Calmodulin Binds RalA and RalB and Is Required for the Thrombin-induced Activation of Ral in Human Platelets*

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Ral GTPases may be involved in calcium/calmodulin-mediated intracellular signaling pathways. RalA and RalB are activated by calcium, and RalA binds calmodulin in vitro. It was examined whether RalA can bind calmodulin in vivo, whether RalB can bind calmodulin, and whether calmodulin is functionally involved in Ral activation. Yeast two-hybrid analyses demonstrated both Rals interact directly but differentially with calmodulin. Coimmunoprecipitation experiments determined that calmodulin is involved in the regulation of the activation of RalA and RalB underlies possible functional differences between the two proteins and that calmodulin binding domain. Functionally, in vitro pull-down experiments determined that calmodulin binding domain to that previously described, that although RalB binds calmodulin, its C-terminal region is involved in partially inhibiting this interaction, and that in vitro RalA and RalB have an N-terminal calcium-independent and a C-terminal calcium-dependent calmodulin binding domain. Functionally, in vitro Ral-GTP pull-down experiments determined that calmodulin is required for the thrombin-induced activation of Ral in human platelets. We propose that differential binding of calmodulin by RalA and RalB underlies possible functional differences between the two proteins and that calmodulin is involved in the regulation of the activation of Ral-GTPases.

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¶ To whom correspondence should be addressed: 744 Bannatyne Ave., Winnipeg, Manitoba R3E 0W2, Canada. Tel.: 204-789-3703; Fax: 204-789-3913; E-mail: bhullar@ms.umanitoba.ca.

† The abbreviations used are: GEF, guanine nucleotide exchange factors; PLD, phospholipase D; RalBP1, Ral-binding protein 1; RIP1, Ral interacting protein 1; EH, epsin homology; CaM, calmodulin; BD, binding domain; AEBSP, 4-[2-aminoethyl]-benzenesulfonyl fluoride; PVDF, polyvinylidene difluoride; AD, activation domain; GST, glutathione S-transferase; RRBD, RIP1 Ral binding domain; Y2H, yeast two-hybrid; X-α-Gal, 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside; and RGL3 are important downstream effector proteins in Ras signaling pathways (6–16). Activation of Ral appears to be required for Ras-induced oncogenic growth and morphologic transformation (13, 17, 18) and induction of DNA synthesis (19). Phospholipase D (PLD) via its multiple lipid second messengers may be one agent that allows Ral to enhance both Ras- and Ral-induced cellular transformation (13, 20, 21). RalA is involved in the tyrosine kinase- and Src-mediated activation of PLD, perhaps through a direct, constitutive association between the N terminus of Ral and PLD1 (13, 20, 22–24). In quiescent rodent fibroblasts, Ral was shown to be sufficient to induce activation of NF-κB-dependent gene expression and cyclin D1 transcription (25).

Pathways from Ras to Ral through Ral-GEFs may be selectively regulated by other Ras-like GTPases such as Rap1 (26, 27) and TC21 (28). In platelets, Ral and Rap1 were similarly stimulated by platelet agonists α-thrombin and platelet-activating factor (29), and both were rapidly activated by elevated levels of calcium (Ca2+), which was found to be necessary and sufficient (29, 30).

The Ras-RalGEF-Ral pathway may also be involved in the regulation of cell migration through PLD and the Ral effectors, Ral-binding protein 1 (RalBP1) (12, 31), Ral-interacting protein 1 (RIP1) from mouse (32), and 76-kDa Ral-interacting protein from human (RLIP76) (33). The Ral-binding proteins have GTPase-activating protein activity acting upon CDC42 and Rac1 (4). Therefore, Ral may be involved in the negative regulation of CDC42 and Rac1 (31–33) and modulate actin cytoskeletal dynamics by controlling the activities of RalBP1 and PLD (34). Also of relevance to cell migration, Ral may interact directly with filamin-α, an actin cross-linking protein (35–37). Ral may recruit filamin into the filapodial cytoskeleton (38).

