domain, but have distinct biological functions. Among the three Ras isoforms there are clear differences in post-translational regulation, which contribute to differences in localization and signaling output. Modification by ubiquitination was recently reported to activate Ras signaling in cells, but the mechanisms of activation are not well understood. Here, we show that H-Ras is activated by monoubiquitination and that ubiquitination at Lys-117 accelerates intrinsic nucleotide exchange, thereby promoting GTP loading. This mechanism of Ras activation is distinct from K-Ras monoubiquitination at Lys-147, which leads to impaired regulator-mediated GTP

hydrolysis. These findings reveal that different Ras isoforms are monoubiquitinated at distinct sites, with distinct mechanisms of action, but with a common ability to chronically activate the protein in the absence of a receptor signal or oncogenic mutation.

The small GTPase Ras is a signaling switch that regulates cellular growth and differentiation (1). In accord with its essential role in regulating cell growth, point mutations in Ras are common in cancer; about 30% of all human tumors contain an activating Ras mutation (2). To control the activation of essential pathways involved in cell proliferation, differentiation, and apoptosis, Ras binds and hydrolyzes GTP. Ras is active when it is GTP-bound and becomes inactive when GTP is hydrolyzed to GDP (3, 4). On its own, Ras is a poor enzyme and requires regulators to respond to cell signals on an appropriate timescale. The primary regulators of Ras cycling are guanine nucleotide exchange factors (GEFs),[4] which increase the rate of GDP dissociation and GTP loading, leading to Ras activation (5, 6), and GTPase-activating proteins (GAPs), which down-regulate activated Ras by increasing the rate of GTP hydrolysis (4, 7).

There are three isoforms of Ras: K-Ras (two splice variants, K-Ras4A and K-Ras4B), H-Ras, and N-Ras. The core guanine nucleotide binding domain is highly conserved between these isoforms (>90% identical in the first 168 amino acids). However, despite the high sequence identity, Ras isoforms play distinct roles in regulating downstream signaling pathways. In particular, the three proteins exhibit differences in their localization *in vivo*, which alters access to regulatory proteins and downstream effectors (8–10). H-Ras and N-Ras are localized at both the plasma membrane and the Golgi, whereas K-Ras is predominately at the plasma membrane (11). Differences in Ras localization and activity are driven by differences in post-translational modifications (12–19). For example, differential lipidation of the Ras isoforms within their variable C-terminal domain is essential for proper membrane targeting and localization, which is in turn necessary for proper signaling (15, 20–24). Another post-translational modification, phosphorylation, has been shown to modulate K-Ras signaling output by changing its localization from the plasma membrane to the mitochondria and Golgi (25). Both lipid and phosphoryl modifications are dynamically regulated, which in turn dynamically controls Ras localization and activity (25).

Aside from lipidation and phosphorylation, there is mounting evidence that ubiquitination also contributes to the differentiation between Ras isoforms (26). Ubiquitination of an as yet unidentified site in H-Ras promotes its association with the endosome (26, 27). This localization change leads to altered effector interactions and signaling output (25). We and others have shown that monoubiquitination of Lys-147 in K-Ras does

not appear to affect protein localization, but rather acts as a reversible trigger for signal initiation (28, 29). Monoubiquitination of K-Ras at this position impedes GAP-mediated GTP hydrolysis and promotes association with select downstream effectors (28, 29). This mechanism of K-Ras activation appears specific to ubiquitination at Lys-147 because modification of other lysines does not confer a GAP defect (29). Thus, H-Ras and K-Ras display differential regulation by ubiquitination.

In addition to Lys-147, Sasaki *et al.* (28) have identified Lys-117 as a site of ubiquitination in H-Ras and Lys-104 as a secondary site of ubiquitination in K-Ras. Thus, we postulated that different sites of H- and K-Ras ubiquitination have distinct consequences for Ras activity. Here, we show that monoubiquitination at Lys-104 does not affect nucleotide exchange or hydrolysis properties of Ras, whereas modification at Lys-117 promotes Ras activation. However, in this case, the mechanism of activation is unique. Although monoubiquitination of K-Ras at Lys-147 inhibits GAP-mediated hydrolysis, monoubiquitination of H-Ras at Lys-117 greatly enhances GTP-GDP exchange. These findings reveal that K-Ras and H-Ras can each be activated by monoubiquitination, but that activation occurs via distinct molecular mechanisms.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—All Ras constructs were generated with a C118S mutation to eliminate a native solvent exposed cysteine, as described previously (29, 30). The Ras domains (1–166) of H-Ras^{C118S}, H-Ras$^{C118S/K104C}$, and H-Ras$^{C118S/K117C}$ were expressed and purified as described previously (29). The standard Ras protocol was revised for purification of H-Ras$^{C118S/K117C}$ by the addition of 10% glycerol to all buffers to accommodate for the instability of this variant.

