Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 105–109
BBRC
www.elsevier.com/locate/ybbrc

# Calmodulin binding to the small GTPase Ral requires isoprenylated Ral ☆

Ranjinder S. Sidhu [a], Sherif M. Elsaraj [a], Ognjen Grujic [a], Rajinder P. Bhullar [a,b,*]

[a] *Department of Oral Biology, University of Manitoba, Winnipeg, Man., Canada R3E 0W2*
[b] *Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Man., Canada R3E 0W2*

Received 26 July 2005
Available online 18 August 2005

**Abstract**

Ral, a member of the Ras-p21 superfamily of small GTPases, has been shown to require the calcium-signaling protein calmodulin (CaM) for activation. In the present work, we investigated the properties of the Ral-CaM interaction. Using CaM affinity binding assay with lysates from mammalian cells overexpressing various Ral mutants, we found that RalB(V23, Δ*CAAX*) lacking the C-terminal isoprenylation region bound significantly less efficiently to CaM. Binding of other mutants containing critical amino acid changes in the nucleotide or substrate binding regions (residues 23, 28, and 49) was not affected. In addition, all mutants bound significantly better in the presence of calcium versus the calcium chelator EGTA. Using in vitro transcription–translation in the presence of geranylgeranyl pyrophosphate, we demonstrate enhanced Ral binding to CaM. Inhibition of isoprenylation in cells in culture with lovastatin resulted in decreased binding of CaM to Ral. The present results show that post-translational isoprenylation of Ral is important in Ral–CaM interaction.


*Keywords:* Ral; Small GTPase; Calmodulin; Isoprenylation; Geranylgeranyl pyrophosphate

Small GTP-binding proteins belonging to the Ras superfamily are important in cell signaling pathways controlling cell proliferation, differentiation, trafficking, and cytoskeleton organization [1]. Ral, a member of the Ras subfamily, consists of two highly related proteins, RalA and RalB. The two Ral isoforms are 85% identical and most of the difference lies in the C-terminal region [2]. Ral undergoes activation through exchange of GDP for GTP and is inactivated upon hydrolysis of bound GTP to GDP [3].

Ral can be activated in response to high intracellular calcium concentrations [1]. Thus, Ral is activated by the calcium ionophore ionomycin, while activation by lysophosphatidic acid or epidermal growth factor is blocked by phospholipase C inhibitor [4]. A basic/hydrophobic amino acid rich region that forms an amphipathic α-helix is found in the C-terminal region of RalA and has been shown to bind calmodulin [5]. Calcium stimulates GTP binding to RalA while it reduces the binding of GDP to RalA [6]. Binding of GTP to RalA was increased 3-fold in the presence of calcium/calmodulin [7]. In addition, the GTPase activity of Ral was stimulated by calcium/calmodulin [6]. We have shown that calmodulin is required for the thrombin-induced activation of RalA and RalB in human platelets [8]. In addition, both RalA and RalB have a calcium-dependent

☆ *Abbreviations:* CaM, calmodulin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AEBSF, 4-[(2-aminoethyl)]-benzenesulfonyl fluoride; PVDF, polyvinyl difluoride.

* Corresponding author. Fax: +204 789 3913.
*E-mail address:* bhullar@Ms.UManitoba.CA (R.P. Bhullar).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.053

calmodulin binding site in their C-terminal region and a calcium-independent binding site in their N-terminal region [8].

The Ras family proteins are post-translationally modified through prenylation of their conserved C-terminal C*AA*X box (where C is cysteine, *A* is an aliphatic amino acid, and X could be any residue) which targets the GTPase to the plasma membrane [9,10]. Following isoprenylation, the last three amino acids of the C*AA*X box are cleaved, and a methyl group is added at the cysteine residue [11]. The protein is farnesylated when the last amino acid is Ala, Cys, Gln, Met, or Ser, and geranylgeranylated when the last amino acid is Leu or Phe [11,12]. Since the last amino acid of Ral is leucine, Ral is modified through geranylgeranylation. In contrast to Ral, Ras is farnesylated at its C-terminus. In addition to Ras farnesylation, palmitoylation or a polybasic region, found in the hypervariable domain immediately upstream of the C*AA*X box, is required for localization of Ras to the plasma membrane [10]. Since all Ras proteins are polyisoprenylated, but only some are palmitoylated, isoprenylation is the major determinant for membrane localization [9]. Mutation of the Ras C-terminal Cys186 prevents the membrane localization of Ras and blocks transformation, highlighting the importance of post-translational modifications [9]. Thus, the membrane localization may be necessary for high affinity protein–protein interactions. Since Ral is also post-translationally modified through prenylation, we wished to test the hypothesis that the C*AA*X box is required for the interaction of Ral with calmodulin. We used various Ral mutants and found the C-terminal isoprenylated region to be important for calmodulin interaction. In addition, an inhibitor of Ral isoprenylation, lovastatin, reduces binding of calmodulin to Ral from cell lysates. This suggests that post-translational modifications necessary for membrane association of Ral are also required for calmodulin binding.

