The Activation of RalGDS Can Be Achieved Independently of Its Ras Binding Domain

IMPLICATIONS OF AN ACTIVATION MECHANISM IN Ras EFFECtor SPECIFICITY AND SIGNAL DISTRIBUTION*S

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Small GTPases of the Ras family are major players of signal transduction in eukaryotic cells. They receive signals from a number of receptors and transmit them to a variety of effectors. The distribution of signals to different effector molecules allows for the generation of opposing effects like proliferation and differentiation. To understand the specificity of Ras signaling, we investigated the activation of RalGDS, one of the Ras effector proteins with guanine-nucleotide exchange factor activity for Ral. We determined the GTP level on RalA and showed that the highly conserved Ras binding domain (RBD) of RalGDS, which mediates association with Ras, is important but not sufficient to explain the stimulation of the exchange factor. Although a point mutation in the RBD of RalGDS, which abrogates binding to Ras, renders RalGDS independent to activated Ras, an artificially membrane-targeted version of RalGDS lacking its RBD could still be activated by Ras. The switch II region of Ras is involved in the activation, because the mutant Y64W in this region is impaired in the RalGDS activation. Furthermore, it is shown that Rap1, which was originally identified as a Ras antagonist, can block Ras-mediated RalGDS signaling only when RalGDS contains an intact RBD. In addition, kinetic studies of the complex formation between RalGDS-RBD and Ras suggest that the fast association between RalGDS and Ras, which is analogous to the Ras/Raf case, achieves signaling specificity. Conversely, the Ras-RalGDS complex has a short lifetime of 0.1 s and Rap1 forms a long-lived complex with RalGDS, possibly explaining its antagonistic effect on Ras.

Ras is known as a major regulator of cell growth, development, and the cell cycle. Ras binds tightly to GDP or GTP, and this conversion between both nucleotide states is strictly controlled and involves conformational changes in two regions of the protein. Therefore, Ras behaves like a molecular switch. The active GTP state of Ras signals to different protein cascades. In the past Raf, phosphatidylinositol 3-kinase (PI3K), and members of the RalGDS family were described as effectors of Ras, and a similar relationship is proposed for other proteins like AP6 or Rin (1, 2). Because Ras is tethered to the plasma membrane through its lipid modifications, the effectors are recruited to the plasma membrane.

Ras can initiate different effects like proliferation and differentiation. In many systems Raf signaling via extracellular regulated kinase is sufficient for cell transformation (3). Oncogenic properties are also demonstrated for PI3K and RalGDS, albeit weaker (4, 5), and suppression of these protein functions can partly block transformation by Ras (6, 7). In other primary cells the opposite effects, cell cycle arrest and cell differentiation, are induced by active Ras (8). In the neuroblastoma cell line PC12, the activation of the Raf and PI3K branch of Ras signaling transmits the nerve growth factor signal to finally induce neurite outgrowth (9). In contrast, the RalGDS branch blocks this differentiation and keeps the cell in a proliferative state (10).

Members of the RalGDS family have guanine-nucleotide exchange factor (GEF) activity for RalA and RalB (11). The members of this family, RalGDS, Rgl, and Rif, have a C-terminal Ras binding domain (RBD). Rgr, another member, was identified as part of a fusion protein, which confers tumor-forming activity on NIH3T3 cells (12).