Catalytic domains of the GEF, Sos (Sos^{cat}), and GAP, p120^{GAP} (GAP-334), were purified as described previously (29, 31). Full-length ubiquitin^{G76C} was also purified as described previously (29).

Chemical Ubiquitination—The chemical ligation strategy used to link H-Ras^{K104C} and H-Ras^{K117C} to ubiquitin^{G76C} was performed essentially as described in Baker *et al.* (29), except that 10% glycerol was added to buffers containing the Ras^{K117C} variant.

Thermal Stability of Ras—The fast quantitative cysteine reactivity (fQCR) method (32) was employed to measure changes in Ras thermal stability. Briefly, 2 μM Ras^{K104C}, mUbRas^{K104}, Ras^{K117C}, or mUbRas^{K117} was incubated with 1 mM 4-fluoro-7-aminosulfonylbenzofurazan (AnaSpec) at pH 7.0 in the presence of 20 μM GTPγS and 2 mM MgCl$_2$ in a gradient thermocycler (Biometra TProfessional thermocycler) at the desired temperature for 3 min. Reactions were quenched by the addition of 0.02 N HCl (final concentration). Fluorescence intensity was measured on a PHERAstar plate reader (BMG Labtech) with excitation and emission band-pass filters of 400 and 500 nm. The highest fluorescence for each unfolding curve was normalized to one after base lines were corrected for differences in background fluorescence. Data were fit using GraphPad Prism (GraphPad Software; San Diego, CA) to determine the melting temperature (T_m) for each construct.

Nucleotide Dissociation Assay—For the Ras^{K104C} and mUbRas^{K104} variants, the *N*-methylanthraniloyl (MANT)-GDP nucleotide

dissociation assay was performed as described previously (29). The assay was adapted for Ras^{K117C} and mUbRas^{K117} as follows. Ras protein was exchanged into MANT nucleotide exchange buffer (50 mM Tris, pH 8.0, 50 mM NaCl, and 5 mM MgCl$_2$). MANT-GDP (0.5 μM) was added to 1 μM of protein in the cuvette. The Ras protein was determined to be fully loaded when the fluorescence of the spectra no longer increased, ~500 s. Unlabeled GDP (final concentration of 100 μM) was added to initiate dissociation. Sos^{cat} was mixed with H-Ras at a 1:1 molar ratio. MANT-GDP dissociation was measured as a change in fluorescence intensity over time (excitation: 360 nm, emission: 440 nm) (LS50B PerkinElmer luminescence spectrometer). Fluorescence data were fit in GraphPad Prism to a one-phase exponential decay curve to determine rates of nucleotide release.

Single Turnover Nucleotide Hydrolysis—Single turnover nucleotide hydrolysis assays were performed as described in Baker *et al.* (29). The ratio of fluorescence emission was measured at 480 and 530 nm with an excitation of 435 nm on a SpectraMax M5 (Molecular Devices). Fluorescence ratios were converted to phosphate concentrations using a standard curve. The measured rates of phosphate release over time were fit in GraphPad Prism to a one-phase exponential association curve.

Multiple Turnover Nucleotide Hydrolysis—Due to limitations associated with instability of Ras^{K117C} and mUbRas^{K117}, GTP hydrolysis was measured in the presence of excess GTP (multiple turnover). Ras was exchanged (20-fold dilution and concentrated three times) into phosphate-free buffer with 5 mM EDTA. For intrinsic hydrolysis, 20 μM Ras was mixed with 60 μM GTP, and hydrolysis was initiated by the addition of magnesium. For GAP-mediated hydrolysis, GAP-334, at concentrations ranging from 0.0625 to 8 μM, was added to the reaction. The phosphate-binding protein Flippi 5U (Addgene) was used to detect inorganic phosphate released upon GTP hydrolysis (33). Fluorescence emission was measured as a ratio at 480 and 530 nm and an excitation of 435 nm (SpectraMax M5, Molecular Devices). Fluorescence ratios were converted to a phosphate concentration using a standard curve. Data from the first 10 min of hydrolysis were fit in GraphPad Prism to a linear regression and the resulting rates were plotted as a function of GAP concentration.

Mapping GEF and GAP Binding Sites on Ras—The pHinder algorithm was used to calculate a surface between Ras and the catalytic domains of Sos and p120^{GAP}. Information on the triangulation used to define the surface has been described elsewhere (34). Residues were considered to be part of the interaction surface if they were less than 6 Å apart. Interaction surfaces were determined from the crystal structures of Ras with Sos (Protein Data Bank (PDB) 1BKD) and Ras with p120^{GAP} (PDB 1WQ1).

