Effects of Linker Flexibility and Conformational Changes of IP3 Receptor on Split Luciferase Complementation Assay

Maryam Moradi[1], Saman Hosseinkhani[1,*], Seyed Shahriar Arab[2], Anahita Khammari[2]

[1] Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[2] Department of Biophysics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran

Corresponding author: Saman Hosseinkhani, Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, 14115-154, Iran. Tel: +98-2182884407 / Fax:+98-2182884484: E-mail: saman_h@modares.ac.ir

Background: IP3-induced Ca^{2+} release, mediated by IP3R, is one of the most momentous cellular signaling mechanisms that regulate in a wide variety of essential cellular functions. Involvement of disrupted IP3 signaling pathways in numerous pathophysiology conditions is implicated to find the best methods for its measurement. Hence, several different biosensors have developed to monitor temporal changes of IP3 by using the IP3-binding domain of IP3 receptors.

Objectives: Based on a previous study, we developed and characterized a series of bioluminescent biosensors using the human type-II IP3 receptor ligand binding domain (residues 1–604), named LAIRE (luminescent analyzer for IP3 receptor element) to study the effect of flexible and rigid linkers on the luminescence intensity of split luciferase. The effect of a mutation in IP3 binding residues and suppressor domain in the IP3 binding domain on luciferase complementary assay is also investigated.

Materials and Methods: In the present study, first IP3-binding domain (residues 1-604) of IP3-receptor type 2 (LAIRE) was fused between complementary non-functional fragments of firefly luciferase and then the rigid linker sequence (LLRAIEAQQHLL), selected by ProDA database, introduced between Nluc and ligand binding domain and compared with that of the flexible linker ((GGGGS)2) in LAIRE chimera. The IP3-insensitive mutant of the biosensor was constructed using the Stratagene QuikChange® procedure. In order to the analysis of the dynamical movements of selected structures in the large-scale, coarse-graining method of molecular dynamics simulation (1µs) was applied.

Results: As expected, the flexible linker brings two inactive fragments of luciferase together relative to the rigid linker and leads to complementation of luciferase activity, which is detected using luciferin. However, this conformational flexibility in linker increases background to noise ratio and attenuates fold induction.

Conclusions: It seems that the ligand binding properties of IP3 binding core make it more suitable for the design of biosensor than the ligand binding domain.

Keywords: IP3; Molecular modeling; Molecular dynamic simulation; Split Luciferase

1.Background

Inositol trisphosphate or inositol 1,4,5-trisphosphate (IP3), as an important second messenger, involves in many cellular processes. IP3, resulting from PLC-mediated hydrolysis of PIP2, binds to its specific receptors (named as IP3R) located in the endoplasmic reticulum and triggers the opening of the Ca^{2+} channel, and thus release of Ca^{2+} into the cytoplasm (1,2).

The three distinct isoforms or IP3 receptor (IP3R1, IP3R2, and IP3R3), encoded by three different genes, are expressed in mammals and share only 60–80% overall similarity at the amino acid level (sequence identity) (1, 3). These proteins (isoforms) are structurally divided into three functional domains: An N-terminal region that binds IP3 (600 residues), that is separated from a C-terminal channel-forming region (400 residues) by a large central modulatory region (1700 residues) (4).

The opening of IP3Rs was initiated by the conformational change in the ligand binding domain (LBD) and subsequently affect in c-terminal and release of ca^{2+} into the cytoplasm.

Their N-terminal ligand binding site, the most conserved region, consists of some separated motifs. The first 223 residues are identified as the IP3-binding

suppressor region and the critical IP3 binding site are localized between the residues 226 and 576 (5). It has been shown that 10 residues (Arg-241, Lys-249, Arg-265, Arg-269, Arg-504, Arg-506, Lys-508, Arg-511, Arg-568, and Lys-569) are conserved among IP3Rs, within the binding core form a highly positively charged pocket to coordinate IP3 (6, 7).

Because of its fundamental role in intracellular signaling, development of the most efficient and effective methods for monitoring local IP3 level in living cells is crucial. Based on the conformational change of IP3- binding site, as a natural physiological target for IP3, several biosensors that transduce ligand binding to a variety of physical signals have been designed (8,9). These biosensors are composed of the ligand-binding domain of IP3R fused between a pair of fluorescent proteins. Although these FRET biosensors may be useful in drug screening, they need to be improved further, especially in the dynamic range (9).

