Single-molecule view of basal activity and activation mechanisms of the G protein-coupled receptor β_2AR

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Contributed by Kurt Wüthrich, October 9, 2015 (sent for review August 16, 2015; reviewed by W. E. Moerner and David Rueda)

Binding of extracellular ligands to G protein-coupled receptors (GPCRs) initiates transmembrane signaling by inducing conformational changes on the cytoplasmic receptor surface. Knowledge of this process provides a platform for the development of GPCR-targeting drugs. Here, using a site-specific Cy3 fluorescence probe in the human β_2-adrenergic receptor (β_2AR), we observed that individual receptor molecules in the native-like environment of phospholipid nanodiscs undergo spontaneous transitions between two distinct conformational states. These states are assigned to inactive and active-like receptor conformations. Individual receptor molecules in the apo form repeatedly sample both conformations, with a bias toward the inactive conformation. Experiments in the presence of drug ligands show that binding of the full agonist formoterol shifts the conformational distribution in favor of the active-like conformation, whereas binding of the inverse agonist ICI-118,551 favors the inactive conformation. Analysis of single-molecule dwell-time distributions for each state reveals that formoterol increases the frequency of activation transitions, while also reducing the frequency of deactivation events. In contrast, the inverse agonist increases the frequency of deactivation transitions. Our observations account for the high level of basal activity of this receptor and provide insights that help to rationalize, on the molecular level, the widely documented variability of the pharmacological efficacies among GPCR-targeting drugs.

signal transduction mechanisms | agonists and inverse agonists | conformational polymorphism | single-molecule fluorescence spectroscopy | phospholipid nanodiscs

G protein-coupled receptors (GPCRs) mediate a multitude of physiological functions and are the targets for a myriad of drugs (1), many of which elicit different functional outcomes through the same receptor (2). It remains to be rationalized at the molecular level why some drugs stimulate the signaling activity of a GPCR (full or partial agonists), whereas others either repress the receptor (inverse agonists) or have no effect on the intrinsic signaling activity (neutral antagonists). Moreover, the existence of a high basal activity of some GPCRs (3) suggests that the conformational transitions leading to activation may occur spontaneously, even in the absence of ligands, which in turn raises questions about the mechanistic roles of GPCR ligands. Understanding the mechanisms and pathways of receptor activation or deactivation, and how these are linked to the binding of ligands with different chemical structures and pharmacological efficacies, will aid in design of new GPCR-targeted drugs with tailored pharmacological responses and fewer side effects. To attain these goals, new methods are required to visualize the conformational dynamics of GPCRs in the presence and absence of drugs.

The β_2-adrenergic receptor (β_2AR) has been extensively investigated in crystals (4, 5), by NMR in solution (6–11), by bulk fluorescence spectroscopy in solution (12–14) and in cells (2), by single-molecule fluorescence spectroscopy (15–17), and by molecular dynamics simulations (18). Despite the availability of high-resolution crystal structures of β_2AR in inactive (4) and active (5) conformations, it remains unknown how ligands regulate transitions between the two states and why β_2AR exhibits a significant level of ligand-independent, basal signaling activity. To address these questions, we use single-molecule fluorescence spectroscopy to monitor activation-linked conformational transitions of individual receptor molecules in real time over extended time periods. Our results highlight the intrinsically dynamic character of β_2AR and provide insights into the mechanism of receptor activation and the roles of β_2AR ligands.

Results

To visualize individual human β_2AR molecules, we labeled the receptor with a bright fluorescent dye (Cy3) and reconstituted the receptor in phospholipid nanodiscs, under conditions that favor incorporation of just a single receptor per nanodisc (19). The receptor–nanodisc complexes were tethered to a quartz surface and monitored over time for an average period of 70 s by total internal reflection fluorescence (TIRF) microscopy (Fig. 1*A*). The Cy3 label was attached to Cys265 near the cytoplasmic end of helix VI (Fig. 1*B*). Comparison of crystal structures of β_2AR in inactive (4) and active (5) states reveals that the cytoplasmic end of helix VI moves outward by 14 Å and rotates during receptor activation, a conformational change that is expected to alter the fluorophore

Significance

Activation of G protein-coupled receptors (GPCRs) by agonists is the first step of eukaryotic cellular signal transduction. Because GPCRs are expressed in almost all human tissues and play a key role in human physiology, they are the targets for more than 30% of pharmaceutical drugs. Binding of ligands on the extracellular surface of a GPCR induces a conformational change on the cytoplasmic surface, which is recognized by G proteins or other cellular effectors. Here we show that the β_2-adrenergic receptor, a prototypical GPCR, naturally fluctuates between inactive and active conformations, and that agonist or inverse agonist ligands modulate the conformational exchange kinetics in distinct ways, explaining their different pharmacological efficacies. These insights should assist in the design of improved GPCR-targeting drugs.

Author contributions: R.C.S., K.W., and D.P.M. designed research; R.L., J.J.L., G.P., K.L.W., E.v.d.S., and V.K. performed research; J.J.L., G.P., K.L.W., E.v.d.S., and V.K. contributed new reagents/analytic tools; R.L., K.L.W., and D.P.M. analyzed data; and R.L., K.L.W., V.K., R.C.S., K.W., and D.P.M. wrote the paper.

Reviewers: W.E.M., Stanford University; and D.R., Imperial College London.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1519626112/-/DCSupplemental.