Assays in Cell Lysate—Ras activation was measured as described previously (28). The constructs FLAG-His-H-Ras or the mutant FLAG-His-H-Ras^{K117N} were co-expressed with HA-ubiquitin in HEK293T cells. The cells were prepared and lysed as described previously (29). For assays conducted in the presence of either Sos^{cat} or p120^{GAP}, the catalytic domains were purified from bacterially produced protein and incubated with the cell lysate for 20 min at room temperature. The effect of the regulators on the population of active Ras or mUbRas was
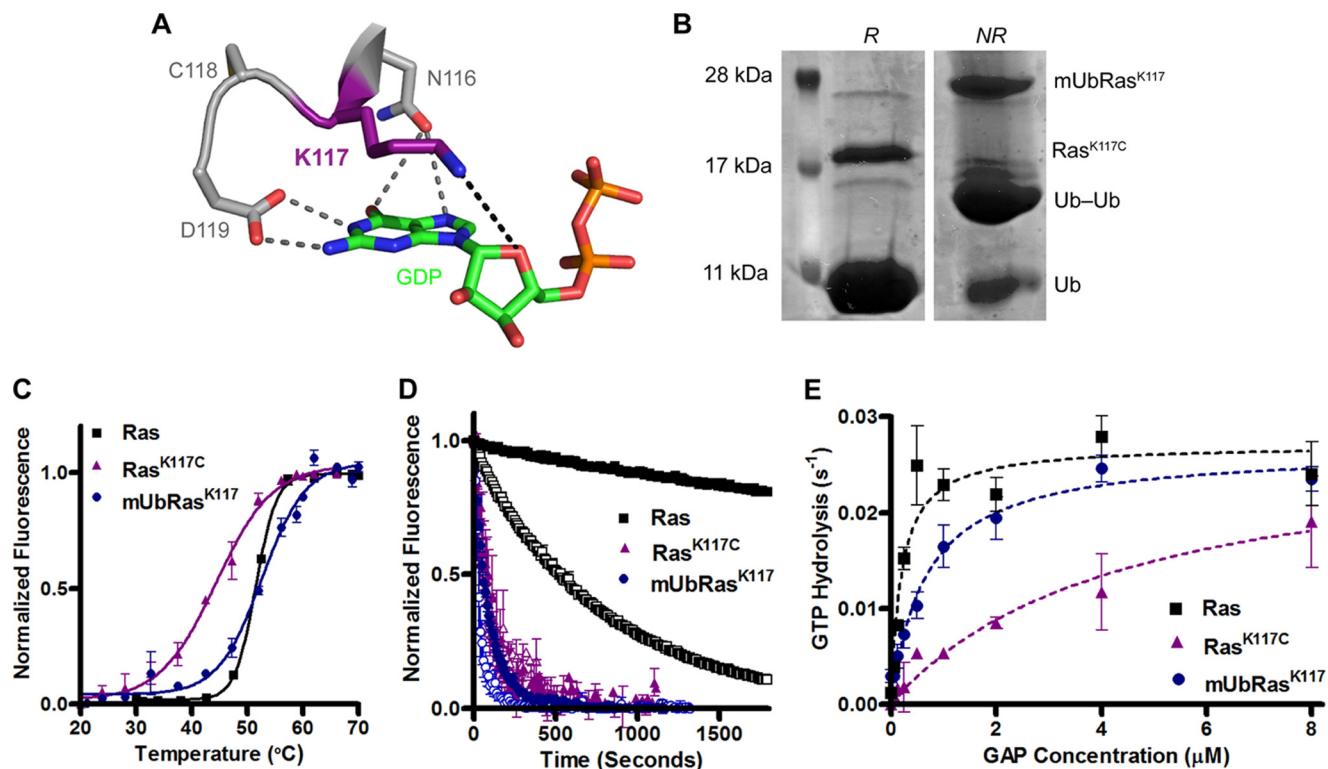
FIGURE 1. **Monoubiquitination at lysine 117 activates H-Ras.** *A*, graphic highlighting interactions formed between the NK*X*D motif of Ras (GDP) and the guanine nucleotide base (PDB 1CRR). Nucleotide is color-coded by element (nitrogen in *blue*, oxygen in *red*, and carbon in *green*), and Lys-117 of Ras is shown in *purple*. *B*, formation of disulfide bond between ubiquitin^{G76C} and Ras^{K117C} under nonreducing (*NR*) and reducing (*R*) conditions. The product of the reaction contains mUbRas, Ras, ubiquitin-ubiquitin dimer (*Ub-Ub*), and free ubiquitin (*Ub*). *C*, the thermal stability of Ras (*black squares* throughout), Ras^{K117C} (*purple triangles* throughout), and mUbRas^{K117} (*blue circles* throughout) measured by fQCR. The data were normalized using the maximum fluorescence intensity. Results are the mean ± S.E. (*n* = 4). *D*, intrinsic nucleotide release (*closed symbols*) for Ras, Ras^{K117C}, and mUbRas^{K117} bound to MANT-GDP. The rate of GDP dissociation was determined by monitoring the decrease in MANT-GDP fluorescence emission over time following the addition of unlabeled GDP. Nucleotide dissociation was also measured in the presence of a 1:1 molar ratio of Ras to Sos^{cat} (*open symbols*). Data were fit to an exponential dissociation curve, and the results are the mean ± S.E. (*n* = 4). *E*, multi-turnover hydrolysis for Ras, Ras^{K117C}, and mUbRas^{K117}. GTP hydrolysis was measured by monitoring the change in fluorescence of the phosphate-binding protein, Flippi, upon binding to free phosphate. Data were converted to a phosphate concentration using a standard curve. Phosphate release was measured for 20 μM Ras in the presence of GAP concentrations ranging from 0.0625 to 8 μM. Results are the mean ± S.E. (*n* = 4). Data were fit to a one site binding model in GraphPad Prism.

