Distinct Utilization of Effectors and Biological Outcomes Resulting from Site-Specific Ras Activation: Ras Functions in Lipid Rafts and Golgi Complex Are Dispensable for Proliferation and Transformation

David Matallanas,† Victoria Sanz-Moreno,‡ Imanol Arozarena,† Fernando Calvo,† Lorena Agudo-Ibáñez,‡ Eugenio Santos,‡ María T. Berciano,‡ and Piero Crespo‡*

Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas (CSIC), Departamento de Biología Molecular,† and Departamento de Anatomía y Biología Celular, Unidad de Biomedicina,‡ CSIC-Universidad de Cantabria, Santander 39011, Spain, and Centro de Investigación del Cáncer, IBMCC, CSIC-Universidad de Salamanca, Salamanca 37007, Spain*

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Ras proteins are distributed in different types of plasma membrane microdomains and endomembranes. However, how microlocalization affects the signals generated by Ras and its subsequent biological outputs is largely unknown. We have approached this question by selectively targeting RasV12 to different cellular sublocalizations. We show here that compartmentalization dictates Ras utilization of effectors and the intensity of its signals. Activated Ras can evoke enhanced proliferation and transformation from most of its platforms, with the exception of the Golgi complex. Furthermore, signals that promote survival emanate primarily from the endoplasmic reticulum pool. In addition, we have investigated the need for the different pools of endogenous Ras in the conveyance of upstream mitogenic and transforming signals. Using targeted RasN17 inhibitory mutants and in physiological contexts such as H-Ras/N-Ras double knockout fibroblasts, we demonstrate that Ras functions at lipid rafts and at the Golgi complex are fully dispensable for proliferation and transformation.

Ras GTPases—H-Ras, N-Ras, and K-Ras 4B/4A—operate as key molecular switches that convey extracellular signals from surface receptors to the interior of the cell, thereby regulating essential processes including proliferation, differentiation, and survival (15, 34). It is well known that Ras must be attached to the inner leaflet of the plasma membrane (PM) to be functional (50). This is accomplished by lipidic additions to the protein C terminus (33), which contains the essential signal for localizing Ras to membranes: the CAAX box (where C is cysteine, A is aliphatic amino acid, and X is serine/methionine). This motif undergoes posttranslational modifications that make it more hydrophobic. The cysteine is farnesylated, the AAX sequence is proteolyzed, and the newly C-terminal cysteine is carboxymethylated (50). However, a second signal is necessary for efficiently positioning Ras in the membrane. This is accomplished by palmitoylation of cysteine 181 in N-Ras, and cysteines 181 and 184 in H-Ras. In the case of K-Ras 4B the second signal is attained by a polybasic motif of six lysines (175 to 180) that interacts electrostatically with the negatively charged membrane (24–26).

Recently, a new twist has been provided by findings indicating that Ras isoforms are distinctively segregated in different PM microdomains with unique biochemical and physicochemical characteristics, H-Ras can be found in bulk membrane and in lipid rafts, both caveolar and noncaveolar. K-Ras is exclusively located in bulk membrane, whereas N-Ras can only be detected in noncaveolar lipid rafts (35, 38–40). Furthermore, recent reports indicate that Ras is also present in endomembranes such as endosomes, endoplasmic reticulum (ER), and the Golgi complex (10, 37, 45). The significance of this distribution seems to go beyond that of a transient event associated to transport and/or recycling. Instead, a pool of Ras appears to reside in these organelles, and therein Ras can productively engage downstream effectors (10, 11, 37, 45). Moreover, at these endomembranes Ras regulation is undertaken by proteins that operate in a location-specific fashion. As such, the guanine nucleotide exchange factor RasGRP specifically regulates Ras activation at the Golgi complex (7, 9), whereas SOS and RasGRF undertake Ras regulation at the ER. Likewise, stimuli such as lysophosphatidic acid preferentially activate the Ras pool at the ER, whereas calcium ionophores are more effective in activating PM Ras (4).

The fact that exogenous stimuli activate Ras distinctively depending on its localization and that Ras regulation at different sites requires the participation of specific intermediaries hints at the necessity for a location-specific control. This, in term, may imply that Ras functions at its different sites may not be totally redundant. Thus, a selective activation of Ras at each of its locations could be intended to generate variability in its biochemical and biological outputs. It is known that Ras regulates numerous cellular functions through the activation of an ever-growing number of effector molecules (15). However, how microlocalization affects the biochemical signals that Ras...
generates and the biological outputs that it regulates is just beginning to be unfolded (23). Herein, we have addressed that question. We present for the first time a systematic study on the differences in effectors utilization resulting from Ras activity at specific cellular sites. In addition, we also demonstrate how activation or inhibition of Ras restricted to defined membrane systems and microdomains affects biological outcomes such as cellular proliferation, survival, and transformation. Overall, the present study moves one step beyond into understanding how compartmentalization of Ras signals affects its performance and identifies those cellular sites where Ras is essential for cellular proliferation and transformation.

MATERIALS AND METHODS

Constructs. pEXV H-RasV12 C181,184 S (SS) was provided by J. F. Hancock; pGEX-Ral-RBD was provided by J. L. Bos; and pCEFL-m1 was provided by J. S. Gutkind. Plasmids encoding for v-Sis and v-Src have been described (29). pCEFL-pGEX-Ral-RBD was provided by J. L. Bos; and pCEFL-m1 was provided by J. S. Gutkind. Plasmids encoding for v-Sis and v-Src have been described (29). pCEFL-pGEX-Ral-RBD was provided by J. L. Bos; and pCEFL-m1 was provided by J. S. Gutkind. 