Cell Calcium 57 (2015) 1–13

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Combined single channel and single molecule detection identifies subunit composition of STIM1-activated transient receptor potential canonical (TRPC) channels

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ARTICLE INFO

Article history:
Received 15 October 2014
Received in revised form 19 October 2014
Accepted 21 October 2014
Available online 31 October 2014

Keywords:
STIM1
TRPC
Store-operated channels
Single channel recording
Single molecule detection

ABSTRACT

Depletion of intracellular calcium ion stores initiates a rapid cascade of events culminating with the activation of the so-called Store-Operated Channels (SOC) at the plasma membrane. Calcium influx via SOC is essential in the initiation of calcium-dependent intracellular signaling and for the refilling of internal calcium stores, ensuring the regeneration of the signaling cascade. In spite of the significance of this evolutionary conserved mechanism, the molecular identity of SOC has been the center of a heated controversy spanning over the last 20 years. Initial studies positioned some members of the transient receptor potential canonical (TRPC) channel superfamily of channels (with the more robust evidence pointing to TRPC1) as a putative SOC. Recent evidence indicates that Stromal Interacting Molecule 1 (STIM1) activates some members from the TRPC family of channels. However, the exact subunit composition of TRPC channels remains undetermined to this date. To identify the subunit composition of STIM1-activated TRPC channels, we developed novel method, which combines single channel electrophysiological measurements based on the patch clamp technique with single molecule fluorescence imaging. We termed this method Single ion Channel Single Molecule Detection technique (SC-SMD). Using SC-SMD method, we have obtained direct evidence of the subunit composition of TRPC channels activated by STIM1. Furthermore, our electrophysiological-imaging SC-SMD method provides evidence at the molecular level of the mechanism by which STIM1 and calmodulin antagonize to modulate TRPC channel activity.

1. Introduction

Calcium influx is a key process responsible for initiating a wide variety of cellular functions. In the phenomenon known as Store-Operated Calcium Entry (SOCE) [1], the influx of calcium is triggered by the release of calcium from intracellular calcium storage compartments (essentially, the Endoplasmic Reticulum (ER)) [1]. The channel at the plasma membrane involved in SOCE is known as Store-Operated Channel (SOC). Given the significance of this highly conserved mechanism, there has been an intense search for the molecular identity of SOC, which started over twenty years ago [1].

The initial identification of a novel family of cationic channels, named Transient Receptor Potential (TRP) channels, demonstrating also that some of them were activated by store depletion, positioned some members from this superfamily of channels as potential SOC [2]. However, experimental evidence accumulated over the years has shown conflicting results regarding the mode of activation of TRP channels and their putative role in SOCE (for a review see [3,4]).

Linkage mapping by genome-wide SNP array screening from 23 patients suffering hereditary severe combined immune deficiency syndrome, identified a novel family of channels, named Orai, as responsible for deficient SOCE in T lymphocytes from these

Abbreviations: TRPC, transient receptor potential canonical channel family; SOC, store-operated channel; SOCE, store-operated calcium entry; SOAR, STIM Orai activating region; STIM1, stromal interacting molecule 1; PO, open probability; STIM1-CT, STIM1 cytoplasmic region; SMFI, single molecule fluorescence imaging; SC-SMD, single ion channel single molecule detection; CaM, calmodulin.

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http://dx.doi.org/10.1016/j.ceca.2014.10.011
0143-4160/

A Cholesterol Recognition Amino Acid Consensus Domain in GP64 Fusion Protein Facilitates Anchoring of Baculovirus to Mammalian Cells

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***Baculoviridae* is a large family of double-stranded DNA viruses that selectively infect insects. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the best-studied baculovirus from the family. Many studies over the last several years have shown that AcMNPV can enter a wide variety of mammalian cells and deliver genetic material for foreign gene expression. While most animal viruses studied so far have developed sophisticated mechanisms to selectively infect specific cells and tissues in an organism, AcMNPV can penetrate and deliver foreign genes into most cells studied to this date. The details about the mechanisms of internalization have been partially described. In the present study, we have identified a cholesterol recognition amino acid consensus (CRAC) domain present in the AcMNPV envelope fusion protein GP64. We demonstrated the association of a CRAC domain with cholesterol, which is important to facilitate the anchoring of the virus at the mammalian cell membrane. Furthermore, this initial anchoring favors AcMNPV endocytosis via a dynamin- and clathrin-dependent mechanism. Under these conditions, efficient baculovirus-driven gene expression is obtained. In contrast, when cholesterol is reduced from the plasma membrane, AcMNPV enters the cell via a dynamin- and clathrin-independent mechanism. The result of using this alternative internalization pathway is a reduced level of baculovirus-driven gene expression. This study is the first to document the importance of a novel CRAC domain in GP64 and its role in modulating gene delivery in AcMNPV.**

Most viruses have developed, over many years of evolution, sophisticated mechanisms to internalize in the host and take control of the protein synthesis machinery in order to generate thousands of new progeny viruses. Not surprisingly, most viruses possess highly selective mechanisms for internalization into the host cell (1). Many such mechanisms involve the participation of a specific receptor at the host cell surface. These selective internalization mechanisms result in the tropism shown by most viruses toward specific tissues (2).

Baculoviridae is a large family of viruses that selectively infect insects (3). These double-stranded circular DNA viruses possess rod-shaped capsids, giving the family its name. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the best-studied baculovirus. AcMNPV has been extensively used as an efficient gene expression vector in insect cells for massive protein production (4, 5). Since the initial studies conducted many years ago, it is well established that AcMNPV can transduce mammalian cells with a suitable promoter (6).

Unlike most viruses studied so far, most interestingly, AcMNPV can enter a wide variety of cells from different organisms and drive the expression of foreign genes under the control of mammalian promoters (7, 8, 9).

The fact that baculovirus can enter a wide variety of mammalian cells poses interesting questions. For instance, is AcMNPV using the same mechanism to enter insect and mammalian cells? Is there a receptor involved in this process? Is the same putative receptor present in insects and mammals?

In spite of many years of baculovirus research and dozens of studies showing baculovirus-driven gene expression in mammalian cells, these conundrums remain unsolved to date.

In insect cells, the glycoprotein GP64, a major envelope fusion protein, is essential for virus budding from the cells, virus internalization into a new host cell, and virus escape from the early endosome inside the infected insect cell (10).

Different studies have demonstrated that GP64 is essential for baculovirus transduction of mammalian cells (11). In mammalian cells, it has been shown that electrostatic interactions, heparan sulfate, and phospholipids are necessary for baculovirus binding to the mammalian cell surface (12).

