The Linker Domain of the Ha-Ras Hypervariable Region Regulates Interactions with Exchange Factors, Raf-1 and Phosphoinositide 3-Kinase*

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Ha-Ras and Ki-Ras have different distributions across plasma membrane microdomains. The Ras C-terminal anchors are primarily responsible for membrane microlocalization, but recent work has shown that the interaction of Ha-Ras with lipid rafts is modulated by GTP loading via a mechanism that requires the hypervariable region (HVR). We have now identified two regions in the HVR linker domain that regulate Ha-Ras raft association. Release of activated Ha-Ras from lipid rafts is blocked by deleting amino acids 173–179 or 166–172. Alanine replacement of amino acids 173–179 but not 166–172 restores wild type micro-localization, indicating that specific N-terminal sequences of the linker domain operate in concert with a more C-terminal spacer domain to regulate Ha-Ras raft association. Mutations in the linker domain that confine activated Ha-RasG12V to lipid rafts abrogate Raf-1, phosphoinositide 3-kinase, and Akt activation and inhibit PC12 cell differentiation. N-Myristoylation also prevents the release of activated Ha-Ras from lipid rafts and inhibits Raf-1 activation. These results demonstrate that the correct modulation of Ha-Ras lateral segregation is critical for downstream signaling. Mutations in the linker domain also suppress the dominant negative phenotype of Ha-RasS17N, indicating that HVR sequences are essential for efficient interaction of Ha-Ras with exchange factors in intact cells.

The guanine nucleotide-binding protein Ras acts as a molecular switch connecting extracellular signals with a complex network of intracellular signal transduction pathways that mediate a variety of cellular responses including proliferation and differentiation (1, 2). The three mammalian ras genes encode for four proteins of 188 and 189 amino acids (Ha-Ras, N-Ras, Ki-RasA, Ki-RasB) that are identical over the N-terminal 165 residues and 90% homologous over the next 80 residues. These N-terminal 165 residues are sufficient for binding guanine nucleotides and interacting with effector and exchange factor proteins in vitro. The divergence between the Ras isoforms is largely confined to the final 23 and 24 C-terminal amino acids, the so-called hypervariable region (HVR) which less than 10–15% of residues are identical between any pair of Ras proteins.

The localization of Ras to the inner surface of the plasma membrane is essential for its biological activity (3). The two signals responsible for the correct plasma membrane localization of Ras are contained in the Ras HVR. The first signal sequence, common to all Ras proteins, is the C-terminal CAAX box (in which A = aliphatic amino acid and X = serine or methionine) that is sequentially farnesylated, -AXX proteolysed, and carboxyl-methylated (3–6). This series of posttranslational modifications is completed on the cytoplasmic surface of the endoplasmic reticulum. The second signal comprises palmitoylation of two upstream cysteines (Cys161 and Cys184) in Ha-Ras, one cysteine (Cys183) in N-Ras, and a polybasic sequence of multiple lysines (Lys175–190) in Ki-Ras2 (7, 8). These targeting signals direct the trafficking of Ras to the cell surface via alternative routes. After palmitoylation, probably also in the endoplasmic reticulum, Ha- and N-Ras traffic through the classical exocytic pathway via the Golgi to the plasma membrane (9, 10). Ki-Ras in contrast bypasses the Golgi and reaches the plasma membrane by an unknown mechanism that may involve transport or simple diffusion (9–13). For convenience we refer to the sequences of the Ras HVR that are involved in trafficking and plasma membrane attachment as the membrane-targeting domain and the remainder of the Ras HVR as the linker domain.

A number of studies have shown that the membrane-targeting domains localize Ha-Ras and Ki-Ras to different microdomains of the plasma membrane. A dominant negative mutant of caveolin, CavΔCov, which blocks delivery of cholesterol to the plasma membrane (14), completely blocks Ha-Ras-dependent Raf activation but does not affect Ki-Ras signaling (15). The inhibition is reversed by repleting plasma membranes with cholesterol. In addition, chemically depleting plasma membrane cholesterol with cycloextrin selectively inhibits Ha-Ras but not Ki-Ras function (15). Taken together, these data suggest that Ha-Ras but not Ki-Ras function is dependent on the integrity of lipid rafts in the plasma membrane. More recently we used electron and fluorescent microscopy coupled with biochemical fractionation to examine the plasma membrane microdomain distributions of Ha- and Ki-Ras (16). The results were unexpected but clear. The C-terminal minimal membrane-targeting sequences of Ha-Ras localize GFP to caveolae and lipid rafts, whereas the equivalent targeting signals for phosphoinositide 3-kinase; GFP, green fluorescent protein; GEF, guanine nucleotide exchange factor; BHK, baby hamster kidney; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; RBD, Ras-binding domain.