Cell culture. HEK293T, COS-7, MDCK, and NIH 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM)--10% fetal calf serum (calf serum and adriamycin were from Preprotech. Interleukin-1 recepтор constructs, respectively. SSS mutants were generated by PCR-directed mutagenesis. Oligonucleotides encoding LCK myristoylation signal were cloned in pCEFL-HA, directly upstream of the HA tag. Tethering constructs expressing FLAG, instead of HA tag, were also synthesized. H-RasV12 SS and H-RasN17 SS were amplified by PCR and cloned into pCEFL-HA or pCEFL-FLAG targer constructs, respectively. SSS mutants were generated by PCR-directed mutagenesis (QuickChange; Promega), introducing the C186S mutation into the targeted V12 constructs. All constructs were verified by DNA sequencing. Sequences of the oligonucleotides utilized are available upon request. Interleukin-1 and adriamycin were from Preprotech.

Antibodies. Mouse monoclonal anti-HA was from Babco; rabbit polyclonal anti-FLAG was from Invitrogen. Rabbit polyclonal anti-calreticulin was from Calbiochem. Antigiantin mouse monoclonal antibodies were supplied by H. P. Hauri (Basel, Switzerland). Mouse monoclonal anti-transferrin receptor was from BD Biosciences. Rabbit polyclonal anti-Akt and rabbit polyclonal anti-Raf have been previously described (16). Goat polyclonal antibody anti-Ral A and rabbit polyclonal antibodies anti-HA anti-ERK2, anti-RasGRF1, anticaveolin, anti-PARP, anti-TC21, anti-H-Ras, anti-K-Ras, and anti-N-Ras were from Santa Cruz Biotechnology.

Measurement of proliferation and survival rates. Cells were plated at low density in six-well plates (10,000 cells/per plate) and grown in different serum conditions as indicated. Cells were detached and scored by standard cell counting techniques at the indicated intervals.

RESULTS

Selective tethering of H-Ras V12 to defined sublocalizations. To investigate the differences in Ras functions at sites where it is present, it was necessary to activate it precisely at, and only at, the desired subcellular localizations. For this purpose we generated constructs that encoded for constitutively active H-RasV12 fused to specific tethering signals. First, we generated a palmitoylation-deficient H-RasV12 by mutating cysteines 181 and 184 to serines (H-RasV12 SS). This mutant is not efficiently retained at the PM and exists in a dynamic equilibrium shuttling between the ER and cytoplasmic pools (10). The palmitoylation signal was then substituted by alternative cues that would specifically direct H-RasV12 SS to the desired locations. To deliver it to the ER, we fused to its N terminus amino acids 1 to 66 of the avian infectious bronchitis virus M protein (M1) (49) (referred to as M1-V2). We sent H-RasV12 SS to the Golgi complex, by means of an N-terminally fused KDEL receptor (KDELr) (9). Since KDELr is an itinerant protein that recycles to the ER, mutation N193D was introduced. This mutation prevents it from redistributing to the ER and renders it a resident Golgi protein (13) (named KDELr-V12). At the PM, we wanted to discriminate between events regulated from bulk membrane and from lipid rafts. As such, H-RasV12 SS was tethered to bulk membrane by placing in its N terminus the transmembrane domain of the CD8a receptor, known to locate exclusively in this type of PM (2) (referred to as CD8-V12 hereafter). Finally, to target H-RasV12 SS to lipid rafts,
we utilized an N-terminal myristoylation signal. Unpalmitoylated myristoylation signals, like that of Rasheed sarcoma virus, cannot retain farnesylated Ras at the membrane (8), whereas palmitoylated myristoylation signals, such as the one of LCK (42), act as effective lipid raft anchors (48). An HA tag was included to enable the detection of these proteins.

These constructs were stably expressed in NIH 3T3 cells, and polyclonal pools, in which more than 80% of the cells stained positive for HA (data not shown), were utilized for further experimentation. In these, the localization of the targeted Ras proteins was ascertained by immunofluorescence with anti-HA antibodies. Cells expressing H-RasV12 exhibited a typical spindle-shaped morphology. In these, H-RasV12 was evident at the PM outlining the cells, in addition to endomembranes such as the Golgi complex (Fig. 1A, arrowhead) and the ER network, more prominently in the perinuclear area. In contrast, in cells expressing H-RasV12 SS Ras was absent from peripheral PM, and it exhibited a diffuse staining characteristic of cytoplasmic proteins, although staining at the nuclear envelope, typical of ER localization, was also clear (Fig. 1B). On the other hand, ER-tethered M1-V12 displayed a typical reticular distribution with a complete absence of cytoplasmic staining and was undetectable at the PM and at the Golgi complex (Fig. 1C). KDELr-V12 expression was clearly restricted to the Golgi apparatus and was absent from the PM and the ER (Fig. 1D). CD8-V12 was localized in patches throughout the PM, as shown in a tangential confocal plane in Fig. 1E and, within the cell body, in vesicular structures, resembling endosomes. However, it was undetectable at the ER (not shown). Finally, LCK-V12 also exhibited a peripheral membrane staining at defined patches. Small vesicles, probably lipid raft-derived endosomes, were also apparent, but no ER staining was evident (Fig. 1F). Even though Ras proteins are known to be subject to a fast, dynamic shuttling between PM and the Golgi complex (11), we could not detect LCK or CD8-tethered Ras proteins at the Golgi complex (Fig. 1E and F and data not shown). These results pointed to the correct localization of our targeted Ras proteins. Interestingly, these staining patterns, with virtually no mislocalization, were also detected in COS-7 and 293T cells, even though in these cells our plasmids yielded much higher expression levels (data not shown).