Ral proteins are also intimately involved in endocytosis and exocytosis. RalBP1 regulates endocytosis of epidermal growth factor and insulin receptors (39) by binding to two highly related epsin homology (EH) domain proteins, RalBP1-associated EH domain protein and POB1 (partner of RALBP1) (40, 41). It has been suggested (38) that a Ral-RalBP1-POB1 complex transmits signals from receptors to epsin and Eps15, which bind directly to the EH domain of POB1 (39, 42) and to the plasma membrane and clathrin adaptor protein complex-2 (43, 44), thereby regulating ligand-dependent, receptor-mediated endocytosis (45). Activated Ral may also play a central role in directing sites of exocytosis, because eight specific proteins that comprise the mammalian exocyst complex were found to associate with RalA in a GTP-dependent manner in rat brain (46). The exocyst complex is required for exocytosis and neurite outgrowth, and it localizes to filapodia and neurite growth cones. Therefore, RalA may regulate the integration of receptor and Ca2+ signaling with neurite outgrowth, endocytosis, and directing sites of exocytosis (46).

Ral activation is controlled by both Ras-dependent and Ras-independent events (47). Ras-independent activation of Ral
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EXPERIMENTAL PROCEDURES

Materials and Chemicals—AD202 cells were kindly supplied by Dr. N. Whitehead (Yale University). pGEX-4T-2-RIP1 was generously provided by Dr. R. A. Weinberg (Whitehead Institute, Cambridge, MA). The cDNA for rat calmodulin was obtained from Dr. J. P. Adelmen (Oregon Health Sciences University, Portland). Matchmaker Two-hybrid System 3, pGBK7T, pGADT7, pGBK7T [murine p53], pGBK7T [lamin C], pGADT7 [large T-antigen], pG71 expression plasmids, AH1109 and 187 yeast strains, Yeast Transformation Kit, all yeast media, and X-gal were from CLONTECH. Pure bovine brain calmodulin, W7-HCl, and W54HI were obtained from Calbiochem. Anti-RalA monoclonal and anti-RalB polyclonal antibodies were from Transduction Laboratories, and anti-Ral5A and anti-Ral6B polyclonal antibodies and protein A Plus-agarose were from Santa Cruz Biotechnology. TnT Coupled Reticulocyte Lysate System was purchased from Promega. [35S]Methionine (specific activity >1000 Ci/mmol), Kodak X-Omat-AR and BIO-MAX-MR film, ECL reagents, Sepharose 4B, and calmodulin-Sepharose 4B were from Amersham Biosciences. Klenow fragment of DNA polymerase 1 was from Promega or New England Biolabs. Rapid DNA Ligation kit was from Roche Diagnostics or Quick Ligase kit was from New England Biolabs. Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore. Restriction enzymes were from Promega, Roche Diagnostics or Quick, and New England Biolabs. All other chemicals were obtained from Sigma.