The small Ras-related GTPase Rap1 was originally identified as a Ras antagonist (13), and this finding was confirmed in other studies (14–19). Recent findings show that direct functions are the control of development and cell morphology (20). The interaction between Rap1 and RalGDS-RBD is the tightest interaction seen for a Ras family member and an effector RBD (21). In reconstituted lipid vesicle systems a stimulation of the RalGDS effector pathway could be achieved and was interpreted as the Rap1-induced co-localization of RalA and RalGDS on the artificial lipid membranes (22). In experiments with COS7 cells it was not possible to demonstrate any stimulatory effect of Rap1 on RalGDS (7). This was interpreted with different localizations of Rap1 and RalA (23). However, other studies have clearly shown that Rap1 is found at the plasma membrane and membranes of specialized vesicles (24–27). Furthermore, RalA is not solely localized at the plasma membrane but is also found in intracellular vesicles (28). Therefore, cellular localizations of Rap1 and Ral do not exclude a functional cascade of Rap1, RalGDS, and Ral. Another explanation for the inability of Rap1 to activate RalGDS could be seen in an acti-
viation mechanism as described for Raf kinase (29) and PI3K (30). In such a scenario, RalGDS could be recruited to the plasma membrane by Ras and additionally activated; Rap1 would only be able to bind RalGDS. To test this hypothesis we have analyzed different constructs of RalGDS and demonstrated an RBD-independent activation mechanism, which involves the switch II region of Ras. Kinetic studies support the notion that the Ras switch has evolved to control the association speed of effector complex formation (31). The latter can be reconciled with the finding that the lower affinity interaction between Ras and RalGDS is sufficient for signal transmission. A comparison with other RBDs prompted us to conclude that the high affinity between Rap1 and RalGDS could be important for the inhibition of this signaling branch of Ras.

MATERIALS AND METHODS

**Plasmids**—pBSK:mRalGDS, containing the RalGDS cDNA from mouse, was kindly provided by Dr. R. A. Weinberg. The whole cDNA, including the 5′-untranslated region was sub-cloned with EcoRI/AatII after removal of the 5′-overhang from the AatII restriction site into EcoRI/BamHI-digested pCDNA3 to yield pCDNA3:mRalGDS. The K835A variant of RalGDS was generated according to the PCR mutation protocol of Picard et al. (32).

pCDNA3:RalGDS, which contained a Myc-tag, was obtained with two PCR templates between the primers 5′-GGCGAATTCGCGGCCGCG/5′-CCGGGATCTTATGCTTCGCGGGATCCTGCTTGTCGACCCTCCTGAGCCCGG and 5′-CCGGAAATTCGCGGAATTCGAGATTTGACCGCCAGGCTGCA and 5′-CGGGGTACCTCTAGAGGAGGC. Fragments were amplified from pBSK:mRalGDS and sub-cloned via EcoRI/KpnI digestion into pBSK. The first PCR template was ligated in front of the second with EcoRI/EcoO109 yielding an N-terminal construct of RalGDS, which contains the whole 5′-untranslated region, and a c-Myc-tag at the N terminus. The two original Met start codons of RalGDS are deleted in this construct. This fragment was sub-cloned with EcoRI/XhoI digestion into pCDNA3:RalGDS using only the N-terminal XhoI restriction site in pCDNA3:RalGDS.

To generate pCDNA3:RalGDS2ARBD the XhoI restriction site in pBSKSmRalGDS was removed with a XhoI/SalI digestion followed by religation (= pBSKSmRalGDSΔXhol). The RB was removed from this construct with PstI/AatII digestion and an oligonucleotide linker comprising 5′-GCTACAGGTTAATCTACTGAGAGTTGACCGCCAGGCTGCA and 5′-GCTCGGTTAATCTACTGAGAGTTGACCGCCAGGCTGCA was ligated into this plasmid (= pBSKSmRalGDS2ARBD). Sub-cloning of a fragment from pBSK:RalGDS2ARBD into pCDNA3:RalGDS with PstI/XhoI digestion resulted in pCDNA3:RalGDS2ARBD.