Because of the high sensitivity and flexibility, noninvasiveness, high dynamic range and the very low background in biological systems, bioluminescence-based complementation assay techniques gain a prominent position among Protein-Protein Interaction methods (10-12).To design intracellular biosensor using the firefly luciferase complementation assay, the interested protein which undergoes a conformational change under specific cellular conditions such as ligand binding was sandwiched between two non-functional fragments of split luciferase (N-terminal and C-terminal fragments). The restored luminescence activity should result from reconstituting the active site of the luciferase enzyme following protein conformational changes in response to ligand binding (upon ligand binding).

In a previous study, a bioluminescent reporter based on the interaction of the N- and C-terminal domains of luciferase that results from an IP3-induced conformational change in IP3R has been characterized. This was achieved by insertion of two non-functional luciferase fragments (Nluc 1-416aa and Cluc 395-550aa) to the N- and C-terminals of IP3 binding core domain (residues224–604) of human type 2 IP3R, respectively linked by a flexible linker($(G4s)_2$). Although the luminescent signal increased after the addition of IP3, the signal detected in the absence of a ligand was considerable. To overcome this problem, a library of different linkers and of luciferase splitting sites should be constructed in order to minimize background noise (13).

2. Objectives

Here, based on a previous study, we developed and characterized a series of bioluminescent biosensors using the human type-II IP3 receptor ligand binding domain (residues 1–604), named LAIRE (Luminescent Analyzer for IP3 Receptor Element). It should be noted that a bioluminescent reporter based on the interaction of the N- and C-terminal domains of luciferase with the IP3-binding core (IBC) without a suppressor (LAIREΔS) has been introduced in previous work (13). Additionally, an IP3-insensitive sensor (LAIREΔSm) is generated by deletion of suppressor domain and mutation of alanine (A) to Arginine (R) and lysine (K) at positions 504, 508(R504A, K508A）in the IP3-binding domain of LAIRE.

Moreover, an important consideration in the design of multi-domain chimeric proteins is a selection of proper linker in terms of flexibility that interconnect the various domains in these large proteins. In complement with experimental efforts for probing protein dynamics at atomic resolution, molecular dynamics simulation can be a straightforward way to elucidate the fine details of protein conformational motions. Here we used a novel database (ProDA) with the aim to design helical linker for domain fusion. The database could be searched for fragments with a certain length, secondary structures, the number and type of amino acids, hydrophobicity and accessible surface area (14). The effect of linker flexibility on conformational change and luciferase complementation has been investigated.

3.Materials and Methods

3.1. Materials

The following materials were obtained from the indicated sources:

IP3 and U73122 were purchased from Sigma; ATP (Roche); D-luciferin potassium salt (Resem); PrimeSTAR DNA polymerase was purchased from Takara (Japan); the pCDNA-NLuc-IBC-CLuc construct was as previously described (13).

3.2. Molecular Modeling

Briefly, the human IP3R2 (accession number:**Q14571**) and luciferase protein sequences (accession number:AAA89088.1) were first searched on the National Center for Biotechnology Information's (NCBI) BLAST program using protein data bank (PDB) to find template protein highly homologous to these proteins. The closed structure of IP3R1 and *Phonituspyralis* luciferase as the template structures were downloaded from the RSCB Protein Data Bank (PDB IDs: 3UJ0, 1N4K(for IP3R1) that show74%, 73% identity and 99%, 62% query cover with human IP3R2, respectively and 1LCI(for luciferase) and exploited to derive 5 initial

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starting three-dimensional models using MODELLER software, version 9.17. To build the homology model of these five recombinant fusion proteins, the multi-template approach was applied.

The ProDA database (14) has the ability to search the database containing more than 400 million peptide fragments extracted from about 70,000 protein structures taken from the Protein Data Database (PDB). ProDA can suggest more peptide fragments by considering some inputs, such as the content of the amino acids, the length of the sequence, the secondary structure, the polarity and the accessibility to the solvent optionally(http://bioinf.modares.ac.ir/software/proda). The database was searched to find a non-buried helix with the size of 12 residues and length about 16-17A^0. We replaced Nluc fragment in LAIRE construct with a different N-terminal fragment encompassing the 1-436 residues for more analysis (LAIRE-1). Furthermore, the fragment with helix-forming peptide (LLRAIEAQQHLL) was then selected and introduced between Nluc and ligand binding domain (LAIRE-2 construct) and compared with that of the flexible linker in LAIRE chimera.

Finally, the best-constructed models were selected based on the lowest DOPE score, which is a statistical potential optimized for model assessment and calculated based on the standard MODELLERenergy function. In this research, five constructed models (named LAIRE,LAIREΔS,LAIREΔSm,LAIRE-1,and LAIRE-2) were selected and MD simulation was performed on every selected model.