## Methods

*Plasmids and chemicals.* The pRK5-[RalB] mutant vectors [RalB(V23), RalB(N28), RalB(V23, D49), RalB(V23, ΔN11), and RalB(V23, ΔC*AA*X)] were generously provided by Dr. M. White (University of Texas Southwestern Medical Center, Dallas, Texas, USA). The pGBKT7 plasmids containing RalA and RalB were described previously [8]. Sepharose 4B coupled Calmodulin and [$^{35}$S]methionine (specific activity >1000 Ci/mmol) were purchased from Amersham Biosciences. Lipofectamine 2000 and cell culture media were obtained from Invitrogen. TnT coupled rabbit reticulocyte lysate system was from Promega. Polyclonal RalB antibody was obtained from Transduction. All other reagents were from Sigma unless otherwise indicated.

*Cell culture.* HeLa cells were maintained in DMEM supplemented with 10% FBS (v/v), 1.5 g/L $NaHCO_3$, and 100 U penicillin/streptomycin solution at 37 °C in 5% $CO_2$ and 95% air (v/v) on 24-well, 35 or 100 mm plates.

*Cell transfections and lysis.* Mammalian expression vectors containing mutant RalB proteins were transfected into HeLa cells using Lipofectamine 2000 in accordance with the manufacturer's instructions. For transfection, 4 μg of DNA plus Lipofectamine was added to HeLa cells (~70% confluent) in 35 mm plates. Proteins were expressed overnight and lysed in buffer containing 50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 0.1% NP-40, and 10% glycerol plus protease inhibitor cocktail with 500 μM $Ca^{2+}$ or 5 mM EGTA.

*In vitro transcription–translation.* The TnT Coupled Rabbit Reticulocyte Lysate System was used for in vitro transcription and translation of [$^{35}$S]Met-labeled RalA or RalB following the manufacturer's instructions with slight modifications. That is, 1 μg of pGBKT7-[RalA] or -[RalB] plasmid was used in the presence or absence of 1 μM geranylgeranyl pyrophosphate, the substrate for isoprenylation.

*CaM affinity binding assay.* CaM binding assays were performed essentially as described previously [8]. For experiments using in vitro transcribed and translated Ral, 50 μl of Calmodulin–Sepharose 4B beads or blank Sepharose 4B beads were equilibrated in binding buffer containing 20 mM Hepes, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, 20% glycerol, and 0.55% Triton X-100. Binding reactions were carried out overnight at 4 °C using 7.5 μl TnT reaction mix with 500 μM $Ca^{2+}$ or 5 mM EDTA. CaM–Sepharose beads were washed 3× with binding buffer and bound proteins were recovered in Laemmli's sample buffer by heating at 100 °C. Proteins were separated on 13% SDS–PAGE gels and fixed for 1 h in gel fixer solution (50% methanol and 10% acetic acid). Following two washes with double-distilled water, the gel was incubated with 1 M sodium salicylate to enhance radioactive signal. Just prior to drying, the gel was incubated for 10 min in pre-drying buffer (7% methanol, 7% acetic acid, and 1% glycerol). Dried gels were exposed to autoradiography film for 2–3 days at −70 °C.

To determine the binding preference of Ral to CaM, HeLa cells overexpressing the various Ral mutants were lysed in buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 0.1% NP-40, and 10% glycerol plus protease inhibitor cocktail) and incubated with 50 μl CaM–Sepharose beads for 2 h at 4 °C as described previously [8]. Proteins were separated on 13% SDS–PAGE gels and transferred to PVDF membrane. Western blotting with polyclonal anti-RalB antibody was used to demonstrate the binding to CaM–Sepharose.