Plasmid Constructs—The RalA and RalB cDNAs and their deletion constructs were inserted in-frame with the GAL4 DNA BD in the pGBK7T expression vector, and the CaM cDNA was inserted in-frame with the GAL4 activation domain (AD) in the pGADT7 expression vector as follows. pUC219[RalA] was restricted with HindIII, and the resulting 900-bp Ral fragment was filled in to create blunt ends and ligated into pGBK7T as above to create pGBK7T[RalA]. pLex10[RalB] was restricted with BamHI and SalI, and the resulting 621-bp Ral fragment was filled in with Klenow and ligated into pGBK7T as above to create pGBK7T[RalB]. pBF(CaM) was restricted with SalI and BglII, and the 450-bp CaM fragment was filled in with Klenow fragment and ligated into pGBK7T as above to create pGBK7T[CaM]. Klenow-trimmed pGADT7 was restricted with HindIII, and the resulting 900-bp Ral fragment was further restricted with BsoI, which cleaved Ral at bp 549. This 3’-truncated Ral cDNA was filled in with Klenow fragment and ligated into SfI restricted, Klenow-blunted pGBK7T to create pGBK7T[RalA(1–549)]. pGBK7T[RalB] was restricted with SmaI, which removed the terminal 3’ 139 bp. This construct was self-ligated to create pGBK7T[RalB(1–482)]. pLex10[RalB] was restricted with BglII and SalI, and the resulting 621-bp Ral fragment was filled in with Klenow fragment and ligated into pGBK7T as above to create pGBK7T[RalB]. pUC219[RalA] was restricted with HindIII, and the resulting 900-bp Ral fragment was further restricted with BsoI, which cleaved Ral at bp 264. The 5’ half-fragment of RalA was filled in with Klenow and ligated into SfI restricted, Klenow-blunted pGBK7T to create the pGBK7T[RalA(1–264)] construct. The 3’ half-fragment of RalA was made blunt with Klenow and ligated into NdeI restricted and Klenow-filled pGBK7T to create the pGBK7T[RalA(1–265–621)] construct. pGBK7T[RalB] was restricted with EcoRI. The resulting pGBK7T[RalB(1–316)] fragment was self-ligated to create the pGBK7T[RalB(1–316)] construct. The 3’-187 restriction fragment of RalB, produced by the above restriction, was ligated into EcoRI restricted pGBK7T to produce the pGBK7T[RalB(1–317)] construct. The 3’-187 restriction fragment of RalB, produced by the above restriction, was digested with NdeI and XhoI, and the resulting 100-bp fragment was ligated into pGEX-4T-2, creating the pGEX-4T-2[RBBD] construct. All cDNA inserts were verified to be in the correct orientation by restriction enzyme analysis and to be in-frame by sequence analysis.

Isolation of GST, GST-RalA, GST-RalB, and GST-RBBD Fusion Proteins—GST and GST fusion proteins were purified as described previously (55). GST-RBBD was purified from AD202 cells after stimulation with isopropyl-1-thio-β-D-galactopyranoside (55). The purity of the final preparation of the proteins was assessed using SDS-PAGE.
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Platelet Washing—Partially purified platelets obtained from the Canadian Blood Services (Winnipeg, Manitoba, Canada) or freshly drawn platelets were gently rocked and treated for 30 min with 0.1 volume of ACD buffer (1.5% citric acid, 2.5% trisodium citrate, 2% glucose) (29). The platelets were centrifuged at 600 × g for 15 min to remove any remaining erythrocytes and supernatants. Portions were resuspended in the indicated buffer containing the protein concentrations based on the protocol of Jullien-Flores et al. (33). Briefly, 50 μl of Sepharose-CNBr-coupled proteins (CaM BD of RaA (amino acids 183–200) and full-length RalB) and control Sepharose beads as negative control were washed twice in ice-cold in vitro binding buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 200 mM NaCl, 0.5% Nonidet P-40, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF). The beads were incubated overnight at 4°C with equal amounts (−10 μl) of in vitro transfected/translated [35S]CaM in 100 μl of binding buffer. After sedimentation of the beads, the supernatant was removed, and the beads were washed three times with ice-cold binding buffer containing 1% dithiotreitol. The bound proteins were recovered by boiling the beads in 1× Laemmli’s sample buffer and separated by 15% SDS-PAGE. The gels were fixed for 1 h in 50% methanol, 10% acetic acid, and 40% water, washed twice in double-distilled water for 15 min each, treated for 1 h with 1% sodium salicylate, pH 7.0, and soaked in pre-drying buffer (7% methanol, 7% acetic acid, 1% glycerol) for 10 min. The gel was dried, and the presence of [35S]-CaM was detected by autoradiography at −70°C for 1–7 days. In the reverse reaction, the in vitro binding reaction was repeated with in vitro transfected/translated [35S]methionine-labeled full-length and truncated Ral constructs. The labeled proteins were incubated with 50 μl of CaM-Sepharose or blank Sepharose 4B beads as control. In all assays, the binding buffer was used with no additions or with 0.5 mM CaCl₂ or 5 mM EGTA/EDTA added. 

