Identification of a Novel Domain of Ras and Rap1 That Directs Their Differential Subcellular Localizations*

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The small GTPase Ha-Ras and Rap1A exhibit high mutual sequence homology and share various target proteins. However, they exert distinct biological functions and exhibit differential subcellular localizations; Rap1A is predominantly localized in the perinuclear region including the Golgi apparatus and endosomes, whereas Ha-Ras is predominantly localized in the plasma membrane. Here, we have identified a small region in Rap1A that is crucial for its perinuclear localization. Analysis of a series of Ha-Ras-Rap1A chimeras shows that Ha-Ras carrying a replacement of amino acids 46–101 with that of Rap1 exhibits the perinuclear localization. Subsequent mutational studies indicate that Rap1A-type substitutions within five amino acids at positions 85–89 of Ha-Ras, such as NNTK585–89TAQST, NN85–86TA, and TAAQST, are sufficient to induce the perinuclear localization of Ha-Ras. In contrast, substitutions of residues surrounding this region, such as FA182–84YSI and FEDI90–93FNDL, have no effect on the plasma membrane localization of Ha-Ras. A chimeric construct consisting of amino acids 1–134 of Rap1A and 134–189 of Ha-Ras, which harbors both the palmitoylation and farnesylation sites of Ha-Ras, exhibits the perinuclear localization like Rap1A. Introduction of a Ha-Ras-type substitution into amino acids 85–89 (TAQST85–89NNTKS) of this chimeric construct causes alteration of its predominant subcellular localization site from the perinuclear region to the plasma membrane. These results indicate that a previously uncharacterized domain spanning amino acids 85–89 of Rap1A plays a pivotal role in its perinuclear localization. Moreover, this domain acts dominantly over COOH-terminal lipid modification of Ha-Ras, which has been considered to be essential and sufficient for the plasma membrane localization.

The mammalian Ras family of small GTPases consists of more than 20 members including Ras, Rap, R-Ras, Ral, Rin, and Rheb (1, 2). As molecular switches, these GTPases cycle between two conformational states depending on whether GDP or GTP is bound. The GTP-bound form represents the active conformation, which interacts with and stimulates downstream target proteins. In the case of Ras as a representative of the small GTPases, two regions, designated switch 1 (amino acids 32–40) and switch 2 (amino acids 60–76), undergo conformational change upon GDP/GTP exchange. These two regions have been implicated in interaction of Ras with an array of its regulatory and target molecules (3, 4). In particular, the switch 1 region binds to the RBD of the serine/threonine kinase Raf-1 or the Ras/Rap1-associating domains of various other effectors in a GTP-dependent manner and therefore is also called the effector region. In addition to the binding through the effector region, a second GTP-independent interaction between Ras and its effector is required for full activation of the effectors (5).

Ras acts as a molecular switch of a wide variety of signaling pathways that direct cell cycle progression, survival and differentiation depending on cell types (6–8). Being localized in the plasma membrane as well as in the endoplasmic reticulum and the Golgi apparatus, Ras is activated by GEFs, including Sos1, Sos2, Ras-GRF1, Ras-GRF2, and Ras-GRP-1 to 4, in response to signals triggered by various membrane-spanning receptors. Activated Ras, in turn, interacts with and stimulates a variety of target proteins. Signaling pathways downstream of Ras ultimately modulate specific gene expression in the nucleus. Once its GTP-hydrolyzing activity is impaired by mutation, Ras becomes oncogenic as a constitutive GDP-bound form. Such mutations, in fact, have been identified in various human cancers as well as in carcinogen-induced mouse tumors, implying that genetic alteration at the ras locus is a critical step in carcinogenesis.

