Identification of Essential Interacting Elements in K-Ras/Calmodulin Binding and Its Role in K-Ras Localization

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We previously showed that K-Ras is a calmodulin-binding protein. Involvement of this interaction in anterograde and retrograde transport of K-Ras was then suggested. To test this we have analyzed here the domains of K-Ras essential for the interaction with calmodulin. At least three different regions in the K-Ras molecule were important; they are the hypervariable region, the α-helix between amino acids 151 and 166, and the Switch II. Within the hypervariable region, both the hydrophobic farnesyl group and the positive-charged amino acids were essential for the interaction between K-Ras and calmodulin in cellular extracts. Consistently, K-Ras S181D, which mimics phosphorylation of Ser-181 of K-Ras, also completely abolished binding to calmodulin. K-Ras mutants correctly farnesylated that did not bind calmodulin were all located at plasma membrane, showing that calmodulin interaction was not required for the transport of K-Ras to plasma membrane. In NIH3T3 cells, K-Ras and calmodulin colocalized mainly in the plasma membrane even after the addition of Ca2+ ionophore, indicating that interaction did not directly lead to K-Ras internalization. Furthermore, using a K-Ras with impaired binding to calmodulin but with membrane localization, we could demonstrate in striatal neurones that interaction between K-Ras and calmodulin was not required for Golgi K-Ras translocation induced by Ca2+ influx.

Ras GTPases are key regulators of signal transduction pathways controlling cell proliferation, differentiation, survival, and apoptosis (1–3). The molecular basis for such a wide variety of cell responses controlled by Ras proteins is that Ras transduces signals from various extracellular stimuli, including growth factors, hormones, and cell-extracellular matrix contacts to many downstream effectors (4, 5). As a molecular switch, Ras cycles between a GTP-bound active and an inactive state when GTP is hydrolyzed to GDP. Active Ras interacts with and modulates the activity of effector proteins. Their role in transformation and oncogenesis is highlighted by the fact that more than 10% of human cancers harbor point mutations on Ras proteins (6–8). There are three ubiquitously expressed Ras isoforms, H-, N-, and K-Ras 4B (K-Ras). The high degree of homology between the different Ras isoforms suggests that they are functionally identical, but accumulating evidence points to a preferential activation of specific effectors by different Ras isoforms (9). Furthermore, experiments with mice knocked out selectively for each Ras isoform showed that K-Ras, but not H-Ras or N-Ras, is essential for development (10, 11).

Those Ras isoforms have a conserved globular domain between positions 1 and 165, whereas the carboxyl-terminal 23–24 amino acids show considerable variation (hypervariable region: HVR). Within the conserved domain there are several motifs important for Ras functions including GTP binding, and Switch I and Switch II regions responsible for effector, guanine nucleotide exchange factor, and GTPase-activating protein interactions (see Fig. 1) (12). The crystal structure of Ras reveals that this protein exists in different conformations in the GDP-versus GTP-bound states, this being the most important change in the Switch I and II regions. Consequently, GDP- and GTP-bound states have different affinity for effectors, guanine nucleotide exchange factors, and GTPase-activating proteins (13–15). The HVR contains sequences important for post-translational modification, including the CAAX box responsible for targeting lipid modification.

The three isoforms are anchored to the inner surface of the plasma membranes, and their location is believed to be essential to physiological activity. Although H-, N-, and K-Ras are mostly found in the plasma membrane, their location and signaling in endomembranes has also been described (16). A carboxyl-terminal S-farnesyl cysteine carboxyl methyl ester together with a

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5 The abbreviations used are: HVR, hypervariable region; CaM, calmodulin; CaMBP, CaM-binding protein; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RBD, Ras binding domain; TBS, Tris-buffered saline; BSA, bovine serum albumin; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; ROI, regions of interest; HIV, human immunodeficiency virus; Seph, Sepharose; WT, wild type; MARKS, myristoylated alanine-rich C kinase substrate.
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second signal directs their location to the membrane (17). The second signal in K-Ras comprises a polybasic domain of six lysine residues. Those basic residues are believed to associate with the anionic head groups of inner leaflet phospholipids. In contrast, the second signal in H- and N-Ras comprises palmitoylation of cysteine residues. This two-signal membrane-targeting mechanism serves to determine the trafficking pathway of the different Ras proteins to the plasma membrane and the microlocation at different plasma membrane domains. All Ras proteins are synthesized at the cytosol on free polysomes. They are immediately prenylated and consequently targeted to the endoplasmic reticulum. N-Ras and H-Ras are palmitoylated, directed to Golgi, and sent to the membrane via vesicular transport (18). Retrograde N-Ras and H-Ras traffic back to Golgi occurs after depalmitoylation (19). In contrast, K-Ras moves directly from the endoplasmic reticulum to the plasma membrane through an unknown mechanism, which may imply a chaperone protein to hide the farnesyl group, whereas K-Ras is in the cytosol. Furthermore, it has been recently suggested that K-Ras can also be redirected from the plasma membrane to Golgi apparatus in hippocampal neurons stimulated with glutamate through a CaM-dependent pathway (20) and to the mitochondria in other cellular types after protein kinase C-dependent phosphorylation (21).