3.3. Coarse-Grained Molecular Dynamic Simulations

In order to the analysis of the dynamical movements of selected structures in the large-scale, coarse-graining method of molecular dynamics (MD) simulation was applied. The dynamics simulation of large movements of constructs was studied using the Martini coarse-grained (CG)force field model (15,16) implemented in GROMACS 5.0.1 (17) Software.CG models were built based on the MARTINI force field. The MARTINI CG model was developed for the simulations of lipids, proteins, and carbohydrates and widely used to extend the accessible simulation timescale significantly by employing a 4:1 heavy-atom/CG-bead mapping and a reduced water model.

To initiate a simulation using the MARTINI model, the secondary structure should be first assigned to the protein structures, and the assigned secondary structure remains fixed during the simulation (15, 16). It is noted that secondary structure changes of proteins cannot be modeled in Martini force field, and explicit hydrogen bonds are also absent in this CG model, although changes in tertiary structure are unrestricted (16,18,19). In our CG-MD simulations, the secondary structure of every residue of these constructs was determined using the program DSSP (20). The proteins were initially placed in a cubic box and solvated with water. After that%10 of the CG water model has been replaced with the "antifreezing" model to avoid the freezing effect that sometimes occurs with the MARTINI model in all the systems.

After mapping atoms into beads energy-minimized in vacuum was performed for each structure and then solvated with CG water beads (Each CG water bead corresponds to four water molecules). Next, counter ion beads were added to neutralize the system. To study domain Movements of these Large Proteins, a 1-μs CG-MD simulation was performed in NPT ensemble for the systems, with the pressure and temperature fixed to 1 atm and 300 K, respectively, using the Nose–Hoover thermostat (21,22) and Parrinello–Rahman barostat (23,24).

This Martini CG model not only reduces the number of degrees of freedom significantly but also allows a large integration time step between 20 and 40 fs in the simulation, thus assuring a more efficient sampling scheme. Here we used a time step of 20 fs. Van der Waals (vdW) and Electrostatic, treated by particle-mesh Ewald summation method, interactions were used in their shifted forms with a cutoff at a distance of 1.2 nm. The vdW interaction was shifted from 0.9 to 1.2 nm and the electrostatic interaction was shifted from 0.0 to 1.2 nm. The neighbor-list was updated every 10 steps with a distance cutoff of 1.4 nm.

3.4. Plasmid Constructions

Construction of biosensor has been described previously. Briefly, a type 2 IP3R biosensor were assembled using the IP3-binding domain encompassing the amino-terminal 224-604 residues terminally fused with N- and C-terminal fragments of firefly (*Photinus pyralis*) luciferase at the amino and carboxyl termini, respectively. Using this construct, Plasmid for expression of ligand binding domain of IP3R2(1-604) was created by PCR amplification of the ligand binding domain with flanking *MluI* and *SalI* sites (using forward primer 5'- GAC**ACGCGT**AT-GACTGAGAAAATGTCCAGC-3' and reverse primer5'- GTA**GTCGAC**GCTACCTCCACCTCCACTTC-CGCCACCACCTTTTCTGTTGTTGTGC-3'and replacement in *MluI*/*SalI*-digested pCDNA-NLuc-IBC-CLuc vector producing plasmid pCDNA-NLuc-LBD-CLuc. Toanalysis the best combination for this biosensor, luciferase in pGL3 was used as a template to amplify two different lengths of N terminal fragments (1-411 and

1-435) with a rigid linker (LLRAIEAQQHLL) on the C-terminus using a common forward primer5'-CTAG-**GGATCC**ACCATGGAAGACGCCAAAAAC-3'and reverse primers5'- GTT**ACGCGT**CAGTAGGTGCT-GCTGGGCTTCAATAGCTCTTAACAATGCATTG-GTCGCCTCAGG-3'and5'-   CAA**ACGCGT**CAG-TAGGTGCTGCTGGGCCTCAATAGCTCTTAA-CAAAACGATGAAGAAGTGCTC- 3' respectively (cleavage sites by *BamHI/*and *MluI* are shown in bold letters).Then the PCR products were digested with *Bam-HI/MluI* and ligated into similarly digested pCDNA3.1+ vector to form LAIRE-1 and LAIRE-2 vectors. The IP3-insensitive mutant of the biosensor was constructed by site-directed mutagenesis of the IP3-binding domain of plasmid by using the Stratagene QuikChange® proce-dure. The forward PCR primer used for mutagenesis was 5'-CCAAAT**GCA**GAACGCCAAG**GCA**TTGATGAG-GGAACAAAAC-3' and the reverse PCR primer was 5'-GTTTTGTTCCCTCATCAA**TGC**TTGGCGTTC**T-GC**ATTTGG-3' (bold letters represent mutated codons). Sequencing results confirmed the fidelity of Plasmid Constructions.