RalGDS-RBD was introduced into pCDNA3:RalGDS2ARBD with a PCR fragment amplified from pBSK:RalGDS-RBD between the primers 5′-GGCGAATTCGCGGCCGCG/5′-CCGGGATCTTATGCTTCGCGGGATCCTGCTTGTCGACCCTCCTGAGCCCGG and 5′-GCTCGGTTAATCTACTGAGAGTTGACCGCCAGGCTGCA. The PCR fragment was then ligated with PstI/XhoI into pBSKSmRalGDS2ARBD. The resulting pBSKSmRalGDS* does not contain the last 30 amino acids of RalGDS. Sub-cloning a pBSK:Al/XhoI fragment from pBSKSmRalGDS* into pCDNA3:Myc-mRalGDS led to pCDNA3:RalGDS*.

The C terminus from Ki-Ras, including the CAAAX box, was attached to the RalGDS sequences via a PCR fragment amplified from ptac-K-ras (provided by Dr. A. Wittinghofer) between the primers 5′-CCGGGATCTCAGGTAACAGATGGTA and 5′-CCGGGATCTCAGGTAACAGATGGTA. The amplified sequence was ligated to pBSKSmRalGDS* and pBSKSmRalGDS2ARBD between the restriction sites HpaI and XhoI. The CAAAX-modified RalGDS sequences were sub-cloned with PstI/XhoI into pCDNA3:RalGDS to yield pCDNA3:RalGDS*CAAX and pCDNA3:RalGDS2ARBD-CAAX, respectively.

pMT2:Rala was kindly provided by Dr. G. J. T. Zwartkruis, pSVK3: Ras G12V and the double mutants G12V/Y64W and G12V/E37G in the same expression vector were provided by Dr. C. Block. pCDNA3:Rap1A G12V was obtained from Dr. A. Wittinghofer. All constructs were verified by sequence analysis.

**Cell Culture and Transfection Assay**—COS7 cells were grown in DMEM (without pyruvate, with pyridoxin and Glutamin-L, Invitrogen) and 10% FCS (Pan-Biotech). The cells were transfected with activated dendraimers (SuperFect, Qiagen) in 6-cm dishes. 5–6 μg of DNA was diluted with 125 μl of DMEM, and 20 μl of SuperFect reagent was added. The cells were incubated with this transfection solution for 4 h and then incubated in medium with 10% FCS for 24 h. The Rala loading assay was carried out as described previously (5). Briefly, 24 h after transfection the COS7 cells were grown overnight in DMEM with 1.5% FCS. Then the cells were washed with DMEM without serum and incubated in DMEM (without phosphate) containing 0.3 μCi of [32P]orthophosphate for 5 h. The cells were then lysed and the hemaglutinin-tagged Rala immunoprecipitated with the 12CA5 antibody (Roche Molecular Biochemicals) coupled to protein G-Sepharose (Amersham Biosciences, Inc.). The Sepharose resin with the 12CA5 antibody and the precipitated Rala was extensively washed, and the remaining GTP/GDP was eluted. Both guanine nucleotides were separated by TLC with polyethyleneimine-cellulose (Merck), and their amounts were quantified by phosophoimaging analysis (Bio-Rad).

**Immunofluorescence and Western Blots**—COS7 cells were directly cultured on coverslips and transfected with SuperFect as described above. 1 μg of DNA was used for transfection. After transfection and expression of the constructs, the cells were fixed and incubated with antibodies as described previously (34). For the recognition of c-Myc-tagged RalGDS constructs, the monoclonal 9E10 antibody was employed (Roche Molecular Biochemicals), and for detection of the primary antibody Alexa 594 goat c-mouse (Molecular Probes) was used. Fluorescence was observed with a Zeiss confocal microscope at 1-μm resolution and ×100 magnification.

Western blotting was performed according to standard procedures with a secondary antibody conjugated to horseradish peroxidase. For the recognition of RalGDS a-Myc (Santa Cruz Biotechnologies) a-RalGDS antibody (Amersham Biosciences, Inc.) was used. The band was visualized with ECL Chemiluminescence (Amersham Biosciences, Inc.). Expression levels of RalGDS in the Ral loading assay were determined in separate transfections.

