カルモジュリンを用いたイオノクロミックナノデバイスによるバイオナノマシンの制御

The control of bionanomachine using calmodulin-based ionoresponsive nanodevice

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SYNOPSIS

Small GTPase is one of the nucleotide-driven Bionanomachine. Interestingly mechanical function and the catalytic core of small GTPase resemble ATP-driven motor proteins. Ras is a small G-protein that stimulates downstream pathways and plays an important role in cell proliferation and differentiation, involving Several factors, like GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), regulating the activity of RAS proteins. GAPs stimulate the hydrolysis of GTP to GDP, converting RAS back to its inactive state, while GEFs promote the exchange of GDP for GTP, activating RAS.

Calmodulin (CaM) is involved in intracellular signaling and regulatory events in many calcium-dependent processes in almost every eukaryotic cell. CaM target proteins have relatively short CaM-binding peptides, such as the M13 peptide, which bind tightly to the M13 peptide in the presence of calcium, activating the enzymatic activity. CaM is thus classified as an ionoresponsive molecular device. In this study. Modified and non-modified CaM were employed as regulatory devices, to dependently control Ras/Modified Ras with calcium ions as an activating factor. Firstly, CaM with GAP binding inhibitory peptide and M13 peptide, CaM-Gb-M13, was designed and prepared. The fusion protein exhibited H-Ras cycle ionoresponsive based regulation. Secondly, the modified Ras control part was designed by incorporating M13 peptide to the N-terminal and C-terminal of Ras to control by WT Calmodulin with Ca2+ as an activator.

Keywords: Ionoresponsive, Calmodulin, Small GTPase, Ras, GAP, GEF

1. introduction

Recent research reveals structural and functional similarities between kinesin and myosin motor domains, as well as between motor proteins and other nucleotide-binding proteins with phosphate loops. Previous Studies suggest that kinesin and myosin, as well as possibly G proteins, are likely connected through divergent development from a similar core nucleotide-binding motif. The topology of these proteins uses analogous chemical and physical mechanisms to sense nucleotide status in in the active site and send this information to protein partners.[1]

Chromic chemicals, including photochromic, thermochromic, ionoresponsive, electrochromic, and piezoceramic switches, are sensitive to color change or absorption[2]. This paper focuses on chromic compounds involving a protein with calcium-binding domains, which are negatively charged amino acid residues that interact with positively charged ions, causing a conformational shift in its structure called ionoresponsive.

Organic compounds like Metal-organic frameworks (MOFs) bridging ligands that link metal ions or clusters. are some examples of ionoresponsive compounds used in biochemistry, In our case, I focus on another ionoresponsive bionanodivice called calmodulin, a calcium-based nanoswitch that targets another peptide called M13 when calmodulin binds to 4 Ca^{2+}ions.

Calmodulin (CaM) plays an important role in signaling and regulating numerous calcium-dependent processes in almost all eukaryotic cells. The structure of CaM has been particularly conserved throughout eukaryotic evolution. Many of the target proteins of CaM enzymes activity is known to be stimulated by Ca^{2+}-dependent association with CaM[3,4];

Ca2+-dependent interaction with CaM activates enzymes like smMLCK, crucial for muscle contraction regulation. CaM, a 148 amino acid protein, has EF-hand motifs in two domains, with four EF-hands in CAM binding to four Ca2+. causes

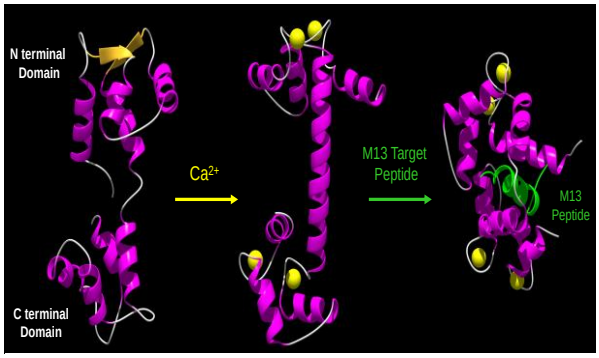

Fig. 1 Calmodulin based bionanodivice Calmodulin works as a Ca2+ -binding protein and is one of the key regulatory proteins of cell signal transduction pathways. Ca^{2+} /Calmodulin binds to the well-conserved binding region M13 in Calmodulin-dependent enzymes, resulting in the stimulation of the enzyme activities.