Rap1, also called Krev-1 and smg p21, is a close relative of Ras, sharing the identical effector region with Ras (2, 9). In fact, Rap1 associates with a subset of Ras effectors including Raf-1, B-Raf, phosphoinositide 3-kinase, Ral GEFs, and PLCε. Still, the interaction with Rap1 does not cause, for instance, Raf-1 activation and even antagonizes Ras signaling, thereby suppressing Ki-Ras-induced transformation of NIH3T3 cells. These activities are proposed to be ascribable to the tight binding of Rap1 to the cysteine-rich domain, the second Ras/Rap1-binding site, of Raf-1 (5). Contrary to the downstream targets, Rap1 does not share its GEFs, including C3G, Epac, cAMP-GEF, CalDAGGEF1, RA (PDZ)-GEF-1, and RA (PDZ)-GEF-enhanced green fluorescent protein; HA, haemagglutinin; GST, glutathione S-transferase; MBE, maltose-binding protein; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonic acid; mant-GDP, 2′,3′-(5′-azido-3′-deoxy-5′-thriAMYTHRENOYL)guanosine 5′-diphosphate.

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GEF-2, with Ras. Although Rap1 exhibits the Ras-antagonizing activity under certain conditions, Rap1 is localized predominantly in the perinuclear compartments including the Golgi apparatus and late endosomes in a marked contrast to Ras, a large population of which exists in the plasma membrane (10–13). Thus, it is feasible that Rap1 exerts its own functions in addition to inhibition of the Ras pathways. Indeed, Rap1 has been reported to mediate activation of integrins and subsequent cell adhesion following extracellular stimulations such as T-cell receptor or CD31 ligation and lipopolysaccharide treatment (9). However, subcellular location of Rap1 pertinent to these functions remains obscure.

Both Ras and Rap1 undergo a series of postranslational modifications in the COOH-terminal hypervariable region, which are thought to be crucial for determination of their subcellular localization (2). Initially, a lipid tail (a farnesyl moiety for Ras and a geranylgeranyl moiety for Rap1) is attached to a cysteine residue at the fourth position from the COOH terminus. This is an obligatory event to elicit subsequent processes including proteolytic removal of the COOH-terminal three amino acids and methylation of the COOH terminus. In Ha-Ras, Ki-Ras4A, and N-Ras, an additional membrane-targeting signal involving one or two palmitolytized cysteine residues within the COOH-terminal portion cooperate with the farnesyl moiety. In Ki-Ras4B and Rap1, a polybasic motif consisting of multiple lysine residues near the COOH terminus enhances the membrane attachment. Although the farnesyl moiety is required for membrane attachment of Ha-Ras, it alone does not suffice for targeting this protein to the plasma membrane. In fact, a Ha-Ras mutant that lacks the palmitolytated sites is localized and activated in the endoplasmic reticulum and the Golgi apparatus (14, 15).

Although the association of Ras and Rap1 with the membrane is primarily because of the COOH-terminal lipid modifications, the mechanisms whereby Ras and Rap1 are localized in specific subcellular membrane compartments remain to be clarified. Here, as a step toward understanding this issue, we have attempted to identify a specific region of Ras and Rap1 responsible for determination of their differential subcellular localizations by employing a series of chimeric constructs between them.

**EXPERIMENTAL PROCEDURE**

**Plasmids—**cDNAs for wild-type human Ha-Ras (GenBank™ accession number NM_002884) and human Rap1A (GenBank™ accession number NM_002884) and their mutants were subcloned between HindIII and BamHI sites of pFLAG-CMV-2 (Sigma) for expression as EGFP fusions in COS-7 cells and into a BamHI site of pEF-BOS (16) for expression with an NH2-terminal triple HA tag in COS-7 cells. cDNAs for wild-type human Ha-Ras and its mutants were subcloned into a BamHI site of pGEX-6P-1 (Amersham Biosciences) for expression as GST fusions in Escherichia coli. The cDNA for EGFP-tagged Raf-1 BBD (amino acids 50–131) was subcloned between HindIII and BamHI sites of pFLAG-CMV-2. The expression plasmid for an MBP fusion Raf-1 BBD was previously described (17). Site-directed mutagenesis was carried out by using the QuikChange site-directed mutagenesis kit (Stratagene) and appropriate oligonucleotides. For the construction of cDNAs for Ha-Ras/Rap1A chimeras, the following restriction enzyme cleavage sites were generated by site-directed mutagenesis: MuI sites were generated by the introduction of a “CCG” to “CCG” mutation at the 102nd codons of Rap1A and Ha-Ras. SalI sites were generated by the introduction of an “ATTGAT” to “GTGCAC” mutation in the region of 46th and 47th codons of Ha-Ras and the introduction of a “GAT” to “GAC” mutation at the 47th codon of Rap1A (1–101)-Ha-Ras, and a BssHII site was generated by the introduction of a “GCCCGA” to “GCCGCC” mutation in the region of 134th and 135th codons of Rap1A (1–101)-Ha-Ras. The cDNA for Rap1A(1–134) with BssHII sites at both ends was amplified by the polymerase chain reaction. All the recombinant cDNAs were confirmed by sequencing.