To ascertain beyond doubt that our targeted Ras proteins were correctly localized, we analyzed their colocalization with bona fide markers of the aimed compartments. As we have previously demonstrated, M1- and KDELr-tethered Ras proteins displayed a diffuse staining coexisting with the ER marker calreticulin and the Golgi marker giantin, respectively (4; data not shown). In the same fashion, CD8-V12 and LCK-V12 were...
detected at, and only at, the expected PM microdomains. As such, CD8-V12 markedly colocalized with the transferrin receptor, a marker for disordered membrane (Fig. 1G), but an absolute lack of colocalization with the lipid raft marker 5′ nucleotidase was evident (Fig. 1H). Conversely, LCK-V12 strongly merged with the raft marker ganglioside GM1, as shown in a tangential confocal plane (Fig. 1I), but no costaining with the transferrin receptor was apparent (Fig. 1J). Further proof for the correct localization of these constructs was provided by experiments in which membranes of the targeted-Ras-transfected cells were solubilized in low concentrations of Triton X-100 and fractionated in sucrose gradients. As ex-
lack of contamination of the peripheral membrane with the endomembrane-targeted constructs (Fig. 2A). Solubilization in sodium carbonate yielded identical results (data not shown). Overall, these results clearly indicated that our targeted H-RasV12 proteins were specifically localized at the desired microdomains.

It was important to acquire some notion of the relative expression levels that each targeted protein attained. By immunoblotting for HA expression it was found that, as expected, all of the targeted V12 proteins were expressed at lower levels than those of H-Ras V12, widespread throughout all of the sublocalizations, or of H-Ras V12 SS, which accumulated to high levels in the cytoplasm. Noticeably, the PM proteins, LCK-V12 and CD8-V12, were expressed at slightly higher levels than those of M1-V12 and KDELr-V12, located at endomembranes (Fig. 2B). As such, we wanted to know whether these differences were a consequence of variations in the intrinsic expression potentials of each of the constructs. When expressed in vitro in a reticulocyte system, these constructs yielded very similar expression levels (data not shown), something that argued against this concept. Alternatively, the possibility existed that the expression levels of the targeted proteins were a reflection of how much Ras could be inserted on each type of membrane. In our unpalmitoylated constructs, the remaining Ras-specific determinant for membrane localization was their farnesylo moiety. Thus, we introduced the mutation C186S that rendered them farnesyl deficient as well. It was found that, regardless of the targeting signal, these proteins (termed SSS) achieved much higher expression levels than their respective palmitoylation-deficient (SS) constructs, as shown for M1-V12 (Fig. 2B, lower panel). Interestingly, a significant proportion of the SSS proteins had lost their specific localization and was detected at the cytoplasm (data not shown). Overall, these results indicated that a Ras membrane localization determinant was important for defining both the expression levels and the localization of our targeted proteins.

Targeted Ras proteins can effectively engage effectors in vitro and in vivo. It was essential to verify that the addition of a more or less bulky targeting signal to the N terminus of H-RasV12 did not compromise its GTP-bound state and therefore its ability to engage effectors. Initially, we tested the behavior of the targeted Ras proteins in a standard in vitro effector binding assay; these were expressed in 293T cells, and the GTP-bound fractions were affinity pulled-down by using the c-Raf Ras binding domain (RBD) bound to glutathione beads. It was found that untargeted H-RasV12 and the different location-specific RasV12 proteins bound to GST-RBD with very similar efficiencies (data not shown). Next, we ascertained whether the targeted Ras proteins could also bind to effectors in vivo. In the NIH 3T3 cell lines we examined whether the presence of site-specific RasV12 proteins brought about colocalization with c-Raf at defined sites. In cells expressing H-Ras wild-type, H-Ras could be detected at PM, ER, and Golgi complex, as expected. c-Raf displayed a diffuse staining, typical of a cytoplasmic protein, but no colocalization was apparent at any of these sites under serum starvation conditions (Fig. 3A). In sharp contrast, under the same conditions, in M1-V12-expressing cells a clear Ras/c-Raf colocalization was present throughout the reticular network (Fig. 3B). A marked costaining was also apparent in LCK-V12- and CD8-
V12-expressing cells, but in these cases, merging took place primarily at the peripheral PM and to a lesser extent in endosomes (Fig. 3C and D). Surprisingly, in KDELr-V12-expressing cells the colocalization between Raf and Ras at the Golgi complex was very poor (Fig. 3E), even though, as stated above, KDELr-V12 could effectively bind to c-Raf RBD in vitro. Overall, our results demonstrated that the addition of tethering signals did not compromise RasV12 functionality, both in