An elegant recent study highlights the role of cholesterol- and dynamin-dependent endocytosis as the mechanism for virus internalization into mammalian cells (13). However, other studies have shown that baculovirus internalization into mammalian cells also involves dynamin-independent macropinocytosis (14, 15). Many studies, however, have positioned GP64 as an essential element for virus internalization into mammalian cells (13).

Baculovirus GP64 forms trimers in its pre- and postfusion states, similar to vesicular stomatitis virus (VSV) G and herpes simplex virus type 1 (HSV-1) gB glycoproteins. All three proteins resemble each other, belonging to the domain III fusion proteins (16).

Received 23 May 2013 **Accepted** 22 August 2013
Published ahead of print 28 August 2013
Address correspondence to Luis Vaca, lvaca@ifc.unam.mx.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.01356-13.

doi:10.1128/JVI.01356-13

Hindawi Publishing Corporation
ISRN Cell Biology
Volume 2013, Article ID 237192, 13 pages
http://dx.doi.org/10.1155/2013/237192

Review Article

A Microscopic View of the Store-Operated Calcium Entry-Pathway

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Received 30 August 2013; Accepted 24 September 2013

Academic Editors: S. Bruzzone and M. Estrada

Orai and STIM are the basic components of a highly complex and regulated mechanism for Ca^{2+} entry into the cell, known as store-operated calcium entry (SOCE). The activation of plasma membrane G-protein-coupled receptors associated with the phospholipase C cascade results in the rapid and massive production of inositol 1,4,5-triphosphate (IP_3). This second messenger triggers the massive efflux of Ca^{2+} from the endoplasmic reticulum and into the cytosol, resulting in the oligomerization of the stromal interacting molecule (STIM1), a sensor of ER Ca^{2+}. STIM1 oligomers (the so-called puncta) activate Orai channels at the plasma membrane, triggering the influx of Ca^{2+} into the cytosol. Several microscopy techniques have been implemented to study SOCE, resulting in stunning images of protein complexes assembling in real time. However, little attention has been paid to the findings about this complex mechanism from the imaging point of view, some of which appear to produce contradictory results. In the present review we gathered all the information about SOCE obtained with imaging techniques and contrast these findings with those obtained with alternative methods.

1. Introduction

Calcium (Ca^{2+}) is a key and ubiquitous second messenger, controlling a wide variety of cellular functions from cell proliferation to apoptosis [1]. Hence, Ca^{2+} signaling has to be highly regulated both in time and space [2]. To accomplish this task, cells rely on numerous pumps and channels, comprising the machinery to generate and regulate spatial-temporal Ca^{2+} signals [3].

There are two principal Ca^{2+} sources in the cell: (i) the extracellular medium and (ii) the intracellular stores, most notably the endoplasmic reticulum (ER), which plays a central role in Ca^{2+} homeostasis, not only as the major intracellular store, but also as the controller of the cytosolic entry of Ca^{2+} across the plasma membrane (PM). The modulation of calcium entry from the ER begins when phospholipase C (PLC) is activated by G-protein-coupled receptors on the cell surface, leading to the generation of the second messenger inositol 1,4,5-trisphosphate (IP_3), which releases Ca^{2+} from the ER after binding the IP_3 receptor (IP_3R) located at the ER membrane [4, 5]. IP_3R activation results in the massive efflux of Ca^{2+} from the ER and into the cytosol. This decrease of ER-Ca^{2+} content triggers the influx of extracellular Ca^{2+} via plasma membrane Ca^{2+} channels in a process known as store operated calcium entry (SOCE) [4].

The importance of SOCE in the immune system has been well established, where it plays a pivotal role in B- and T-cell activation, by triggering antigen recognition through activation of the transcription factor, NFAT [6, 7]. The molecular identities of the proteins involved in the SOCE were discovered recently in 2005, through a strategy of large-scale RNAi-based screening. dSTIM was initially identified in *Drosophila* as well as its human homologue STIM1 [8]. One year later Orai was identified almost simultaneously by a wide-genome screen strategy and by linkage analysis with single nucleotide polymorphism arrays in patients with a form of hereditary severe combined immune deficiency (SCID) [9, 10].

In the last seven years there have been major advances in our understanding of SOCE at the molecular level. The mechanism of gating and regulation of the Orai channel has been almost elucidated; inter- and intra-molecular dynamic

Cell Calcium 54 (2013) 246–256

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

A relay mechanism between EB1 and APC facilitate STIM1 puncta assembly at endoplasmic reticulum–plasma membrane junctions☆

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ARTICLE INFO

Article history:
Received 9 April 2013
Received in revised form 19 June 2013
Accepted 21 June 2013
Available online 18 July 2013

Keywords:
STIM1
EB1
APC
SOCE
Orai

ABSTRACT

The assembly of STIM1 protein puncta near endoplasmic reticulum–plasma membrane (ER-PM) junctions is required for optimal activation of store-operated channels (SOC). The mechanisms controlling the translocation of STIM1 puncta to ER-PM junctions remain largely unknown.

In the present study, we have explored the role of the microtubule binding protein adenomatous polyposis coli (APC), on STIM1 puncta and store-operated calcium entry (SOCE). APC-depleted cells showed reduced STIM1 puncta near ER-PM junctions, instead puncta is found at the ER surrounding the cell nucleus. Reduced STIM1 puncta near ER-PM junctions in APC-depleted cells correlates with a strong inhibition of SOCE and diminished Orai whole-cell currents. Immunoprecipitation and confocal microscopy co-localization studies indicate that, upon depletion of the ER, STIM1 dissociates from EB1 and associates to APC. Deletion analysis identified an APC-binding domain in the carboxyl terminus of STIM1 (STIM1 650–685).

These results together position APC as an important element in facilitating the translocation of STIM1 puncta near ER-PM junctions, which in turn is required for efficient SOCE and Orai activation upon depletion of the ER.