conformational changes within each EF hand[3,4]; this assumed conformation is significantly flexible, with exposed hydrophobic clefts available for target binding[7]. CaM target proteins have CaM-binding peptides that are relatively short, such as the M13 peptide and the IQ motif. The M13 peptide, which is part of the skeletal muscle myosin light chain kinase (skMLCK), has been

shown to form a 1:1 complex with Ca2+/CaM[8,9]

The H-Ras signaling system is essential for cellular proliferation and survival. This pathway is generally dysregulated in a variety of human diseases, including cancer. Highlighting the necessity of discovering an effective way to adjust H-Ras activity[10]. Through its interactions with their accelerators GTPase-activating proteins (GAPs), calmodulin (CaM), is a ubiquitous calcium-binding protein, has emerged as a crucial regulator of Ras signaling. through its interaction with GTPase-activating proteins (GAPs). negative regulator of Ras

by stimulating the hydrolysis of GTP to GDP, thus promoting

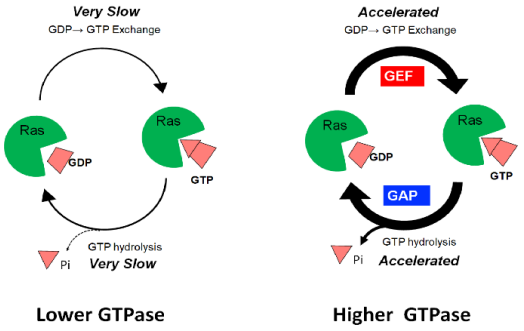

Fig. 2 Ras GTPase cycle H-RAS cycle in the presence of GAP and GEF shows an accelerated GTPas circle compared to ras in the absence of the regulators. The crucial role that GEF and GAP play in accelerating the Ras cycle involving GDP/GTP exchange is shown in this figure.

the inactivation of Ras and terminating downstream signaling[11]. However, the traditional approach of using exogenous GAPs to modulate Ras activity has faced challenges due to its limited efficacy and specificity[12]. Therefore, there is a need for alternative strategies to inhibit the H-Ras cycle with improved precision and potency. As a result, developing effective strategies to modulate H-Ras activity is crucial for therapeutic interventions.

In this study, as the first part, I propose an innovative approach to modulate the H-Ras cycle pathway by introducing a modified structure of calmodulin. I hypothesize that incorporating a GAP-like sequence within the calmodulin structure and incorporation of calmodulin target peptide into the functional sites of Ras regulatory proteins may enable direct competition with endogenous GAP proteins, leading to the inhibition of H-Ras signaling. For the second part. and knowing that WT Calmodulin (CaM) acts as an ionoresponsive molecule. The molecular mechanisms underlying the structural changes induced by calcium binding to target proteins have been clarified. It acts as a regulatory molecular device for proteins. The well-known CaM target-binding region will be introduced as a control device in our biomolecular machines using genetic engineering. Previously, we demonstrated that incorporating a CaM target peptide into the N-terminus of Ras facilitates the control of Ras function in the presence or absence of calcium ions with CaM[13]. In this study, we created Ras fusion proteins by incorporating a CaM target peptide at either the C-terminus or both N- and C-termini of Ras to efficiently control Ras function. Subsequently, we examined the ionoreversible control of Ras fusion proteins by CaM.

2 Material and methods

First part:

Protein design (CaM.Gb.M13).

expression and purification were expressed and purified as follows.

The fusion protein was synthesized by Funakoshi Coltd,

CLC sequence viewer 8.0 and Eurofins genomic were respectively used to design. and order The CaM.Gb.M13 and DNA sequence structures was modeled using Pymol software monitoring the crystal structures of CaM.

I constructed plasmid pET15b (Novagen, Madison, WI, USA), peptide sequence RWKKNFIAVSAANRFKKIS, with a C-terminal 6 His tag. E. coli BL21 (DE3) cells were transformed with the plasmid (pET15b: CaM.Gb.M13) for expression. (CaM.Gb.M13) was then purified starting with E. coli treatment, lysate by co-chelating chromatography followed by dialysis against 100 mM NaCl, 30 mM Tris–HCl (pH 7.5), 0.3 mM $MgCl_2$, 0.2 mM ATP, and 1 mM DTT. purified ant stored at -80 °C until use, to determine GTPase activity. H-Ras (2 μM) was preincubated for 5 minutes in the presence of 2 μM GAP coexisting and competing with our Gap-based inhibitor (CaM.Gb.M13 or CaM.M13.Gb) in a concentration of 5μM in the presence of Ca^{2+} ($CaCl_2$ 0.5mM) and in the absence of Ca^{2+} (EGTA 0.5mM) and GEF 2 μM in GTPase activity assay buffer (30 mM Tris-HCl pH 7.5, 60 mM NaCl, 2 mM $MgCl_2$). Then, 1 mM GTP was added to start the GTPase assay at 25 °C for 30 min before stopping with 10% trichloroacetic acid (TCA). BioMol Green Reagent was added to the supernatant and, after centrifugation at 15000 rpm for 5 min at 4 °C, incubated at 25 °C for 30 min to determine the amount of Pi produced by GTP hydrolysis.