In fact, we showed previously that CaM down-regulates Ras activation in fibroblasts and that CaM binds specifically to GTP-loaded K-Ras in a Ca\(^{2+}\)-dependent way (22). But the interaction between K-Ras and CaM has not been analyzed in detail, and consequently its role in the K-Ras intracellular localization has not been directly studied.

CaM is a small (148 amino acids) and well conserved Ca\(^{2+}\)-binding protein (23). The crystal structure of CaM in the Ca\(^{2+}\)-bound form shows a dumbbell-shaped molecule with two globular domains arranged in a trans configuration. These domains are connected by a long extended central \(\alpha\)-helix, the middle portion of which is highly mobile and acts as a flexible tether. Each domain consists of two helix-loop-helix motifs (EF hands), with each binding one molecule of Ca\(^{2+}\). Ca\(^{2+}\) binding changes the orientation of the two EF hands of each domain, inducing the appearance of hydrophobic patches that interact with proteins known as CaM-binding proteins (CaMBPs). Binding of CaM to CaMBPs modulates the function of these proteins and, in consequence, affects many aspects of cell regulation. The carboxyl-terminal lobe binds Ca\(^{2+}\) with high affinity (\(K_d \approx 10^{-7} \text{ M}\)), whereas the amino-terminal lobe binds it with lower affinity (\(K_d \approx 10^{-6} \text{ M}\)). The fact that the \(K_d\) values fall within the range of intracellular Ca\(^{2+}\) concentration exhibited for most cells (10\(^{-7}\)–10\(^{-8} \text{ M}\)) makes it a good sensor for changes in Ca\(^{2+}\) intracellular levels (24–26).

The CaM binding domain of some of the CaMBPs with high affinity for CaM (nm range) consists of a 20-amino acid sequence that has an amphiphatic \(\alpha\)-helix conformation (27). CaM binding domains with lower affinity for CaM (\(\mu\)M range) have also been described (28). Recently, it was found that some proteins like MARCKS and CAP-23/NAP-22 use the myristoyl group to interact with CaM (29, 30). As well as K-Ras, diverse Ras superfamily GTPases like Kir/Gem (31), Ric (32), Rin (33), Rab3A (34), and RaIA (35) have been shown to bind to CaM. We analyzed here which elements of K-Ras are essential for its interaction with CaM to assess whether this interaction is important for anterograde or retrograde K-Ras traffic.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—The truncated forms of K-Ras (from amino acid 1 to 150 and from amino acid 1 to 166) were obtained by PCR and cloned into a pGEXKG plasmid with the restriction enzymes BamHI and XhoI to obtain the GST-K-Ras-(1–150), GST-K-Ras-(1–166), GST-K-Ras-(1–166)R161D, and GST-K-Ras-(1–166)R164D fusion proteins. The truncated K-Ras-(1–166) and H-Ras-(1–166) protein with amino-terminal hemagglutinin (HA) tag was obtained by single PCR and cloned into a pEFHA plasmid. All mutants (except Switch II) were obtained by single PCR but with reverse or forward oligonucleotides carrying the appropriate mutations. The forward oligonucleotide incorporated a BamHI site, and the reverse one incorporated a ClaI site to allow subcloning into the pEFHA and BamHI and XhoI, respectively, to subclone into pGEXKG. Switch II mutants were obtained by point mutation using QuikChange\textsuperscript{\textregistered} site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA), and we used as a double-stranded DNA template the construction K-Ras or K-RasV12 cloned into a pEFHA plasmid. This pEFHA-K-Ras plasmid was a gift from R. Marais (UK Cancer Research Centre, London, England), and the effector domain K-Ras mutants cloned in a pCEFLAU5 plasmid was a gift from J. M. Rojas (Instituto de Salud Carlos III, Madrid, Spain). K-Ras and K-RasV12 were cloned into pEYFP-C1 plasmid (Clontech) by BglII/Sall sites. CaM was cloned into pECFP-C1 and pEGFP-C1 plasmids (Clontech) by Smal site.

Protein Expression and Purification—GST fusion proteins were expressed in Escherichia coli BL21 carrying the pLysS plasmid. Purification and loading with the GTP\(\gamma\)S nucleotide were performed as indicated elsewhere (22).