**Proteins**—RalGDS-RBD (formerly termed RGF97) and Rap1A were prepared as described by Herrmann et al. (21), and the preparation of Ras is described by Tucker et al. (33). Loading of the Ras proteins with GppNHp (Sigma Chemical Co.) and mantGppNHp, respectively, was performed in a buffer containing 200 mM ammonium sulfate and 2 units of alkaline phosphatase (Roche Molecular Biochemicals) per milligram of protein. mantGppNHp was synthesized according to John et al. (34). In the case of Ras a 2-fold excess of the non-hydrolyzable nucleotide was added, and in the case of Rap1A a 5-fold excess and 10 mM EDTA were added. Mant solutions were dialyzed at 20 °C for 3 days. Protein concentrations were measured using the dye assay as described by Bradford (35). Bovine serum albumin was used for calibration.

**In Vitro Assays**—All experiments were carried out in a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, and 1 mM dithioerythritol. Fluorescence spectra were recorded on a spectrofluorometer (Fluoromax, Spex) at 25 °C with an experimental error smaller than 5% when the spectra were reproduced. For stopped-flow measurements, an SM17 apparatus (Applied Photophysics) was used. Rate constants can reliably be measured up to 500 s−1, because the dead time for the mixing of two solutions is in the range of 1–2 ms. Mant nucleotides were excited at 360 nm, and the fluorescence was recorded with a 480-nm cut-off filter. Ras or Rap1A bound to mantGppNHp was mixed with RalGDS-RBD in more than 5-fold molar excess to have pseudo-first-order conditions. Binding of effectors to Ras proteins is detectable by a change of the fluorescence of the mant nucleotide as described earlier (31, 36).

**RESULTS**

Ras G12V-mediated RalGDS Activation Can Be Impaired by a Single Point Mutation within the RBD—Previous experiments have shown the activation of proteins of the RalGDS family by Ras (11). From the structure of the complex between Ras and RalGDS-RBD, it became evident that K835 (originally termed K48 in the RBD of RalGDS (37)) is one of three major amino acids involved in the complex formation between RalGDS-RBD and Ras proteins (38, 39). The K835A mutation reduced the binding affinities by a factor of 2000 and 7000 for Ras and Rap1A, respectively (40). This mutation was tested in the Ral loading assay (Fig. 1). We chose to analyze the ratio of GTP over total guanine nucleotide bound to Rala, because this does not depend on changes in the expression levels of Rala as compared with methods, which detect only the GTP form. RalGDS and its K835A mutant were transiently expressed in COS7 cells together with Rala and a constitutive active version of Ras (Ras G12V). The activity of RalGDS was measured...
indirectly via the GTP load on immunoprecipitated RalA. In accordance with the results from Urano et al. (7), it is shown that, upon co-expression of RalGDS and RalA in COS7 cells, the GTP content on RalA is significantly increased over the basal GTP content when RalA is expressed either alone, or together with Ras G12V in COS7 cells. However, the 23% GTP bound to RalA in the presence of RalGDS was increased to 37% with Ras G12V. In contrast, the K835A mutant, which was expressed at a slightly higher level than the wild type, was hardly stimulated by Ras G12V. These results underscore the important contribution of the RBD for the stimulation of RalGDS by Ras.

**RalGDS Can Be Activated by Ras Independently of Its RBD**—In agreement with recent reports (5, 7) the experiments described above showed that members of the RalGDS family are activated by Ras. Rap was not able to activate RalGDS, although the affinity between both proteins is two orders of magnitude higher than that of RalGDS and Ras (21). To understand the difference between Ras and Rap regarding the activation of RalGDS, we tested whether additional activation steps are involved during the interaction between Ras and RalGDS. To discriminate between recruitment and activation of RalGDS by Ras, different constructs of the exchange factor were used (Fig. 2A). The C-terminal 30 amino acids, which do not belong to the C-terminal RBD fold, were deleted (RalGDS*), and the Ki-Ras CAAX box was attached (RalGDS*CAAX). In addition, the RBD was deleted from RalGDS (RalGDSΔRBD), and again the Ki-Ras CAAX box was tethered to the newly formed C terminus (RalGDSΔRBD-CAAX).