1. Introduction

Store-operated calcium entry (SOCE) is the mechanism by which the depletion of intracellular calcium stores (essentially the endoplasmic reticulum or ER) is conveyed to a plasmalemmal calcium channel to trigger calcium influx from the extracellular space and into the cell cytosol [1].

SOCE is a widespread phenomenon present in the majority of (if not all) the mammalian cells studied so far. This calcium entry mechanism has been observed in nematodes, insects and mammals [2].

SOCE can be induced upon stimulation of G-protein coupled receptors associated to the inositol-triphosphate (IP3) and phospholipase C cascade, resulting in the release of calcium from ER, via the IP3 receptor (IP3R) [2]. The emptying of the ER initiates the dissociation of ER luminal calcium from the EF hand of the stromal interacting molecule (STIM1). This protein is a sensor of the calcium content in the ER [3].

The dissociation of calcium from the EF hand of STIM1, results in the oligomerization of this protein and the formation of the so-called STIM1 puncta [4].

Puncta formation occurs at junctions between the ER and the plasma membrane (named ER-PM junctions). The movement of STIM1 (upon depletion of the ER) toward ER-PM junctions is a poorly understood phenomenon, but necessary for the interaction between STIM1 and the plasma membrane calcium influx channel Orai, or between STIM1 and TRPC1 channels [5–8].

Recent studies have shown that STIM1 is a microtubule plus end tracking protein, involved in remodeling of the ER [9]. With the ER filled with calcium, STIM1 remains attached to end binding 1 (EB1) microtubule protein, tracking growing microtubules [6,9]. This moving of STIM1, pulling portions of the ER, plays a relevant role in the continuous remodeling the ER [9,10]. After depletion of the ER, STIM1 dissociates from EB1 and moves to the ER-PM junctions, forming the puncta described above. The final result is the activation of Orai channel at the plasma membrane, via a STIM1–Orai direct interaction [10]. STIM1 associates to and activates Orai via its SOAR (STIM1–Orai activating region) domain [11].

End binding protein 1 (EB1) is one of three members (EB1–EB3) from a family of proteins, which track the positive end of growing microtubules [12]. EB proteins interact with many partners

☆ This work was supported by grants from CONACyT 127822, DGAPA and ICyTDF to L.V.

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0143-4160/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ceca.2013.06.008

JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

Dynamics of Site Switching in DNA Polymerase

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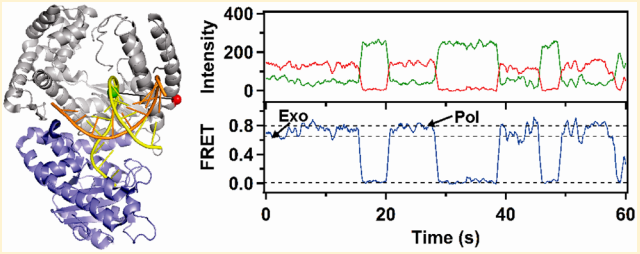
Supporting Information

ABSTRACT: DNA polymerases replicate DNA by catalyzing the template-directed polymerization of deoxynucleoside triphosphate (dNTP) substrates onto the 3′ end of a growing DNA primer strand. Many DNA polymerases also possess a separate 3′-5′ exonuclease activity that is used to remove misincorporated nucleotides from the nascent DNA (proofreading). The polymerase (pol) and exonuclease (exo) activities are spatially separated in different enzyme domains, indicating that a mechanism must exist to transfer the growing primer terminus from one site to the other. Here we report a single-molecule Förster resonance energy transfer (smFRET) system that directly monitors the movement of a DNA substrate between the pol and exo sites of DNA polymerase I Klenow fragment (KF). FRET trajectories recorded during the encounter between single polymerase and DNA molecules reveal that DNA can channel between the pol and exo sites in both directions while remaining closely associated with the enzyme (intramolecular transfer). In addition, it is evident from the trajectories that DNA can also dissociate from one site and subsequently rebind at the other (intermolecular transfer). Rate constants for each pathway have been determined by dwell-time analysis, revealing that intramolecular transfer is the faster of the two pathways. Unexpectedly, a mispaired primer terminus accesses the exo site more frequently when dNTP substrates are also present in solution, which is expected to enhance proofreading. Together, these results explain how the separate pol and exo activities of KF are physically coordinated to achieve efficient proofreading.



INTRODUCTION

DNA polymerases replicate DNA rapidly and with extraordinary accuracy. The high fidelity is a result of selection of the correct deoxynucleoside triphosphate (dNTP) substrate (complementary to the template base) during each cycle of template-directed polymerization, coupled with post-synthetic proofreading of misincorporated nucleotides by a separate 3′-5′ exonuclease activity.[1] Proofreading enhances the overall fidelity of DNA synthesis by a factor of 10−300, depending on the specific DNA polymerase and the nature of the primer terminal mispair.[2,3] The functional importance of proofreading is highlighted by observations that reduced 3′-5′ exonuclease activity of DNA polymerases is correlated with an increased mutation frequency *in vivo*.[4−6]

Structural studies of several DNA polymerases, including members of the A, B, and C polymerase families, indicate that the polymerase (pol) and 3′-5′ exonuclease (exo) activities are spatially separated in different enzyme domains,[7−12] raising the question of how they work together to achieve accurate and efficient DNA replication (Figure 1). Previous biochemical studies performed under conditions that enable observations of a single encounter between the polymerase and DNA substrate imply that the enzyme can switch from polymerization to 3′-5′ exonuclease activity without dissociation of the complex, suggesting that the primer 3′ terminus can channel between the respective active sites.[13−17] However, such channeling has not been directly observed within a single enzyme−DNA complex.