The major expressed Ki-Ras protein is Ki-RasB, referred to as Ki-Ras hereafter.

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1 The abbreviations used are: HVR, hypervariable region; PI3K, phosphoinositide 3-kinase; GFP, green fluorescent protein; GEF, guanine nucleotide exchange factor; BHK, baby hamster kidney; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; RBD, Ras-binding domain.
2 The major expressed Ki-Ras protein is Ki-RasB, referred to as Ki-Ras hereafter.
Ki-Ras do not. Wild type GDP-bound Ha-Ras is distributed equally between lipid raft and non-raft membrane, but constitutively activated GTP-bound Ha-Ras is almost completely excluded from lipid rafts and caveolae. In contrast, GDP- and GTP-loaded Ki-Ras are both absent from caveolae and co-fractionate with markers of disordered plasma membrane. The data suggest that Ha-Ras is in a GTP-regulated equilibrium between lipid rafts and non-raft plasma membrane (16, 17). Interestingly, the Ha-Ras linker domain is necessary for the GTP-dependent release of Ha-Ras from lipid rafts and for biological activity (16). Other studies have also shown that manipulation of the C-terminal membrane anchor of Ha-Ras decreases Ras biological activity in NIH3T3 cells and PC12 cells by changing effector pathway utilization (18, 19). Together these observations demonstrate that the integrity of the HVR is essential for normal Ha-Ras signaling.

The aim of the present study was to investigate in more detail the role of the HVR linker domain in Ha-Ras function. In particular we wanted to establish what structural features and/or specific sequences in the linker domain contribute to the modulation of Ha-Ras raft association and to examine whether raft association alone determines the efficiency with which Ha-Ras interacts with specific effectors and activators. These are important questions because differential distribution of Ras isoforms, exchange factors, effectors, and effector co-activators across plasma membrane microdomains is the simplest mechanism that can account for the increasing number of biological and in vivo biochemical differences that are being identified between Ha-, Ki-, and N-Ras.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Ha-RasG12V cDNA sequences encoding His166 through Asn172 or Pro through Pro279 were deleted or replaced with polyalanine using oligonucleotide-directed mutagenesis to generate Ha1-H2a-G12V, Ha1a-G12V, Ha2-G12V, and Ha2alpha-G12V, respectively. Mutated cDNAs were fully sequenced and cloned into the expression vector pEXV3, Gb12, and Asn172 versions of the HVR mutants were constructed by recombination with pEXV-Ha-Ras and pEXV-Ha-RasS17N, respectively. EXV-Hahvhr-G12V and EXV-Mys-Ha-Ras have been described previously (16, 19).

**Cell Culture and Transfection**—BHK cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum. Cells were plated onto 10-cm plates at 60% confluence and transfected with 5 × 10^6 bovine calf serum. Cells were plated onto 10-cm plates at 60% confluency and transfected with 5 × 10^6 cells. After 5 h, medium containing 10% calf serum and 500 µl AMINE (Invitrogen) according to the manufacturer’s instructions. The hypervariable region and Ha-Ras function

**Toxicity Assays**—BHK cells were transfected with 10 µg of pC1 (which carries a G418-selectable marker). After 5 h, medium containing 20% calf serum was added for 24 h, and then the transfected cells were split between eight 10-cm plates into medium containing 10% calf serum and 500 µg/ml G418. Cells were maintained for 16 days and then fixed with cold methanol and stained with 10% Giemsa stain, and the colonies were counted.
cDNA sequences encoding His166 though Asn172 or Pro173 though Pro179, 1-G12V and H9004/1-H9004/1 plasma membrane. A respectably. Mutants H9004/1ala-G12V and H9004/2ala-G12V were generated by substituting the amino acids 166–179 with an equivalent number of alanine residues to generate H9004/1ala and H9004/2ala (Fig. 1). The membrane-targeting domain of the HVR is left intact in all these constructs, and Fig. 1B shows that as expected, all of the Ha-Ras HVR-mutated proteins localized normally to the plasma membrane when expressed in BHK cells.

