The Function of Embryonic Stem Cell-expressed RAS (E-RAS), a Unique RAS Family Member, Correlates with Its Additional Motifs and Its Structural Properties*§

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**Background:** E-RAS contains additional motifs and regions with unknown functions.

**Results:** Biochemical analysis reveals that effector selection of E-RAS significantly differs from H-RAS.

**Conclusion:** E-RAS selectivity and consequently cellular outcomes depend on its unique switch and interswitch regions.

**Significance:** E-RAS possesses specific sequence fingerprints and therefore no overlapping function with H-RAS.

E-RAS is a member of the RAS family specifically expressed in embryonic stem cells, gastric tumors, and hepatic stellate cells. Unlike classical RAS isoforms (H-, N-, and K-RAS4B), E-RAS has, in addition to striking and remarkable sequence deviations, an extended 38-amino acid-long unique N-terminal region with still unknown functions. We investigated the molecular mechanism of E-RAS regulation and function with respect to its sequence and structural features. We found that N-terminal extension of E-RAS is important for E-RAS signaling activity. E-RAS protein most remarkably revealed a different mode of effector interaction as compared with H-RAS, which correlates with deviations in the effector-binding site of E-RAS. Of all these residues, tryptophan 79 (arginine 41 in H-RAS), in the interswitch region, modulates the effector selectivity of RAS proteins from H-RAS to E-RAS features.

Small GTPases of the RAS family act as molecular switches within the cell, cycling between a GTP-bound (active) and a GDP-bound (inactive) state (1, 2). These molecules trigger intracellular responses by sensing the extracellular signals through their interacting receptors or intermediate proteins and passing the signal to downstream targets. Therefore, they play a key role in various cellular processes, including gene expression, metabolism, cell cycle progression, proliferation, survival, and differentiation. Somatic or germ line mutations in genes related to members of the RAS family or their regulators are commonly associated with cancer progression or developmental disorders (3–9).

The best investigated RAS proteins are H-, N-, and K-RAS4B, which share overlapping functions, including cell proliferation, differentiation, and apoptosis (10–13). However, different RAS isoforms exhibit a particular pattern of expression, different regulators, and specific microdomains or subcellular localization, indicating their functional specificity as well as redundant roles (10–17). The individual roles of other members of the RAS family, such as R-RAS, TC21, M-RAS, AGS-1, or the embryonic stem cell-expressed RAS (E-RAS), have not been fully described. E-RAS was identified in 2003 as a new member of the RAS family, which is specifically expressed in undifferentiated mouse embryonic stem cells (18). In addition to stem cells, E-RAS has been detected in the several adult cynomolgus tissues (19) and in gastric cancer and neuroblastoma cell lines (20, 21).

Plasma membrane localization of the classical RAS isoforms (H-, N-, and K-RAS4B) has been shown to be critical for their functionality (22–24). The membrane association is achieved by post-translational modifications (PTMs) at the C terminus of RAS proteins. H-RAS and N-RAS undergo two types of PTMs, farnesylation at a cysteine residue in CAAX (where C is cysteine, A is any aliphatic amino acid, and X is any amino acid) motifs and palmitoylation of one or two cysteine residues in the hypervariable region (HVR) (23, 25–27). K-RAS4B lacks the cysteine residues in its HVR; instead it has a basic sequence of six lysines that maintains its strong association with the plasma membrane (24, 28, 29).

RAS proteins are inefficient GTP-hydrolyzing enzymes. Such an intrinsic GTPase reaction requires stimulation through GTPase-activating proteins (GAPs) by orders of magnitude (30–32). However, GAP dissociation is also a very slow

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§ The abbreviations used are: PTM, post-translational modification; RBD, RAS-binding domain; aa, amino acid; GEF, guanine nucleotide exchange factor; PLC, phospholipase C; PDB, Protein Data Bank; MDCK, Madin-Darby canine kidney cell; HVR, hypervariable region; PIP3, phosphoinositide 3,4,5-trisphosphate; RA, RAS association; GAP, GTPase-activating protein; EYFP, enhanced YFP.
reaction that needs acceleration by guanine nucleotide exchange factors (GEFs) (33, 34). RAS proteins share a highly conserved GTP-binding (G) domain with five essential motifs, termed G1 to G5 (supplemental Fig. S1) (35, 36). G1 or the P-loop (10GXXGXK(S/T)7; H-RAS numbering) binds the β- and γ-phosphates of GTP (37). Substitution of glycine 12 to any other amino acid (except for proline) is most frequently found in human cancers. These mutations render RAS protein GAP-insensitive and consequently hyperactive (7, 38). G2 and G3, also referred to as switch I and switch II, respectively, are dynamic regions that sense the nucleotide state and provide the regulator and effector-binding sites (1, 39). G4 and G5 are important for determining the guanine base-binding specificity of the G domain (40, 41). Sequence analysis revealed that E-RAS contains a G domain with five fingerprint sequence motifs almost identical to classical RAS proteins indicating that it is a functional GTP-binding protein (supplemental Fig. S1). However, E-RAS contains a serine instead of glycine 12 (H-RAS numbering), making it GAP-insensitive (18).

H-, N-, and K-RAS4B share an identical effector binding regions (switch I and II; supplemental Fig. S1), suggesting that they may share the same downstream effectors. In contrast, E-RAS revealed significant differences in the effector binding regions (supplemental Fig. S1). This implicates that it may utilize other effectors as compared with known H-RAS effectors and may consequently have different cellular functions. However, the downstream effectors selective for E-RAS are not fully identified yet. A known H-RAS effector is phosphoinositide 3-kinase (PI3K) that has also been reported to be activated by E-RAS (18, 27, 42).