3.5. Cell Culture

HEK293T cells were grown in Dulbecco's Eagle's medium nutrient mixture F-12 Ham (Sigma).
Supplemented with 10% heat-inactivation fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S) at 37 °C in a 5% CO_2 incubator.

3.6. Cell Transfection and Luciferase Assay

HEK293T cells seeded at 400,000 cells/six-well plate were transiently transfected with noted plasmids using a transfection reagent, Polyethylenimine (PEI). The cells were incubated (5% CO_2 at 37 °C) for 48 h (medium changed after 24 h) prior to assay. Subsequently, the cells were lysed for 20 min on ice in CCLR lysis buffer (150 mM NaCl / 2 mM EDTA / 1%Triton-X 100 / 10% glycerol / 20 mM Tris pH 7.5) and then used for the following experiments. For bioluminescence assay, a 1:1 vol/vol ratio of lysate to Luciferase Assay Reagent (Promega)was added and Bioluminescence was measured immediately by using the luminometer, and luminescence was normalized to protein content in samples as determined by Bradford assay. The response rate for the IP3-dependent increase in luciferase activity was examined by application of 25 μM IP3 to the experimental chamber containing lysate biosensors-expressing cells.

4. Results

4.1. Molecular Modeling and Molecular Dynamics Simulation

As mentioned in the materials and methods section, the first step in homology modeling is to identify template structures that have high similarity to the targets. This identification is achieved by scanning the sequences of LBD, IBC, and luciferase against a library of sequences extracted from known protein structures in the Protein Data Bank (PDB). For IP3R modeling two incomplete reported crystal structure of type 1 IP3R, with PDB entry codes 3UJO and 1N4K, were used as templates to build the complete structure. Then dynamic conformational changes were studied through Molecular Dynamic simulations. The molecular dynamics (MD) simulations described in this study were performed using the GROMACS Software package.

The Root Mean Square Deviation (RMSD). The structural stability of all models was monitored by computing of the RMSD of the backbone Cα atoms relative to starting structures during the 1μs simulations
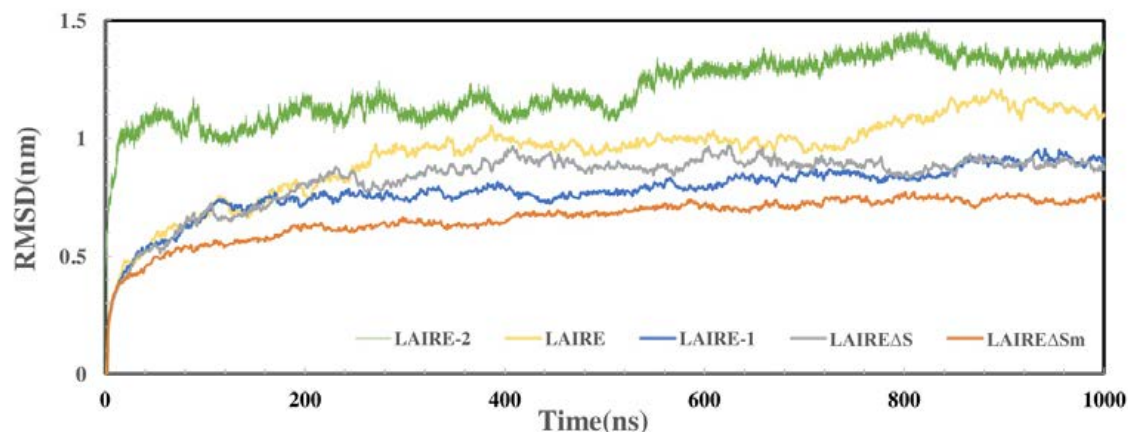

Figure 1. Time dependence of different RMSD values over MD trajectories for all five models. The reference structures were the starting energy-minimized structures.

time. As shown in **Figure 1**, all models seem stable throughout the simulation time, reaching equilibrium after the first 40 ns of the simulation. LAIRE-2 and LAIRE exhibit a rise in RMSD after 550 and 800ns, respectively, which is likely to indicate a structural transition.