Cell Culture and Transfections—NIH3T3 cells were grown in Dulbecco’s minimum essential medium supplemented with 10% donor calf serum. Immortalized striatal neurons (STHdh) (a gift of S. Ginés, University of Barcelona, Spain) were cultured in Dulbecco’s minimum essential medium with 10% fetal calf serum. Transient expression of the different K-Ras mutants was achieved by transfecting cells with the appropriate expression vector and using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

CaM-Sepharose Pulldown Assays—For pulldown assays with cellular lysates, cells (1 \(\times\) 10\(^6\)) were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed with 0.4 ml of pulldown buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM dithiothreitol) plus protease and phosphatase inhibitors (0.1 mM Na\(_2\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, 10 mM \(\beta\)-glycerophosphate, 2 \(\mu\)g/ml aprotinin, and 10 \(\mu\)g/ml leupeptin) for 30 min at 4 °C and clarified by centrifugation. Lysates (equalized for protein content) were incubated with 30 \(\mu\)l of CaM-Sepharose (Amersham Biosciences) for 2 h at 4 °C in the presence of 0.1 mM CaCl\(_2\) or 1 mM EGTA. The unbound fraction was collected by centrifugation, and the remaining bound fraction was washed four times with pull-down buffer with either CaCl\(_2\) or EGTA. An aliquot (25–50 \(\mu\)l)
of the unbound fraction and the input fraction and the entire bound fraction were analyzed by electrophoresis and Western blotting. A lysate from NIH3T3-transfected cells was always loaded in the same gel as a control for the transfection. For in vitro binding experiments with purified proteins, these were incubated for 1 h at room temperature with 20 μl of CaM-Sepharose (previously blocked for 30 min with 10% bovine serum albumin). In this case, pulldown buffer also contained 300 mM NaCl with either 1 mM CaCl2 or 5 mM EGTA. Unless otherwise indicated in the figure legend, total bound and unbound fractions and equivalent amounts of input were obtained as indicated above and analyzed by Western blotting.

In the competition assay between K-RasV12 and Ras15-(1–166) α-helix peptide, this peptide was diluted in pulldown buffer and preincubated with CaM-Sepharose for 30 min at 4 °C. After this preincubation, the beads were washed two times with pulldown buffer and incubated with a lysate from NIH3T3 cells previously transfected with K-RasV12 for 2 h at 4 °C in the presence of 0.1 mM CaCl2.

Ras Binding Domain (RBD) Pulldown Assay—To analyze the capacity of Ras mutants to be loaded with GTP in cell lysates, a RBD (of Raf-1) pulldown assay was performed. Cells (5–10 × 106) were lysed in the culture dish with CaM-Sepharose (previously blocked for 30 min with 10% bovine serum albumin). In this case, pulldown buffer also contained 300 mM NaCl with either 1 mM CaCl2 or 5 mM EGTA. Unless otherwise indicated in the figure legend, total bound and unbound fractions and equivalent amounts of input were obtained as indicated above and analyzed by Western blotting.

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the fluorescence intensity at any time, and \( F_0 \) was the base-line fluorescence intensity. Image treatment and movie assembly were performed using the Image Processing Leica Confocal Software.

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Colocalization was quantified using the Image J Software and the Highlighting Colocalization plugin (Pierre Bourdoncle, Institut Jacques Monod, Service Imagerie, Paris). This plugin highlights the colocalized points of two images. Two points were considered colocalized if their intensities were higher than the threshold of their channels and the ratio of their intensity was higher than 50%. The Highlighting Colocalization plugin generated an image of colocalized pixels. With the Image Calculator plugin the values of the colocalized points were converted to the real value of red (HA-K-Ras protein). Then membrane and cytoplasmatic ROIs were defined in each cell, and the integrity density of both the colocalized points with the real red value (HA-K-Ras colocalized with GFP-CaM) and the red image (total HA-K-Ras) was calculated in each ROI. The percentage of colocalization was obtained in each ROI by dividing the two intensities. At least 10 cells were analyzed for each condition. The mean and S.D. were determined.

The perinuclear region (PN)/plasma membrane (PM) ratio was calculated drawing different ROIs of PM and PN, PM regions where movement or shape changes were observed during the experiment were discarded. The average fluorescence intensity of a selected ROI was then logged onto an Excel spreadsheet, normalized, and plotted over time. Only images of cells that showed a medium overexpression of the proteins were analyzed.