*In vivo inhibition of Ral isoprenylation and cell fractionation.* HeLa cells at 80% confluence were serum-starved for 24 h prior to treatment with lovastatin (50 or 100 μM) or DMSO (0.2% or 0.4%). Following 24 h treatment with lovastatin, cells were washed in PBS and collected in buffer containing 20 mM Hepes, pH 7.4, 200 mM KCl, 1 mM $MgCl_2$, and 1 mM PMSF. Cells were lysed by sonication (Heat Systems ultrasonicator, Microson) using 3 pulses of 15 s each. The soluble cytosolic fraction, which was used in affinity binding experiments with CaM–Sepharose as described above, was collected by centrifugation at 100,000*g* for 2 h at 4 °C. Binding was performed at 4 °C for 2 h and Western blotting using monoclonal RalA antibody was carried out to demonstrate binding of CaM to Ral.

## Results and discussion

Previous studies have shown that calcium ($Ca^{2+}$) and $Ca^{2+}$/calmodulin ($Ca^{2+}$/CaM) play a role in the activation of the small GTPase Ral following certain physiological stimuli [5,8]. We have previously shown that both RalA and B contain regions capable of binding CaM in a $Ca^{2+}$-dependent and -independent manner, and raised the possibility that the C-terminal

region of RalB may act as an inhibitory domain [8]. In a more recent work, we have shown that Ral and CaM can bind and regulate the enzymatic activity of the $Ca^{2+}$-dependent enzyme phospholipase C-δ1 [13]. The importance of RalA in $Ca^{2+}$-dependent intracellular signaling pathways was observed when cells treated with the $Ca^{2+}$ ionophore ionomycin had elevated levels of active GTP-bound Ral [4]. In addition, increases in intracellular $Ca^{2+}$ due to release from intracellular stores using thapsigargin could also activate Ral while depletion of $Ca^{2+}$ using the chelator BAPTA-AM inhibited thrombin-mediated activation of Ral in platelets [14]. In in vitro experiments, $Ca^{2+}$ was found to stimulate the binding of GTP and reduce the binding of GDP to Ral in a dose-dependent manner [6]. Taken together, RalA activation could be mediated by a common signaling event that involves $Ca^{2+}$ [14]. The goal of the present study was to determine the role of post-translational lipid modifications in the CaM–Ral interaction.

It has been shown that the carboxy-terminal C*AA*X box of Ral is a substrate for geranylgeranylation [15,16], and that the post-translational modification by the addition of lipids plays an important role in its targeting to cell membranes [17]. A mechanism for CaM-mediated regulation of Ral has been suggested through experiments showing the dissociation of RalA from synaptic vesicle membranes by exogenous CaM in a $Ca^{2+}$-dependent manner [18]. A similar regulatory mechanism was seen for K-RasB [19] and the Ras-related Rab3A and -3B small GTPases [20,21]. Interestingly, although the GTPase activity of RalA was reported to be stimulated by $Ca^{2+}$/CaM, the $Ca^{2+}$/CaM-mediated dissociation of RalA from membranes was independent of nucleotide status [18].

In order to further define the interaction between Ral and calmodulin, in the present study we used cultured HeLa cells that were transfected with five different RalB mutant clones. Using CaM affinity binding assay we found that the Ral mutants containing critical amino acid changes in the nucleotide or substrate binding regions (residues 23, 28, and 49) and the mutant lacking the N-terminal 11 amino acids RalB(V23, ΔN11) did not alter binding to CaM (Fig. 1, top panel). However, the binding of CaM to the constitutively active mutant lacking the C-terminal isoprenylated region RalB(V23, Δ*CAA*X) was significantly reduced. Binding of all mutants to CaM was reduced in the presence of the calcium chelator EGTA compared to samples containing calcium (Fig. 1, bottom panel). The cysteine residue in the C-terminal region is the site for the addition of the lipid isoprenoid group. Removal of the last four amino acids prevents the incorporation of Ral into the membrane. It has been previously suggested that CaM causes dissociation of Ral from the membrane [18]. The observed weakened interaction of CaM with non-isoprenylated