**Cell Culture and Confocal Laser Microscopy—**COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected with expression plasmids by using the Superfect transfection reagent (Qiagen) according to the manufacturer’s instructions. After culture for 48 h, cells were fixed in PBS supplemented with formaldehyde (3.7% (v/v)) for 30 min. For immunofluorescent staining, fixed cells were washed three times with PBS and permeabilized with methanol for 1 min. After washing three times with PBS, cells were incubated in PBS supplemented with horse serum (10% (v/v)) and then in PBS supplemented with horse serum (10% (v/v)) and primary antibody (an antibody against the HA tag (12CA5, Roche Applied Science), the mannose 6-phosphate receptor (Calbiochem), or lysosome-associated membrane protein-1 (Santa Cruz Biotechnology)) for 2 h. Subsequently, cells were washed three times with PBS, and incubated in PBS supplemented with horse serum (10% (v/v)) and an anti-mouse IgG antibody conjugated with AlexaFluor488 or AlexaFluor546 (Molecular Probes) for 1 h, followed by washing three times with PBS. Staining of the Golgi apparatus and the endoplasmic reticulum with BODIPY TR ceramide (Molecular Probes) and AlexaFluor594-conjugated concanavalin A (Molecular Probes), respectively, was performed according to the manufacturer’s instructions as described previously (18). Fluorescent labels were visualized by a confocal laser scanning microscope (LSM510 META, Carl Zeiss). The protein farnesyltransferase inhibitor FTI-277 and the protein geranylgeranyltransferase inhibitor GGTT-286 were purchased from Calbiochem.

**Preparation of Recombinant Ha-Ras Proteins—**Wild-type and mutant Ha-Ras proteins (Ha-Ras, Ha-Ras(NTKSS85–89TAQST), Ha-Ras(NNS85–86TA), Ha-Ras(89TAQST), Ha-Ras(89TAQST)) were expressed as GST fusions in an E. coli strain BL21 in the presence of isopropyl-β-D-thiogalactopyranoside (0.5 mM) at 30 °C for 3 h. Cells were harvested, resuspended in buffer A (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 0.8% (w/v) CHAPS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM diethiothreitol) supplemented with 5 mM MgCl2, and 10 μg/ml GTP, and disrupted by sonication (15 s x 5 times). Supernatants of centrifugation at 100,000 × g for 1 h were incubated with glutathione-Sepharose 4B (Amersham Biosciences) at 4 °C for 3 h. After washing with buffer B (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM MgCl2, 1 mM EDTA), glutathione-Sepharose beads were treated with PreScission protease (125 units/ml, Amersham Biosciences) at 4 °C overnight. Recombinant Ha-Ras proteins without GST were collected and subjected to further experiments.