assessed by immunoblotting using GST-Raf-RBD (Ras binding domain) to detect active Ras. For the Sos^{cat} assay, 25 μM of GTPγS was added during the incubation. Samples were prepared from SDS-PAGE gels (Invitrogen, NuPAGE), and Ras was detected by varying exposure times.

RESULTS

Monoubiquitination at Lys-117 Activates H-Ras by Increasing Intrinsic Exchange—It was recently shown that H-Ras can be monoubiquitinated at Lys-117 (28). Lysine 117 is part of the conserved NK*X*D motif that contributes to nucleotide affinity and specificity by forming interactions with the guanine nucleotide base and ribose (Fig. 1*A*) (35). Conservative mutations at Lys-117 (K117R and K117N) are present in human cancers and developmental disorders and lead to Ras activation by reducing nucleotide binding affinity, resulting in increased rates of nucleotide exchange (36–38). Given these observations, we postulated that ubiquitination of Lys-117 is likely to disrupt interactions between the lysine and the guanine nucleotide base and activate Ras by a mechanism similar to mutation at the same site.

We chemically ubiquitinated Ras at position 117 (mUbRas^{K117}) using a cysteine ligation approach reported by us previously (Fig. 1*B*) (29). Mutation at a nearby site in the conserved NK*X*D motif,