In addition to effector binding regions, E-RAS is distinguished from the classical RAS isoforms due to its unique extended N terminus (Fig. 1A and supplemental Fig. S1). This may provide a putative interaction site for a new group of proteins, which may determine its subcellular localization. For instance, it contains a PXXP motif that may serve as a putative binding motif for interaction with Src homology 3-containing proteins. In this study, we comprehensively investigated human E-RAS and its variants regarding their cellular localization and functional and structural properties in direct comparison with H-RAS wild-type and its G12V hyperactive variant. We found that N-terminal extension of E-RAS is important for E-RAS signaling activity. E-RAS protein most remarkably revealed different effector selectivity as compared with H-RAS, which is influenced by deviations in the effector-binding site of E-RAS. Data presented in this study implicate that in addition to switch regions, the interswitch region of E-RAS also contributes to high affinity binding to PI3Kβ and low affinity to other RAS effectors, including RASSF5/Nore1, RAF1, Ral guanine nucleotide dissociation stimulator (RalGDS), and phospholipase Cε (PLCε).

Materials and Methods

Constructs—Human E-RAS cDNA was obtained from pCMV6-AC-hsE-RAS (Origene). Human H-RAS was obtained from ptcH-RAS (43). H-RASVal-12, E-RASSer-226/Ser-228, and E-RASSer-226/Ser-228 were generated by PCR-based site-directed mutagenesis as described (32). The E-RAS with the N-terminal deletion, lacking the first 38 amino acids (aa) (E-RASΔN), was designed using primers to amplify E-RAS cDNA starting from aa 39 and ending with aa 233 (supplemental Fig. S1). The same primers were used to generate E-RASΔN/Ser-226/Ser-228 (palmitoylation-dead variant of E-RAS lacking the N terminus) using E-RASSer-226/Ser-228 as template. To generate E-RAS constructs with mutations in their effector binding regions, we used E-RASWT cDNA as template. First, E-RASWild (H70V/Q75E; Tyr-32 and Glu-37 in H-RAS), E-RASArg-79 (W79R; Arg-41 in H-RAS), and E-RASWild (A10E/Ser-226/Ser-228) were generated. These constructs were used to generate E-RASWild/Arg-79, E-RASWild/SerII, and E-RASWild/Arg-79/SerII, respectively. All cDNAs were amplified via PCR and subcloned via BamHI/Xhol in pcDNA 3.1 vector with an N-terminal FLAG tag or EcoRI/BamHI in pEFYFP-C1.

Cell Culture and Transfection—MDCK II and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 50 units of penicillin/streptomycin (Gibco® Life Technologies, Inc.). Transfection was performed by using TurboFect transfection reagent, according to manufacturer’s protocol (Life Technologies, Inc.).

Immunostaining—Cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, the cells were permeabilized with 0.25% Triton X-100/PBS for 5 min and washed again. For blocking, the cells were treated 1 h with PBS containing 0.25% Triton X-100 and 3% bovine serum albumin (BSA, Merck) at room temperature, then incubated with primary antibodies for 1 h, then washed three times, followed by incubation with secondary antibodies for 2 h at room temperature. The coverslips were mounted using ProLong® Gold antifade reagent containing DAPI dye (Life Technologies, Inc.). Primary antibodies were rabbit anti-FLAG (1:700, catalog no. F7425 Sigma) and mouse anti-Na+/K+ATPase (1:100, catalog no. A275 Sigma), and secondary antibodies Alexa 488-conjugated goat anti-rabbit IgG (1:500, catalog no. A11008, Life Technologies, Inc.) and Alexa 546-conjugated goat anti-mouse IgG (1:500, catalog no. A11003, Life Technologies, Inc.). The images were taken by using an LSM 510-Meta microscope (Zeiss) at excitation wavelengths of 364, 488, and 546 nm.

Live Cell Imaging—MDCK II cells were seeded on Permanox 8-well chambered slides (Lab-Tek, Nunc). LSM 510-Meta microscope (Zeiss) was equipped with ×63 immersion objective, and fluorescent fusion proteins were excited using lasers with 504 nm (YFP) wavelength. An environmental chamber holds the temperature at 37°C, and the cells were maintained in imaging medium.

Pulldown Assay and Immunoblotting—The RAS-binding domain (RBD) of RAF1 (aa 51–131), the RAS association (RA) domain of RalGDS (aa 777–872), the RA domain of PLCε (aa 1270–2240), the RBD of p110α (aa 127–314), the catalytic subunit of PI3Kα, and the RA domain of RASSF5 (aa 200–358) were inserted in pGEX-4T vector and expressed in Escherichia coli to obtain GST-fused proteins. Bacterial lysates were used to pulldown GTP-bound RAS proteins from total cell lysates. GST pulldown and immunoblotting using rabbit anti-FLAG (1:5000, catalog no. F7425 Sigma) and rat anti-α-tubulin (1:2000, SM 568, Acris) were carried out as described previously (44).
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parallel, the cell lysates were used to visualize phospho-MEK1/2, phospho-ERK1/2, and phospho-AKT proteins states, respectively, using antibodies against MEK1/2 (Cell Signaling™), ERK1/2 (Cell Signaling™), AKT (Cell Signaling™), phospho-MEK1/2 (Ser-217/S221, Cell Signaling™), phospho-ERK1/2 (Thr-202/Thr-204, Cell Signaling™), and phospho-AKT (Ser-473 and Thr-308, Cell Signaling) in immunoblotting. All antibodies were diluted in 5% nonfat milk (Carl Roth GmbH).

**Structural Methods**—The structures of H-RAS were used in our study because no E-RAS structure was available to date. The G domains of H-RAS and E-RAS share 48% identity and were originally described to be structurally very similar, if not identical (18). The interactions with potential binding partners were analyzed on the basis of the structures of H-RAS in complexes with p120RASGAP (PDB code 1WQ1) (30), the RASGEF SOS1 (PDB codes 1NVV (45) and 4NYI), and the downstream effectors RAF1-RBD (PDB codes 1C1Y and 3KUD) (46, 47), PI3Kγ (PDB code 1HE8) (48), BYR2-RBD (PDB code 1K8R) (49), RapGDS (PDB code 1LFD) (50), PLC1 (PDB code 2C5L) (51), Grb14 (PDB code 4K81) (52), and RASSF5 (PDB code 3DDCS) (53).