Second part:

HRas catalytic domain (1–166 aa) at the N-terminus and M13 peptide at the C-terminus were connected

with a GS linker (LESGGSGGGS). The designed DNA sequence was synthesized by IDT. The synthesized HRas-M13 DNA was inserted into the MCS of pET21a using BamHI and XhoI and

prepared using DH5α. M13-HRas-M13 was prepared using HRas-M13 as a template, and M13-HRas was prepared using M13-HRas-M13 as a template.

Next, the cDNA of the GST-cRaf RBD plasmid, kindly provided by Dr. Sako (RIKEN), was amplified via polymerase chain reaction and ligated into the pET42c vector. CaM, HRas (1–166 aa), GEF (Sos cat; 564–1049 aa), and GAP (NF1 GRD domain; 1195–1528 aa) cDNAs were prepared, as previously described[15, 16, 17]

GTPase assay in the presence of GEFs and GAPs GTPase activity was determined as previously described. H-Ras (2.5 μM) or HRas fusion proteins with M13 (2.5 μM), CaM (5 μM), and CaCl2/EGTA (0.5 mM) in the GTPase activity assay buffer (30 mM Tris-HCl, 120 mM NaCl, and 2 mM MgCl2, pH 7.5) were pre-incubated in the presence of 2.5 μM GEF and GAP for 10 min. Subsequently, 1 mM GTP was added to initiate the GTPase assay at 25 ℃ for 30 min, and the reaction was stopped by adding 10% trichloroacetic acid. After 5-min centrifugation at 17360 × g at 4 °C, the supernatant was mixed with the BioMol Green Reagent and incubated at 25 °C for 30 min to quantitatively determine the amount of Pi generated by GTP hydrolysis

.3 Results and discussion

3-1 Ionoresponsive regulation using CaM fused with inhibitory peptide and M13 peptide.

I designed the two CaM fused with inhibitors to show which conformation is more responsive in terms of controlling the H RAS cycle. The linker SG was enlarged, giving more flexibility to the calmodulin/M13 target peptide and allowing a larger binding angle. Subsequent GTPase results will provide further details.

In accordance with the data previously published, Structural and biochemical studies show that the residues of GAP are involved in the GTPase reaction of Ras, supporting the arginine finger theory. The neutralization of an increasing charge in transition state mechanisms is required for faster phosphoryl transfer.[14]

The expected mechanism shows Ras cycle control by CAM-Gb-M13 Gap competition avoiding GTP to GDP exchange
I can deduce that the H-Ras Gap interaction will be less

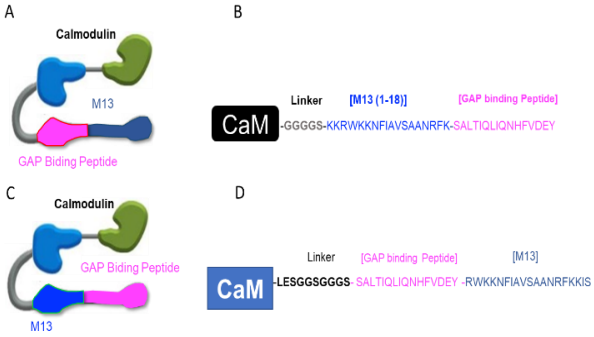

Fig. 3 DNA Encoding peptide sequence: (A) represents CaM.Gb.M13 with a shorter GS linker and (C) represents CaM.M13. Gb with a larger SG linker for better flexibility and an inverted Gb/M13 position compared to A. The modification is formed by four starting with the calmodulin (black (B)/ blue (D)) parts, the linker (grey (B) /black(D)), the Gap binding peptide (pink B and D), and the calmodulin target peptide M13 as represented in the figure, different colors were used to distinguish the two nanodevices.

consistent due to the competitivity, H-RAS-Gap and Gap CAM-Gb-M13 leading to a potential inhibitory control of the H-Ras cycle as shown in figure 4.