Western Blot Analysis and Antibodies—Fractions of the pull down experiments were resolved in SDS-polyacrylamide gels and transferred to Immobilon-P membranes for 2 h at 60 V. The sheets were pre-incubated in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), 0.05% Tween 20, and 5% BSA for 1 h at room temperature and then incubated for 1 h at room temperature in TBS, 0.05% Tween 20, 1% BSA containing the appropriate antibodies: Pan-Ras (Oncogene Science OP40, 1:100 dilution), anti-HA (Roche Applied Science 1583816, clone 12CA5 1:1000 dilution), anti-AU5 (Berkeley Antibody Co., Inc., 1:1000). After washing in
K-Ras/CaM Interaction and Localization

Analysis of the CaM-interacting Domains of K-Ras—To better understand regulation of K-Ras activity by CaM, we analyzed the domains of K-Ras that are essential for the interaction with CaM. We previously showed that truncated GST-K-Ras-(1–166) (amino acid 1–166), expressed in bacteria and purified, was able to bind to CaM-Sepharose in a Ca\(^{2+}\)-dependent way, specifically when loaded with GTP (22). To analyze which domains in GST-K-Ras-(1–166) were responsible for the binding to CaM, interaction of a further truncated version of K-Ras (GST-K-Ras-(1–150)) completely lacking the last α-helix α5 (Fig. 1) was analyzed. Both truncated proteins were expressed in bacteria, purified, and GTP-loaded before analysis of their interaction with CaM. To confirm GTP loading, the ability of both truncated proteins to bind to RBD-Sepharose (Ras binding domain of Raf-Sepharose) was analyzed. As shown in Fig. 2A, both truncated forms were GTP-loaded since they bound equally to RBD-Sepharose. However, although GST-K-Ras-(1–166) interacted with CaM in the presence of Ca\(^{2+}\), GST-K-Ras-(1–150) did not. These results suggested the presence of a CaM-interacting region in the last α-helix of K-Ras, between amino acids 151 and 166. To further confirm that, surface plasmon resonance technology was used to determine the affinity of K-Ras helix α5 to CaM. Our data showed that K-Ras-(151–166) peptide had a clear affinity for CaM (K_D = 470 nM), whereas with the same analysis two unrelated peptides that had a similar proportion of hydrophobic and basic amino acids showed no significant binding to CaM. As a positive control CaM kinase II-(290–309) peptide (the CaMBD of CaMKII) was used and showed as already described a very high affinity for CaM (K_D = 20 nM).

Binding kinetics of purified GST-K-Ras constructs was also analyzed and are as shown in Fig. 2B. Although GST-K-Ras-(1–166) had an affinity of 630 nM, the deletion of the helix α5 notably increased the K_D, indicating a reduction of the affinity for CaM.

But the presence of another CaM-interacting region in the HVR has also been reported (20). To test whether in a cell context the HVR was essential for the interaction with CaM, we expressed both full-length HA-K-Ras and HA-K-Ras-(1–166) in NIH3T3 cells and performed pulldown assays with CaM-Sepharose directly with the cellular extracts. Extracts were performed with a buffer that allowed GTP loading in the lysate (22) and, as shown in Fig. 2C (upper panel), both forms of K-Ras were GTP-loaded. Although full-length HA-K-Ras interacted with CaM, deletion of the HVR completely abolished binding to CaM (Fig. 2C, lower panel). Thus, although the HVR was not required for the interaction of purified K-Ras to CaM, it was essential when the binding was performed from cellular extracts. Most probably, under these conditions the concentration of HA-KRas-(1–166) present in the cell lysate was below the K_D, and consequently, binding could not be observed in the absence of the HVR region that would have a lower affinity for CaM.

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RESULTS

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Two different pieces of data allowed as to prove that the 151–166 α-helix, although not by itself sufficient for binding when using cell extracts, also contributed in vivo to the inter-
action. First, binding of HA-K-RasV12 from NIH3T3 cellular extract to CaM-Sepharose decreased if CaM-Sepharose was previously incubated with growing concentrations of the 151–166 K-Ras peptide (Fig. 2D). Second, point mutation in the 151–166 α-helix reduced binding to CaM (Fig. 3). Because interaction with CaM is favored by basic amino acids, mutants were designed to substitute Arg-161, Arg-164, or Lys-165 to aspartic. All HA-K-Ras mutants were expressed in NIH3T3, and their GTP-loaded quality and binding to CaM were analyzed directly from cellular extracts. All mutants were equally loaded with GTP and in a growth factor-dependent way, indicating that they were not unfolded proteins (supplemental Fig. S1 and data not shown). As shown in Fig. 3, A and B, replacement of Lys-165 by an acidic amino acid did not alter binding to CaM, but mutation of Arg-164 to an aspartic reduced binding by 50% and mutation of Arg-161 to aspartic almost completely inhibited binding to CaM. The same results were obtained with oncogenic K-RasV12 (data not shown). Furthermore, we confirmed that binding of K-RasK165D to CaM was GTP-dependent as previously showed by K-Ras (data not shown). K-RasR161D was not located at the plasma membrane when analyzed by immunocytochemistry and, in addition, was not partitioned within the hydrophobic Triton X-114 phase, indicating that it was normally farnesylated (Fig. 3, C and D). Therefore, the lack of interaction with CaM could be due to the absence of the farnesyl group instead to the introduction of a negative charge in this α-helix. In contrast, K-RasR164D was located at the plasma membrane and partitioned as the wild type in the hydrophobic Triton X-114 phase, indicating that it was normally farnesylated (Fig. 3, C and D). In addition, binding of bacterially expressed, purified, and GTP-loaded GST-K-Ras(1–166)R164D to CaM-Sepharose was drastically reduced compared with GST-K-Ras(1–166), whereas the mutation R161D did not affect binding to CaM (Fig. 3E). These results indicate that, although the 151–166 α-helix of K-Ras was not sufficient for the binding of K-Ras to CaM in a cell extract context, some of the amino acids, such as Arg-164, participated in the interaction. Furthermore, our results indicate that Arg-161 is an essential amino acid for K-Ras farnesylation, and consequently, mutation of this amino acid indirectly affected binding of full-length K-Ras to CaM.