**Results**

**N Terminus Is an Important Factor for E-RAS Function**—The cellular localizations of FLAG-tagged and EYFP-tagged wild-type E-RAS (E-RAS WT) were investigated in direct comparison with H-RAS WT in MDCK II cells. Confocal imaging revealed that E-RAS, very similar to H-RAS, is mainly associated with the plasma membrane (Fig. 1B) as it is co-localized with the basolateral membrane marker of sodium/potassium-ATPase (Fig. 1D). This result clearly suggests that E-RAS undergoes post-translational modifications, e.g. farnesylation and palmitoylation, at the very C-terminal cysteines (supplemental Fig. S1). Accordingly, a palmitoylation-deficient E-RAS Ser-226/Ser-228 variant clearly exhibited a cytoplasmic accumulation, which supports the notion that E-RAS also underlies a palmitoylation/depalmitoylation mechanism as was shown previously for H-RAS (Fig. 1C) (25).

Another question addressed in this study was the role of the 38-amino acid unique N-terminal extension in E-RAS, which does not exist in other RAS proteins (Supplemental Fig. S1). This extension contains motifs, which may act either as a PXXP motif-binding site for specific Src homology 3-containing proteins or as an electrostatic interaction site (RRR motif) with a negatively charged region of proteins or with a lipid membrane. Thus, one function of the N-terminal extension and its motifs could be providing an additional signal for subcellular localization of E-RAS. Hence, we generated the N-terminal truncated E-RAS N (aa 39–233), putative PXXP motif variant E-RASSer-7 (aa 1–233), and a triple arginine motif variant E-RASAla-31/Ala-32/Ala-33-less plasma membrane localization (Fig. 1C) but not significant differences.

**Effector Selection of E-RAS Significantly Differs from H-RAS**—Before investigating the specific function of E-RAS in cells, it was important to gain insights into the E-RAS effector selectiv-
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FIGURE 2. Different effector selection of E-RAS and H-RAS. A, effector-binding residues of H-RAS, obtained from various crystal structures, are highlighted with blue letters and yellow background. RAF1 (PDB code 1C1Y), PLCε (PDB code 2CSL), RalGDS (PDB code 1LFQ), PI3Kα (PDB code 1HE8), and RASSF5 (PDB code 3DZC). B, effector binding regions (in yellow and orange) of H-RAS and E-RAS were structurally analyzed on the basis of the H-RAS structure in complexes with p120RASGAP (PDB code 1WQ1). The orange amino acids indicate the sequence deviation between H-RAS and E-RAS. C, schematic view of RAS effector pathways and their cellular functions. D, E-RAS and H-RAS pulldown (PD) with various RAS effectors using COS-7 cell lysates transiently transfected with FLAG-tagged E-RASwt, H-RASwt, and H-RASVal-12 using GST-fused effector proteins, such as RAF1-RBD, RalGDS-RA, PLCε-RBD, and RASSF5-RA. RAS proteins were analysis by immunoblot using an anti-FLAG antibody. Immunoblots (IB) of total cell lysates were used as a control to detect FLAG-RAS. Exp. time stands for exposure time. RAF, rapidly accelerated fibrosarcoma; MEK, mitogen-activated protein kinase/ERK kinase; ERK, extracellular signal-regulated kinase; PLCε, phospholipase Cε; PKC, protein kinase C; RalGDS, Ral GDP dissociation stimulator; RLP76, Ral-interacting protein 76 kDa; PI3K, phosphoinositide 3-kinase; PIP3, phosphoinositide 3,4,5-trisphosphate; MST1/2, mammalian Ste20-like kinases 1, 2.

Effectors. Effector interactions with H-RAS have been investigated both biochemically and structurally in great detail. Various amino acids of H-RAS undergo selective contacts with the effectors, including RAF1, RalGDS, RASSF5, and PLCε (Fig. 2A, blue residues with yellow background). These residues, mainly switch I, interswitch, and partially in the switch II region, are conserved among common RAS proteins but vary in E-RAS proteins (supplemental Fig. S1). This suggests that classical RAS family members, except the E-RAS, are in principle able to recognize and activate various effectors. Importantly, these effector-binding residues are highly variable between H-RAS and E-RAS (supplemental Fig. S1; Fig. 2A). Structural analysis of the effector binding regions of E-RAS was performed according to H-RAS complexes with p120RASGAP (PDB code 1WQ1). In comparison with H-RAS, the exposed residues along the effector-binding surface of E-RAS revealed significant sequence deviations (Fig. 2B). This strongly indicates a differential effector selectivity of the RAS proteins.

The members of the RAS family are known to interact with a wide range of effectors (5, 54–61) and therefore stimulate various cellular responses. Regarding their physical interaction with E-RAS and H-RAS proteins, five RAS effectors (RAF1, RalGDS, PLCε, PI3Kα, and RASSF5), with defined cellular functions (Fig. 2C), were investigated in this study. In pulldown experiments, GST-fused RAS-binding domain of RAF1 (RAF1-RBD), the RAS association domain of RalGDS (RalGDS-RA), PLCε-RA, PI3Kα-RBD, and RASSF5-RA were used as baits to pulldown FLAG-tagged E-RASwt, H-RASwt, and H-RASVal-12 overexpressed in COS-7 cells. We found that H-RASwt and H-RASVal-12 strongly bind RAF1 and weakly bind to PI3Kα. Importantly, E-RASwt clearly showed an opposite pattern of these interactions, where it binds very tightly to PI3Kα and very weakly to RAF1, RalGDS, PLCε, and RASSF5 (Fig. 2D). These data confirm that the amino acid deviations in effector-binding sites (Fig. 2, A and B) make E-RAS a unique member of the RAS family and a potent activator of the PI3K-PIP3-signaling pathways.