Here we describe a single-molecule Förster resonance energy transfer (smFRET) system that directly monitors movement of a DNA substrate between the spatially distinct pol and exo sites of DNA polymerase I Klenow fragment (KF). Our results reveal two distinct mechanisms for DNA site switching during proofreading. We have also quantified the microscopic rate constants for each transfer pathway and elucidated the influence of mismatch location within the DNA substrate on the switching kinetics. We further demonstrate that dNTPs specifically accelerate pol-to-exo switching of a mispaired DNA terminus, suggesting an expanded role for nucleotides in DNA polymerase function.

RESULTS

Detection of pol and exo Site Complexes by smFRET.

The smFRET system utilizes a primer/template duplex labeled with an Alexa-Fluor 488 (A488) donor dye in the primer strand (Table 1) and a KF construct labeled with an Alexa-Fluor 594 (A594) acceptor at a mutated residue (K550C) in the thumb subdomain (Figure 1). The KF construct also contains a C907S mutation to remove the single native cysteine and a D424A mutation that eliminates 3′-5′ exonuclease activity.[18] This

Received: November 28, 2012
Published: February 14, 2013

   dx.doi.org/10.1021/ja311641b | J. Am. Chem. Soc. 2013, 135, 4735−4742

COMPARTMENTALIZED SIGNALING

Direct Binding Between Orai1 and AC8 Mediates Dynamic Interplay Between Ca^{2+} and cAMP Signaling

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The interplay between calcium ion (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) signaling underlies crucial aspects of cell homeostasis. The membrane-bound Ca^{2+}-regulated adenylyl cyclases (ACs) are pivotal points of this integration. These enzymes display high selectivity for Ca^{2+} entry arising from the activation of store-operated Ca^{2+} (SOC) channels, and they have been proposed to functionally colocalize with SOC channels to reinforce crosstalk between the two signaling pathways. Using a multidisciplinary approach, we have identified a direct interaction between the amino termini of Ca^{2+}-stimulated AC8 and Orai1, the pore component of SOC channels. High-resolution biosensors targeted to the AC8 and Orai1 microdomains revealed that this protein-protein interaction is responsible for coordinating subcellular changes in both Ca^{2+} and cAMP. The demonstration that Orai1 functions as an integral component of a highly organized signaling complex to coordinate Ca^{2+} and cAMP signals underscores how SOC channels can be recruited to maximize the efficiency of the interplay between these two ubiquitous signaling pathways.

INTRODUCTION

Signaling via Ca^{2+} in nonexcitable cells is typically initiated by the inositol 1,4,5-trisphosphate (IP_3)–mediated release of Ca^{2+} from the endoplasmic reticulum (ER) Ca^{2+} stores in response to G protein (heterotrimeric guanosine triphosphate–binding protein)–coupled receptor stimulation of phospholipase C. Subsequent depletion of these stores triggers the entry of Ca^{2+} through store-operated Ca^{2+} (SOC) entry (SOCE) channels. SOCE, attributed to the activation of SOC channels within the plasma membrane, plays a critical role in the control of a number of cellular functions (*1*). Orai1, a 33-kD protein with four transmembrane domains found in numerous cell types, has been identified as the pore-forming subunit of SOC channels [including the well-characterized Ca^{2+} release–activated Ca^{2+} (CRAC) channel] (*2*–*4*). Clinically, mutations of the human *Orai1* gene have been linked to severe immunodeficiency and various myopathies (*5*). The highly Ca^{2+}-selective SOC channels, formed by a tetrameric assembly of Orai1 subunits (*6*–*8*), are activated by the ER Ca^{2+} sensor, stromal interaction molecule 1 (STIM1) (*9*, *10*), upon ER Ca^{2+} store depletion and clustering of STIM1 at junctions between the ER and the plasma membrane (*11*, *12*).

Of the nine membrane-bound adenylyl cyclases (ACs), the enzymes that convert adenosine triphosphate to cyclic adenosine monophosphate (cAMP), four isoforms respond to submicromolar Ca^{2+} concentrations in vitro (*13*). These Ca^{2+}-sensitive ACs are found in various tissues and modulate such processes as cardiac rhythmicity, pulsatile hormone release, and hippocampal memory formation (*14*–*16*). AC1 and AC8 are stimulated through an interaction with Ca^{2+}-calmodulin (CaM) (*17*–*21*), whereas AC5 and AC6 are directly inhibited by Ca^{2+} (*22*, *23*). In cells, Ca^{2+}-sensitive ACs show a marked selectivity for SOCE over other modes of increasing cytosolic Ca^{2+} (*24*, *25*), even in excitable cells, and they reside in cholesterol- and sphingolipid-enriched domains of the plasma membrane known as lipid rafts, unlike their Ca^{2+}-insensitive congeners (*1*, *26*).

The role of Orai1 and STIM1 in mediating the selective regulation of ACs has been demonstrated in cultured human embryonic kidney (HEK) 293, pancreatic, and colonic cell lines, where their overexpression potentiates the Ca^{2+}-dependent stimulation or inhibition of AC8 and AC6, respectively (*27*). STIM1-dependent Ca^{2+} store depletion has also been linked to AC activation, independently of any change in cytosolic Ca^{2+} (*28*). Data obtained with a genetically encoded Ca^{2+} sensor fused to AC8 (GCaMP2-AC8) indicated that the Ca^{2+}-stimulable AC8 resides in a discrete Ca^{2+} microdomain that experiences rapid fluctuations in Ca^{2+} during SOCE, but is shielded from other modes of Ca^{2+} increase (such as Ca^{2+} release from the ER or ionomycin-mediated Ca^{2+} entry) (*29*). These findings support the existence of a proposed functional colocalization between AC8, Orai1, and STIM1 in the plasma membrane (*25*, *27*).