The localization of the different RalGDS constructs was investigated via immunofluorescence with confocal microscopy and compared with the wild type (Fig. 2B). For RalGDS a typical cytosolic stain could be observed in the confocal plane (image 1). A similar staining pattern was seen for the Myc-RalGDS* and -ΔRBD constructs, indicating that the truncated constructs localize to the same cellular compartments as RalGDS (images 2 and 4). In contrast, when the CAAX-box modified versions of RalGDS were analyzed, a rim stain was observed that demonstrates the artificial recruitment to the plasma membrane (images 3 and 5).

Next, the modified RalGDS constructs were transiently expressed in COS7 cells, and their activity was probed by the determination of the GTP content of co-transfected RalA. In
addition, the capability of Ras G12V to stimulate the nucleotide exchange rate of RapA via the RapGDS variants was investigated. In Fig. 3 it is seen that the RapGDS* construct, which lacks the C-terminal amino acids, behaves similarly to RapGDS wild type in Fig. 1, i.e., the GTP content of RapA when co-transfected with WT* can be stimulated from 25% to 35% in the presence of Ras G12V. Western blot analysis demonstrated comparable expression levels of RapGDS probed with the α-Myc antibody. B, the switch II region of Ras is involved in the activation of RapGDS. As under A, pMT2:RapA was co-transfected with RapGDSΔRBD-CAAX as indicated. In addition, Ras G12V and Ras G12V, Y64W mutants were co-expressed and the GTP content of RapA was quantified after immunoprecipitation as described in Fig. 1. The experiments were repeated three times, and the mean and standard deviation of the GTP content on RapA are given. The Western blot (WB) shows the expression levels of RapGDS probed with the α-Myc antibody.

**Fig. 3. Activation of RapGDS variants by Ras.** A, RBD-independent activation of RapGDS. 1 μg of pMT2:RapA was co-transfected with the indicated RapGDS and Ras expression vectors (1 μg each). The GTP content of RapA was quantified after immunoprecipitation as described in Fig. 1. The experiments were repeated three times, and the mean and standard deviation of the GTP content of RapA are given. The Western blot (WB) shows the expression levels of RapGDS probed with the α-Myc antibody. B, the switch II region of Ras is involved in the activation of RapGDS. As under A, pMT2:RapA was co-transfected with RapGDSΔRBD-CAAX as indicated. In addition, Ras G12V and Ras G12V, Y64W mutants were co-expressed and the GTP content of RapA was quantified. The data show a representative experiment from three repetitions.

**Influence of Rap1 on the Activation of RapGDS**—Rap1 had been described as an antagonist of Ras (13–19). Therefore, we tested whether it can block the RapGDS-mediated activation of RapGDS. When constitutively active Rap1A G12V was transiently transfected together with RapGDS-WT* or RapGDSΔRBD-CAAX, it is seen that the RBD is required for a decrease of the exchange activity (Fig. 4). The block of signal transmission was seen in the non-stimulated and Ras G12V-stimulated case. Western blot analysis of the RapGDS expression levels showed comparable levels in all assays. The presence of the RBD is crucial for an Rap1-mediated effect on Ras signaling via RapGDS.