FIG. 3. Colocalization of c-Raf with targeted Ras proteins. Serum-starved NIH 3T3 cells expressing the indicated Ras constructs were costained with anti-HA and anti-Raf antibodies. (A) No colocalization with c-Raf is observed in cells expressing H-Ras wild type (wt). (B) M1-V12 displays high colocalization with c-Raf at the ER. (C and D) Colocalization of Raf with LCK- and CD8-V12 at the PM and endosomes. (E) Limited colocalization of c-Raf with KDELr-V12 at the Golgi complex. Confocal sections at the level of the nucleus are shown in all panels. In panels B to D, insets highlight areas of prominent colocalization (arrows). Bars: A, C, and D, 20 μm; B, 10 μm; E, 15 μm.
Distinct pools of RasV12 elicit different biochemical signals. It was of interest to investigate whether signaling pathways triggered by Ras were equally activated irrespective of Ras localization or, alternatively, different cellular pools of Ras evoked distinct signals. For that purpose, in the NIH 3T3 lines stably expressing the targeted RasV12 constructs we analyzed...
the activation status of several well-known Ras effector pathways. When we studied the activation of the ERK cascade, pronounced differences were detected depending on the cellular site from which the Ras signal originated. H-RasV12 SS, a protein loosely held to the ER, showed a reduced ability to activate ERKs (Fig. 4A), as previously demonstrated (8). However, firmly anchoring Ras to the ER, as was the case for M1-V12, elicited a potent activation of ERKs. LCK-V12, at lipid rafts, was capable of activating ERKs to similar levels, while bulk membrane CD8-RasV12 displayed a lower, though significant, ability to activate this route. Interestingly, very little activation of ERKs was detected when the Ras signal emanated from the Golgi complex, as that one generated by KDELr-V12, in agreement with the low colocalization of Ras and Raf in this organelle. A similar situation was encountered when we studied the PI3K/Akt pathway. In this case, Ras signals originating at the ER and at lipid rafts were the most effective for activating this route (Fig. 4B). Ras capacity to activate Akt from the disordered membrane was noticeably reduced. Once more, more Ras located at the Golgi complex was the least efficient for activating Akt. Curiously, this pattern was reversed when we looked at the activation of Ral-GDS. In this case, the Golgi complex was the platform from which Ral-GDS was most effectively activated, followed by the bulk membrane (Fig. 4C). Even though the JNK pathway is not considered a bona fide Ras effector route, in NIH 3T3 cells JNK can be activated by Ras to some extent (14). Thus, we also tested how Ras compartmentalization affected the activation of this route. Interestingly, it was found that endomembranes, both ER and Golgi, were the sites preferred by Ras to activate JNK (Fig. 4D), whereas the Ras pools present at PM microdomains were considerably less effective for activating this mitogen-activated protein kinase. Assays

FIG. 5. Compartmentalized Ras activation effects on cellular proliferation. (A) Proliferation rate of the NIH 3T3 cells lines expressing the indicated Ras constructs growing in media supplemented with 5% calf serum. (B and C) Proliferation rates of three clones (C1 to C3) isolated from the M1-V12 (B) and the LCK-V12 (C) polyclonal cell lines compared to their respective total pools. (D) Effects of targeted Ras V12 constructs on colony growth. NIH 3T3 fibroblasts were transfected with the indicated constructs (0.25 µg) and selected in the presence of G418 (750 µg/ml). After 2 weeks in culture, colonies were stained, and those with a diameter greater than 0.05 mm were scored. In all cases, results show the average ± the SEM of at least three independent experiments.
similar to those just described were also performed in 293T cells under transient-transfection conditions. Noticeably, the results obtained were identical to those just described (data not shown).

We needed to ascertain that the signals elicited by the targeted RasV12 proteins were directly generated by Ras itself and were not secondary signals, consequences of autocrine loops induced by Ras activation. To test this, conditioned media from cells expressing the site-specific Ras constructs were collected after 12 and 24 h and added to parental NIH 3T3 cells, and effector activation was assayed. In no case was the conditioned medium able to induce any significant ERK, Akt, or Ral-GDS activation (data not shown), thus demonstrating that the signals detected in the aforementioned experiments were a consequence of RasV12 direct effects.

**Distinct pools of RasV12 differentially regulate cellular proliferation, survival, and transformation.** We wanted to determine whether variations in effector usage and in signal magnitude at the distinct Ras signaling platforms translated into different biological responses. First, we evaluated how cellular proliferation was affected by location-restricted Ras signaling by comparing the proliferation rates of the cell lines expressing the targeted Ras constructs when growth occurred under standard culture conditions. We found that most of the pools displayed similar growth curves (Fig. 5A), with a proliferating profile that resembled that of H-RasV12-transfected cells (doubling time of 13.4 h). The remarkable exception was the line expressing Golgi complex-tethered KDELr-V12, which exhibited much slower growth kinetics (doubling time of 19.6 h), similar to those encountered in control cells. It was important to ascertain to what extent the behavior of the polyclonal cell lines was representative of the clonal population that they were made up of. To this end, the growth kinetics of three isolated clones extracted from each of the polyclonal lines was compared to those of their respective pools. As shown for M1-V12 (Fig. 5B) and for LCK-V12 (Fig. 5C), no significant differences were encountered among the proliferation rates of the pools and of the isolated clones. Likewise, no substantial variations were apparent in the cases of KDELr-V12 and CD8-V12 either (data not shown).

The ability of the tethered Ras proteins for supporting proliferation was tested further by colony growth assays. As such, the targeted Ras constructs were transfected into NIH 3T3 cells and after G418 selection the arising colonies were scored. In line with the previous data, all of the targeted Ras construct yielded a number of colonies similar to that of H-RasV12 (Fig. 5D), with the exception of KDELr-V12, for which the potential to form colonies was similar to that of vector-transfected cells and significantly lower than that of H-Ras wild type.

We also wanted to investigate how compartmentalized Ras activity affected cell survival. To this aim, the NIH 3T3 cell lines were deprived of serum, and their ability to survive in these conditions was monitored. As shown in Fig. 6A, in the absence of growth factors RasV12, irrespective of its localization, supported proliferation for almost two complete doubling cycles. Eventually, all lines reached crisis, and cell numbers started dropping with similar kinetics, with the exception of cells harboring ER-tethered M1-V12, in which the rate of cell death was remarkably lower. This behavior was mirrored to some extent by the cell line expressing H-RasV12 SS. Even though this cell line reached crisis before the rest, its death kinetics were noticeably slower.