Communoprecipitation—Freshly drawn and outdated human platelets were prepared as above and divided into 1-ml aliquots. Fresh platelets were treated with 0.2 units/ml thrombin for 60 s at 37°C without stirring. The outdated platelets were lysed 1:1 v/v with ice-cold 2× platelet RIPA buffer (2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS, 316 mM NaCl, 2 mM EGTA, 20 mM Tris, pH 7.6) containing protease inhibitors at a final concentration of 1 mM AEBSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 μg benzamidine, plus 1 mM sodium orthovanadate. Fresh platelets were lysed by adding 1:1 v/v of 2× platelet RIPA buffer minus Triton X-100, deoxycholate, and SDS but with protease inhibitors added. The mixtures were put on ice for 30 min (RIPA-lysed) or sonicated (fresh platelets) at 4°C. The lysates were cleared by centrifugation (17,000 × g for 60 min), and the supernatants were precleared with 20 μl of protein A/G Plus-agarose and 1 μg of goat anti-rabbit IgG (RIPA-lysed) or 0.2 μg of rabbit anti-mouse IgG (sonicated) antibodies. Following the initial immunoblotting, the blots were visualized using ECL and X-Omat-AR or BIO-MAX-MR film for 8 h at −80°C. To test that the CaM-Sepharose bound protein was the appropriate protein was quantitated using an scintillation counter.

In Vitro Transcription/Translation—The pGADT’[CaM] and full-length and truncated pGBKKT[Ral] constructs, plus empty plasmids as controls (1 μg of each), were subjected to in vitro transcription/translation using the TnT’Coupled Reticulocyte Lysate System according to manufacturer’s instructions. The reaction mixtures included an RNA transcript with minus methionine and [35S]methionine (−1000 Ci/mmol at 10 μCi/ml). The reactions were incubated for 90 min at 30°C. To test that the correct proteins were translated and in equal amounts, 8 μl of reaction mixture was added to 20 μl of 1× Laemmli’s sample buffer. This gel was boiled for 2 min, and 8 μl of this was subjected to 15% or 15% SDS-PAGE. The gel was subsequently fixed, dried, and exposed on Kodak X-Omat-AR or BIO-MAX-MR film for 8–16 h at −70°C, and the radioactivity associated with the appropriate protein was quantitated using a scintillation counter.
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RESULTS

Calmodulin Binds to and Regulates Activation of RalA/RalB—It has been shown previously (53) that RalA has a C-terminal CaM BD. To determine, for the first time, whether both RalB and RalA interact with CaM, an in vitro GST fusion protein pull-down experiment, 15 μg of agarose GST-RalA (lanes 1–3) and -RalB beads (lanes 4–6) or 15 μg of control agarose-GST beads (lanes 7–9) were incubated with platelet cytosol fractions under various conditions as described under “Experimental Procedures.” Bound proteins were separated by SDS-PAGE, transferred to PVDF membrane, and probed with anti-CaM monoclonal antibody to monitor CaM binding by GST-RalA (lane 1), GST-RalB (lane 4), and control GST (lane 7) beads in incubation buffer alone, and in the presence of 0.2 mM CaCl₂ (lanes 3, 6, and 9, respectively) or 5 mM EGTA (lanes 2, 5, and 8, respectively). Platelet cytosol was also probed with anti-CaM antibody (lane 10). These experiments were repeated at least five times and gave identical results.