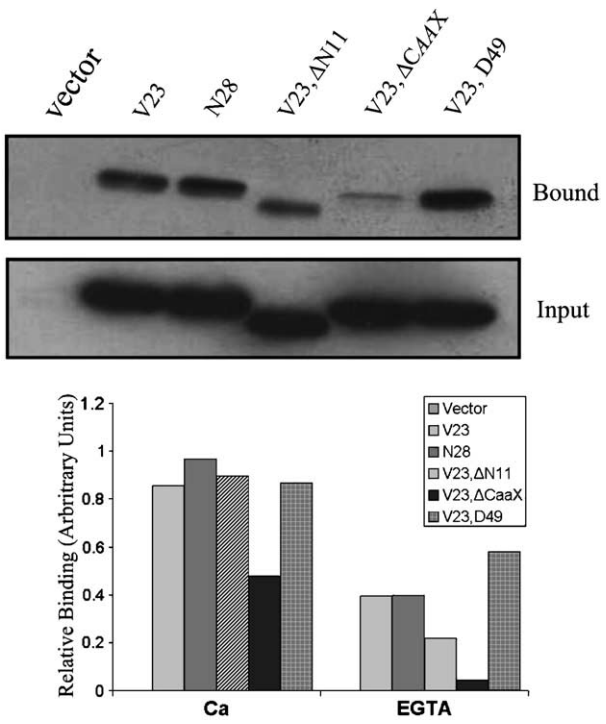


Fig. 1. CaM binding to C-terminal truncated Ral is reduced. CaM–Sepharose was incubated with HeLa cell lysates (500 μg protein) expressing the Ral mutants RalB(V23), RalB(N28), RalB(V23,ΔN11), RalB(V23,Δ*CAA*X) or RalB(V23,D49) in the presence of 500 μM $Ca^{2+}$ or 5 mM EGTA. Ral bound to CaM–Sepharose was determined by Western blotting (top) and quantified (bottom) using Quantity One. The blot shows data in the presence of $Ca^{2+}$ from a representative experiment repeated three times.

Ral in this study suggests that the isoprenoid group is important in Ral–CaM interaction.

We next wished to determine whether the addition of the isoprenyl group alone is necessary and sufficient to improve the affinity of CaM for Ral. Therefore, we used the rabbit reticulocyte lysate system to in vitro transcribe and translate (TnT system) recombinant Ral protein in the presence or absence of the substrate geranylgeranyl pyrophosphate (GPP). We found that in the presence of GPP in vitro transcription and translation of Ral was improved and that binding to CaM relative to input was also significantly enhanced (Fig. 2). Addition of the geranylgeranyl lipid moiety provides hydrophobic character to Ral, which could strengthen interactions with the C-terminal CaM binding domain of Ral. Thus, it is possible that CaM plays a role in the recycling of Ral from the membrane to the cytosol and that isoprenyl group is essential in this process.

In order to establish whether inhibition of Ral isoprenylation alters binding of CaM to Ral in cultured mammalian cells, we used HeLa cells treated with lovastatin. Lovastatin inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, leading to decreased isoprenylation of small GTPases [22]. We performed

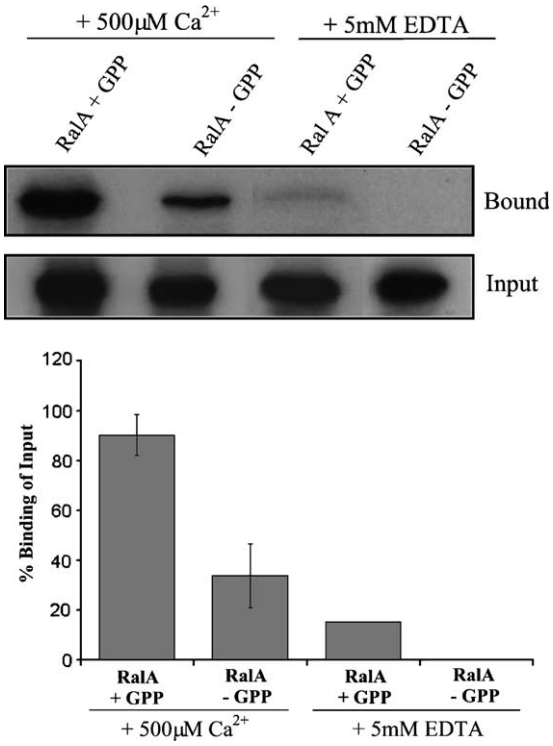


Fig. 2. CaM binds preferentially to isoprenylated Ral in vitro. Recombinant RalA was in vitro transcribed and translated in the presence or absence of the Ral isoprenylation substrate geranylgeranyl pyrophosphate plus [$^{35}$S]Met. Ral bound to CaM–Sepharose was separated on SDS–PAGE and visualized by exposing dried gels to autoradiography film for 2–3 days at −70 °C.