We next examined the distribution of the Ha-Ras linker domain mutants between raft and non-raft membranes. Detergent-free lysates prepared from transiently transfected BHK cells were fractionated over sucrose gradients. 10 fractions were collected from the top to the bottom of each gradient, and the membranes contained in each fraction isolated by centrifugation. Membrane pellets were then analyzed by immunoblotting using anti-Ras antibodies. We have previously extensively characterized this gradient protocol and shown that lipid raft-enriched membranes are readily resolved from non-raft membranes (16, 22). Specifically, lipid raft markers are found in the top 5 fractions of the gradients, which contain light membranes, and non-raft markers are confined to the denser bottom 5 fractions (16, 22).

In accordance with our previous results, more than 80% of activated Ha-RasG12V was present in the high density fractions (6–9) and was totally excluded from the top fractions of the gradient (Fig. 2). In contrast, the truncated proteins H9004/1-G12V and H9004/2-G12V and the alanine-substituted protein H9004/1ala-G12V fractionated almost exclusively to the lipid raft-enriched fractions (2–5) as seen previously for H9004/1-hvrG12V (see Fig. 2 and Ref. 16). Interestingly, however, the distribution of the alanine-substituted protein H9004/2ala-G12V more closely resembled that of full-length Ha-RasG12V with a small peak in lipid raft fractions 2 and 3 and a more major peak in fractions 7–10. One interpretation of these results is that the whole Ha-Ras linker domain is required for correct lateral segregation, but although the specific amino acid sequence of 166–172 is critical, residues 173–179 may just operate as an essential spacer element, the specific sequence of which is not important.

**RESULTS**

**Identification of Two Regions within the Ha-Ras HVR Linker Domain That Regulate Raft Association—**Our previous results showed that deletion of the entire linker domain of Ha-Ras (166–179) totally confines activated H9004/1-hvrG12V to lipid rafts and partially abrogates the ability of H9004/1-hvrG12V to activate Raf-1 (16). To further characterize the HVR linker domain, we constructed four additional mutants of this region. We deleted amino acids 166–172 or 173–179 from activated Ha-RasG12V, *i.e.* the N-terminal or C-terminal amino acids of the linker domain, to create the mutants H9004/1-D1 and H9004/2-D2, respectively (Fig. 1). In addition, we generated two alanine replacement mutants by substituting the amino acids 166–172 or 173–179 with an equivalent number of alanine residues to generate H9004/1ala and H9004/2ala (Fig. 1). The membrane-targeting domain of the HVR is left intact in all these constructs, and Fig. 1B shows that as expected, all of the Ha-Ras HVR-mutated proteins localized normally to the plasma membrane when expressed in BHK cells.

Membrane fractions were then assayed for endogenous PI3K activity using a coupled MEK-ERK assay. Fig. 3, A and B, shows that the ability of H9004/1-D1 and H9004/2-D2 to activate Raf-1 was severely compromised compared with that of full-length Ha-RasG12V. The abrogation of activity was similar to that seen with H9004/1-hvrG12V. The ability of H9004/1ala-G12V to activate Raf-1 was also compromised but not to the same extent as H9004/1-D1, H9004/2-D2, or H9004/1-hvrG12V (Fig. 3, A and B). In contrast, the alanine replacement mutant H9004/2ala-G12V activated Raf-1 as efficiently as full-length Ha-RasG12V (Fig. 3, A and B). We next examined the levels of activated MEK and ERK in the S100 fractions of the same lysates by immunoblotting with phospho-specific antisera. Fig. 3A shows that MEK phosphorylation was significantly compromised only in cells expressing H9004/1-hvrG12V. Moreover, levels of phosphorylated ERK were essentially equivalent in all of the mutant-expressing cells. Thus recruitment of Raf to lipid rafts by mislocalized Ha-Ras compromises activation but also apparently interferes with the communication between Raf, MEK, and MAPK, perhaps by preventing appropriate negative feedback within the kinase cascade.

Membrane fractions were then assayed for endogenous PI3K activity in an *in vitro* lipid kinase assay using phosphatidylinositol as substrate. Expression of H9004/1-hvrG12V, H9004/1-D1, and H9004/2-D2 failed to activate PI3K, whereas expression of H9004/2ala-G12V or full-length Ha-RasG12V generated substantial membrane-associated PI3K activity (Fig. 3, C and D). Ex-
expression of H31ala-G12V generated low PI3K activity in the majority of our experiments (as shown in Fig. 3C), but in some experiments, higher levels of PI3K activity were detected. The reasons for this variability are unclear. To verify the results of the in vitro lipid kinase data, we therefore assessed the activation status of Akt in the same cell lysates by quantitative immunoblotting using phospho-specific antisera. Cells expressing H3hrvG12V, H31-G12V, H3Δ2-G12V, or H31ala-G12V had low levels of phosphorylated Akt both in cell membranes and in cytosol consistent with the profile of PI3K activation (Fig. 3E). H3Δ2ala-G12V was the only mutant protein capable of activating Akt to the same extent as full-length Ha-RasG12V (Fig. 3E).