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Fig. 2. GST-RalA and GST-RalB bind to purified bovine brain CaM in a Ca\textsuperscript{2+}-dependent manner. In a GST fusion protein pull-down experiment, 15 μg of agarose-GST-RalA (lanes 1–3) and -RalB beads (lanes 4–6) were incubated with purified CaM under various conditions as described under “Experimental Procedures.” Bound proteins were separated by 13% SDS-PAGE, transferred to PVDF membrane, and probed with anti-CaM monoclonal antibody to monitor the interaction between CaM and GST-RalA (lane 1) and GST-RalB (lane 4) in incubation buffer alone and in the presence of 0.2 mM CaCl\textsubscript{2} (lanes 3 and 6 respectively) or 5 mM EGTA (lanes 2 and 5 respectively). Bovine brain CaM (0.5 μg) was also probed with the anti-CaM antibody (lane 7). These experiments were repeated at least five times and gave identical results.

Fig. 3. Endogenous RalA and RalB in platelet particulate fraction interact with CaM-Sepharose. Solubilized platelet particulate proteins (500 μg) were subjected to a CaM affinity binding assay as described under “Experimental Procedures.” To demonstrate Ral/CaM interactions and determine the Ca\textsuperscript{2+} dependence of such, the particulate proteins were incubated with 50 μl of Sepharose 4B beads (A and B, lane 1) as control, with 50 μl of CaM-Sepharose 4B beads in buffer alone (A and B, lane 2), or with 5 mM EGTA (A and B, lane 3) or 0.2 mM CaCl\textsubscript{2} (A and B, lane 4) added to incubation buffer. Bound proteins were subjected to 13% SDS-PAGE, transferred to PVDF membranes, and probed with either anti-RalA (A) or anti-RalB (B) antibodies. An aliquot of platelet particulate fraction (3 μg) was also probed with anti-RalA (A, lane 5) and anti-RalB (B, lane 5) antibodies. These experiments were repeated at least five times and gave identical results.

Calmodulin Interacts Specifically with RalA and RalB in Vivo—To relate these findings to mammalian cells, it was also important to demonstrate this Ral/CaM interaction in vivo in a eukaryotic system. Therefore, a Y2H assay was employed, which demonstrated, for the first time, specific interaction of the Ral proteins with CaM in vivo. Positive blue colonies, caused by the protein-protein-induced activation of the yeast MELI gene and subsequent synthesis of α-galactosidase and digestion of X-a-Gal, resulted when AH109 cells were transfected with pGADT7[CaM] and pGBK7[RalA] or pGBK7[RalB] (Fig. 6B, RalA + CaM and RalB + CaM). Positive controls (i) pCL1 (Fig. 6B, pCL1) and (ii) pGBK7[murine p53] plus pGADT7[SV40 large T-antigen] (Fig. 6B, T + Lam) also produced blue colonies, whereas negative controls (i) pGBK7[lamin C] plus pGADT7[T-antigen] (Fig. 6B) and (ii) pGBK7[lamin C] plus pGADT7[CaM] (data not shown) and the Ral (Fig. 6B, RalA and RalB) and CaM (Fig. 6B, CaM) constructs by themselves did not activate the MELI gene. This showed that the interactions between Ral and CaM were specific and that the plasmid constructs themselves did not activate the MELI gene autonomously. To show further this specific interaction in yeast, AH109 cells transfected with pGBK7[RalA] or pGBK7[RalB] constructs were mated with Y187 yeast cells transfected with pGADT7[CaM].

Both RalA and RalB interacted with CaM to induce synthesis of α-galactosidase (data not shown). The blue phenotype in both the yeast transfection and mating experiments persisted upon restreaking 3–4 times on high stringency drop-out media. In all cases, the positive RalA/CaM colonies grew more quickly and developed a more intense blue coloration than the RalB/CaM colonies, even though Western blotting showed, qualitatively, similar levels of protein were expressed by the yeast (data not shown). These results demonstrate that both RalA and RalB directly bind CaM in vivo in a eukaryotic system and that RalA may bind CaM more readily than RalB.