Farnesylation of K-Ras and the Polybasic Region Are Both Essential for the Interaction Between K-Ras and CaM—Because the HVR, as shown above, is required for the interaction of K-Ras with CaM in cell extracts, we wanted to analyze the involvement of the farnesyl group in this interaction. To this end, a non-farnesylatable Ras mutant (HA-K-RasC185A) lacking the cysteine that is farnesylated, was expressed (either WT or V12) in NIH3T3 cells. As expected, this mutant did not localize at the plasma membrane (Fig. 4B) and was not partitioned into the hydrophobic phase of Triton X-114.

**FIGURE 4.** Farnesylation of K-Ras is essential for its binding to CaM and its intracellular localization. The indicated HA-K-Ras mutants were expressed in NIH3T3 cells. A, CaM-Sepharose pulldown assays were performed using cellular lysates in the presence of either Ca²⁺ (Ca) or EGTA (E) as indicated under “Experimental Procedures.” B, immunofluorescence using anti-HA antibody to analyze the intracellular localization of the indicated HA-K-Ras mutants was performed.

**FIGURE 5.** Effect of deletion of lysines from the polybasic sequence on the interaction of K-Ras with CaM and on its intracellular localization. The indicated HA-K-Ras mutants were expressed in NIH3T3 cells. A, CaM-Sepharose pulldown assays were performed using cellular lysates in the presence of either Ca²⁺ (Ca) or EGTA (E) as indicated under “Experimental Procedures.” B, the amount of the different K-Ras mutants bound to CaM was quantified from three different experiments as in A and expressed as a % of the binding of K-RasV12 to CaM. C, Immunofluorescence using anti-HA antibody to analyze the intracellular localization of the indicated HA-K-Ras mutants was performed.
CaM binding to RBD-GST indicated that its ability to be GTP-loaded was not altered (data not shown). As shown in Fig. 6, A and B, pseudophosphorylation of Ser-181 completely inhibited binding of K-Ras to CaM. In contrast, replacement of Ser-181 by Ala, a non-phosphorylatable residue, did not alter the binding of K-Ras to CaM. Western blotting was performed this time using a pan-Ras antibody to simultaneously detect endogenous Ras binding to CaM. The lack of interaction of K-RasS181D with CaM was not due to lack of farnesylation because, as shown in Fig. 6C, HA-K-RasS181D was also partitioned in the Triton X-114 hydrophobic phase, like HA-K-Ras and HA-K-RasS181A. In addition, both HA-K-RasS181D and HA-K-RasS181A were equally located at the plasma membrane (Fig. 6D). The same results were obtained with the oncogenic K-RasV12 form (data not shown). These observations confirm the essential contribution of the HVR of K-Ras in the interaction with CaM and that this interaction can be modulated by K-Ras phosphorylation at Ser-181.

K-Ras Switch II Region Also Participates in the Interaction with CaM—We have shown that GTP loading of K-Ras was essential for its binding to CaM. This suggested that Switch I or II regions may also participate in this interaction. To test that, different K-Ras mutants were used. Any of the known K-Ras switch-I effector-domain mutants, G34, C35, G37, or C40 disrupted the binding of K-Ras to CaM (supplemental Fig. S2). The following Switch-II mutants of K-RasV12 were tested: Q61L (Ras mutant, in which GTPase activity cannot be stimulated by GTPase-activating protein) (38), A66D-M67D, and R68D-R73D. As shown in Fig. 7, although all mutants could load GTP in a growth factor-dependent way (Fig. 7C and supplemental Fig. S1) and are located at the plasma membrane (data not shown), substitution of Arg-68 and Arg-73 for acidic amino acids clearly reduced binding to CaM (80% reduction). The participation of the Switch II region in the interaction between K-Ras and CaM could explain its GTP dependence.