**Biological Activity of HVR Linker Domain Mutants**—To assess the biological activity of the Ha-RasG12V HVR linker domain mutants, the constructs were tagged with N-terminal GFP and transfected into PC12 cells. After 48 h, the cell cultures were fixed. The morphology of the cells was compared with cells expressing full-length Ha-RasG12V that underwent extensive neurite outgrowth and differentiation and cells expressing membrane-targeted GFP-IH that do not differentiate (GFP-IH consists of the C-terminal 10 amino acids of Ha-Ras cloned onto the C terminus of GFP) (Fig. 4). To quantify the extent of differentiation, mean neurite length was calculated from a random sample of ~50 cells expressing each Ha-Ras protein (Fig. 4). Cells expressing H3hrvG12V, H31-G12V, or H3Δ2-G12V showed some enlargement of the cell body and in some cases the formation of very short neurite-like structures, but the extent of these changes and the complexity of the differentiation was markedly reduced compared with full-length Ha-RasG12V (Fig. 4). The alanine-substituted mutant H31ala-G12V induced slightly more neurite outgrowth than the cognate-deletion mutant. However, only the differentiation induced by H3Δ2ala-G12V resembled that observed with full-length Ha-RasG12V. It is clear that the profiles of PC12 differentiation observed in these experiments correlate closely with the biochemical and raft micro-localization data shown in Figs. 2 and 3.

**N-Myristoylation Confines Activated Ha-Ras to Lipid Rafts and Abrogates Raf-1 Activation**—The simplest conclusion to be drawn from the preceding experiments is that preventing the access of activated Ha-Ras to the disordered plasma membrane blocks biological activity. An alternative interpretation is that the linker domain modulates raft association but also directly interacts with plasma membrane-localized Raf-1 and PI3K. We therefore used another mechanism to confine activated Ha-Ras to lipid rafts that did not involve mutating the HVR linker domain. We have previously shown that the addition of an N-myristoylation signal to Ha-RasG12V does not interfere with plasma membrane localization. However, sucrose gradient analysis showed that Mys-Ha-RasG12V is extensively localized to lipid rafts (Fig. 5A; compare with Fig. 2). Consistent with this observation, assays of raft association based on detergent insolubility showed that membrane-associated Mys-Ha-
RasG12V was resistant to extraction in 0.05% Triton X-100, whereas 75% of Ha-RasG12V was solubilized in 0, 0.05, 0.25, or 0.5% Triton X-100 (Fig. 5B). P100 fractions were then reisolated by centrifugation and immunoblotted for Ras. The graph shows the mean fraction (% of Ras (± S.E., n = 3) that was detergent-insoluble in each concentration of Triton X-100. P100 fractions from COS cells transfected with Ha-RasG12V or with Mys-Ha-RasG12V were assayed for Raf kinase activity in a coupled MEK-ERK assay (Fig. 5C). The graph shows mean relative Raf activity (± S.E.), normalized for Ras expression, that was calculated from 4 independent experiments.

The Interactions of Plasma Membrane-associated Ha-Ras with Exchange Factors Is Altered by Mutations in the HVR Linker Domain—The S17N mutation results in a Ras protein that forms an irreversibly complex with RasGEFs and renders RasS17N dominant negative for cell growth (24). To investigate whether the HVR influences the ability of Ha-Ras to interact with RasGEFs in vivo, we introduced the S17N mutation into the Ha-Ras linker domain mutants and assayed them for growth inhibition. BHK cells were co-transfected with S17N-substituted Ha-Ras constructs and pC1, a neomycin resistance plasmid, and selected for 16 days in G418. The number of surviving cells was then compared with control plate transfected with an equivalent amount of empty Ras expression vector. Fig. 6A shows that the growth inhibitory phenotype of Ha-RasS17N is markedly reduced in the context of all five linker domain deletions or alanine substitutions. This result strongly suggests that efficient interaction between membrane-associated Ha-Ras and RasGEFs requires an intact HVR linker domain. To extend this analysis, we assessed directly whether the linker domain influences the sensitivity of Ha-Ras to activation by a specific exchange factor, Sos1. COS cells were transfected with Ha-Ras or the HVR linker domain mutants, all wild type at codon 12, with or without mSos1. The amount of GTP-loaded Ha-Ras protein in the membrane fraction was then determined by a GST-RBD pull-down assay. The basal levels of GTP loading of H2 and H2ala were significantly lower (p < 0.01) than wild type Ha-Ras (Fig. 6B). Similarly, the levels of GTP loading of H2 and H2ala in cells co-expressing an equivalent low level of mSos1 were all significantly lower (p < 0.05) than the GTP loading of full-length Ha-Ras (Fig. 6B). These results are what would be expected if the efficiency of interaction of H2 and H2ala with Sos1 is compromised as suggested by the S17N toxicity assays. The basal and Sos1-stimulated GTP loading of H2 and H2ala with Sos1 is compromised as suggested by the S17N toxicity assays. The basal and Sos1-stimulated GTP loading of H2 and H2ala with Sos1 is compromised as suggested by the S17N toxicity assays. The basal Sos1-stimulated GTP loading of H2 and H2ala was significantly lower (p < 0.05 or p < 0.01) than that seen with the corresponding wild type Ha-Ras control (Fig. 6B).