CaM Interacts with C-terminally Truncated Ral Proteins in a Ca\textsuperscript{2+}-dependent Manner—A CaM target data base (calcium.
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RALA AND RALB HAVE AN N-TERMINAL Ca2+ -DEPENDENT AND A C-TERMINAL Ca2+ -DEPENDENT CA2+ BD—To confirm further the presence of more than one CaM BD in Ral, [35S]Met-labeled N- and C-terminal halves of RalA and RalB were tested for interaction with CaM-Sepharose. [35S]Met-labeled RalA-(1–264) (Fig. 8, upper panel, lanes 1, 3, 5, and 7) and RalB-(1–316) (Fig. 8, lower panel, lanes 1, 3, 5, and 7), both containing the approximate N-terminal half of each protein, strongly bound CaM-Sepharose in the presence of either 0.5 mM CaCl2 (Fig. 8, lanes 1) or 5 mM EDTA (lanes 3), whereas [35S]Met-labeled RalA-(265–621) (Fig. 8, upper panel, lanes 2, 4, 6, and 8) and RalB-(317–621) (Fig. 8, lower panel, lanes 2, 4, 6, and 8), both containing the approximate C-terminal half of each protein, strongly bound CaM-Sepharose in the presence of Ca2+ (Fig. 8, lanes 2) but significantly less or not at all in the presence of EDTA (Fig. 8, lanes 4). The reaction was specific because neither Rals bound to the control Sepharose 4B beads (Fig. 8, upper and lower panels, lanes 5 and 6). These results suggest that the C-terminal halves of RalA and RalB contain a Ca2+ -dependent CaM BD motif, whereas the N-terminal halves contain a Ca2+ -independent CaM BD. [35S]Met-labeled RalB-(1–482), which also bound CaM-Sepharose in a Ca2+ -independent manner, indicate that the last 40 or so amino acids that partially inhibit, in some way, the RalB/CaM interaction in vitro, also contain the Ca2+ -dependent CaM BD (results not shown).

CaM IS REQUIRED FOR THE THROMBIN-INDUCED ACTIVATION OF RALA AND RALB—We used the RRBD to determine whether CaM plays a role in the activation of Rals. Initially, to check specificity of our GST-RRBD, 1 ml of thrombin-stimulated (75 s) and unstimulated freshly drawn human platelets were subject to GST-RRBD and control GST pull-down experiments (as described under “Experimental Procedures”). The Ral BD motif of RIP1 selectively binds GTP-bound but not GDP-bound Ral (29). Only thrombin-stimulated platelets showed significant amounts of Ral-GTP, with unstimulated platelets showing negligible amounts. The reaction was specific because GST control beads did not bind activated Ral in either unstimulated or thrombin-stimulated platelets (data not shown). The next step was to determine whether CaM was involved in the activation of Ral. Thrombin led to full activation of RalA (Fig. 9, upper panel) and RalB (Fig. 9, lower panel) within 10 s. This activation was eliminated in the presence of the CaM inhibitor W7-HCl but not W5-HCl. W5 was used at a low non-CaM-inhibiting concentration to show that CaM inhibitors do not autonomously prevent Ral activation. These results suggest that CaM functions in the activation and regulation of RalA and RalB.