Effectors Selection by E-RAS Is Largely Determined by Cryptophan 79—To identify the residues determining the specificity for effector binding and activation, we next analyzed the impact of deviating residues in E-RAS on its interaction with different effectors by replacing the E-RAS residues in switch I (His-70 and Gln-75, collectively named here SwI), interswitch (Arg-79), and switch II (Ala-100, Ile-101, His-102, and Arg-103, collectively named here SwII) for the equivalent residues in H-RAS (supplemental Fig. S1). The corresponding variants, E-RASSwI, E-RASSwII, E-RASSwI/SwII, and E-RASSwI/Arg-79/SwII, were analyzed for their interaction abilities with different effectors using E-RASwt and the constitutive active variant of H-RAS, H-RASVal-12, as controls. These constructs were transiently transfected in COS-7 cells, and the GTP-bound forms of these RAS variants were pulled down using GST-fused effector proteins under the same conditions as described above. Data
obtained revealed that substitution of Trp-79 for arginine in E-RAS (E-RASArg-79) rescued the low affinity of E-RAS for PLC interacting with H9280, RAF1, and RalGDS, and no effect was observed on RASSF5 binding (Fig. 3B). In contrast, W79R-containing variants (E-RASArg-79, E-RASSwI, and E-RASSwI/Arg-79), when compared with E-RASWT, exhibited a significant reduction in binding affinity for PI3K interacting with H9251, which is comparable with the levels with H-RASVal-12. Collectively, all mutations in three regions, especially W79R, affected E-RAS interaction for PI3K interacting with H9251 (Fig. 3B). Mutations in the switch I region (E-RASSwI, E-RASSwI/Arg-79, RASwI/SwII, and E-RASSwI/Arg-79/SwII) exclusively compromised E-RAS interaction with RASSF5. However, switch II variants (E-RASSwII, E-RASArg-79/SwII, and RASwI/SwII) more strongly diminished affinity for RalGDS and RAF1 (Fig. 3B).

These results raised the following question. How does the Trp-79 interaction with effectors affect the binding affinity of E-RAS for these proteins? To address this question, we inspected available H-RAS structures in complexes with investigated effector proteins and created corresponding structural models of E-RAS with particular focus on Trp-79 in E-RAS (Arg-41 in H-RAS). Data obtained pointed to an unexpected and potentially significant role of Glu-3 (Glu-41 in E-RAS) in effector selection by RAS proteins (Fig. 3C; supplemental Fig. S1). Arg-41 is stabilized by intramolecular interactions with Glu-3 (Glu-41 in E-RAS) and side-chain contacts directly at Lys-65 of RAF1 among the analyzed H-RAS effector complexes but not PI3K. Tryptophan replacing Arg-41 in E-RAS would, because of its hydrophobic nature, be expelled from Glu-41, Glu-54, and Asn-92. This generates a new conformation in the effector region of E-RAS and accounts for a shift in effector selectivity. The highest probability for such adopting provides an empty space around the Arg-41 in the case of the PI3K complex thus yielding higher affinity of PI3K to E-RASWT. Trp-79 interacts best in a hydrophobic environment with PI3K as compared with RAF1. Reciprocal scenario applies in the case of RAF1 and PLC interacting with H9280 causing lower affinity of these effectors to E-RASWT. One example is the repulsion of Lys-65 of RAF1 by the W79R mutation that might be responsible for a weak reconstitution of E-RASArg-79 binding to RAF1.

We next examined the consequences of the affected effector interaction of the E-RAS variants regarding activation of the corresponding downstream cascades (see Fig. 3C). Interestingly, impaired PI3Kα binding of E-RAS variants, particularly W79R and SwII, also strongly influenced downstream signals of PI3K monitored by pAKT levels but not that of RAF1 analyzed by pMEK/pERK levels (Fig. 3C). Remarkably, AKT phosphorylation at both sides, Thr-308 (PDK1) and Ser-473 (mTORC2), were impaired (see below). The E-RASArg-79 variant lost its ability to signal via the PI3K/AKT cascade almost completely, indicating a key role of tryptophan 79 in E-RAS and E-RAS-like proteins in effector association and activation. An interesting observation is that a gain of RAF1 binding to E-RAS variants, especially SwI and W79R, did not result in RAF1 activation and
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in turn phosphorylation of MEK1/2 and ERK1/2 (Fig. 3, B and C).

Distinct Downstream Signaling Pathways of E-RAS via PI3K—The data presented above shed light on the specificity-determining residues for direct E-RAS-effector interaction and the consequent activation of downstream pathways. The next question we addressed was to understand the role of additional motifs within the N-terminal extension and HVR of E-RAS (see Fig. 1A) as potential molecular and cellular determinants required for signal transduction through PI3K- AKT-mTORC and RAF1-MEK1/2-ERK1/2. Therefore, we first investigated the ability of E-RAS variants to directly interact with PI3Kα and RAF1. In this experiment, FLAG-tagged E-RAS variants, H-RASWT and H-RASVal-12, transiently transfected in COS-7 cells, were pulled down with GST-fused PI3Kα-RBD and RAF1-RBD from the cell lysates (Fig. 5A). Similar to E-RASWT, the interactions of E-RAS variants were much stronger with PI3Kα-RBD as compared with RAF1-RBD, although hyperactive H-RASVal-12 mainly bound to RAF1-RBD. Moreover, this assay was used to visualize the amounts of the GTP-bound state of the E-RAS variants. Fig. 5A shows that all E-RAS variants exist in the active, GTP-bound forms.