DISCUSSION

Numerous studies published over the last few years have challenged the view that the highly homologous Ha-, Ki-, and N-Ras are functionally redundant. Transgenic experiments have shown that only Ki-Ras is required for normal mouse development (25–27), whereas primary cell culture experi-
ments have shown that N-Ras may have a specific role in suppressing apoptosis (28). In addition, different Ras isoforms transform established fibroblasts with varying efficiencies and exhibit different phenotypes in cell motility and migration assays (29, 30). These biological differences must result from different signal outputs from activated Ha-, N- or Ki-Ras and/or different sensitivities to activation by exchange factors. All three Ras proteins share a common set of GEFs and effectors, yet there are marked quantitative differences in their use of these interacting proteins in intact cells. For example, Ras-GRF1 activates Ha-Ras more efficiently than N- or Ki-Ras (31), RasGRP2 activates N- and Ki-Ras but not Ha-Ras (23), and mSos1 activates Ras in the hierarchy Ha > N > Ki-Ras. Also, with respect to effector interactions, Ki-Ras is more potent than Ha-Ras at activating Raf-1 and Rac1, whereas Ha-Ras is more potent than Ki-Ras at activating PI3K (29–32). Analysis of Ha/Ki-Ras chimeras has shown that the biological phenotype of the chimeric protein correlates with the origin of the C-terminal HVR (30). Others have shown that perturbing the mechanism of interaction of the C-terminal anchor with the plasma membrane alters the efficiency with which Ha-Ras activates Raf-1 and PI3K (18, 19). These data, taken together with our observations that the Ha-Ras and Ki-Ras C-terminal anchors target to different plasma membrane microdomains, lead to the attractive hypothesis that biochemical and biological differences between the Ras isoforms are due to differences in plasma membrane micro-localization.

The interaction of full-length Ha-Ras with lipid rafts is regulated by GTP loading and is dependent on sequences in the HVR linker domain (16). In the present study, we investigated whether the linker domain modulates Ha-Ras function by directly altering interactions with effectors and GEFs and/or indirectly by altering plasma membrane micro-localization. The deletion analysis shows that, with respect to regulating raft interactions, there are two distinct requirements of the linker domain. Amino acids 173–179 operate as a spacer element because their replacement with alanine residues in HΔ2ala allowed access to the disordered plasma membrane and restored the diminished effector function of H22 back to wild type levels. In contrast, deletion of amino acids 166–172 compromised the normal raft/non-raft equilibrium and effector function of Ha-Ras, and this was not restored by alanine replacement. This suggests that the specific sequence of 166–172 rather than its length is important for regulating Ha-Ras raft association and function.

In Ki-Ras, the sequence corresponding to amino acids 173–179 is taken up by the polybasic domain. This sequence interacts electrostatically with the negatively charged plasma membrane and is in close proximity with the inner leaflet; it is therefore tempting to speculate that the catalytic domains of Ha-Ras may be spaced further away from the inner surface of the plasma membrane than those of Ki-Ras. Interestingly a recent analysis of the HVR of Ki-RasG12V described two constructs in which a short or long polybasic domain plus a CAAAX motif was appended directly onto the N-terminal 165 amino acids. Both proteins had a much lower transforming activity than full-length Ki-RasG12V despite both being efficiently localized to the plasma membrane (33). These mutant Ki-Ras constructs effectively have a deletion of the linker domain sequence 166–174; thus specific residues in this region of both Ha- and Ki-Ras appear to be essential for the effector interactions of both Ras proteins. It has not yet been determined, however, whether the microdomain localization of Ki-Ras is altered by deletion of these sequences, as is the case for Ha-Ras.