DISCUSSION

It has been reported previously (7, 13, 29, 48, 49, 51, 52) that elevated levels of Ca2+ activate RalA and that CaM binds RalA in vitro (51, 53), leading to the proposal that RalA is associated with Ca2+/CaM-dependent intracellular signaling pathways (53). As well, platelet agonists (e.g. platelet-activating factor, thromboxane A2, and α-thrombin) activate RalA in platelets in a Ca2+-dependent manner, suggesting that RalA activation is mediated by a common signaling event that may involve Ca2+ (29). We propose that CaM is an essential component in this process. We have shown, for what appears to be the first time, by coimmunoprecipitation in human platelets and yeast two-hybrid experiments, that RalB binds CaM and that both RalA and RalB interact specifically and directly with CaM in vitro in a eukaryotic system. These results were confirmed and shown to be Ca2+-dependent by in vitro binding assays, as well as GST and Sepharose fusion protein pull-down assays in human platelet lysates (results from the Y2H assays appear to show that RalA binds CaM more readily than RalB. This was based solely on the qualitative finding that positive blue RalA/CaM colonies formed more quickly and had a more intense blue coloration than RalB/CaM colonies, even though Western analysis showed similar protein expression levels. We speculate that this differential binding of CaM by the Ral proteins may determine functional differences between RalA and RalB, as related to Ca2+/CaM signaling pathways. Further study is
Calmodulin interacts specifically with RalA and RalB in vivo. A. full-length and truncated RalA and RalB constructs. Arrowheads denote cleavage sites, and numbers represent nucleotide base pairs. B, calmodulin interacts specifically with various RalA and RalB constructs in vivo. RalA and RalB cDNAs and their deletion constructs were subcloned into GAL4 BD expression plasmid pGDKT7, and CaM cDNA was subcloned into GAL4 AD expression plasmid pGADT7. To identify specific protein-protein interactions, yeast two-hybrid and yeast mating assays were performed as described under “Experimental Procedures.” Colonies were monitored for blue coloration caused by the direct interaction of the GAL4 BD and AD fusion proteins. Interactions tested were pGBK7[RalA] + pGADT7[CaM] (RalA + CaM), pGBK7[RalB] + pGADT7[CaM] (RalB + CaM), pGBK7[RalA-1(549)] + pGADT7[CaM] (RalA-0.1, T-antigen + CaM), and pGBK7[RalB-0(100–482)] + pGADT7[CaM] (RalB-0.1, T-antigen + CaM). To ensure specificity of the systems, positive controls pCL1 (pCL1), and pGBK7[murine p53 + pGADT7[SV40 large T-antigen] (T + 53)], and negative control pGBK7[lamin C] + pGADT7[T-antigen] (T + Lamin), were also tested. AH109 yeast transformed with pGBK7[RalA] (RalA), pGBK7[RalB] (RalB), or pGADT7[CaM] (CaM) were also tested for autonomous activation of the MELI gene. In all cases, the positive interaction phenotype persisted upon restreaking at least 3 times on high stringency drop-out medium.

needed to examine these preliminary findings of differential binding and to determine the functional significance.

We have shown that in human platelets, thrombin induces the activation of both RalA and RalB in a CaM-dependent manner. This would suggest that the Ca2+-dependent platelet agonist-induced (29) and Ca2+-induced (29, 30) activation of RalA reported previously may have in fact been both CaM- and Ca2+-dependent. It is likely that Ral is activated by many of the CaM-regulated intracellular pathways by forming a complex with CaM and its associated proteins. Several studies propose Ca2+-involved Ral activation by various mechanisms (4, 29, 50). We speculate that CaM may be required for some, if not all, of these Ca2+-dependent regulatory pathways. Ca2+/CaM induces GTP binding to RalA in human erythrocyte plasma membrane (48, 51, 53), and the cycling of RalA in synaptic vesicle membranes by CaM is Ca2+-dependent (50, 54). Further study is needed to determine exactly which of these CaM-regulated pathways are involved with Ral, the mechanism by which CaM induces Ral activation, and the results of such CaM-induced Ral activation on downstream effectors and their pathways. Our results demonstrating the thrombin-induced, CaM-dependent activation of RalA and RalB in platelets, plus results cited from the literature, suggest that Ca2+/CaM activates both RalA and RalB and that the signaling pathways of Ca2+/CaM and RalA (54) and RalB are directly linked.