We extended this observation by analyzing the consequences of compartmentalized Ras activity when cells were challenged with defined apoptogenic stimuli. For this purpose, the NIH 3T3 cell lines were treated with interleukin-1 (10 μg/ml) for 24 h and with adriamycin (5 μM) for 16 h, respectively. PARP degradation was monitored in total lysates by anti-PARP immunoblotting. (C) Cellular transformation induced by site-specific Ras constructs. NIH 3T3 and MDCK cells stably expressing the Ras targeted constructs were transfected with the indicated constructs (100 ng) by the calcium phosphate technique. Foci were stained and scored after 2 weeks in culture. Numbers indicate average number of foci per microgram of DNA ± the SEM of at least three independent experiments.
limited to a given cell type, we generated lines of MDCK epithelial cells stably expressing the targeted Ras constructs. In this cellular model, Ras activation restricted to the ER also conferred a remarkable resistance to cell death, as induced by treatment with the proapoptotic drug adriamycin (Fig. 6B, bottom panel).

Finally, we looked at whether sublocalization affected H-RasV12 transformation potential. For this purpose, the ability of the targeted RasV12 constructs to generate transformed foci in NIH 3T3 cells was tested. It was found that H-RasV12 was able to robustly induce cellular transformation from most of the cellular localizations where it was targeted (Fig. 6C), slightly more prominently from PM microdomains, both lipid rafts and bulk membrane, than from endomembranes such as the ER. Conversely, Golgi complex-tethered KDELr-V12 was incapable of promoting cellular transformation to any extent. Summing up, these sets of experiments clearly demonstrated that, depending on the subcellular compartment at which it was located, activated H-Ras could trigger proliferation, survival, and transformation to different extents.

**Effects of site-specific Ras inhibition on cellular proliferation and transformation.** The aforementioned results clearly supported the notion that sublocalization dictates Ras signal output and its biological consequences. However, these sets of experiments explored how compartmentalization influenced the effects of constitutive Ras signals and did not provide an answer as to how necessary Ras is at its different localizations for the conveyance of upstream signals that drive cellular proliferation or...
transformation. To address this important point, we designed a strategy to selectively inhibit Ras activity at defined sites. We have previously demonstrated that the inhibitory specificities of Ras N17 dominant inhibitory mutants are related to their microlocalization, in such a way that a N17 mutant acting at a defined microdomain, will inhibit all Ras molecules therein irrespective of the isoform (35). Thus, we reasoned that specifically targeting H-RasN17 to defined localizations, by using the aforementioned tethering cues, could be an effective method to block Ras functions in a site-specific fashion.

To begin with, we verified the site inhibition specificity of our targeted H-RasN17 constructs. Inhibition at the ER was tested in COS-7 cells by assaying GTP loading in ER-tethered M1-H-Ras, induced by the guanine nucleotide exchange factor RasGRF1, in the presence of the inhibitory constructs M1-N17 and CD8-N17. It was found that GDP/GTP exchange in M1-H-Ras was abrogated by ER-tethered M1-N17, but not by bulk membrane-targeted CD8-N17 (Fig. 7A, left panel). As a negative control, M1-green fluorescent protein (GFP) was used to validate that the inhibition was specific for RasN17 and that it was not an unspecific effect due to M1 tethering signal. Conversely, when GTP loading was assayed on bulk membrane-tethered CD8-H-Ras, this was inhibited by CD8-N17 but not by M1-N17 (Fig. 7A, right panel). Similar results were obtained when testing for the cross-inhibitory effects of most N17 constructs (data not shown), thereby demonstrating that the inhibitory effects of the targeted N17 mutants were site specific. Only in one instance cross-inhibition was evident. This was when nucleotide exchange was assayed in lipid raft-tethered LCK-H-Ras that could be inhibited both by LCK-N17 and by CD8-N17 (Fig. 7B, left panel). Likewise, GTP loading in bulk membrane-tethered CD8-H-Ras could be inhibited by CD8-N17 and, to a lesser extent, by LCK-N17 as well (Fig. 7B, right panel).

Next, we studied the effects of the targeted N17 mutants on cellular proliferation by testing their ability to interfere with colony growth. As such, NIH 3T3 cells were transfected with the targeted N17 constructs and, after culture under G418 selection, the resulting colonies were scored. As shown in Fig. 7C, ER-targeted M1-N17 and bulk membrane-targeted CD8-N17 robustly restricted growth, showing more than 66% reduction compared to the number of colonies formed by H-Ras wild-type, a decrease comparable to that one brought about by untethered H-RasN17. On the other hand, Golgi complex-tethered KDELr-N17 had minimal effects on colony proliferation. Interestingly, LCK-N17, in spite of its cross-interference with Ras activation at bulk membranes, was considerably less efficient than CD8-N17 for restricting colony numbers, reducing it by only 30%. These results hinted that only Ras functions at the ER and at bulk membrane were essential for supporting cellular growth.

We then investigated the effects of site-restricted Ras inhibition on cellular transformation. To do this, we analyzed in NIH 3T3 fibroblasts the transforming potential of different types of oncogenes, when Ras was inhibited at some specific site by cotransfection with the targeted N17 mutants. Since Ras N17 mutants function by unproductively sequestering exchange factors (19), they should not affect Ras V12-evoked events. As such, the concentration of the constructs encoding for the N17 mutants was adjusted so as to minimally interfere with transformation induced by H-RasV12, thereby avoiding nonspecific, inhibitory effects. Under these conditions, transformation by a PM-bound tyrosine kinase such as v-Src proved to be particularly sensitive to Ras inhibition at the PM, in particular at the bulk membrane. Noticeably, Ras inhibition at the ER had little effects on v-Src-induced transformation (Table 1). In the case of v-Sis, encoding for a growth factor, platelet-derived growth factor (PDGF), which acts through a tyrosine kinase receptor, blocking Ras functions at the PM markedly reduced its transforming potential, 73% blockade by bulk membrane CD8-N17 and 53% by LCK-N17 at lipid rafts. On the other hand, inhibiting Ras ER pool had only moderate consequences on v-Sis transformation, preventing it by only 25%. A very similar situation was encountered when we evaluated transformation by the G protein-coupled receptor, m1 muscarinic receptor, an agonist-dependent oncogene, in the presence of its ligand carbachol (20). Interestingly, blocking Ras activation at the Golgi complex by KDELr-N17 had very modest effects on the transforming potential of all of the oncogenes tested (Table 1).