Our data show a clear correlation between confinement of Ha-Ras to lipid rafts and defective effector function as measured in Raf-1 and PI3K assays and a cell biological assay of PC12 cell differentiation. Our preferred interpretation is that these observations are related, but it is formally possible that they represent separate phenomena, i.e. the linker domain regulates Ha-Ras microdomain association and also has a separate direct role in regulating effector interactions. The results with myristoylated Ha-Ras, however, argue against this alternative hypothesis. N-myristoylated plasma membrane-localized Ha-RasG12V is confined to lipid rafts, in this case because the additional saturated fatty acid facilitates partitioning into liquid-ordered membrane. The linker domain is intact in myristoylated Ha-Ras; therefore the observed defective effector function is most likely due to raft association.

Although Raf activation was compromised by confinement to lipid rafts, downstream activation of MEK and MAPK was apparently unaffected. A similar observation was made recently by Chen and Resh (34), who targeted Raf to lipid rafts using the Src or Fyn N-terminal motifs or to disordered membrane using the Ki-Ras C-terminal targeting motifs. Raf kinase activity per se was not assayed in their study, but all three Raf constructs activated MEK and Erk with equal efficiency. A complete explanation of these data is not possible at the present time, although it has been demonstrated by others that the correct coordination of activity within the Raf/MEK/Erk cascade is critically dependent on raft structure and function (35).

In addition, it has recently been proposed that internalization of Ras-Raf complexes may be required for efficient activation of MEK and Erk (36). If so, the data in Fig. 3A could be rationalized by speculating that raft-associated Raf is more extensively endocytosed than Raf associated with disordered membrane. Notwithstanding the phospho-MEK and phospho-Erk results, the PC12 experiments clearly show that the biological activity of Ha-Ras correlates closely with raft dissociation.

Suppression of the S17N dominant negative phenotype in the context of all five linker domain deletions or alanine substitutions demonstrates that the interaction of RasGEFs with membrane-associated Ha-Ras is critically dependent on the integrity of the linker domain. Unlike the requirements for normal effector function, we cannot safely conclude that any of the Ha-Ras linker domain acts simply as a spacer element for RasGEF binding given that deleted and alanine-substituted proteins were equally compromised. In a co-crystal of the Sos1 catalytic domain with Ha-Ras, the C terminus of Ha-Ras is orientated away from the contact surfaces (37, 38). However, the C-terminal 25 amino acids of Ha-Ras and the non-catalytic domains of Sos-1 were absent from the solved protein complex, so it remains possible that some direct interaction between the Ha-Ras HVR and Sos1 is important for their efficient functional interaction at the plasma membrane. Indeed we have shown previously that prenylated Ras is a better substrate for Sos1 than unmodified Ras in vitro (39), observations that also demonstrate that C-terminal Ras epitopes influence N-terminal protein/protein interactions.

Although there was good correlation between the two assays of RasGEF/Ha-Ras interaction with respect to three of the HVR linker domains mutants (Fig. 6, A and B), this was not the case for HΔ1ala and HΔ1. The increased basal and Sos1-stimulated GTP loading is at variance with the reduced levels expected, as seen with HΔ2ala and HΔ2. One explanation for these results is that the ability of GAP to interact with membrane-associated HΔ1ala and HΔ1 is also compromised. If so, then despite a compromised interaction with Sos1, as demonstrated by the S17N toxicity assay result, HΔ1ala and HΔ1 proteins that are wild type at codon Gly12 would still accumulate in the GTP-
bound state. Wild type H41ala and H41, however, do not drive differentiation of PC12 cells (data not shown), presumably because, although GTP-loaded, their effector interactions are also compromised to the same extent as the cognate G12V-substituted proteins. The interaction of p120GAP with the Ha-Ras linker domain mutants is currently being investigated.

In summary, we have shown here that the HVR linker domain modulates the effector, GEF, and possibly GAP interactions of plasma membrane-localized Ha-Ras. There are still some unanswered questions as to how the linker domain mediates these effects, but it is clearly essential for normal Ha-Ras biological function.

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The Linker Domain of the Ha-Ras Hypervariable Region Regulates Interactions with Exchange Factors, Raf-1 and Phosphoinositide 3-Kinase
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