Asp-119, reduces protein stability and increases the rate of nucleotide exchange (39). We therefore investigated whether perturbations at Lys-117 could also alter protein stability. We measured the thermal stability of Ras using the fQCR assay (32). This assay measures protein unfolding as a function of temperature and chemical labeling of exposed cysteines. Because ubiquitin does not contain native cysteines, fQCR can be used to specifically determine whether ubiquitination affects Ras thermal stability. We found that mUbRas^{K117} retains the thermal stability observed for unmodified Ras (Fig. 1*C*) (T_m = 52.5 ± 0.5 °C). However, ubiquitination at Lys-117 is distinct from a Ras^{K117C} mutation, which leads to a 10° decrease in the thermal stability of Ras (Ras^{K117C} T_m = 43.8 ± 0.9 °C). Relative to unmodified Ras, Ras^{K117C} and mUbRas^{K117C} exhibited less cooperative unfolding transitions (Fig. 1*C*) and increased rates of nucleotide dissociation (Fig. 1*D*). We attribute this correlation to loss of a key interaction (Lys-117) that impairs nucleotide binding, resulting in reduced cooperativity of the unfolding transition (40). Although either ubiquitination or mutation of Ras at position 117 alters the lysine side chain, which is crucial for forming interactions with the guanine nucleotide, these distinct alterations differentially affect the thermal stability of Ras.
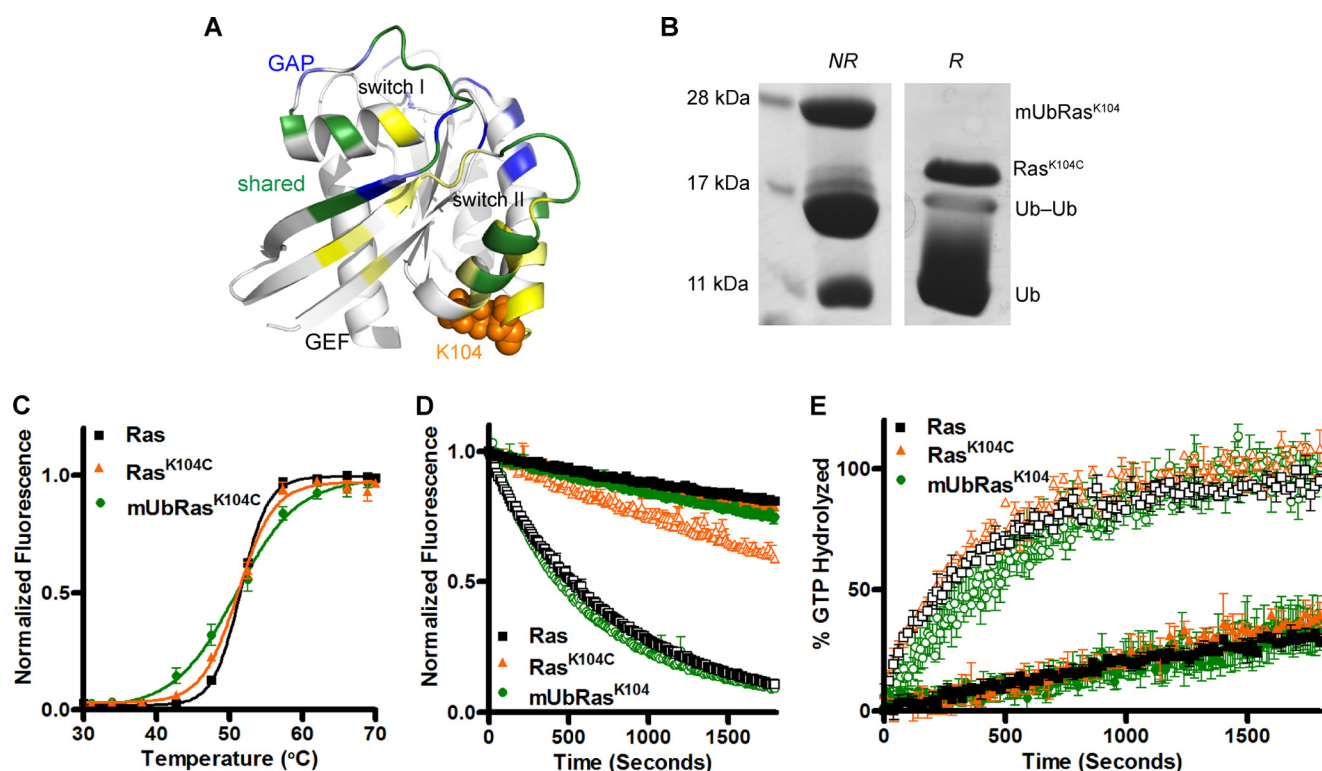
FIGURE 2. **Ras monoubiquitinated at lysine 104 retains the activity of unmodified Ras.** *A*, structure of Ras-GDP (PDB 1CRR) with the Lys-104 side chain highlighted in *orange*. Backbone residues in *yellow* make contact with the GEF Sos (PDB 1BKD), backbone residues in *blue* make contact with the GAP p120^{GAP} (PDB 1WQ1), and backbone residues in *green* are contacts common for both the GEF and the GAP. *B*, formation of disulfide bond between ubiquitin^{G76C} and Ras^{K104C} under nonreducing (*NR*) and reducing (*R*) conditions. The product of the reaction contains mUbRas, Ras, ubiquitin-ubiquitin dimer (*Ub-Ub*), and free ubiquitin (*Ub*). *C*, the thermal stability of Ras (*black squares* throughout), Ras^{K104C} (*orange triangles* throughout), and mUbRas^{K104} (*green circles* throughout) measured by fQCR. The data were normalized using the maximum fluorescence intensity. Results are the mean ± S.E. ($n = 4$). *D*, intrinsic nucleotide dissociation (*closed symbols*) for Ras, Ras^{K104C}, and mUbRas^{K104} loaded with MANT-GDP. The rate of GDP dissociation was determined by monitoring the decrease in MANT-GDP fluorescence emission over time following the addition of unlabeled GDP. Nucleotide dissociation was also measured in the presence of a 1:1 molar ratio of Ras to Sos^{cat} (*open symbols*). Data were fit to an exponential dissociation curve, and the results are the mean ± S.E. ($n = 4$). *E*, single-turnover GTP hydrolysis for Ras, Ras^{K104C}, and mUbRas^{K104} in the absence (*closed symbols*) and presence (*open symbols*) of GAP-334 (1:500 GAP:Ras). Hydrolysis was initiated by the addition of Mg^{2+} and monitored by the change in fluorescence of the protein, Flippi, upon binding free phosphate. Data were converted to a phosphate concentration using a standard curve. Results are the mean ± S.E. ($n = 6$).