Cells devoid of Ras at lipid rafts and at the Golgi complex proliferate normally and can be transformed. The fact that our N17 constructs tethered to bulk membrane and to lipid rafts exhibited some degree of cross-inhibition precluded us from gaining a clear picture of the necessity for Ras functions at these microdomains. In order to clarify this point, we resorted to an alternative experimental model. This was provided to us by the existence of double-knocked-out mice for H-Ras and N-Ras (18). Cells derived from these mice express only the isoform K-Ras, but since K-Ras is not found in lipid rafts (39) these are completely devoid of all Ras isoforms, thus constituting an unambiguous model for our purposes. In addition, cells derived from H-Ras/ N-Ras double-knockout mice also lack Ras at the Golgi complex, since K-Ras is also absent from this organelle (1, 11).

First of all, we ascertained that in MEFs from H-Ras/N- Ras−/− mice, K-Ras had not undergone a redistribution process due to adaptive pressures and lipid rafts and the Golgi complex were indeed devoid of every Ras isoform. Double immunofluorescence analysis of H-Ras with the lipid raft marker GM1 and the Golgi marker giantin verified the complete lack of expression of H-Ras in these cells (Fig. 8A and B). Identical results were obtained when we analyzed the expression of N-Ras (data not shown). When the distribution of K-Ras was analyzed, it was found that this isoform was notoriously present at the ER and at some PM localizations (Fig. 8C). However, upon double staining with GM1 an absolute lack of colocalization was evident, a finding indicative of the existence of double-knocked-out mice for H-Ras and N-Ras (18). However, KDELr-N17 had very modest effects on colony proliferation, 73% blockade by bulk membrane CD8-N17 and 53% by LCK-N17 at lipid rafts. On the other hand, inhibiting Ras ER pool had only moderate consequences on v-Sis transformation, preventing it by only 25%. A very similar situation was encountered when we evaluated transformation by the G protein-coupled receptor, m1 muscarinic receptor, an agonist-dependent oncogene, in the presence of its ligand carbachol (20). Interestingly, blocking Ras activation at the Golgi complex by KDELr-N17 had very modest effects on the transforming potential of all of the oncogenes tested (Table 1).
the additional participation of a nuclear oncogene, c-Myc was cotransfected in all cases. As expected, H-Ras V12 displayed a potent focus-forming activity. Interestingly, this was also the case for v-Src and for m1 receptor (Fig. 8F). Furthermore, these oncogenes were able to transform H-Ras/N-Ras +/− MEFS in proportions similar to those of the wild-type MEFS (data not shown). Therefore, these results clearly corroborated that the functions undertaken by Ras at lipid rafts and the Golgi complex were fully dispensable for cellular growth and transformation.

**DISCUSSION**

Today, a large body of evidence supports the notion that Ras proteins are segregated throughout different PM microdomains and internal membrane systems (23). Determining how compartmentalization influences Ras signaling and its subsequent biochemical and biological effects undoubtedly represents a conceptual milestone in the long quest for unraveling the fine points of Ras mechanics. Ideally, one would attempt to selectively activate or inhibit endogenous Ras at a particular compartment. However, our current state of knowledge and technical limitations preclude such an untainted approach. Thus, we are forced to resort to alternative strategies that, though less sophisticated, will enable us to gain a timely insight into this conundrum.

We have approached the problem by selectively targeting H-RasV12 to those cellular compartments where Ras is localized under physiological conditions. This we have achieved by substituting H-Ras palmitoylation signal for N-terminally fused peptides encoding for alternative cues that specifically direct Ras to the desired locations. These proteins have been...
stably expressed in NIH 3T3 cells, and their correct localization has been extensively corroborated. It is noteworthy the fact that we have encountered very little mislocalization and unwanted intercompartment spillage. This was particularly striking in the case of experiments using transient transfections in COS-7 or 293T cells, in which plasmid episomal replication yields very high protein levels, in spite of which mislocalizations were seldom detected. In this respect, we have observed that there are slight variations in the levels of targeted Ras proteins present at the different localizations. This probably reflects that distinct types of membranes support different amounts of Ras. In support of this notion we have observed that the expression levels of our targeted Ras constructs are dependent on the Ras farnesylation signal. A likely explanation for the absence of mislocalizations could be that once Ras-binding sites at a given location are saturated, excess targeted Ras proteins, bearing a farnesyl moiety that cannot be inserted into a membrane, are rapidly degraded. In support of this notion we have observed that farnesylation-deficient, targeted Ras proteins accumulate in the cytoplasm when overexpressed. Regardless of the targeting signal utilized, there will be a limit to how much protein can be inserted into a given site, after which surplus will accumulate in the cytoplasm and/or will be degraded. In the case of our constructs that limit is set by the availability of Ras-binding sites.