Here, we used a multidisciplinary approach involving fluorescence resonance energy transfer (FRET), glutathione *S*-transferase (GST) pulldown, coimmunoprecipitation, and peptide array analyses to identify a direct protein-protein interaction between AC8 and Orai1. We used live-cell imaging with high-resolution AC-targeted Ca^{2+} and cAMP biosensors, together with small interfering RNA (siRNA) knockdown of Orai1, to demonstrate a role for Orai1 in generating dynamic changes in Ca^{2+} concentration within the AC8 microdomain to stimulate cAMP production. Comparisons were made with an N-terminally truncated form of AC8, 8M1, which was unable to bind Orai1 directly. Our data suggest that SOC channels can function as integral components of highly organized signaling complexes to facilitate the efficient and dynamic regulation of Ca^{2+}-dependent cAMP signaling events.

RESULTS

FRET reveals an association between AC8 and Orai1

We used FRET to assess direct, or indirect, interactions between fluorescently tagged AC8 and Orai1 constructs in plasma membrane regions

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JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

Single-Molecule Förster Resonance Energy Transfer Reveals an Innate Fidelity Checkpoint in DNA Polymerase I

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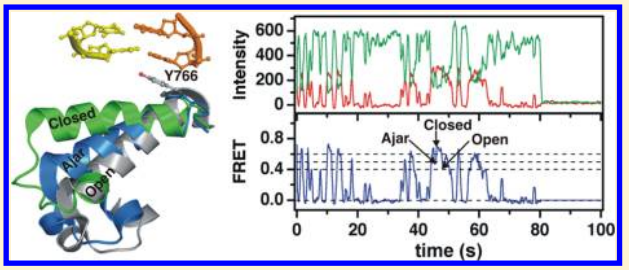
Supporting Information

ABSTRACT: Enzymatic reactions typically involve complex dynamics during substrate binding, conformational rearrangement, chemistry, and product release. The noncovalent steps provide kinetic checkpoints that contribute to the overall specificity of enzymatic reactions. DNA polymerases perform DNA replication with outstanding fidelity by actively rejecting noncognate nucleotide substrates early in the reaction pathway. Substrates are delivered to the active site by a flexible fingers subdomain of the enzyme, as it converts from an open to a closed conformation. The conformational dynamics of the fingers subdomain might also play a role in nucleotide selection, although the precise role is currently unknown. Using single-molecule Förster resonance energy transfer, we observed individual *Escherichia coli* DNA polymerase I (Klenow fragment) molecules performing substrate selection. We discovered that the fingers subdomain actually samples through three distinct conformations—open, closed, and a previously unrecognized intermediate conformation. We measured the overall dissociation rate of the polymerase−DNA complex and the distribution among the various conformational states in the absence and presence of nucleotide substrates, which were either correct or incorrect. Correct substrates promote rapid progression of the polymerase to the catalytically competent closed conformation, whereas incorrect nucleotides block the enzyme in the intermediate conformation and induce rapid dissociation from DNA. Remarkably, incorrect nucleotide substrates also promote partitioning of DNA to the spatially separated 3′−5′ exonuclease domain, providing an additional mechanism to prevent misincorporation at the polymerase active site. These results reveal the existence of an early innate fidelity checkpoint, rejecting incorrect nucleotide substrates before the enzyme encloses the nascent base pair.



INTRODUCTION

DNA replication is a finely tuned and precisely regulated process. High-fidelity DNA polymerases replicate DNA with an extremely low error rate by selecting the correct nucleotide substrate during each cycle of nucleotide incorporation. While the selection is based on complementarity of the incoming nucleotide and templating base, the high degree of accuracy that is achieved (1 error in 10^5 to 10^8 correct incorporations) significantly exceeds the fidelity threshold expected solely on the basis of the free energy difference between correct and incorrect base pairings.[1] Hence, DNA polymerases must actively contribute to the rejection of incorrect nucleotide substrates.

Rapid chemical kinetic studies have provided important mechanistic insights into the elementary steps in the nucleotide incorporation cycle and the origin of polymerase fidelity.[2−4] These studies have revealed the existence of one or more noncovalent steps, occurring after binding of deoxynucleoside triphosphate (dNTP) substrates and preceding the covalent step of phosphoryl transfer. The noncovalent steps appear to have a functional role in nucleotide selection, serving as kinetic checkpoints before the chemical step of nucleotide incorporation.[5]

DNA polymerases have an overall architecture that resembles a half-open human right hand, with fingers, palm, and thumb subdomains.[6] The fingers provide a binding site for dNTP substrates, while the palm and thumb grip the duplex DNA product. Crystal structures of DNA polymerases show that the fingers subdomain adopts an open conformation in binary complexes with DNA.[7,8] However, in structures of ternary complexes containing complementary nucleotide substrates, the fingers are observed in a closed conformation enveloping the nascent base pair.[7,8]

The open and closed conformations of DNA polymerases correspond to distinct functional states on the nucleotide insertion pathway. The open conformation allows binding of an incoming dNTP substrate, which is then delivered to the polymerase active site as the fingers switch to the closed conformation.[9−11] While in the closed conformation, the triphosphate group of the incoming nucleotide is aligned for in-line attack from the 3′-hydroxyl of the extending DNA strand, resulting in covalent incorporation of the nucleotide.

Received: April 20, 2012
Published: May 31, 2012

   dx.doi.org/10.1021/ja3038273 | J. Am. Chem. Soc. 2012, 134, 11261−11268

Biochimica et Biophysica Acta 1801 (2010) 147–155

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

A novel form of Total Internal Reflection Fluorescence Microscopy (LG-TIRFM) reveals different and independent lipid raft domains in living cells

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ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 8 October 2009
Accepted 12 October 2009
Available online 17 October 2009

Keywords:
TIRFM
Lipid raft
p75 receptor
Decay accelerating factor
Cholera toxin
Imaging

ABSTRACT

In the present study we have applied a novel form of Total Internal Reflection Fluorescence Microscopy (LG-TIRFM) in combination with fluorescently labeled cholera toxin to the study of lipid rafts dynamics in living cells. We demonstrate the usefulness of such approach by showing the dynamic formation/disaggregation of islands of cholera toxin on the surface of cells. Using multicolor LG-TIRFM with co-localization studies we show for the first time that two receptors previously identified as constituents of lipid rafts are found on different and independent "raft domains" on the cell plasma membrane. Furthermore, LG-TIRFM studies revealed limited association and dissociation of both domains overtime on different areas of the plasma membrane. The implications of different "raft domains" on cell physiology are discussed.