To provide further insights to the downstream signaling activity of the above-mentioned E-RAS variants, we investigated the phosphorylation status of AKT (Thr-308 and Ser-473), MEK1/2 (Ser-217/Ser-221), and ERK1/2 (Thr-202/Thr-204), which are representative cellular targets of PI3K and RAF1 (Fig. 5B). Although the pulldown showed almost no significant difference between E-RAS variants in binding to RAS effectors, we found E-RASΔN, E-RASSer-226/Ser-228, and E-RASΔN/Ser-226/Ser-228 were strongly impaired in the activation of the PI3K-AKT-mTORC axis and clearly exhibited lower phosphorylation levels for AKT, especially at Thr-308. All E-RAS variants, including E-RASΔN, were inefficient in stimulation of MEK1/2 and ERK1/2 phosphorylation in comparison with H-RASWT and H-RASVal-12 that actively contributed to the activation of both PI3K and RAF1. Furthermore, the identification of tryptophan 79 as a specificity-determining amino acid in RAS effectors. Multiple amino acid sequence alignments of the RBD of human RAF isoforms and the catalytic subunits of human PI3K isoforms are illustrated with major focus on the some RAS-binding residues. The corresponding sequences are RAF-1 (P04049; aa 51–131), A-RAF (P10398; aa 14–91), B-RAF (P15056; aa 105–227), PI3Kα (P42336; aa 184–276), PI3Kβ (P42338; aa 191–272), PI3K γ (P48736; aa 214–296), and PI3Kδ (O00329; aa 184–226). X highlights residues interacting in β-β manner with switch I. # highlights additional residues interacting with switch I. Ø shows residues interacting with Tyr-64 in switch II. * shows residues close to Arg-41 in H-RAS or Trp-79 in E-RAS.

Discussion

In this study, we have investigated cellular localization and the signaling activity of human E-RAS regarding its physical interaction with RAS effectors and the roles of both its unique features, the N terminus and PTM by palmitoylation in direct comparison with human H-RAS. The structure-function relationship of the effector interaction sites of E-RAS resulted in the identification of tryptophan 79 as a specificity-determining amino acid of E-RAS, which is critical for its strong association with PI3K. In the cell, this interaction additionally requires the presence of both a functional N-terminal extension and palmitoylation at cysteines 226 and 228 that collectively lead to the precise activation of the PI3K-AKT-mTORC pathway.
Palmitoylation Modification and E-RAS Trafficking—To transduce signals, RAS proteins should be associated with the lipid membranes. They are compartmentalized by PTMs at their C terminus, with the CAAX motif at the farnesylation site, and additional upstream cysteine residues at the palmitoylation site(s) in the case of H- and N-RAS (supplemental Fig. S1) (23–25,29). We found that like the mouse E-RAS (42), substitution of two cysteine residues Cys-226/Cys-228 in HVR of human E-RAS with serines clearly impaired the plasma membrane localization of protein. This is a strong indication that human E-RAS undergoes palmitoylation at these sites, as described for the first time for H-RAS (62). Yamanaka and co-workers (42) reported that these cysteine residues are important for endomembrane localization of mouse E-RAS and only signals if HVR of H-RAS can rescue endomembrane localization of E-RASSer-226/Ser-228. Our confocal microscopy data revealed that in contrast to plasma membrane localization of E-RASWT, palmitoylation-deficient E-RASSer-226/Ser-228 is mainly localized, with a clear pattern, in cytoplasm and also in endomembranes. Our data clearly support proposed reports demonstrating that H-RAS and N-RAS cycle between Golgi and the plasma membrane via reversible and dynamic palmitoylation-depalmitoylation reactions (25, 63, 64).

N-terminal Extension and C-terminal Insertion of E-RAS—A sequence comparison between E-RAS and other RAS isoforms highlighted additional regions and motifs, such as the unique N terminus of E-RAS that is not present in other RAS-like proteins. We propose that the N-terminal extension of E-RAS might modulate its localization through interaction with potential adaptor/scaffold proteins via putative PXXP and RRR motifs. With our co-localization studies, we did not observe significant differences in localization of the N-terminal mutants of E-RAS. However, considering our results, we cannot exclude the role of the E-RAS N terminus as a putative protein interaction site, because E-RAS is not expressed endogenously in the MDCK II cells, and therefore its binding partner may not be available in this cell line. To confirm our hypothesis, we need to study a different cell line, like embryonic stem cells (42), gastric tumors (65), neuroblastoma cells (20), and also hepatic stellate cells,3 where E-RAS is endogenously expressed (unpublished data).

Imaging methods used in this study did not allow visualizing microdomain localization of E-RAS variants. The plasma membrane is not a homogeneous lipid bilayer and includes a set of microdomains, such as lipid raft and caveolea (66, 67). The HVR at the C-terminal end of RAS proteins is critical for lateral sorting and is divided into two separate domains, membrane-targeting domain and linker domain (68). Membrane targeting domain contains a CAAX box and one or two upstream cysteines that are palmitoylation sites. Palmitoylated proteins can be targeted to lipid rafts. Because H-, N-, and K-RAS are dipalmitoylated, monopalmitoylated, and nonpalmitoylated, respectively, they exhibit different lateral segregation across the plasma membrane microdomains (69). GDP-bound H-RAS is associated with the lipid raft, but when it is activated and GTP-loaded, it moves laterally to nonlipid raft regions (68, 70, 71). E-RAS, like H-RAS, is dipalmitoylated suggesting that it may favor the lipid rafts. On the contrary, E-RAS is mainly GTP-

loaded, which makes it difficult to compare it with wild-type H-RAS. It is reported that the active GTP-loaded H-RAS
\textsuperscript{Val-12} variant occupies the nonlipid rafts so the constitutively active E-RAS may also be clustered in this region. The second domain in HVR, termed linker domain, releases GTP-loaded H-RAS from the lipid rafts. Linker domain can be divided in N- and C-terminal regions in a way that the C-terminal region is a spacer, which seems not to be important (68). Human E-RAS has an insertion in this C-terminal spacer (aa 173–179, H-RAS numbering) that may also affect microdomain migration of E-RAS. Taken together, we propose that three factors most likely modulate the microdomain targeting of E-RAS, such as an extended N terminus, a C-terminal insertion, and the GTP-loaded state due to a prominent deviation at position Ser-50 (Gly-12 in H-RAS).