**Pull-down Assay for Ras**

**Fig. 1. Schematic representation of Ha-Ras/Rap1A chimeras and Rap1A deletion mutants used in this study.** Blue and red bars represent Ha-Ras and Rap1A, respectively. Numbers above and below the bars depict amino acid residues of Ha-Ras and Rap1A, respectively. (v/v) fetal bovine serum and transfected with expression plasmids by using the Superfect transfection reagent (Qiagen) according to the manufacturer’s instructions. After culture for 48 h, cells were fixed in PBS supplemented with formaldehyde (3.7% (v/v)) for 30 min. For immunofluorescent staining, fixed cells were washed three times with PBS and permeabilized with methanol for 1 min. After washing three times with PBS, cells were incubated in PBS supplemented with horse serum (10% (v/v)) and an anti-mouse IgG antibody conjugated with AlexaFluor488 or AlexaFluor546 (Molecular Probes) for 1 h, followed by washing three times with PBS. Staining of the Golgi apparatus and the endoplasmic reticulum with BODIPY TR ceramide (Molecular Probes) and AlexaFluor594-conjugated concanavalin A (Molecular Probes), respectively, was performed according to the manufacturer’s instructions as described previously (18). Fluorescent labels were visualized by a confocal laser scanning microscope (LSM510 META, Carl Zeiss). The protein farnesyltransferase inhibitor FTI-277 and the protein geranylgeranyltransferase inhibitor GGTT-286 were purchased from Calbiochem.
The supernatant of centrifugation at 15,000 × g for 1 h was collected, and MBP-Raf-1 RBD in the supernatant was immobilized on amylose resin (Bio-Rad). COS-7 cells transfected with expression plasmids for HA-tagged wild-type and mutant Ha-Ras proteins (Ha-Ras, Ha-Ras(G12V), Ha-Ras(G12V, NNTKS85–89TAQST), Ha-Ras(G12V, NN85–86TA), Ha-Ras(G12V, TK87–89QST)) were incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum for 24 h and then serum-starved for another 16 h. Cells were lysed in buffer C (50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 2.5 mM MgCl2, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM dithiothreitol, 1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride), and centrifuged at 15,000 × g for 15 min. The resultant supernatants were incubated with MBP-Raf-1 RBD immobilized on amylose resin at 4 °C for 3 h. After washing with buffer C four times, samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using an anti-HA antibody (12CA5).

**Preparation of Soluble and Particulate Subcellular Fractions**—COS-7 cells transfected with expression plasmids were suspended in PBS, sonicated, and then centrifuged at 100,000 × g for 1 h. The supernatant and the precipitate were used as soluble and particulate fractions.

**RESULTS**

**Subcellular Localization of Ha-Ras/Rap1A Chimeric Constructions**—To determine the region that is important for the perinuclear localization of Rap1A, a series of chimeric constructs between Ha-Ras and Rap1A as well as various Rap1A deletion mutants were expressed as EGFP fusions in COS-7 cells, and their subcellular localization was assessed by confocal laser microscopy (Fig. 1). Wild-type Ha-Ras and Rap1A were localized in the cell surface membrane and the perinuclear region, respectively (Figs. 2, A and B, and 3, E and F). The perinuclear location of Rap1A matched well with that of the Golgi apparatus, which was stained with BODIPY TR ceramide (18). A chimera composed of the NH2-terminal half of Rap1A and the COOH-terminal half of Ha-Ras (termed Rap1A(1–101)-Ha-Ras) (Fig. 1), which is anticipated to undergo Ha-Ras-type posttranslational modifications at its COOH terminus, was predominantly localized in the perinuclear region, suggesting that the NH2-terminal portion of Rap1A contains a signal that directs the perinuclear localization of the protein (Fig. 2C).

To further delineate the region responsible for the perinuclear localization, the subcellular localization of a Ha-Ras-derived protein, in which a central region (amino acid residues 46–101) was replaced by the corresponding Rap1A sequence (termed Rap1A(101–46)-Ha-Ras) (Fig. 1), was examined. This construct indeed localized in the perinuclear region, which was stained with BODIPY TR ceramide, supportive of a role of the sequence between amino acid residues 46 and 101 of Rap1A for the perinuclear localization (Figs. 2, D–F, and 3G).

We next constructed a series of NH2-terminal deletion mutants of Rap1A as shown in Fig. 1, and their subcellular localization was examined. Rap1A(ΔN30) and Rap1A(ΔN60) were mainly localized in the perinuclear region, whereas Rap1A(ΔN90) was localized in the plasma membrane as well as in the cytoplasm (Fig. 2, G–I). Therefore, posttranslational modifica-
tions at the COOH terminus of Rap1A are not sufficient for its perinuclear localization, and a region spanning amino acid residues 60–90 may also be required.