I investigated the complicated regulation of the H-Ras cycle consequently I used a two-pronged approach: first, I regulated the H-Ras/Gap interface, and then I manipulated the modified H-Ras with a double M13 incorporation, as described in the Materials and Methods section.

Our research into the H-Ras/Gap interaction taught us important insights into how I might manipulate this interaction, possibly altering Ras signaling pathways.

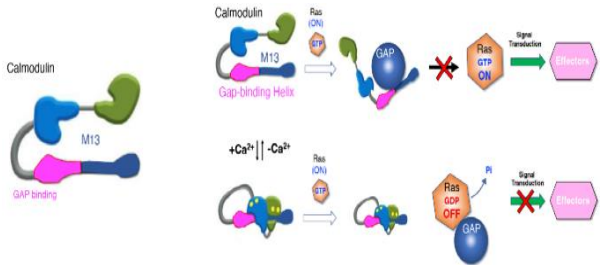

Fig4 Representing the CAM-Gb-M13 bionanodevice: showing the expected mechanism for RAS activity inhibition. The inhibitor structure is represented by the GAP binding part (pink) and the calmodulin attached with a linker (grey) to the Inhibitor and M13 CAM target protein (dark blue)

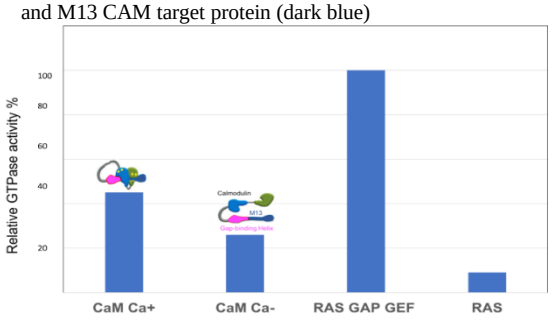

Fig5 GTP Hydrolysis inhibition of H-RAS in the presence and absence of CAM-Gb-M13, 5µM A) GTPase was inhibited in the condition of 2µM H-Ras with the presence of 2µM GAP and 2µM GEF by 5µM CAM-Gb-M13 in active form Ca- (absence of Ca2+) and a little less inhibition in its inactive form Ca+ (presence of Ca2+) in both cases in the presence of Ras and GEF at 25C absorption of 630 nm. H-RAS cycle in the presence of GAP and GEF shows an accelerated GTPas circle compared to Ras in the absence of the regulators, however, the incorporation of our CAM-Gb-M13 showed a considerate decrease in the RAS activity in the presence of

These discoveries are significant because they shed insight into our capacity to alter crucial Ras interactions, potentially opening the door to therapeutic approaches and a better