1. Introduction

The classical model postulated by Singer and Nicholson to explain how cell membranes are formed invoked a uniform lipid bilayer randomly studded with floating proteins (the so-called fluid-mosaic model) [1]. However, experimental data obtained over the last few years shows that this model is not accurate, and that clusters of lipids in a more ordered state exist within the generally disordered lipid milieu of the membrane (for review see [2]). These clusters of ordered lipids are now referred to as lipid rafts.

Lipid rafts are specialized membrane domains composed mainly of cholesterol and sphingolipids, and are relatively poor in poly-unsaturated lipids such as glycerophospholipids. This composition makes lipid rafts insoluble in non-ionic detergents at 4 °C, a condition that yields detergent-resistant membranes (DRMs).

Researchers have taken advantage of these properties to isolate DRMs and study the proteins present in these preparations. However, this type of studies provides limited information about protein function and dynamics, since they obtain information using isolated membranes mixed with detergents. There is also the question about the effect of using detergents on biological membranes, and how they may tamper the results obtained with DRMs.

Lipid rafts appear to be dynamic structures within the plasma membrane, and they play important roles in several aspects of cell physiology, including activation and protein sorting processes (for review see [3]). They function as microdomain signaling centers, and a key property of lipid rafts lies in their ability to include or exclude certain proteins to a variable extent. Recent studies even show that the same protein may have different functions inside and outside the raft, by coupling to different signaling mechanisms and effectors [4].

The development of new methodologies to approach the complex process of studying lipid rafts would provide relevant information on the function of lipid rafts within their biological milieu. Recent advances in high resolution imaging may provide avenues to utilize or develop such novel methodologies. Such is the case of, for instance, Total Internal Reflection Fluorescence Microscopy (TIRFM). TIRFM is a surface selective imaging technique that renders information only from a shallow area near the bottom of the dish where cells are plated (for review see [5,6]). In fact, the optical slicing in TIRFM can be as narrow as 50 nm.

Using TIRFM it might be possible to study the distribution of proteins in and out of lipid rafts in intact cells. Furthermore, multicolor TIRFM may provide information about association and dissociation complexes within the rafts, by monitoring different fluorescently tagged proteins simultaneously. One of the proteins that have been recently shown to associate almost exclusively with rafts is the beta subunit from the cholera toxin (β-ChTx) [7]. In fact, fluorescently

Abbreviations: TIRFM, Total Internal Reflection Fluorescence Microscopy; LG-TIRFM, lightguide-based TIRFM; TAC, TIRFM fiber optics adapter and beam conditioner; O-TIRFM, through-Objective TIRFM; β-ChTx, beta subunit from the cholera toxin; DAF, decay accelerating factor; p75, low affinity neurotrophin receptor

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doi:10.1016/j.bbalip.2009.10.004

Cell Calcium 45 (2009) 439–446

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Visualizing the store-operated channel complex assembly in real time: Identification of SERCA2 as a new member

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ARTICLE INFO

Article history:
Received 24 January 2009
Received in revised form 19 February 2009
Accepted 26 February 2009
Available online 26 March 2009

Keywords:
STIM1
Orai
SOC
SOCE
TIRFM

ABSTRACT

Depletion of intracellular calcium stores leads to the activation of calcium influx via the so-called store-operated channels (SOCs). Recent evidence positions Orai proteins as the putative channels responsible for this process. The stromal interacting molecule (STIM1) has been recently identified as the calcium sensor located at the endoplasmic reticulum (ER), and responsible for communicating the deplete state of calcium stores to Orai at the plasma membrane (PM). However, recent experimental findings suggest that Orai and STIM1 are only part of a larger molecular complex required to modulate store-operated calcium entry (SOCE). In the present study we describe the assembly of the several of the components from the SOC complex in real-time, utilizing a novel imaging method. Using FRET imaging we show that under resting conditions (with calcium stores replenished) STIM1 travels continuously through the ER associated to the microtubule tracking protein, EB1. Upon depletion of the ER STIM1 dissociates from EB1 and aggregates into macromolecular complexes at the ER which includes the microsomal calcium ATPase. This association follows the assembly of Orai into macromolecular aggregates at the PM. We show that STIM1–Orai association follows a similar time course as that of Orai aggregation at the PM. During this last step of the process, calcium-selective, whole-cell inward currents developed, simultaneously. We show that this process is fully reversible. Replenishing intracellular calcium stores induces STIM1–Orai complex dissociation and shuts down inward currents. Under these conditions STIM1 re-associates to EB1, and reinitiates its travel through the ER.

1. Introduction

Even though the role of Orai proteins as part of store-operated channels (SOCs) has been recently firmly established (for reviews see [1,2]), solid experimental evidence appears to indicate that these proteins are only part of a larger macromolecular complex, required for proper function of the store-operated calcium entry (SOCE) [3,4].

Another important member of the complex is the stromal interacting molecule (STIM1), a protein originally identified as a tumor suppressor [5], and most recently as the endoplasmic reticulum (ER) sensor responsible for communicating the deplete state of calcium stores to the plasma membrane channel complex [6,7]. However, STIM1 is also a microtubule (MT) tracking protein, which associates to the end binding protein (EB1) at the tip of the growing microtubule [8].

The role of STIM1 as a MT-tracking protein in relation to its function as a calcium sensor is puzzling. Using a novel method for multicolor, multi-FRET total internal reflection fluorescence microscopy (lgTIRFM) we have visualized in real-time the association–dissociation of several members from the store-operated channel complex (SOCC). We show that under resting conditions (with calcium stores replenished) STIM1 travels continuously through the ER associated to EB1. Upon store depletion, STIM1 dissociates from EB1 and associates to a novel member from the SOCC we identified here for the first time, the sarco-endoplasmic reticulum calcium ATPase (SERCA2A). The association between SERCA2A–STIM1 appears to be a slower event when compared to the association between STIM1–Orai. This later association, as previously shown, involves what it is been called "ER–PM junctions". This ER–PM association results in the aggregation of Orai into puncta, similar to those formed by STIM1 at the ER.