Calmodulin and K-Ras Colocalize at the Plasma Membrane in NIH3T3 Cells—We previously showed co-immunoprecipitation of CaM and K-Ras from NIH3T3 cell extracts, suggesting their interaction in vivo. To find where this interaction may occur in vivo, we analyzed here colocalization of these proteins by confocal microscopy. NIH3T3 cells were co-transfected with either HA-tagged wild type K-Ras (HA-K-RasWT) or V12 K-Ras (HA-K-RasV12) together with GFP-CaM expressing vectors. Cells expressing both GFP-CaM and HA-K-Ras were analyzed by immunofluorescence with an anti-HA antibody. CaM was ubiquitously distributed in the cells, including the plasma membrane. HA-K-Ras and HA-K-RasV12 were mainly located at the plasma membrane.
K-Ras/CaM Interaction and Localization

**A**

| HA-KRasV12 | Not Bound | Bound |
|------------|-----------|-------|
| CaM-Seph:  | Input     | Ca    | E    | Ca | E |
| Q61L       | -         |       |      |    |   |
| A66D-M67D  |           |       |      |    |   |
| R68D-R73D  |           |       |      |    |   |

**B**

![Graph showing binding to CaM relative to HA-KRasV12.](image)

**C**

![Graph showing RBD-Sephrase binding to HA-KRasV12.](image)

**FIGURE 7. Effect of the K-Ras Switch II region mutations on its binding to CaM.** HA-KRasV12 or harboring the indicated mutations were expressed in NIH3T3, and after lysis CaM-Sepharose (A) or RBD (C) pulldown assays were performed as indicated under "Experimental Procedures." In A CaM pulldown assays were performed in the presence of either Ca” (Ca) or EGTA (E). The amount of each HA-KRasV12 mutant bound to CaM was quantified from three different experiments as in A and expressed as a % of the binding of K-RasV12 to CaM.

expressed in the plasma membrane in NIH3T3 cycling cells and also in cells treated with the Ca” ionophore (Fig. 8A). As also shown in Fig. 8A, colocalization of GFP-CaM with K-RasWT or K-RasV12 was observed mainly at specific areas of the plasma membrane. Quantification of colocalization indicated that 34 ± 3 and 38 ± 5% of total plasma membrane HA-K-Ras and HA-K-RasV12, respectively, colocalized with GFP-CaM, whereas in the cytoplasm this value was very low (8 ± 2 and 12 ± 2%, respectively). Furthermore, upon 5 min of Ca” ionophore treatment (to ensure CaM activation), colocalization of GFP-CaM with K-RasV12 was still observed at the plasma membrane (51 ± 2%), and no significant colocalization increase was found in the cytoplasm (17 ± 3%). In vivo colocalization of GFP-CaM and YFP-K-Ras (WT or V12) expressed in NIH3T3 cells was also analyzed after Ca” ionophore treatment. As shown in Fig. 8B and in supplemental Figs. S2 and S3, colocalization was always observed at the plasma membrane. Fluorescence analysis was performed as a control of Ca” concentration increase in cytoplasm after ionophore treatment (data not shown).

K-Ras takes place primarily at the plasma membrane, and it does not directly lead to K-Ras internalization.

**K-Ras Is Transported to Golgi in Striatal Neurons Independently of Its Interaction with CaM and Its Phosphorylation at Ser-181**—In agreement with what has been described in hippocampal neurons, an increase of Ca” in striatal neurons induced translocation of YFP-K-Ras from the plasma membrane to Golgi as shown in fixed cells (Fig. 9A) and also in vivo (Fig. 9B and D, and supplemental Fig. S4). Fluorescence analysis was performed as a control of Ca” concentration increase after ionophore treatment (Fig. 9C). To test whether this translocation was dependent on the interaction between K-Ras and CaM, striatal neurons were transfected with a K-Ras mutant with impaired binding to CaM (YFP-KRasΔK) and its translocation to Golgi analyzed upon Ca”-ionophore treatment. In control conditions K-RasΔK was located at the plasma membrane, but interestingly, translocation to Golgi induced by Ca” ionophore was also observed in this mutant (Fig. 9A, B, and D, and supplemental Fig. S5). Consequently, interaction with CaM is not required to induce K-Ras translocation to Golgi.

To assess the participation of K-Ras phosphorylation at serine 181 on the retrograde transport of K-Ras to Golgi, cells were transfected with YFP-K-RasS181A. As shown in Fig. 9A, B, and D, and supplemental Fig. S6, Ca” ionophore addition induces translocation of this mutant to Golgi. This occurred even in the presence of the CaM inhibitor W13 (Fig. 9B and supplemental Fig. S7). Consequently, translocation to the Golgi induced by cytosolic Ca” increase can occur in the absence of CaM binding to K-Ras and independently of the phosphorylation of K-Ras at Ser-181.