**Dynamics of the Interaction between RBDs and Ras Proteins**—Depending on its GTPase binding partner, RapGDS is either activated or inhibited. Rap seems to sequester RapGDS into an inactive complex. To further understand the result of a parallel activation of Ras and Rap, we investigated the specificity of the RapGDS-RBD with stopped-flow experiments. To follow the binding between the GTPases and RapGDS-RBD, we employed a fluorescent nucleotide (mantGppNHp) as described previously (31, 36). Upon binding of RapGDS-RBD to Ras or to Rap complexed with mantGppNHp, a decrease in fluorescence was observed (Fig. 5, upper panel). The RapGDS-RBD concentration was in more than 5-fold molar excess over the GTPases; therefore, the fluorescence traces could be fitted according to pseudo-first order kinetics by a single-exponential equation yielding the observed rate constant $k_{\text{obs}}$. The bioluminescence association constant $k_{\text{on}}$ was obtained from the slope of the linear regression of $k_{\text{obs}}$ plotted versus RapGDS-RBD concentration (Table I). The dissociation rate constant $k_{\text{off}}$ corresponds to the intercept value. $k_{\text{off}}$ is 10 s$^{-1}$ for the dissociation of the Ras-RapGDS-RBD complex and close to zero for the Rap-RapGDS system. To ensure that determination of $k_{\text{on}}$ is not obscured by saturation at higher RapGDS-RBD concentrations, only concentrations up to 15 μM RBD were included in the calculations above. Similar to other effector RBDs (31, 36), saturation of the binding kinetics was observed at higher concentrations (Fig. 5, lower panel), and these data can be described with the hyperbolic equation 1 yielding $k_{\text{on}}$ and $K_{1}$. $k_{\text{off}}$ corre-
was fitted with an exponential curve to determine $k_{\text{off}}$ above, the dissociation equilibrium constant for the first step is $K_D = 61 \mu M$. The overall rate is limited to 300 s$^{-1}$ by the rate constant $k_{\text{on}}$ for the second step, which presumably corresponds to a conformational rearrangement. These observations reflect the structural similarity of effector-RBD/Ras complexes. The experimental error for the $k_{\text{on}}$ value is estimated to 10%, whereas for $K_D$ the error is 20%, because errors, both in the fit and in the determination of the concentration, must be considered. For the Rap1-RalGDS-RBD system, no saturation of $k_{\text{on}}$ was observed up to rates of 500 s$^{-1}$ at 100 M RaGDS-RBD, suggesting a much weaker initial complex.

Due to its small value, the dissociation rate constant of RBD-GTPase complexes cannot be determined precisely from the intercept as described above. Therefore we employed displacement experiments to obtain this value more reliably. The equilibrated complex Ras-mantGppNHp-RaGDS-RBD was rapidly mixed with a large excess (>100-fold) of Ras bound to non-labeled GppNHp to sequester RaGDS-RBD. Under these conditions the change of the fluorescence intensity is governed by the dissociation of RalGDS-RBD from Ras-mantGppNHp, and $k_{\text{off}}$ can be derived thereof with a 10% error. The corresponding dissociation rate constant for the Rap1 complex was determined as well.

Table I summarizes the results of the interactions between RaGDS-RBD and Ras or Rap1 at 25 °C and compares them with AF6- and Raf-RBD. Despite the large differences in the $K_D$ values, which vary by a factor of 400, it can be seen that the association rate constants lie close together (within a 5-fold variation). The differential affinities of the complexes are achieved mainly by the dissociation rate constants, which vary by a factor of 600. These results show that the different effector-GTPase complexes form at similar rates and that the varying affinities are due to largely different dissociation rates leading to different lifetimes (defined as inverse dissociation rate constant) of the complexes. The lifetime of the low affinity Ras-RalGDS-RBD complex is about 0.1 s. In contrast, the Rap1-RalGDS-RBD complex is more stable with a lifetime of 10 s.