The regulation of Ral function by CaM appears to be complex. Initially, the CaM BD in RalA has been shown to be at the C terminus (53). Because the two Ral proteins differ mainly at the C terminus, we wished to determine whether there was a CaM BD in another region of RalB and perhaps RalA. The sequences of RalA and RalB were analyzed using the CaM target data base (calcium.oci.utoronto.ca/). This revealed the presence of a potential N-terminal CaM BD in RalB (average propensity for α-helix formation, 1.052). Examination of RalA showed a second high scoring putative N-terminal CaM BD (average propensity 0.957) when the C-terminal 30 residues containing the predicted CaM BD (53) were removed to allow for a pattern search to identify any additional potential CaM Bds. RalA and RalB have almost identical values for various parameters obtained from the analysis of the predicted N-terminal CaM-binding sites. The mean hydrophobicity (~0.125, RalA, and 0.135, RalB) and hydrophobic moments (0.302, RalA, and 0.628, RalB) for the N-terminal regions of both proteins are within the range of values of most CaM Bds (53). The major difference between the putative N-terminal CaM Bds and the predicted C-terminal CaM BD of RalA is that the latter forms a hydrophilic α-helical wheel (53), and the N-terminal regions form hydrophilic α-helical wheels. The interaction of CaM with its target proteins is predominantly hydrophobic (53). Both N- and C-terminal regions carry a net positive charge (+3 for N-terminal and +9 for C-terminal) because binding of CaM with target proteins also involves strong electrostatic interactions (53). Therefore, the possibility of more than one CaM BD being present in the Ral proteins was
examined. To test what effect on CaM binding removal of the C-terminal regions of the Ral proteins would have, the appropriate regions were deleted from RaIA and RaIB, and the subsequent truncated proteins were tested for interaction with CaM. Results demonstrated that RaIA-(1–549), which lacks the predicted CaM BD (53), and RaIB-(1–482), which lacks the region equivalent to that of the CaM BD of RaIA, interacted specifically and directly with CaM in the Y2H and in vitro binding assays. The in vitro results showed the interaction to be Ca$^{2+}$-dependent. It is realized that deleting large portions of a protein may alter its three-dimensional structure and, hence, its ability to interact normally with other proteins. However, in vitro binding assays using N- and C-terminal halves of each Ral protein suggest that their C-terminal halves contains a Ca$^{2+}$-dependent CaM BD motif, whereas their N-terminal halves contain a Ca$^{2+}$-independent CaM BD. Further experiments (e.g. site-directed mutagenesis) are underway to define the CaM binding sequence in the N- and C-terminal regions of Rals.

Results therefore indicate that there is an additional CaM BD in RaIA from that described in the literature (53) and at least two CaM Bds in RaIB. This is supported by the in vitro binding assay results with the N- and C-terminally truncated RaIB construct which failed to bind CaM. The in vitro results appear to show C-terminally truncated RaIB binds CaM much more readily than full-length RaIB and that the deleted portion contains the Ca$^{2+}$-dependent CaM BD. These C-terminal 40 or so amino acids of RaIB may normally be involved in inhibiting CaM/RalB interactions. This may partly explain the differential binding of CaM by RaIA and RaIB. We speculate that in the cell, the putative RaIB CaM-binding inhibitory region is masked or inhibited by another molecule or by a change in conformation. Because this inhibitory region contains an apparent Ca$^{2+}$-dependent CaM BD, the suggested molecular or conformational regulation may be controlled by Ca$^{2+}$. We propose that the inhibitory area is masked at high intracellular Ca$^{2+}$ concentrations and is open and fully active at low Ca$^{2+}$ levels. In addition, optimal binding of CaM to RaIA and RaIB may require both Ca$^{2+}$-dependent and Ca$^{2+}$-independent CaM Bds. For instance, once CaM binds to the C-terminal CaM BD, the N-terminal domain may bind CaM with greater affinity.

It will be necessary to demonstrate RaIA/CaM interactions in mammalian cells and to determine how Ca$^{2+}$, CaM, and other agents (e.g. agonists of platelets and cell transformation) affect the activation of Ral in cells. Identification of additional Ral-interacting proteins is required to discover more CaM-regulated pathways that activate Ral. Examination of suspected...
differences in CaM binding between the Ral proteins warrants further attention and also whether the guanine nucleotide status of Ral affects its CaM binding affinity. How Ral-GTPases regulate, or are regulated themselves, by Ca\(^{2+}\)/CaM-dependent pathways will shed light on important cellular processes in health and disease.

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