After replenishing calcium stores, STIM1 dissociates from the SERCA2A and re-associates to EB1, to initiate its constant

Abbreviations: STIM1, stromal interacting molecule 1; SOC, store-operated channel; SERCA, sarco-endoplasmic reticulum calcium ATPase; EB1, end binding protein 1; SOCE, store-operated calcium entry; TIRFM, total internal reflection fluorescence microscopy; SAFM, shallow angle fluorescence microscopy.

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doi:10.1016/j.ceca.2009.02.010

Sensors **2012**, *12*, 1800-1815; doi:10.3390/s120201800

OPEN ACCESS

http://www.mdpi.com/1424-8220/12/2/1800/
See video in Supplementary Materials

sensors
ISSN 1424-8220
www.mdpi.com/journal/sensors

Article

A Platform for Combined DNA and Protein Microarrays Based on Total Internal Reflection Fluorescence

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Received: 9 December 2011; in revised form: 13 January 2012 / Accepted: 2 February 2012 / Published: 9 February 2012

Abstract: We have developed a novel microarray technology based on total internal reflection fluorescence (TIRF) in combination with DNA and protein bioassays immobilized at the TIRF surface. Unlike conventional microarrays that exhibit reduced signal-to-background ratio, require several stages of incubation, rinsing and stringency control, and measure only end-point results, our TIRF microarray technology provides several orders of magnitude better signal-to-background ratio, performs analysis rapidly in one step, and measures the entire course of association and dissociation kinetics between target DNA and protein molecules and the bioassays. In many practical cases detection of only DNA or protein markers alone does not provide the necessary accuracy for diagnosing a disease or detecting a pathogen. Here we describe TIRF microarrays that detect DNA and protein markers simultaneously, which reduces the probabilities of false responses. Supersensitive and multiplexed TIRF DNA and protein microarray technology may provide a platform for accurate diagnosis or enhanced research studies. Our TIRF microarray system can be mounted on upright or inverted microscopes or interfaced directly with CCD cameras equipped with a single objective, facilitating the development of portable devices. As proof-of-concept we applied TIRF microarrays for detecting molecular markers from *Bacillus anthracis*, the pathogen responsible for anthrax.

Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry xxx (2006) xxx–xxx
Anal Biochem. 2006 Oct 15;357(2):159-66
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

A label-free continuous total-internal-reflection-fluorescence-based immunosensor

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Received 24 November 2005

Abstract

In this study, we continuously monitored, second-by-second, concentration changes of two different carbohydrates (maltose and panose) by using monoclonal antibodies in an optical immunosensor based on total internal reflection fluorescence. Earlier studies have demonstrated that these antibodies increase their intrinsic tryptophan fluorescence upon binding of carbohydrate antigens. Using the four immobilized monoclonal antibodies with low affinities ($K_d > 10^{-6}$ M), fast kinetics ($k_{off} > 1\ s^{-1}$), and high reversibility gave opportunities for developing a continuous immunosensor without any need for regeneration. Since intrinsic fluorescence was used, no extrinsic labeling was necessary. Sensitivity was in the range of 1–5 μM for panose, and 10–15 μM for maltose and the loss of intensity was as low as 3.5% per hour during measurements. Calculations of $\Delta H°$ and $\Delta S°$ from the temperature dependence of K_d indicated an enthalpic driven antigen–antibody binding event that is diminished upon antibody immobilization. We feel certain that weakly interacting antibodies can be used in future applications for continuous monitoring where there is a need to achieve instantaneous information on the concentration of an analyte.

Keywords: Biosensor; Carbohydrate; Evanescent wave; Fluorescence spectroscopy; Hapten; Immunosensor; Monoclonal antibody; Optical biosensor; Protein–carbohydrate interactions; Total internal reflection fluorescence; Transient binding; Weak affinity

Affinity-based biosensors are used to measure binding events using biological molecules such as antibodies, receptors, enzymes, or nucleic acids interfaced to a signal transducer [1]. Popular affinity-based ligands are antibodies and the resulting sensor is known as an immunosensor [2]. They can be categorized based on the detection principle, e.g., electrochemical [3] and optical [4] immunosensors. Commonly optical immunosensors utilize the evanescent wave to form the sensing device and different transducers can be used for creating an optical change, e.g., grating couplers [5], resonant mirror [6], surface plasmon resonance (SPR)[1] [7], interferometry [8], reflectrometric interference spectroscopy [9], ellipsometry [10], and total internal reflection fluorescence (TIRF) [11]. The specific binding in all these optical immunosensors is typically characterized by high affinity (dissociation constant (K_d) < 10^{-6} M) with slow off rates. The analysis is based on end-point determinations where the biosensor surface has to be regenerated to start a new measurement cycle. Assays can be very specific and sensitive enough to detect for example trace components in

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[1] *Abbreviations used:* a.u., arbitrary unit; ΔF, change in fluorescence intensity; ΔF_{max}, maximum change in fluorescence intensity; $\Delta G°$, standard Gibbs free energy change; GOPS, 3-glycidoxypropyltrimethoxysilane; $\Delta H°$, standard enthalpy change; K_a, association constant (M^{-1}); K_d, dissociation constant (M); PBS, phosphate-buffered saline; R, gas constant (8.314 J/mol · K); r^2, correlation coefficient from linear regression; R^2, correlation coefficient from nonlinear regression; SPR, surface plasmon resonance; $\Delta S°$, standard entropy change; T, temperature (K); TIRF, total internal reflection fluorescence; CDR, complementary-determing region.

0003-2697/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2006.03.058