**DISCUSSION**

We have characterized a series of mutants to elucidate the reason for the specificity of the activation of RaGDS by Ras. A single point mutation within the RBD of RaGDS, which weakens the interaction to Ras more than 25-fold, almost completely disrupted the stimulation of RaGDS by Ras. On the contrary, a RaGDS protein, which was artificially targeted to the plasma membrane and lacked the RBD, could be activated by Ras, and the switch II region of Ras was involved in the activation of the effector. When Rap1A G12V is co-transfected with RaGDS, only the constructs containing the RBD are inhibited in their ability to activate RaA by Rap. Stopped-flow measurements demonstrate that the formation of the complex between effector RBDs and the GTP-form of Ras or Rap1 occurs in a comparable time frame, although large variations in the affinity exist. This binding specificity is achieved via largely differing dissociation rates. Active Rap1 can hold RaGDS-RBD especially long in the complexed state.

The observations, that Ras but not Rap1 is able to stimulate Ras-mediated activation. The major effect of this interaction is thought to be the recruitment of the effector to the plasma membrane. However, the observed stimulation of the membrane-targeted RaGDS-RBD-C4AAX construct by Ras shows that recruitment of RaGDS to the plasma membrane is not the

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**Table I**

| GTPase | RBD       | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_D$ |
|--------|-----------|-----------------|------------------|-------|
| Ras    | RaGDS     | 8.8             | 11               | 1.3   |
| Raf1   | AF6       | 45              | 7.4              | 0.16  |
| Rap1   | RaGDS     | 17              | 0.12             | 0.0071|
| Rap1   | AF6       | 35              | 7.0              | 0.20  |

As with other effectors like AF6- and Raf-RBD (31, 36), this behavior may be interpreted as a two-step complex formation. For the interaction of Ras-mantGppNHp and RaGDS-RBD, the dissociation equilibrium constant for the first step is $K_D = 61 \mu M$. The overall rate is limited to 300 s$^{-1}$ by the rate constant $k_{\text{on}}$ for the second step, which presumably corresponds to a conformational rearrangement. These observations reflect the structural similarity of effector-RBD/Ras complexes. The experimental error for the $k_{\text{on}}$ value is estimated to 10%, whereas for $K_D$ the error is 20%, because errors, both in the fit and in the determination of the concentration, must be considered. For the Rap1-RalGDS-RBD system, no saturation of $k_{\text{on}}$ was observed up to rates of 500 s$^{-1}$ at 100 M RaGDS-RBD, suggesting a much weaker initial complex.

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The observations, that Ras but not Rap1 is able to stimulate Ras-mediated activation. The major effect of this interaction is thought to be the recruitment of the effector to the plasma membrane. However, the observed stimulation of the membrane-targeted RaGDS-RBD-C4AAX construct by Ras shows that recruitment of RaGDS to the plasma membrane is not the
only mechanism of RalGDS activation. We were able to demonstrate this RBD-independent activation of RalGDS by Ras, because the recruitment of RalGDS by Ras via the RBD was separated from the subsequent activation. In contrast, RalGDS*-CAAX showed an elevated basal activity and could only be stimulated to a small degree. This effect might be explained by the fact that the GDP form of the Ras proteins still has some affinity for the effectors (40). The artificial membrane recruitment of RalGDS*-CAAX most likely facilitates the association between endogenous Ras in its GDP form and RalGDS*-CAAX, thereby explaining the high basal activity and the apparent unresponsiveness to Ras G12V. The nature of the additional function of Ras in this activation process involves the switch II region of Ras. It is conceivable that there are low affinity contacts between the switch II region of Ras and RalGDS, which enhance the exchange activity of the GEF domain. Such contacts were proposed in a complex between RalGDS RBD and Ras (38) but could not be confirmed by us (39). However, this activation scenario would parallel the findings for Raf-kinase and PI3K, which also became activated when bound to Ras (30, 41, 42). Alternatively, there could be an unidentified factor, which is controlled by Ras and acts positively on RalGDS.

The stimulatory effect observed in addition to membrane recruitment could rely on relieving inhibitory signals (37). Support for inhibitory signals acting on the GEF moiety of RalGDS comes from Rsc (12, 43). It has been demonstrated that transforming properties of this fusion protein are due to the Rgr region. Rgr lacks both N and C termini as observed in the other RalGDS family members and, therefore, may have lost an autoinhibitory signal.