We have thoroughly ascertained the specificity of Ras targeting to bulk membrane and to lipid rafts. This we have monitored by immunofluorescence, detergent solubilization, and lysis in sodium carbonate. The three methods have demonstrated specific targeting and a complete absence of cross-

FIG. 8. MEFs devoid of Ras at lipid rafts can be transformed. H-Ras/N-Ras−/− MEFs are devoid of Ras isoforms at lipid rafts and the Golgi complex. MEFs were cotransfected with anti-H-Ras antibodies (red, panel A; green, panel B) or anti-K-Ras (red, panels C and D) and cholera toxin to stain lipid rafts (green, panels A and C) or the Golgi marker antigiantin (red, panel B; green, panel D). All panels show equatorial sections at the cell nucleus level. Bars: A and C, 20 μm; B and D, 10 μm. (E) Specificity of the antibodies utilized. Lysates from COS-7 cells transfected with H-Ras (H), K-Ras (K), N-Ras (N), and TC-21 (TC) were immunblotted with the indicated antibodies. (F) H-Ras/N-Ras−/− MEF transformation induced by oncogenes. MEFs were transfected with vectors encoding for H-Ras V12, v-Src, or m1 muscarinic receptor (1 μg each), in addition to c-Myc (1 μg). After 3 weeks, cells were stained, and the number of foci was scored. m1-transfected plates were grown in the presence of 10 μM carbachol.
localization. We have observed that the constructs that are targeted to bulk membrane and to lipid rafts can also be detected in small cytoplasmic vesicles, probably bulk membrane and lipid raft-derived endosomes. This phenomenon is highly representative of the internalization and recycling processes that take place at the PM. However, it prevents us from finely discriminating between events strictly taking place at these microdomains and those that occur at their derived endosomes, which would constitute the next level of complexity in our future investigations. At this stage, we have limited ourselves to gain an overall view of the processes regulated by Ras proteins that reside in these membrane subtypes, irrespective of the stage of the membrane turnover cycle. Without interfering with the physiological trafficking and recycling processes that these subdomains are subject to, that could lead to confusing situations.

We have verified that our constructs are functional. In spite of the variable-size peptides that were fused to their N termini, all RasV12 constructs bound with similar efficiencies to an effector molecule in vitro, indicating that the N-terminal structural alterations did not affect their GTP-bound state. This was further corroborated in vivo by their ability to recruit c-Raf to those sites where they had been tethered. A notorious exception was the case of the Golgi complex, in which very little colocalization of c-Raf and KDELr-V12 was observed. This observation is in full agreement with our results showing that

<table>
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<tr>
<th>Oncogene</th>
<th>Vector</th>
<th>N17</th>
<th>M1 N17</th>
<th>Lek N17</th>
<th>CD8 N17</th>
<th>KDELr N17</th>
</tr>
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<tbody>
<tr>
<td>H-Ras V12</td>
<td>3,270 ± 126</td>
<td>3,002 ± 165** (−9)</td>
<td>2,981 ± 90** (−9)</td>
<td>3,182 ± 125** (−3)</td>
<td>2,943 ± 79** (−10)</td>
<td>3,197 ± 105** (−3)</td>
</tr>
<tr>
<td>v-Src</td>
<td>402 ± 31</td>
<td>304 ± 25** (−25)</td>
<td>414 ± 46** (−3)</td>
<td>246 ± 47*** (−39)</td>
<td>194 ± 61*** (−52)</td>
<td>433 ± 38** (8)</td>
</tr>
<tr>
<td>v-Sis</td>
<td>414 ± 83</td>
<td>221 ± 29*** (−47)</td>
<td>322 ± 40* (−23)</td>
<td>179 ± 34*** (−57)</td>
<td>112 ± 48*** (−73)</td>
<td>378 ± 48** (−9)</td>
</tr>
<tr>
<td>m1 MACHR</td>
<td>774 ± 92</td>
<td>487 ± 42*** (−38)</td>
<td>534 ± 75** (−23)</td>
<td>480 ± 30*** (−32)</td>
<td>464 ± 93*** (−41)</td>
<td>825 ± 33** (−10)</td>
</tr>
</tbody>
</table>

*NIH 3T3 fibroblasts were cotransfected with H-RasV12 (100 ng), v-Src (1 μg), v-Sis (1.5 μg), or m1 receptor (0.5 μg) in addition to the different N17 targeted constructs (0.5 μg) where indicated. After 2 weeks, cells were stained, and foci were scored. Figures, expressed as foci per micrograms of oncogene DNA, indicate the average ± the SEM of at least four independent transfections. The percent inhibitions relative to vector-transfected plates are shown in parentheses. m1-transfected plates were grown in the presence of 10 μM carbachol. Analysis of variance was determined by Bonferroni multiple comparisons test relative to the respective “vector” values. P values: *, P < 0.05; **, P < 0.01; ***, P < 0.001; NS, P > 0.05 with a 95% confidence interval.
activation of the ERK cascade at the Golgi complex was very poor and may reflect either the lack of c-Raf at Golgi microlocalizations where Ras is present or, alternatively, the absence therein of scaffold proteins that intervene in the assembly of the Ras-Raf complex. Even though during mitosis the presence of Raf at the Golgi complex and its participation in Golgi fragmentation has been reported (12), whether Raf is directly activated therein or elsewhere and then shuttles to that organelle has not been demonstrated.