We next measured the effect of monoubiquitination on the rate of guanine nucleotide dissociation. We find that the intrinsic rate of nucleotide dissociation was 70-fold faster for mUbRas^{K117} as compared with unmodified Ras ($8.3 \pm 0.3 \times 10^{-3}$ s^{-1}) (Fig. 1*D*). This finding is consistent with the 100-fold increased rate of nucleotide release observed when Ras is mutated (Ras^{K117C}) at this same position ($11.0 \pm 0.4 \times 10^{-1}$ s^{-1}) (Fig. 1*D*). We also observed a small increase in GDP dissociation for mUbRas^{K117} in the presence of the GEF, Sos^{cat}, indicating that the nucleotide release can be further stimulated by GEFs. Even in the absence of GEF-mediated activation, the increased rate of intrinsic nucleotide dissociation should be sufficient to promote GTP exchange and mUbRas^{K117} activation *in vivo* (41).

We then measured intrinsic and GAP-mediated GTP hydrolysis using a multi-turnover GTP hydrolysis assay, as described previously (41). When Ras is monoubiquitinated at Lys-117, the rate of intrinsic GTP hydrolysis is similar (within 2-fold) to that of unmodified Ras (Fig. 1*E*). Furthermore, mUbRas^{K117} retains sensitivity to p120^{GAP} (GAP-334), albeit with a reduced binding affinity. As seen in Fig. 1*E*, at 8 μM GAP-334 (5:2 ratio of Ras to GAP-334), the rate of GTP hydrolysis is not affected by monoubiquitination (0.024 ± 0.003 and 0.023 ± 0.002 s^{-1} for Ras and mUbRas^{K117}, respectively). Therefore, mUbRas^{K117} is still sensitive to GAP-mediated down-regulation. Similar results were observed for both Ras^{K117C} (Fig. 1*E*) and Ras^{K117R} variants (41). Taken together, these data demonstrate that monoubiquitination of H-Ras at Lys-117 causes activation by enhancing the rate of nucleotide exchange.

Ras Monoubiquitinated at Lys-104 Retains the Activity of Unmodified Ras—Mass spectrometry characterization of K-Ras and H-Ras in HEK293T cells identified two major sites of ubiquitination, Lys-147 (K-Ras and H-Ras) and Lys-117 (H-Ras only) (28). Our data indicate that these modifications result in Ras activation, but act through distinct mechanisms. An additional minor site of ubiquitination was observed at position Lys-104 in K-Ras (28). Although Lys-147 and Lys-117 are in a conserved nucleotide binding motif, Lys-104 is located in helix 3 adjacent to the GEF binding interface (Fig. 2*A*). Substitutions at this position (K104Q) have been proposed to alter GEF-mediated dissociation by disrupting an electrostatic interaction that the Lys-104 side chain forms with the α2 helix. Moreover, acetylation at this position has recently been shown to impair GEF-mediated regulation of Ras (42). Therefore, we
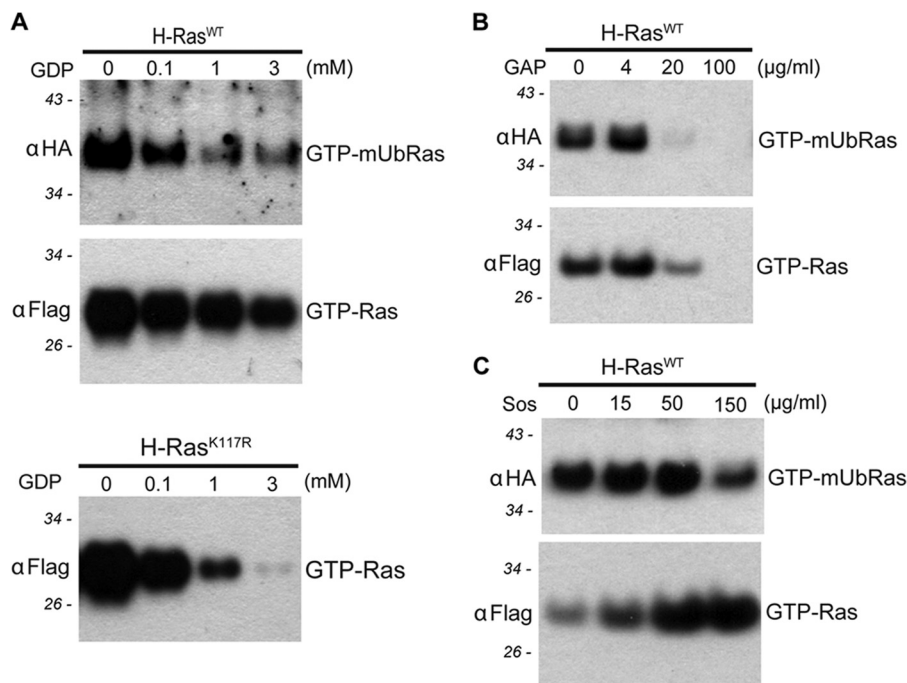
A