A Small Region Consisting of Amino Acid Residues 85–89 of Rap1A Is Sufficient for Golgi Localization—Toward identifying residues of Rap1A that determine the perinuclear localization, various Ha-Ras mutants that contain Ha-Ras to Rap1A-type substitutions of several consecutive amino acids within the region of residues 46–101 were generated, and their localization was examined (Fig. 4). Among them, a substitution of five amino acids (NNTKS85–89TAQST) altered the subcellular localization of Ha-Ras from the plasma membrane to the perinuclear region (Fig. 5, A–D). In marked contrast, amino acid substitutions in adjacent regions (FAI82–84YSI and FEDI90–93FNDL) virtually unaffected the plasma membrane localization of Ha-Ras (Fig. 5, E–L). All other substitutions tested also did not show any effects on the subcellular localization (data not shown). We attempted to further specify residues responsible for the perinuclear localization employing two Ha-Ras mutants with double or triple amino acid change (NN85–86TA and TKS87–89QST). Interestingly, both of these mutants were localized in the perinuclear region like Ha-Ras(NNTKS85–89TAQST) (Fig. 5, M–T). However, all single amino acid substitutions between residues 85–89 failed to convert the subcellular localization of Ha-Ras to the perinuclear region (data not shown).

The subcellular localization of Ha-Ras(NNTKS85–89TAQST) was further analyzed employing several markers for specific perinuclear endomembrane structures (Fig. 6). Ha-Ras(NNTKS85–89TAQST) as well as wild-type Rap1A were colocalized with the mannose 6-phosphate receptor, a marker for the trans Golgi network (19). In contrast, Ha-Ras(NNTKS85–89TAQST) and wild-type Rap1A were localized in neither the endoplasmic reticulum nor lysosomes as revealed by staining with fluorescent dye-conjugated concanavalin A and an anti-lysosome-associated membrane protein-1 antibody (21), respectively.

Biochemical Properties of Perinuclear Region-localized Ha-Ras Mutants—Wild-type Ha-Ras and three Ha-Ras mutants that are localized in the perinuclear region (Ha-Ras(NNTKS85–89TAQST), Ha-Ras(NNTKS85–89TAQST), and Ha-Ras(NNTKS85–89TAQST)) were produced in E. coli, and dissociation rate constants for mant-GDP were determined (Fig. 7). Dissociation rate constants of three Ha-Ras mutants varied from 3.3 to 3.8 s\(^{-1}\), which are comparable with that of wild-type Ha-Ras (3.4 s\(^{-1}\)). Thus, the interaction of Ha-Ras with mant-GDP in vitro remained unaffected upon amino acid substitutions in the region of residues 85–89.

GTP binding of Ha-Ras mutants in cells was also examined by pull-down assays using GTPase-deficient versions of Ha-Ras (NNTKS85–89TAQST) and wild-type Rap1A were localized in neither the endoplasmic reticulum nor lysosomes as revealed by staining with fluorescent dye-conjugated concanavalin A (20) and an anti-lysosome-associated membrane protein-1 antibody (21), respectively.
Fig. 5. Subcellular localization of Ha-Ras mutants containing Ha-Ras to Rap1A-type substitutions in the region of amino acid residues 82–93. COS-7 cells were transfected with pFLAG-CMV-2-EGFP-Ha-Ras(NNTKS85–89TAQST) (A–D), pFLAG-CMV-2-EGFP-Ha-Ras(FAI82–84YSI) (E–H), pFLAG-CMV-2-EGFP-Ha-Ras(FEDI90–93FNDL) (I–L), pFLAG-CMV-2-EGFP-Ha-Ras(NN85–86TA) (M–P), and pFLAG-CMV-2-EGFP-Ha-Ras(TKS87–89QST) (Q–T), and the subcellular localization of the expressed proteins was examined. (A, E, I, M, and Q). The Golgi apparatus was stained with BODIPY TR ceramide (B, F, J, N, and R). Quantitative data as in Fig. 3 are shown in D, H, L, P, and T. Scale bar, 10 μm.
Mechanism of Distinct Subcellular Localization of Ras/Rap1