Our cell-based studies revealed that the N-terminal extension of E-RAS is critical for PI3K-AKT-mTORC activation, and N-terminal truncated E-RAS variants (E-RAS\textsuperscript{ΔN} and E-RAS\textsuperscript{ΔN/Ser-226/Ser-228}) remarkably had a lower signaling activity. One explanation may be the role of the unique N terminus in the lateral segregation of E-RAS across the membrane that consequently specifies association with and activation of its effectors in a manner reminiscent to microdomain localization of H-RAS that regulates its interaction with effector proteins of RAF1 and PI3K (68). In addition, E-RAS was found in membrane ruffles (data not shown), which may be induced by Rac1 activated by the E-RAS-PI3K-PIP\textsubscript{3}-RacGEF axis (72–74). Such a scenario has been reported for the R-RAS N-terminal 26-amino acid extension, which has been proposed to positively regulate Rac activation and cell spreading (75).

Constitutively Active Form—GAPs accelerate the GTP hydrolysis reaction of RAS proteins by orders of magnitude by supplying a highly conserved, catalytic arginine finger (31, 32). H-RAS glycine 12 mutations to any other amino acid interfere with insertion of arginine finger in the GTPase active site and therefore make the enzyme GAP-insensitive (30). Interestingly, E-RAS has a deviation in the corresponding position and carries a serine instead of a glycine indicating that E-RAS is hyperactive and GAP-insensitive. Our stopped-flow data revealed that p120RASGAP was not able to accelerate the GTP hydrolysis

![FIGURE 6. Co-localization of E-RAS with PI3Kα](image)

Transfected MDCKII cells with FLAG-tagged E-RAS were incubated with bacterial lysates, containing GST-RBDs of PI3Kα and RAF1 proteins and stained with antibodies raised against GST and FLAG to investigate their co-localization with GTP-bound H-RAS and E-RAS proteins. E-RAS co-localized with PI3Kα. Scale bar, 10 μm.
reaction of E-RAS, although it can act on H-RAS and convert it to the GDP-bound inactive form (data not shown). We have shown that all E-RAS variants exist mostly in GTP-bound forms as shown by a pulldown experiment with PI3K and RAF1. This and the fact that E-RAS is GAP-insensitive suggest that E-RAS may underlie a different and yet undefined control mechanism that negatively regulates E-RAS activity and thus its signal transduction.

It seems that expression of E-RAS is highly regulated at the transcriptional levels and rather limited to special cell types, such as embryonic stem cells (42), gastric tumors (65), neuroblastoma cells (20), and also hepatic stellate cells.\(^5\) Moreover, the unique N terminus of E-RAS may provide specialized protein-protein interaction sites resulting in E-RAS sequestration, degradation, or membrane microdomain localization as shown for R-RAS (75, 76). E-RAS could interact with specific scaffolding proteins that bring it close to its effectors and regulate its activities. It is tempting to speculate that E-RAS may underlie a similar mechanism via serine/threonine phosphorylation and 14-3-3 binding as described for Rnd3 (75, 76), a constitutively active member of the Rho protein family (77). However, there is as yet no evidence for an E-RAS phosphorylation particularly at its N terminus that contains 4 threonines and 2 serines (supplementary Fig. S1).

**Effector Binding Regions**—E-RAS proteins transduce extracellular signals to a variety of intracellular signaling pathways through the interaction with a wide spectrum of effector proteins. Upon GDP to GTP exchange, RAS proteins undergo conformational changes in two critical regions, switch I and switch II. Notably, the GTP-bound form of RAS interacts with their target effectors through switch regions and thereby activates various pathways (5). A detailed study of structure-sequence relationships revealed a distinctive effector binding region for E-RAS in comparison with RAS isoforms (H-, N-, and K-RAS). Subsequent interaction analysis with five different RAS effectors revealed that effector binding profile of E-RAS significantly differs from H-RAS. E-RAS\(^{WT}\) tightly bound to PI3Kp and revealed very low affinity for other RAS effectors. In contrast, H-RAS showed an opposite pattern with the highest affinity for RAF1. These data were confirmed by investigating the respective downstream signaling cascades (PI3K-AKT-mTORC and RAF1-MEK1/2-ERK1/2) at the level of phosphorylated AKT, MEK1/2, and ERK1/2. Our results are consistent with a previous study of Yamanaka and co-workers (18), who applied another PI3K isoform (PI3K\(\delta\)) and observed differences between H-RAS and E-RAS. It seems probable that E-RAS and H-RAS possess a different affinity for distinct PI3K isoforms, \(\alpha\), \(\beta\), \(\gamma\), and \(\delta\), and this may account for their specific biological outputs (78). Consistently, the catalytic subunit of the PI3K\(\gamma\) isoform, PI3K\(\gamma\), interacts with switch I of H-RAS in anti-parallel \(\beta\)-sheet fashion (48), also approaching RAS-conserved Asp-33 by two lysines. Residues in H-RAS contacting \(\beta\)-strand of PI3K\(\gamma\) and preceding amino acids differ significantly among the PI3K isoforms regarding the primary structures (Fig. 4D).

Although PI3K\(\gamma\) has four hydroxyl-containing amino acid side chains at this place, PI3K\(\beta\) possesses one and PI3K\(\delta\) isoform two negatively charged residues whereby both have in addition two amino acid insertions. In contrast, the PI3Kp isoform has insertion of six residues, and the hydroxyl-containing amino acids are replaced by one asparagine and two lysines (Fig. 4D).