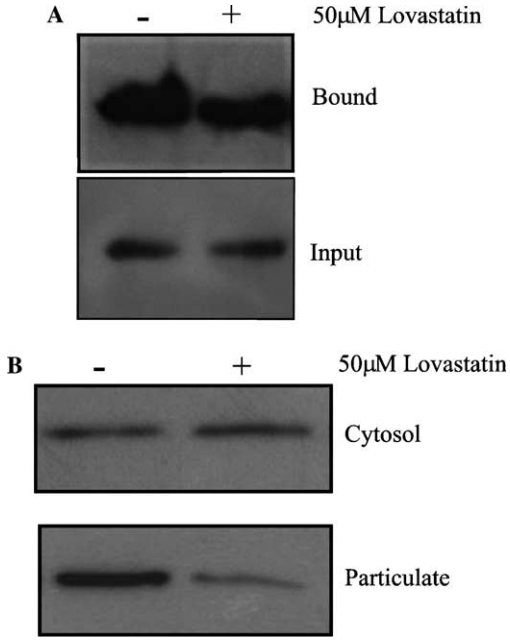


Fig. 3. Inhibition of Ral isoprenylation by lovastatin reduces binding to CaM. (A) Reduced binding of Ral to CaM–Sepharose is seen when isoprenylation is inhibited by 50 μM lovastatin treatment (top). Bottom panel shows an aliquot of the input to highlight equivalent protein in the binding reaction. (B) An increase in Ral in cytosol (top) and a corresponding decrease in membrane (bottom) fraction in the presence of lovastatin is shown to confirm inhibition of Ral isoprenylation.

CaM–Sepharose affinity binding assay using cell lysates from lovastatin treated HeLa cells and control DMSO treated cells (Fig. 3A). Reduced binding of Ral to CaM–Sepharose is seen when isoprenylation is inhibited by lovastatin treatment (50 μM) (top). At higher concentrations of lovastatin (100 μM), we observed a complete inhibition of Ral protein synthesis (results not shown). An increase in Ral in cytosol (Fig. 3B, top) and a corresponding decrease in membrane (Fig. 3B, bottom) fraction in the presence of lovastatin are shown to demonstrate the inhibition of Ral isoprenylation. These experiments show that Ral isoprenylation is necessary for high affinity binding to CaM. Together, these studies highlight the importance of post-translational modification of Ral in $Ca^{2+}$/CaM-dependent signaling pathways.

## Conclusion

In the present work, we demonstrate that binding of CaM to the constitutively active mutant lacking the C-terminal isoprenylated region of Ral (Δ*CAAX* mutant) is significantly reduced compared to all other mutants tested. In addition, the binding of CaM to Ral occurs preferentially to the isoprenylated form of Ral in a $Ca^{2+}$-dependent manner. Using lovastatin to inhibit isoprenylation of endogenous Ral, we found that binding to calmodulin is reduced. The present results show that the Ral/CaM interaction is disrupted by removal of the C-terminal Ral isoprenylated moiety (*CAAX*) and thus post-translational modifications necessary for membrane association are also involved in a CaM-mediated regulatory mechanism for Ral.

## Acknowledgments

This work was supported by a CIHR grant to R.P. Bhullar and a Heart and Stroke Doctoral Research Award to R.S. Sidhu. O. Grujic is the recipient of a CIHR Canada Graduate Scholarships Master's Award.

## References

[1] L.A. Feig, Ral-GTPases: approaching their 15 minutes of fame, Trends Cell Biol. 13 (2003) 419–425.
[2] P. Chardin, A. Tavitian, Coding sequences of human ralA and ralB cDNAs, Nucleic Acids Res. 17 (1989) 4380.
[3] Y. Takai, T. Sasaki, T. Matozaki, Small GTP-binding proteins, Physiol. Rev. 81 (2001) 153–208.
[4] F.R. Hofer, R. Berdeaux, G.S. Martin, Ras-independent activation of Ral by a $Ca^{2+}$-dependent pathway, Curr. Biol. 8 (1998) 839–842.