**Amino Acid Residues 85–89 Are Required for the Perinuclear Localization**—To clarify whether the region of residues 85–89 is required for the perinuclear localization, we first tested the subcellular localization of a Rap1A mutant with a substitution of residues 85–89 for the corresponding Ha-Ras sequence (termed Rap1A(TAQST85–89NNTKS)). Indeed, this Rap1A mutant was not localized in the perinuclear region, supportive of a role of the region of residues 85–89 for the perinuclear localization (data not shown). However, this mutant was distributed uniformly throughout the cytoplasm but not in the plasma membrane, which is presumably ascribed to the lack of COOH-terminal palmitoylation as reported (14). Accordingly, we next examined a chimeric construct consisting of the NH2-terminal portion (residues 1–134) of Rap1A and the COOH-terminal portion (residues 134–189) of Ha-Ras (termed Rap1A(1–134)-Ha-Ras) and its derivative containing a substitution of residues 85–89 for the Ha-Ras sequence (termed Rap1A-(1–134)-Ha-Ras(TAQST85–89NNTKS)) (Fig. 10A). Like Rap1A-(1–101)-Ha-Ras, Rap1A(1–134)-Ha-Ras predominantly existed in the perinuclear region (Fig. 10, B and C). Introduction of a Ha-Ras-type substitution into amino acid residues 85–89 (TAQST85–89NNTKS) caused the plasma membrane localization (Fig. 10, D and E). Therefore, the region of residues 85–89 within the structural context of Rap1A or Ha-Ras is not only sufficient but also required for the localization in the perinuclear region.

**Membrane Attachment and Lipid Modification of the Mutant Proteins**—Membrane attachment of the Golgi-(Ha-Ras(G12V, NNTKS85–89TAQST)) or plasma membrane-(Rap1A(1–134)-Ha-Ras(TAQST85–89NNTKS)) localized mutants was verified by subcellular fractionation. Like wild-type Ha-Ras and Rap1A, both Ha-Ras(G12V, NNTKS85–89TAQST) and Rap1A-(1–134)-Ha-Ras(TAQST85–89NNTKS) were predominantly localized in the particulate fraction but not the soluble fraction (Fig. 11A). Furthermore, prenylation status of these mutant proteins was examined by the use of specific inhibitors for protein farnesyltransferase (FTI-277) or protein geranylgeranyltransferase (GGTI-286) (Fig. 11B). The amounts of the unmodified form of Ha-Ras(G12V), but not Rap1A(G12V), relative to the modified form increased upon treatment with FTI-277, whereas GGTI-286 inhibited prenylation of Rap1A(G12V), but not Ha-Ras(G12V). Both Ha-Ras(G12V, NNTKS85–89TAQST) and Rap1A-(1–134)-Ha-Ras(TAQST85–89NNTKS) were sensitive to the inhibitory effect of FTI-277 but not GGTI-286, leading to the notion that both of the mutants were farnesylated but not geranylgeranylated, as expected from their COOH-terminal amino acid sequence, which is identical to that of Ha-Ras.

**DISCUSSION**

Nascent Ras proteins are not targeted directly from the cytosol to the plasma membrane but instead take an indirect route via the endomembrane system (14, 22). Farnesylation of