H-Ras^{WT}

GDP 0   0.1   1   3   (mM)

43 -
αHA   GTP-mUbRas
34 -

34 -
αFlag   GTP-Ras
26 -

H-Ras^{K117R}

GDP 0   0.1   1   3   (mM)

34 -
αFlag   GTP-Ras
26 -

B

H-Ras^{WT}

GAP 0   4   20   100   (μg/ml)

43 -
αHA   GTP-mUbRas
34 -

34 -
αFlag   GTP-Ras
26 -

C

H-Ras^{WT}

Sos 0   15   50   150   (μg/ml)

43 -
αHA   GTP-mUbRas
34 -

34 -
αFlag   GTP-Ras
26 -

FIGURE 3. **Assays in cell lysate indicate H-Ras is primarily modified at lysine 117.** *A*, immunoblotting of GTP-bound H-Ras, GTP-bound mUbRas, and GTP-bound H-Ras^{K117R} from cell extracts in the presence of the indicated concentrations of GDP. Anti-FLAG and anti-HA antibodies reveal the relative fraction of total Ras and mUbRas, respectively, for all assays. Molecular masses are shown in *italics* for all panels. *B*, immunoblotting of GTP-bound Ras and GTP-bound mUbRas from cell extracts in the presence of the indicated concentrations of recombinant p120^{GAP}, GAP-334. *C*, immunoblotting of GTP-bound Ras and GTP-bound mUbRas in cell extract in the presence of increasing concentrations of recombinant Sos^{cat} and 25 μM GTPγS.

considered whether monoubiquitination at Lys-104 could also alter K-Ras activity.

To determine whether monoubiquitination at position 104 alters Ras activity, we chemically monoubiquitinated the protein at Lys-104 (mUbRas^{K104}) (Fig. 2*B*) and measured thermal stability as well as intrinsic and regulator-mediated enzymatic activity. As seen in Fig. 2*C*, the thermal stability of mUbRas^{K104} is similar to that of Ras ($T_m = 50.8 \pm 0.4\,°C$), consistent with mutation at this same position. We next measured intrinsic and GEF-mediated rates of GDP dissociation using Sos^{cat}. This analysis showed that nucleotide release was unaffected by monoubiquitination or mutation at position 104 (Fig. 2*D*). However, we did not observe a change in Sos^{cat}-mediated dissociation for mUbRas^{K104} (Fig. 2*D*), although the Ras^{K104C} substitution decreased Sos^{cat}-mediated activation by 5-fold, consistent with previous studies (42). Neither monoubiquitination nor mutation at position 104 altered intrinsic or GAP-mediated single-turnover GTP hydrolysis (Fig. 2*E*). Together, these data demonstrate that Lys-104 on Ras can be monoubiquitinated without altering intrinsic or regulator-mediated activity. Moreover, although K-Ras can be ubiquitinated at two distinct lysines *in vivo* (Lys-147 and Lys-104), only the more prevalent site of ubiquitination, Lys-147, leads to a change in the active state of the protein.

H-Ras Is Activated and Primarily Modified at Lysine 117 in HEK293T Cells—Our mechanistic studies indicate that monoubiquitination of H-Ras at Lys-117 enhances guanine nucleotide dissociation, leading to increased GTP loading and protein activation. We previously showed that the mechanism of K-Ras activation determined *in vitro*, using a chemical ubiquitination approach, recapitulated the GAP defect observed for