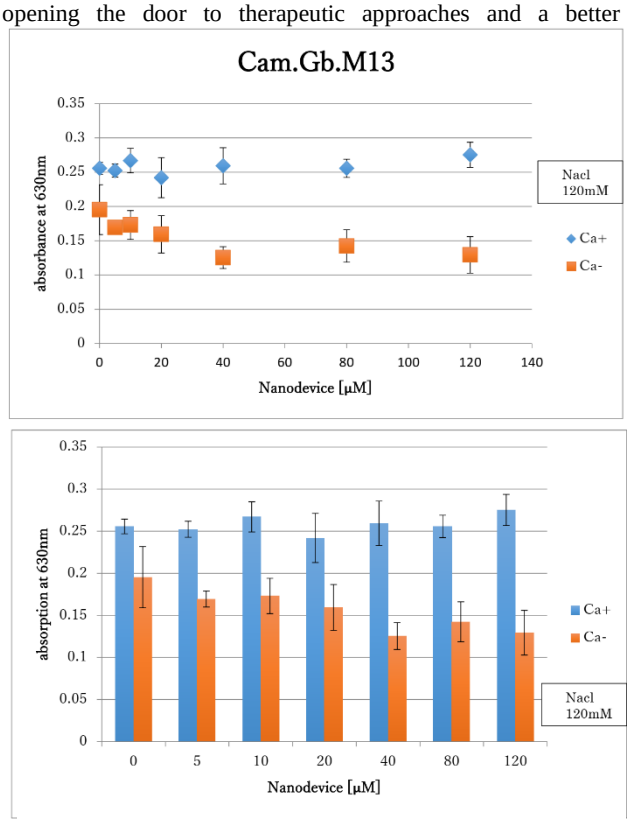

Fig. 6 Modulation of H-Ras GTPase Activity by CAM.Gb.M13: in Response to NaCl Concentration fixed in 120mM This figure illustrates the effective control of H-Ras GTPase activity achieved through modulation of NaCl concentrations showed by two graphs while maintaining a constant concentration of 120 mM. The ionoresponsive control is successfully demonstrated, emphasizing the direct effect of the NaCl on the RAS cycle. The first Graph showing a dose dependent response and the second Barr graph showing the response of CAM-Gb-M13 in active form Ca- (absence of Ca2+) and its inactive form Ca+ (presence of Ca2+) in both cases in the presence of Ras and GEF at 25C absorption of 630 nm. Showing a peak inhibition in the presence of CAM.Gb.M13 at 40µM and 120µM of the same previous conditions 2µM GAP and 2µM GEF in the presence of Ca2+

understanding of Ras-related cellular processes. Accelerated by Gap and allowing a potential H-Ras cycle control.

In Figure 5 the results of the GTPase assay in the presence and absence of Ca2+ showed inhibition of more than 70% in the absence of Ca2+, and surprisingly, the Ras cycle was inhibited even in the presence of Ca2+ but slightly more than CAM -Gb-M13 in the absence of Ca2+, which prompted us to investigate more about the structure and mechanism.

To better understand the phenomenon, I proposed a modification of the structure by using Gb (gap-binding peptide) M13 peptide and decreasing the size of the GS linker for more accuracy. We think that cross-reactivity occurred between two CAM -Gb-M13, resulting in irregular interaction and H-Ras cycle control. On the other hand, I proceeded to control the sodium chloride concentration since it was revealed in previous work its importance and influence in the experiment (Rufiat et al.,2021). After obtaining our second linker CAM -M13-Gb, I proceeded to the GTPase assay experiment, and the GTPase assay under the new conditions was measured. The enzymatic activity and dose dependence in Figure 6 shows the successful ionoresponsive control after modification

This time we observed an improved result with an evident calcium-dependent inhibition and ionoresponsive control.it is accurate to state that the stability of our compound increases however, the free CAM.Gb.M13 and Calcium ions in the solution may interfere into the perfect control of the GTPase activity.

3-2 double incorporation of M13 peptide into the HRas to control by WT Calmodulin

The study focuses on the design and preparation of three fusion proteins, M13-HRas, HRas-M13, and M13-HRas-M13, which use calcium binding to control the function of HRas. The CaM target peptide M13 is linked to the HRas catalytic domain, without the HVR domain. This allows for reversible CaM binding in the presence or absence of calcium ions, mimicking the function of HVR and controlling interaction with GEF.

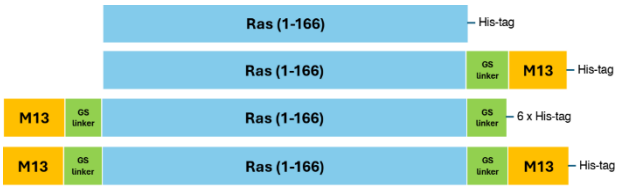

Fig.7. Design of recombinant HRas fusion proteins with M13. Primary structures of the HRas fusion proteins with M13, M13-HRas, HRas-M13, and M13-HRas-M13, in which the calmodulin (CaM) target peptide M13 is linked to the N-terminus, C-terminus, and both termini of the catalytic domain (1–166), excluding the HVR domain, of HRas, respectively.

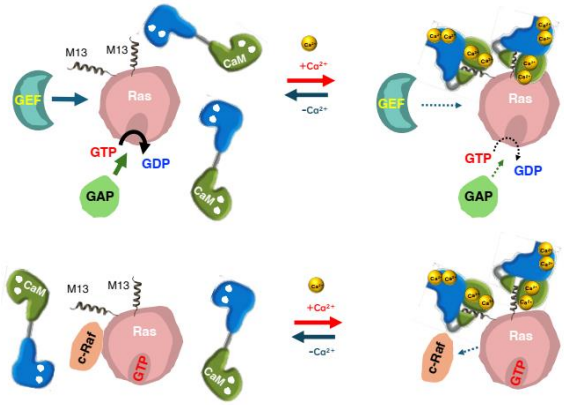

Fig.8. The graphical representation of the modification of HRAS with the M13 peptide at both N- and C-terminals (M13-HRAS-M13) and at the C-terminal only (HRAS-M13), replacing wild-type HRAS (HRAS WT). These modifications enable calcium ion control of Ras function via calmodulin (CaM). The introduction of the M13 peptide at the N- and C-termini near the functional site of Ras allows for the genetic engineering of biomolecular machines. Allows the control of The modified HRAS with the presence of GEF and GAP factors, which regulate the HRAS cycle, to be blocked by CaM intervention, preventing CRaf binding and subsequent downstream operations.