We hypothesize that these differences in PI3K isoforms are of particular importance due to the stabilization of intermolecular \(\beta\)-sheet interaction and especially because the contact site of the crucial Trp-79 in E-RAS (Arg-41 in H-RAS) is highly variable (Lys, Gln, Thr, and Glu; see Fig. 4D).

Substitutions for E-RAS residues in the switch I and II and interswitch regions with corresponding residues in H-RAS provided several interesting aspects and new insights (Fig. 3). One is a shift in effector selection. Strikingly, and in contrast to other investigated effectors, RAF1-RBD undergoes contacts with the switch I and the interswitch regions (Fig. 2A) (46, 47). However, E-RAS\(^{SwI}\), which has an almost identical switch I when compared with H-RAS, showed a reduced binding to RAF1 that was clearly elevated when this was combined with the interswitch mutation W79R (E-RAS\(^{SwI/Arg-79}\)) (Fig. 3B). Consistently, the major difference was observed with E-RAS\(^{Arg-79}\), where a tryptophan was replaced by an arginine (Arg-41 in H-RAS). This variant led to increase in RAF1 binding and partly rescued the low affinity of the wild type and the switch variants (E-RAS\(^{SwI/Arg-79}\) and E-RAS\(^{SwI/Arg-79/SwII}\)). According to the crystal structure (46), Arg-41 in H-RAS (Trp-79 in E-RAS) interestingly forms a hydrogen bond with the backbone oxygen of Asn-64 in RAF1-RBD that very likely enabled E-RAS\(^{Arg-79}\) to make additional electrostatic contacts with RAF1 (Fig. 4, A and B). In addition, E-RAS shares a glutamate (Glu-41) with H-RAS (Glu-3) (supplementary Fig. S1). Glu-3 interacts in intermolecular fashion with Arg-41 and stabilizes the H-RAS/RAF1 complex formation (Fig. 4A). Accordingly, mutation of W79R in E-RAS reconstitutes such intermolecular interaction between Glu-41 and Arg-79, thus increasing significantly the interaction between E-RAS\(^{Arg-79}\) and RAF1 (Fig. 3B). Another important contribution to effector binding concerning Trp-79 originates very likely in its expulsion from the above-mentioned Glu-41 and the ability of bound effector protein to accommodate altered conformation of Trp-79. As mentioned before, Arg-41 of H-RAS is contacted by RAF1 in its complex structure. The space where the tryptophan can be accommodated and hydrophobically interact with the effector is thus limited resulting in diminished affinity of these effectors to E-RAS\(^{WT}\). Moreover, switch II quadruple mutation of E-RAS (E-RAS\(^{SwII}\), see Fig. 3A) showed the largest impairment in RAF1 binding. This was not expected especially because the structural data, reported previously (46, 47), have shown that RAF1-RBD does not physically contact the switch II of RAS. Again, E-RAS\(^{Arg-79/SwII}\) partially restored the loss of RAF1 binding but most remarkably not the E-RAS\(^{SwII}\) variant that actually is almost identical to H-RAS regarding the amino acid sequence of its switch I and II regions (see Fig. 3A). Even though E-RAS\(^{Arg-79}\) binds more tightly to RAF1, it still does not activate the MAPK pathway like E-RAS\(^{WT}\). Note that there was no increase in MEK and ERK phosphorylation, and we detected even the opposite, namely a significant decrease in pMEK1/2 and pERK1/2 as compared with the vector control (Fig. 3C; see E-RAS\(^{WT}\) and E-RAS\(^{Arg-79}\) lanes). An explanation for the absence of E-RAS\(^{Arg-79}\) signaling toward the MAPK pathway is that most probably the additional component, including scaffold proteins such as SHOC2 (79–
PLCɛ contains two RAS association domains, RA1 and RA2. RA2 forms a complex with H-RAS in a GTP-dependent manner by contacting nine different residues of the switch I and II regions, and also Gln-25 and Arg-41 (51), from which four (Glu-37, Arg-41, Glu-63, and Tyr-63) deviate in E-RAS (Fig. 3A). This explains why we observed an extremely weak E-RAS-PLCɛ interaction as compared with H-RAS\(^{\text{Val-12}}\). Most interestingly, the W79R mutation of E-RAS resulted in a strong gain of binding activity (Fig. 3B; see E-RAS\(^{\text{W79R}}\) and E-RAS\(^{\text{Arg-79}}\) lanes). Notably, this effect was not so strong in the case of the switch II mutation (E-RAS\(^{\text{SwII}}\)), and the switch I mutation (E-RAS\(^{\text{SwI}}\)) did not show any change in the E-RAS association with PLCɛ. A combination of the mutations (E-RAS\(^{\text{SwI/SwII}}\)) was hardly detectable and the combinations with W79R (E-RAS\(^{\text{Arg-79/SwI}}\), E-RAS\(^{\text{Arg-79/SwII}}\), and RAS\(^{\text{SwI/Arg-79/SwII}}\)) rather counteracted the gain of binding activity of RAS\(^{\text{Arg-79}}\).

On a molecular level, Trp-79 in wild-type E-RAS can be hydrophobically attracted to Pro-2149 of PLCɛ but not intramolecularly to Glu-41 (data not shown), and the space for its conformational relaxation is limited similarly to RAF1 as mentioned above. We propose that W79R mutation generates stronger intramolecular contact between Glu-41 and Arg-79 and consequently stabilizes the protein complex with PLCɛ. Katan and co-workers (51) have discussed that the H-RAS residues Tyr-64, Ile-36, and Met-67 (His-102, Ile-74, and Leu-105 in E-RAS) in combination with Phe-2138 and Val-2152 from PLCɛ-RA2, provide a hydrophobic clusters. Introduction of another hydrophobic residue in E-RAS as demonstrated with a single point mutation at Trp-79 (E-RAS\(^{\text{Arg-79}}\)) has obviously created an additional and distinct binding site for RAS association domains, such as RA2 of PLCɛ and most likely also RA of RalGDS. The latter, a GEF for Raf, links two RAS family members, RAS and Raf (82). Although the crystal structure of H-RAS/RalGDS-RA has not reported an involvement of Arg-41 (50), our structural analysis predicted a close hydrophobic contact of Arg-41 with Met-819 of RalGDS (3.2 Å). Notably, data obtained from the interaction of RalGDS and RA1 with E-RAS variants appear similar as compared with that for PLCɛ.