[5] K.L. Wang, M.T. Khan, B.D. Roufogalis, Identification and characterization of a calmodulin-binding domain in Ral-A, a Ras-related GTP-binding protein purified from human erythrocyte membrane, J. Biol. Chem. 272 (1997) 16002–16009.
[6] J.B. Park, Regulation of GTP-binding state in RalA through $Ca^{2+}$ and calmodulin, Exp. Mol. Med. 33 (2001) 54–58.
[7] K.L. Wang, B.D. Roufogalis, $Ca^{2+}$/calmodulin stimulates GTP binding to the ras-related protein ral-A, J. Biol. Chem. 274 (1999) 14525–14528.
[8] R.R. Clough, R.S. Sidhu, R.P. Bhullar, Calmodulin binds RalA and RalB and is required for the thrombin-induced activation of Ral in human platelets, J. Biol. Chem. 277 (2002) 28972–28980.
[9] J.F. Hancock, A.I. Magee, J.E. Magee, C.J. Marshall, All ras proteins are polyisoprenylated but only some are palmitoylated, Cell 57 (1989) 1167–1177.
[10] J.F. Hancock, H. Paterson, C.J. Marshall, A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane, Cell 63 (1990) 133–139.
[11] S. Schillo, G. Belusic, K. Hartmann, C. Franz, B. Kuhl, G. Brenner-Weiss, R. Paulsen, A. Huber, Targeted mutagenesis of the farnesylation site of *Drosophila* Gγ disrupts membrane association of the G protein βγ complex and affects the light sensitivity of the visual system, J. Biol. Chem. 279 (2004) 36309–36316.
[12] P.B. Wedegaertner, P.T. Wilson, H.R. Bourne, Lipid modifications of trimeric G proteins, J. Biol. Chem. 270 (1995) 503–506.
[13] R.S. Sidhu, R.R. Clough, R.P. Bhullar, Regulation of phospholipase C-δ1 through direct interactions with the small GTPase Ral and calmodulin, J. Biol. Chem. 280 (2005) 21933–21941.
[14] R.M. Wolthuis, B. Franke, M. van Triest, B. Bauer, R.H. Cool, J.H. Camonis, J.W. Akkerman, J.L. Bos, Activation of the small GTPase Ral in platelets, Mol. Cell. Biol. 18 (1998) 2486–2491.
[15] B.T. Kinsella, R.A. Erdman, W.A. Maltese, Carboxyl-terminal isoprenylation of ras-related GTP-binding proteins encoded by rac1, rac2, and ralA, J. Biol. Chem. 266 (1991) 9786–9794.
[16] O. Jilkina, R.P. Bhullar, Generation of antibodies specific for the RalA and RalB GTP-binding proteins and determination of their concentration and distribution in human platelets, Biochim. Biophys. Acta 1314 (1996) 157–166.
[17] P.J. Casey, Protein lipidation in cell signaling, Science 268 (1995) 221–225.
[18] J.B. Park, J.Y. Lee, J.W. Kim, Dissociation of RalA from synaptic membranes by $Ca^{2+}$/calmodulin, Biochem. Biophys. Res. Commun. 263 (1999) 765–769.
[19] R.S. Sidhu, R.R. Clough, R.P. Bhullar, $Ca^{2+}$/calmodulin binds and dissociates K-RasB from membrane, Biochem. Biophys. Res. Commun. 304 (2003) 655–660.
[20] J.B. Park, C.C. Farnsworth, J.A. Glomset, $Ca^{2+}$/calmodulin causes Rab3A to dissociate from synaptic membranes, J. Biol. Chem. 272 (1997) 20857–20865.
[21] R.S. Sidhu, R.P. Bhullar, Rab3B in human platelet is membrane bound and interacts with $Ca^{2+}$/calmodulin, Biochem. Biophys. Res. Commun. 289 (2001) 1039–1043.
[22] W.A. Maltese, K.M. Sheridan, Isoprenoid modification of G25K (Gp), a low molecular mass GTP-binding protein distinct from p21ras, J. Biol. Chem. 265 (1990) 17883–17890.