We hypothesized that introducing this mechanism into the functional site of the small GTPase Ras allows the calcium ion control of Ras function. CaM and its target peptides can be introduced into the desired biomolecular machines using genetic engineering techniques. Here, we demonstrated that Ras functions can be controlled by introducing the CaM target peptide M13 into the N- and C-termini near the functional site of Ras.

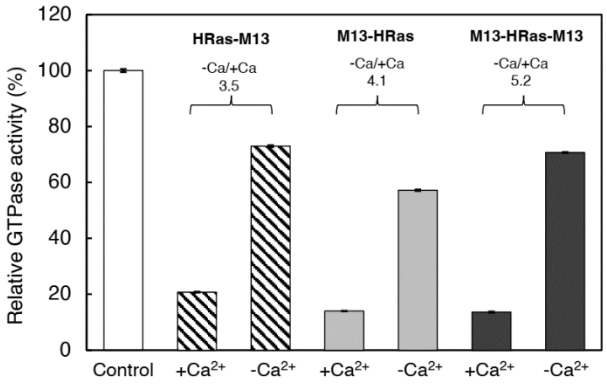

Fig.9. Calcium ion control of GTPase activity of the HRas fusion proteins. GTPase activities of wild-type HRas (1–166) and HRas fusion proteins (HRas-M13, M13-HRas, and M13-HRas-M13) were measured as described in the Experimental Procedures section. Briefly, 2.5 μM of HRas fusion protein with 2.5 μM CaM for HRas-M13 and with M13-HRas and 5 μM CaM for M-13-HRas-M13 were reacted with 1 mM GTP in 30 mM Tris-HCl (pH 7.5) buffer containing 120 mM NaCl, 2 mM MgCl2, and 1 mM GTP in the presence of 0.5 mM CaCl2 or EGTA. The ratios of GTPase activity in the absence and presence of Ca2+ (-Ca/+Ca) were 3.5, 4.1, and 5.2 for HRas-M13, M13-HRas, and M13-HRas-M13 respectively.

The study examined the mechanism by which the GTPase activity of HRas changes with the ionic regulation of CaM binding. Fig. 6, in the absence of calcium ions, GTPase activities of the three M13-Ras fusion proteins, HRas-M13 (73%), M13-HRas (57%), and M13-HRasM13 (71%), were lower than that of the control Ras (1–166). This suggests that interactions with GAPs and GEFs are affected by the presence of M13 at the N- or C-terminus. In the presence of calcium ions, GTPase activity was significantly inhibited by CaM bindingThe GTPase activities of three M13-Ras fusion proteins were assessed in the presence of regulatory factors, GAP and GEF. In the absence of calcium ions, GTPase activities were lower than that of the control Ras. This suggests that interactions with GAPs and GEFs are affected by the presence of M13 at the N- or C-terminus. In the presence of calcium ions, GTPase activity was significantly inhibited by CaM binding. The Ras fusion protein with M13 introduced at both the N- and C-termini, M13-HRas-M13, exhibited the most efficient ionoresponsive control. The study also measured the concentrationdependent GTPase activities of GEF and GAP. GAP affinity for the Ras-M13 fusion protein was not significantly affected by CaM binding, but GEF affinities for M13-HRas-M13 and M13-HRas proteins were significantly decreased by CaM binding.

Conclusion

The bio nanodevice CAM.Gb. M13 showed regular Ras cycle control exhibiting Ras control, nonetheless, our modification of the SG linker added more stability and showed better performance although the calcium dependency seems to be inverted and requires more experiments to clarify the findings.

On the other part of this research demonstrated that the functions of the small GTPase Ras, a molecular machine involved in intracellular signal transduction, can be reversibly controlled by calcium ions using WT CaM as an ion-responsive control device. Our approach can be used to introduce stimulus-responsive control devices into biomolecular machines specific to their unique mechanisms to facilitate artificial control.

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