![Fig. 6 Subcellular localization of Ha-Ras(NNTKS85–89TAQST) in the Golgi apparatus but neither the endoplasmic reticulum nor lysosomes. COS-7 cells were transfected with pFLAG-CMV-2-EGFP-Rap1A (A–D) and pFLAG-CMV-2-EGFP-Ha-Ras(NNTKS85–89TAQST) (J–L), and the subcellular localization of the expressed proteins was examined (A, D, G, J, M, and P). The trans Golgi network, the endoplasmic reticulum, and lysosomes were stained with an anti-mannose 6-phosphate receptor antibody (B and K), concanavalin A (E and N), and an anti-lysosome-associated membrane protein-1 antibody (H and Q), respectively. Scale bar, 10 μm. (Fig. 8). Introduction of the G12V mutation in wild-type Ha-Ras and its perinuclear region-localized mutants did not change the subcellular localization (Fig. 8, A–E). All of the three perinuclear region-localized mutants bound GTP within the cell similarly to Ha-Ras(G12V) (Fig. 8F). To further substantiate that these mutants indeed exist in the perinuclear region as a GTP-bound form, an *in situ* detection assay for GTP-bound Ras was carried out (Fig. 9). EGFP-RBD, which specifically binds to the GTP-bound form of Ras, was detected in the plasma membrane when coexpressed with Ha-Ras(G12V) (Fig. 9, B–D). In marked contrast, EGFP-RBD was colocalized in the perinuclear region with GTPase-deficient Ha-Ras mutants harboring substitutions in residues 85–89 (Fig. 9, E–M).
Ras is thought to occur within the cytoplasm because the responsible enzyme farnesyltransferase is soluble. Prior to plasma membrane expression, farnesylated Ras is transiently localized in the endoplasmic reticulum, where it becomes a substrate for a endoprotease that cleaves the COOH-terminal three amino acids, leaving the prenylcysteine as the new COOH terminus. Subsequently, Ras is methylated and then transported to the plasma membrane. Trafficking of Ras from the endoplasmic reticulum to the plasma membrane requires either palmitoylation or a polybasic motif. Following palmitoylation, Ha-Ras and N-Ras are suggested to transit brefeldin A-sensitive membrane structures, such as the Golgi apparatus, which are known to be the components of the conventional exocytic pathway. Ki-Ras4B, on the other hand, is likely to be transported along an alternative trafficking pathway to the plasma membrane by virtue of its polybasic motif.

In contrast to Ras, the mechanism underlying the determination of the subcellular localization of Rap1 remains largely unknown. Rap1 is found predominantly in the perinuclear membrane structures and endocytic vesicles, although a small portion of Rap1 resides in the plasma membrane (10–13, 23). Geranylgeranyltransferase attaches the prenyl moiety to Rap1 in the cytoplasm, allowing Rap1 to translocate to membrane compartments, such as the Golgi apparatus. Rap1 does not further translocate to the plasma membrane but instead stays in the perinuclear region even though Rap1 has a cluster of basic amino acids like Ki-Ras4B. Thus, a Rap1-specific mechanism that determines the perinuclear localization of Rap1 may exist.

In this study, we identified a small segment consisting of five amino acid residues (TAQST) that causes re-localization of Ha-Ras, which otherwise is mainly localized in the plasma membrane, to the perinuclear region in COS-7 cells. This sequence spanning threonine 85 to threonine 89 of Rap1A is not only sufficient but also required for the perinuclear localization of Rap1A.

FIG. 7. Dissociation rate constants of mant-GDP-loaded wild-type and mutant Ha-Ras proteins. A, the time course of mant-GDP fluorescent change (excitation, 356 nm; emission, 448 nm) upon binding to and release from wild-type Ha-Ras. Mant-GDP (5 μM, Molecular Probes) was mixed with GDP-bound wild-type Ha-Ras (1 μM) in buffer B. EDTA (final concentration = 10 mM) was then added at the indicated time to induce nucleotide exchange. After the equilibrium state, unlabeled GDP (final concentration = 0.5 mM) was added at the indicated time. The solid line represents the fit of the data to first-order kinetics for fluorescent increase and decrease portions of the curve. B–E, determination of dissociation rate constants of mant-GDP-loaded wild-type and mutant Ha-Ras proteins. Plots of \( \ln(F_t - F_0) \) versus incubation time after the addition of unlabeled GDP are shown, where \( F_t \) and \( F_0 \) denote fluorescent intensities at indicated times and the time when unlabeled GDP was added, respectively.
with the putative receptor protein may require only two or three residues given that these amino acids reside within the structural context of Rap1A or Ha-Ras. The KDEL carboxy-terminal sequence is known as a retrieval signal that directs the localization of many soluble proteins in the endoplasmic reticulum (25). However, the TAQST sequence in Rap1A may not act as a signal for the perinuclear localization of diverse proteins because this sequence is found only in Rap1 GTPases (Rap1A and Rap1B).