mUbRas^{K147} in cell lysate assays (29). In the original study by Sasaki *et al.* (28), H-Ras was found to be ubiquitinated at both Lys-117 and Lys-147 in HEK293T cells. Because ubiquitination at Lys-117 and Lys-147 activates Ras by distinct mechanisms, we exploited this difference to determine whether modification at one or the other lysines predominates in HEK293T cells. Using Ras binding domain pulldown assays that specifically detect GTP-bound Ras, we measured intrinsic and regulator-mediated nucleotide exchange and hydrolysis. Monoubiquitinated Ras accounts for ~1% of the total Ras protein and was detected by blotting for HA-ubiquitin. As seen in Fig. 3*A*, as the concentration of exogenously added GDP increases, the population of GTP-bound mUbRas decreases more than unmodified GTP-bound H-Ras. The increased loss of activated mUbRas relative to unmodified Ras indicates that nucleotide exchange is faster when H-Ras is monoubiquitinated. This result is similar to what is observed in the Ras^{K117R} mutant (Fig. 3*A*), which has previously been shown to possesses an 80-fold faster rate of intrinsic nucleotide dissociation relative to unmodified Ras, similar to the 70-fold increased rate of nucleotide exchange observed for mUbRas^{K117} (41). We also determined whether the population of monoubiquitinated H-Ras immunoprecipitated from HEK293T cell lysates was sensitive to Sos-mediated regulation. When H-Ras is monoubiquitinated, the population of active protein does not change significantly in the presence of recombinant GEF (Fig. 3*B*), indicating that mUbRas is not further activated by Sos^{cat}. Ras and mUbRas were equally responsive to the addition of recombinant GAP-334, suggesting that Ras had been modified at a site other than Lys-147 in these cells (Fig. 3*C*). Based on these findings, we propose that cellular H-Ras is predominantly monoubiquitinated at Lys-117 in

HEK293T cells and that monoubiquitination at this site activates the protein through a mechanism distinct from that of K-Ras. Although monoubiquitinated K-Ras is uncoupled from the GTPase-activating protein, monoubiquitinated H-Ras exhibits an increase in intrinsic nucleotide exchange activity.

DISCUSSION

We have shown previously that monoubiquitination of K-Ras at Lys-147 promotes protein activation by rendering the protein insensitive to GAP-mediated GTP hydrolysis (29). This same modification does not alter the intrinsic nucleotide exchange and GTP hydrolysis activities of Ras, and it has only a modest effect on GEF-mediated nucleotide exchange. Here, we characterize two additional sites of monoubiquitination, one acting on K-Ras at Lys-104 and the other acting on H-Ras at Lys-117. We find that *in vitro*, monoubiquitination at Lys-117 uniquely up-regulates H-Ras activity by promoting exchange of GDP for GTP. Furthermore, our cell lysate assays indicate that ubiquitination of H-Ras at Lys-117 predominates in HEK293T cells because monoubiquitinated H-Ras was sensitive to GAP-mediated regulation but displayed an increased rate of intrinsic nucleotide exchange. Activation of Ras by sustained GTP binding (monoubiquitination of K-Ras at Lys-147) may have distinct cellular outcomes from that of constitutive GTP cycling (monoubiquitination of H-Ras at Lys-117). In support of this premise, disease-related Ras mutations that are activated through increased exchange have different phenotypes than those that cause inhibition of GAP-mediated hydrolysis (43).

In contrast to monoubiquitination of Ras at Lys-147 and Lys-117, monoubiquitination of Lys-104 does not affect Ras activity *in vitro*. The absence of any functional difference is striking, particularly because monoubiquitination at a nearby site (Lys-117) was sufficient to fully activate the protein. Nevertheless, the identification of a monoubiquitination site on Ras that does not alter activity is consistent with previous data obtained in CHOK-1 cells, where trafficking to the endosome rather than direct H-Ras activation was the consequence of monoubiquitination (26). Together, these data suggest that site-specific ubiquitination may occur in both an isoform-specific and a cell line-specific manner to regulate Ras localization and signaling. Left unresolved is how the differences in ubiquitination are controlled, although they are likely to depend on differences in E3 ubiquitin ligase and deubiquitinating enzyme expression or localization (28).

It was recently shown that Lys-104 is also a site of acetylation in K-Ras in HEK293T cells (42). Molecular dynamics simulations suggest that acetylation at Lys-104 alters switch II due to a perturbation in electrostatic interaction in the $\alpha2$ helix that populates the Ras switch region in a disordered conformation, which is less favorable for Ras-GEF interaction (42). Acetylation of K-Ras at Lys-104 conferred a defect in GEF-mediated exchange, resulting in its accumulation in the inactive GDP-bound state both *in vitro* and in cells (42). Given that acetylation and monoubiquitination are likely to be mutually exclusive, there may be a role for Lys-104 ubiquitination in protecting Ras from acetylation, or *vice versa*.

Ras proteins are well known to be activated by growth factor receptors and by oncogenic mutations. Although the structure and sequence of the three Ras isoforms are highly conserved, they play distinct roles in signaling and cell regulation *in vivo*. Differences in Ras localization and activity are driven by differences in post-translational modifications (12–19). Our analysis has revealed two distinct mechanisms of Ras activation through the same post-translational modification. Furthermore, our biochemical analysis of site-specific Ras ubiquitination revealed that ubiquitination alters Ras through mechanisms distinct from mutations at the same positions. Assuming that these events are regulated independently, our findings reveal a potential new mechanism for dictating functional hierarchy and conferring isoform-dependent differences in Ras activation and signaling.

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