**DISCUSSION**

We described elsewhere that K-Ras is a CaMBP (22). Different putative roles for this interaction were then put forward, especially in relation to a putative role in its retrograde transport to intracellular membranes (20). To distinguish between the possible roles of CaM/K-Ras interaction, we analyzed here which K-Ras domains are involved in this interaction by searching for K-Ras mutants that do not bind to CaM. Our results indicate that the HVR of K-Ras is essential for the interaction between K-Ras and CaM in cell extracts. Fivaz and Meyer (20) showed that the HVR fused to green fluorescent protein binds to CaM. We show here that this region is in fact essential for the interaction when full-length K-Ras is expressed in eukaryotic cells, and the interaction is assessed from cell extracts. We also show that farnesylation is among the essential elements of this region for the interaction with CaM, since a K-Ras mutant that cannot be farnesylated (K-RasC185A) is unable to bind to CaM. Moreover, we show that the polybasic lysine-rich region in the HVR is also essential for this interaction, since deletion of one or two lysines severely reduced or completely abolished binding to CaM, respectively. Interestingly, although the non-farnesylatable K-RasC185A mutant does not localize to the plasma membrane, both polybasic domain mutants localize properly to the membrane and are farnesylated. This observation indicates that alterations that render K-Ras unable to bind to CaM are not simply consequences of altering K-Ras post-translational processing and...
localization. Although binding of CaM to CaMBPs has mainly been shown to occur through peptides, lipid domains have been recently shown to interact with CaM. For instance, CaM binding to small GTPase Ral requires isoprenylated Ral (39) and CAP-23/NAP-22 (30), MARCKS (29), MARCKS-related protein (40), and the HIV1 Nef (41), which all use myristoyl groups to interact with CaM. Crystallization of a myristoylated CAP-23/NAP22 amino-terminal domain complexed with Ca2+/H11001/CaM showed that the myristoyl moiety of the peptide goes through a hydrophobic tunnel created by the hydrophobic pockets in the amino- and carboxyl-terminal domains of CaM. In addition, several amino acid residues in the peptide are important for CaM binding. Similar to what we showed for K-Ras, near the myristoyl group in CAP-23/NAP22, MARCKS, and HIV1 Nef, there are lysines that are important for the interaction with CaM. The requirement of both the farnesyl group and the polybasic domain for the interaction with CaM explains the isoform specificity of the interaction with K-Ras, since although H-Ras and K-Ras contain a farnesyl group, they do not have the lysines in the HVR and have palmitoyl groups that could further disturb the interaction. Ballester et al. (37) described that K-Ras could be phosphorylated by protein kinase C, pointing to Ser-181 as the most probable residue for this phosphorylation. Because this residue is very close to the polybasic region, it is not surprising that the introduction of a negative charge mimicking a phosphate group on Ser-181 completely inhibited binding to CaM. Apart from the repulsive electrostatic force generated by this phosphate, it would also introduce steric hindrance. Interestingly, this mutant is farnesylated and localizes to membranes, confirming again how disruption of CaM binding capacity is not solely the consequence of incorrect processing of K-Ras but a specific conformational effect.

The exact role of this phosphorylation is not clear, although it has been suggested that it induces internalization of K-Ras and apoptosis (21). We did not detect any significant increase in intracellular localization of pseudo-phosphorylated K-Ras (K-RasS181D) or massive death induction of the cells (data not shown). Independently of the function of this phosphorylation, our results suggest that some of these consequences might be the result of a lack of K-Ras interaction with CaM.

Our data also revealed the existence of another CaM binding domain in K-Ras. Interestingly, MARCKS has also been shown to have two CaM binding domains, the myristoyl group in the amino-terminal region of the protein and a peptide domain in the central part of the molecule (29). Deletion analysis using purified K-Ras indicated that in K-Ras this domain was between amino acids 151 and 166, which is an amphipathic α-helix. Although this peptide showed a noticeable affinity for CaM, the binding constant may be not low enough to allow binding of K-Ras-(1–166) from cellular lysates to CaM. But point mutation analysis of this region allowed us to definitively prove that the K-Ras helix 5 was important for the interaction of K-Ras in vivo. One piece of evidence is the fact that mutation of Arg-164 to aspartic generates a functional K-Ras protein in relation to the ability to bind GTP and to locate at the plasma membrane but with clear reduced binding to CaM. The fact that the Switch II region also participates in the interaction between K-Ras and CaM explains why the interaction occurs preferentially with GTP-loaded K-Ras and opens the possibility that CaM could modulate binding of K-Ras with diverse Ras regulatory proteins such as guanine nucleotide exchange factors or GTPase-activating proteins. The K-Ras mutants described in the present work with impaired binding to CaM...
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will allow us to prove if CaM interaction negatively modulates K-Ras signaling as previously suggested (22, 42).