Ha-Ras and N-Ras have recently been reported to be localized in the endoplasmic reticulum and the Golgi apparatus as well as the plasma membrane, being activated following extracellular stimulation (15). Notably, Ras activation upon T-cell receptor engagement is restricted to the Golgi apparatus, highlighting a significant role of Ras in subcellular compartments other than the plasma membrane (26). The diacylglycerol-responsive GEF RasGRP1 is implicated in the activation of Golgi-localized Ras, which in turn activates downstream molecules, such as ERK, JNK, and Akt, similarly to plasma membrane-localized Ras (26, 27).

Rap1A, when overexpressed, is known to antagonize Ras-induced transformation, which is believed to be ascribed to tight binding of Rap1A to Ras targets such as Raf-1 without activating significantly (5). However, the inhibitory effect of Rap1A may not be accounted for solely by competitive binding to target proteins because the vast majority of Rap1A, unlike Ras, resides in the perinuclear region. Instead, Rap1A-specific signals from the perinuclear region may modulate Ras-dependent signaling pathways. Ha-Ras and Rap1A mutants whose subcellular localization was converted may become a useful tool to test this possibility.

Furthermore, Ha-Ras and Rap1A stimulate common targets or downstream signaling pathways in different subcellular compartments with different time courses. For instance, PLCε interacts with both Ha-Ras and Rap1A being activated in the

![Fig. 8. Subcellular localization and in vivo GTP binding of GTPase-deficient Ha-Ras mutants. COS-7 cells were transfected with pFLAG-CMV-2-EGFP-Ha-Ras, pFLAG-CMV-2-EGFP-Ha-Ras(G12V), pFLAG-CMV-2-EGFP-Ha-Ras(G12V, NN85–86TA), pFLAG-CMV-2-EGFP-Ha-Ras(G12V, TKS87–89QST), and pFLAG-CMV-2-EGFP-Ha-Ras(G12V, NNTKS85–89TAQST). Subcellular localization (A–E) and in vivo GTP binding (F) were examined by confocal laser microscopy and pull down assays, respectively. Scale bar, 10 μm.](image)

![Fig. 9. In situ detection of GTP-bound forms of GTPase-deficient Ha-Ras mutants. COS-7 cells were transfected with pFLAG-CMV-2-EGFP-RBD alone (A) or with pEF-BOS-HA-Ha-Ras(G12V) (B–D), pEF-BOS-HA-Ha-Ras(G12V, NN85–86TA) (E–G), pEF-BOS-HA-Ha-Ras(G12V, TKS87–89QST) (H–J), or pEF-BOS-HA-Ha-Ras(G12V, NNTKS85–89TAQST) (K–M). Subcellular localization of the Ha-Ras mutants (C, F, I, and L) and their GTP-bound forms (B, E, H, and K) were examined by confocal laser microscopy. Scale bar, 10 μm.](image)
plasma membrane and the perinuclear region, respectively (16). In hematopoietic BaF3 cells, Ha-Ras is responsible for rapid and transient activation of PLC upon platelet-derived growth factor treatment, whereas Rap1A induces delayed and prolonged PLC activation (28). Likewise, Ha-Ras mediates nerve growth factor signaling in pheochromocytoma PC12 cells through a transient activation of ERK, whereas Rap1 continues to activate PLC/Ras signaling in pheochromocytoma PC12 cells upon platelet-derived growth factor treatment, whereas Rap1A induces delayed and prolonged PLC activation (28). The possibility of subcellular compartment-specific or GTPase-specific down-regulation is currently being assessed by immunoblotting using an anti-HA antibody. B, COS-7 cells were transfected as described in the legend to A and treated with FTI-277 (300 nM) or GGTI-286 (10 μM) for 2 days. Unprenylated (U) and prenylated (P) forms of the expressed proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting using an anti-HA antibody.

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