Our results clearly demonstrate that the intensity at which an effector route is activated by Ras is greatly dependent on its localization. It is unlikely that differences in Ras concentrations can account for the observed variations. For example, the potential to engage effectors and the robustness of the biological outcomes of M1-V12 are some of the greatest, even though its expression levels are not as high as those of LCK-V12 or of CD8-V12. Furthermore, at any given compartment some effector routes are very efficiently stimulated, while others are not. Probably, the local intensity of a signal is dictated by the limited availability of some or all of the intermediaries that build up a signaling module or of essential scaffold proteins that optimize signal output through that route. In line with this notion, our results demonstrate that the Raf-GDS route is most efficiently stimulated at the Golgi complex where high concentrations of its cognate GTPase, RaLA, are found (31). Likewise, the Raf-ERK pathway is preferentially activated in lipid rafts, reflecting c-Raf high affinity for cholesterol-enriched domains (30). Even though this result may seem contrary to previous reports, indicating that Raf activation is favored in bulk membrane as opposed to lipid rafts (39), Raf activation can be initiated at lipid rafts (44) and lipid raft disruption prevents the activation of the Raf-ERK pathway (43, 47), indicating that the machinery to activate this pathway is readily available at lipid rafts. The fact that we are using fixed Ras constructs, unable to translocate between compartments, could account for the differences in signal intensities reported here and in previous reports (39).

We have found that Ras can effectively support cellular proliferation and transformation from most of its platforms. Since a biological output is the result of the integration of all of the signals generated by a causative stimulus, it is difficult, if not impossible, to discretely associate a biological outcome with the activation of a particular route. However, our results underscore the importance of two essential factors. (i) The first is the signal intensity. How Ras signal strength affects the biological outcome(s) is highlighted by the behavior of the cells harboring M1-V12 and RasV12 SS. Both of them operate at the ER, and both activate the same signaling pathways, M1-V12 more intensively in all cases. As such, M1-V12 is much more effective in promoting proliferation, transformation, and survival. It is noteworthy that signal intensity does not follow a proportional relationship with the resulting biological outcome(s), which hints the existence of signal thresholds over which full biological responses would ensue. (ii) The second factor is the availability of substrates. We show here that only the ER Ras pool can sustain cell survival, even though Ras operating from other platforms, for example, lipid rafts, evokes very similar effectors usage and almost identical signal intensities. One likely explanation for this apparent discrepancy could be that ERK/Akt/Ral substrates mediating in cell survival are confined and available mainly to ER-activated effectors.

The surprising finding of the Ras ER pool being extremely efficient at supporting survival is completely unprecedented. Our results reveal that M1-V12 generates a potent antiapoptotic signal irrespective of the cell type and the apoptogenic stimulus. Interestingly, M1-V12 evokes antiapoptotic events that H-Ras V12 cannot. One possible explanation for this could be that M1-V12 is fixed to the ER, whereas H-Ras V12 is subject to Ras physiological trafficking in and out of the ER. Therefore, it is conceivable that by increasing Ras residence time at this organelle a more efficient pro-survival signaling could be elicited. Even though the exact mechanisms whereby this process takes place are unknown, the ER is a key site directly intervening in the apoptosis-regulating effects of calcium (21). Interestingly, N-Ras, known to provide a potent antiapoptotic signal (51), is particularly abundant at the ER (our unpublished results).

Our results indicate that Ras activation at the Golgi complex cannot promote cellular transformation nor enhance cell proliferation, even though it is competent for sustaining limited cellular growth for short periods under serum starvation conditions. This clearly contrasts with previous reports (7, 10). However, one technical subtlety may likely account for this discrepancy: we utilized here as a Golgi complex-tethering signal KDELr, harboring the mutation N193D that prevents it from translocating to the ER and fixes it to the Golgi complex as a permanent resident protein (13). In contrast, the aforementioned studies used KDELr wild type. As such, proteins tethered with this cue will shuttle between ER and the Golgi complex. Since Ras at the ER can effectively transform, as we and others have shown (10), it is very likely that transformation elicited by KDELr wild-type-tethered Ras is a consequence of its transit through the ER. In full agreement with our results, a former report using yet another Golgi complex-targeting signal, the E1 protein of avian bronchitis virus, detected no transformation induced by Ras at the Golgi complex (28). On the other hand, in light of our data demonstrating the potent transformation of RafGDS by Ras at the Golgi complex and of previous studies reporting the importance of this pathway for human oncogenesis (22, 41), it is conceivable that, in contrast to the murine model, Ras at the Golgi complex could play an important role in the transformation of human cells.

The fact that mutationally activated Ras can effectively exert its effects from most of the sites where it is found, provides little information on the relevance and need for endogenous Ras at its different compartments in processes such as physiological proliferation and transformation by upstream stimuli. We have addressed this issue here, specifically blocking endogenous Ras pools by tethering thereto inhibitory N17 mutants. Remarkably, these mutants have yielded highly specific site-restricted inhibitory effects, the sole exception being N17s targeted to lipid rafts and to disordered membrane, which exhibited some degree of cross-inhibition. However, this should not be surprising. The ER and the Golgi complex are tangible, isolated structures, and most of their extension is spatially separated from each other and from the PM. Thus, it is conceivable that mutants targeted thereto can only exert their effect locally, without interfering with Ras GEFs at other confinements sufficiently separated. On the other
hand, lipid rafts and bulk membrane are contiguous domains, exhibiting diffuse boundaries. As such, it can be envisioned that lipid raft-targeted N17 can deprive adjacent bulk membrane of its GEFs and vice versa. The fact that CD8-N17 displays a greater cross-inhibitory potential than CD8-N17, our data highlights the importance of bulk membrane microdomains in Ras signaling.

Overall, our results underscore for the first time the variability of Ras biochemical signals depending on the signaling platform. Noticeably, we demonstrate that mutationally activated Ras can support enhanced cellular proliferation and transformation from most sites. This has special connotations regarding carcinogenesis, indicating that strategies aimed at inhibiting Ras localization at the PM will not prevent Ras transforming effects elicited from alternative sites such as the ER. In addition, our results highlight the importance of Ras signaling at bulk membrane and provide compelling evidence on the dispensability of Ras functions at lipid rafts and the Golgi complex under physiological conditions.

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