We focused here in analyzing the role of K-Ras and CaM interaction on the intracellular localization of K-Ras. We show that CaM binding to K-Ras is not essential for its transport from the endoplasmic reticulum to the plasma membrane, since K-Ras mutants that do not bind CaM but are farnesylated (K-RasΔKK and K-RasS181D) are located at the plasma membrane. Ras signaling and oncogenesis depend on the dynamic interplay of Ras with distinct plasma membrane microdomains and various intracellular compartments, and the carboxyl-terminal domains play a critical role in the regulation of this Ras distribution and signal output (43–46). Research is focusing on the regulation of the intracellular localization of the different Ras family members. Work on hippocampal neurones indicated, in response to neuronal activity, reversible K-Ras translocation from plasma membrane to Golgi complex and early/recycling endosomes (20). Those authors also showed using W7, as an inhibitor of CaM activity, that this translocation was CaM-dependent and suggested that binding of CaM to the farnesyl group of K-Ras allows the release of K-Ras from the membrane and its intracellular translocation. Furthermore, it has been shown that CaM could dissociate K-Ras from membranes in vitro (20, 47). The fact that the farnesyl group is an essential element for the interaction between CaM and K-Ras favors the hypothesis that CaM modulates interaction of K-Ras with the membranes. But because the only K-Ras mutant described up to now that could not bind CaM was the non-farnesylated one, with an impaired location at the plasma membrane, a direct analysis of the relevance of CaM binding to K-Ras on the retrograde traffic of K-Ras could not be performed. Evidences presented in the present work using mutants that cannot bind CaM but that can interact with the cellular membranes prove that in striatal neurons internalization of K-Ras to Golgi induced by intracellular Ca\(^{2+}\) increase is not dependent on CaM/K-Ras interaction. This may not be a real discrepancy between our results and the ones previously published using hippocampal neurones. In this later case, glutamate was used to stimulate the N-methyl-D-aspartate receptor and consequently increase the intracellular Ca\(^{2+}\) concentration. Maybe the effect of W7 on the translocation of K-Ras could reflect the participation of CaM in another event downstream of N-methyl-D-aspartate receptor and not directly in the induced translocation of K-Ras. We could not prove this in our striatal neurones because, in contrast to the reported with hippocampal neurones, activation with either glutamate or N-methyl-D-aspartate, although inducing an increase in intracellular Ca\(^{2+}\), did not lead to a translocation of K-Ras to intracellular membranes (data not shown). Furthermore, NIH3T3 binding of CaM to K-Ras does not seem enough to release K-Ras from the membrane since strong colocalization at the plasma membrane and very low colocalization in intracellular compartments were observed at 10% fetal calf serum or even in the presence of a Ca\(^{2+}\) ionophore. The same occurs with the myristoylated MARCKS-related protein; although CaM interacts with the MARCKS-related protein (MRP) myristoyl group, it does not interfere with MRP binding to negatively charged vesicles (48). We believe that, although in NIH3T3 CaM interaction with K-Ras does not

FIGURE 9. Effect of CaM interaction on Ca\(^{2+}\)-induced localization of K-Ras to Golgi in striatal neurons. The indicated YFP-K-Ras mutants were expressed in striatal neurones. A, 48 h after transfection cells were treated (+) or not (−) with 2.5 \(\mu\)M ionomycin for 5 min. Cells were fixed and immunostained to detect the Golgi (red) or YFP-K-Ras (green) as indicated under “Experimental Procedures.” Representative cells of each mutant and treatment are shown. B, transfected cells were observed in vivo under the confocal microscope, and then 2.5 \(\mu\)M ionomycin was added. Images of YFP-K-Ras localization were immediately recorded as indicated under “Experimental Procedures,” and the ratio between PN (perinuclear) and PM (plasma membrane) fluorescence intensities were determined and plotted along the time. In the last panel a cell that was preincubated with W13 (15 \(\mu\)g/ml) is shown. Plots of representative cells are shown. C, mean of Fluo4 fluorescence intensity of 10 cells treated as in B. Images were acquired every 700 ms for 5 min. D, translocation index is the fold increase of PN/PM fluorescence intensities before Ca\(^{2+}\) rise (25 s after ionomycin addition) (−) and after Ca\(^{2+}\) rise (150 s after ionomycin addition) (+) of cells treated as in B relative to PN/PM intensity before ionomycin addition (mean and S.D. of 10 cells per mutant is shown).
seem to directly lead to its internalization, it may modify the membrane interactions of K-Ras and its clustering in the plasma membrane. The mechanisms underlying Ras compartmentalization involve a series of protein/lipid, lipid/lipid, and cytoskeleton interactions, resulting in the generation of discrete microdomains from which Ras operates. We propose that, as suggested for other prenyl-interacting proteins such as galectin-1 and galectin-3 (49–52), CaM is a key element in the generation of such K-Ras discrete microdomains. In this way CaM could be regulating K-Ras signaling out-put.

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