P3K is a well known effector of classical RAS proteins and promotes cellular survival (78). In comparison with H-RAS, E-RAS interacts more strongly with P3Kα-RBD and activates the P3K-RAFT-mTORC cascade. Mutagenesis at switch and interswitch regions (E-RAS\(^{\text{SwI}}\), E-RAS\(^{\text{Arg-79}}\), and E-RAS\(^{\text{SwII}}\)), attenuated binding of E-RAS to P3Kα-RBD, demonstrating the role of critical E-RAS residues at effector binding regions. These data are consistent with a previous study that has shown that P3Kγ-RBD contacts both switch I and switch II regions of H-RAS (48). Interestingly, W79R mutation of E-RAS (Arg-41 H-RAS), which has increased binding to RA1, PLCɛ, and RalGDS, dramatically reduced the binding to P3Kα. The affinity of this E-RAS mutant (E-RAS\(^{\text{Arg-79}}\)) for P3Kα-RBD appears similar to that of H-RAS\(^{\text{Val-12}}\) (Fig. 3B; see H-RAS\(^{\text{Val-12}}\) and E-RAS\(^{\text{Arg-79}}\) lanes). We think that the strong interaction between E-RAS and P3K stems from the ability of structure to accommodate altered conformation of Trp-79 and from its hydrophobic contact to P13K (Fig. 4C). In contrast, W79R mutation in E-RAS enables Glu-41 to attract Arg-79 and to interfere with this hydrophobic interaction, resulting in a significant reduction of the binding affinity between P13K and E-RAS (Fig. 4C).

In the same line of evidence, we also observed E-RAS\(^{\text{Arg-79}}\) deficient at the activation of RAS-P3K-RAFT-mTORC2 pathway (9) as monitored with Ser-473 phosphorylation of AKT (see result Fig. 3C). Thus, Trp-79 in E-RAS represents a specificity-determining residue for the proper binding to and activation of P13K.

RASSF members are known as a RAS effector with tumor suppressor functions. RASSF5 have two splice variants NORE1A and RAPl, which share same RBD (53). We applied the RASSF5-RA domain to analyze the interaction of E-RAS variants with this RAS effector. As shown for RA1 and RalGDS, switch I H70Y/Q75E mutation of E-RAS (E-RAS\(^{\text{SwI}}\)) also attenuated the binding to RASSF5, and this was the case for all E-RAS variants harboring switch I mutations (E-RAS\(^{\text{SwI/Arg-79}}\), E-RAS\(^{\text{SwI/SwII}}\), and E-RAS\(^{\text{SwI/Arg-79/SwII}}\)). Switch II and W79R mutations did not affect the binding affinity for RASSF5, emphasizing the importance of the more conserved switch I region in the complex formation of the RAS proteins with RASSF5 (53). It remains to be investigated whether E-RAS is an activator of RASSF5 and thus a regulator of the Hippo pathway.

In summary, we conclude that switch regions of E-RAS act as core effector binding regions that form an E-RAS-specific interaction interface for its effectors, such as P3K. The P3K isoform specificity in E-RAS-expressing cells remains to be investigated. Trp-79 of E-RAS appears to determine the effector selectivity. E-RAS binding to other RAS effectors, such as RASSF5, RalGDS, and RAF1, is weak but may still be of physiological relevance. Improvement of the interaction with RAF1 by mutagenesis, for example, rather exhibited an inhibitory impact on the MAPK pathway. It remains unclear whether protein phosphatases specific for MAPKs were activated. The N terminus of E-RAS is unique and may play a critical role in the interaction with its accessory proteins for positioning E-RAS to subcellular microdomains of the plasma membrane.

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**Note Added in Proof**—Supplemental Fig. 1 comparing mammalian E-RAS and classical RAS sequences was inadvertently omitted from the version of this article that was published May 4, 2015 as a Paper in Press. Supplemental Fig. 1 is now available on line.

**References**


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Functional Properties of E-RAS
Functional Properties of E-RAS


**Supplementary Data**

The function of embryonic stem cell-expressed Ras (E-Ras), a unique Ras family member, correlates with its additional motifs and its structural properties

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Supplementary FIGURE S1. Overall sequence comparison of mammalian E-Ras proteins with classical Ras proteins. E-Ras contains an extended N-terminus (aa 1-38), missing in H-, K-, and NRas, with a putative SH3-binging motif (PxxP). G1 to G5 boxes indicate the presence of five essential GDP/GTP binding (G) motifs. The P-loop (G1) of E-Ras contains a serine instead of a glycine (codon 12, H-Ras numbering), a frequently mutated site within RAS genes in human cancer (Fasano et al., 1984). Several residues in switch I (G2) and switch II (G2) regions that are responsible for effector recognition are different between E-Ras and H-Ras (bold letters). E-Ras contains, like H-Ras, a CAAX motif and two cysteines at the C-terminal hypervariable region (HVR), which is the sites for PTMs by farnesylation and palmitoylation, respectively. The incomplete, terminal sequence of Heterocercus glaber E-Ras is shown by X letters. The secondary structure elements, the α helices (orange) and β sheets (green), of the G domain were deduced from the H-Ras crystal structure (Pai et al., 1990) (PDB code: 5p21). The mutation sites of E-Ras variants, which are used in this study, are highlighted by arrowheads below and asterisk above the sequence.
The Function of Embryonic Stem Cell-expressed RAS (E-RAS), a Unique RAS Family Member, Correlates with Its Additional Motifs and Its Structural Properties

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