The comparison of stopped-flow measurements of the complex formation between RBDs from RalGDS, Raf, and AF6 and the GTPases Ras and Rap1 revealed that the binding specificity is mainly achieved by variation of the dissociation rates. Conversely, for the Ras/Raf-RBD system it has recently been demonstrated that effector recognition by the GTP and GDP forms, respectively, is differentiated by the association process, i.e., the GTPase switch is designed to prevent an association between the GDP form of Ras and an effector (31). Taken together, recognition by the effector of GTPase switch position should be interpreted in terms of association rate constants in which Raf, the paradigm of a Ras effector, differs little from RalGDS. The lifetime of the complex between RalGDS-RBD and Rap1 is about 0.1 s. This should be long enough to enable other processes like small conformational changes and phosphorylation events. It is interesting that RalGDS is indeed phosphorylated (44). So far, no correlation between the activity of RalGDS and its phosphorylation state could be demonstrated.

Rap1 is described originally as a Ras antagonist (13–19). The observed slow dissociation rate between Rap1 and RalGDS-RBD could explain the Ras-antagonistic effect on a molecular level. Currently, no other Ras effector is described that has such a high affinity and, therefore, a slow dissociation rate constant as RalGDS-RBD and Rap1. We therefore speculate that inhibitory effects of Rap1 on Ras might be due mainly to a competition between these proteins for RalGDS. This hypothesis is corroborated by the necessity of the presence of RBD in RalGDS for Rap1 inhibition as observed in this study. In PC12 cells Ras signaling via Raf and PI3K mediates cell cycle arrest and differentiation of the cells into a neural cell type, but signaling via RalGDS leads to proliferation (10). These two opposing effects of Ras effector signaling require additional controls of the effectors. Sequestration of RalGDS by Rap1 would be one mechanism to control the flow of signals coming from Ras and supports observed Rap1 functions in differentiation (45–49). It should be noted that in NIH3T3 and Rat1 fibroblasts no inhibitory effect of Rap1 on RalA was observed (50). It is not clear if these differences are due to the sensitivity of the different assay systems or reflect special features of this kind of fibroblasts. Our data attempt to explain the influence of Rap1 on Ras transformation on a molecular level. However, they do not exclude RalGDS-independent functions of Rap, which might be transmitted by other effector proteins (20).

RalGDS belongs to the group of exchange factors with a Cdc25 homology domain. It has been demonstrated that another member of this family, Sos, is inhibited in its exchange activity via the C and N termini (51). This and our findings could indicate a general mechanism of GEFs homologous to Cdc25. In addition, findings with GEFs of other families of the small GTPases like the Dbl-homology domain containing Rho GEFs (52) and the Sec7-homology domain containing Arf GEFs (53), where PH domains are thought to regulate the exchange domain, give rise to a general picture in which GEF activity is down-modulated in the non-stimulated state. The physiological relevance of an actively inhibited GEF might be a decreased spontaneous activation of Ras.

The data presented here show the importance of the RBD in RalGDS for activation by Ras as well as for the inhibition by Rap1. In addition to RBD-mediated membrane recruitment by Ras, another component of activation, involving the switch II region of Ras, is indicated. Our kinetic data suggest a role of interaction dynamics for specific signal transduction. To further understand signal transmission by RalGDS, it will be necessary to determine the nature of the observed RBD-independent activation. Employing RalGDS variants, which are deficient of either Ras binding or activation, should also shed light on the benefits of an activation mechanism of RalGDS for signaling of Ras proteins under different physiological conditions.

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The Activation of RalGDS Can Be Achieved Independently of Its Ras Binding Domain: IMPLICATIONS OF AN ACTIVATION MECHANISM IN Ras EFFECTOR SPECIFICITY AND SIGNAL DISTRIBUTION

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