The radius of Gyration (Rg). The global changes in the protein structure can be calculated via measurement of the radius of gyration, which determines the mean positional distances of atoms from the center of mass. Usually, a lower radius of gyration represents tighter packing of the structure. We used a radius of gyration

analysis to compare the compactness of our constructs. **Figure 2A, B** shows the calculated radius of gyration for each of the constructs as a function of time (the construct with similar residue compared together). Both LAIRE-1, -2 show a large increase in the radius of gyration relative to LAIRE that suggests a more "open" average structure for LAIRE-1, -2and a higher degree of packing for LAIRE (**Fig. 2A**).

LAIREΔSm exhibit a small but significant increase in the radius of gyration relative to LAIREΔS. This indicates a more expanded structure for LAIREΔSm (**Fig. 2B**).
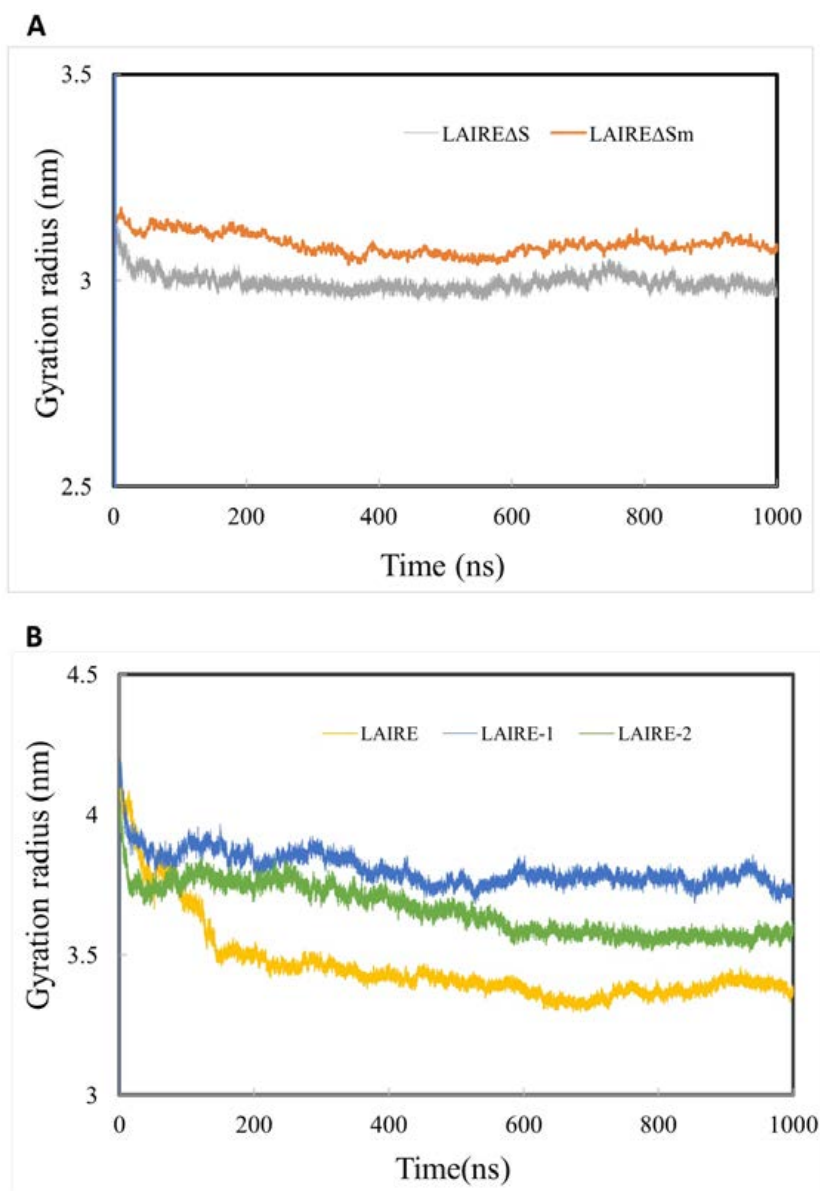

Figure 2. Graph of Radius of gyration (Rg) vs. time. A) plot for the gyration of Cα atoms of LAIREΔS (native) and LAIREΔSmare quite same but LAIRE structure exhibited higher Rg value in comparison to another structure. The Rg of LAIRE was found to be 3.5 nm while that of LAIREΔS and LAIREΔSm were 3 nm after 1000 ns of Simulation. B) The curve reveals that the LAIRE-1 and LAIRE-2 show higher Rg value than LAIRE during the whole simulation time period.

4.2. Construction and Expression of IP3 Biosensors

We constructed a set of luciferase reporter-ligand-binding domain chimeras from the type 2 human InsP3R isoform terminally fused with N- and C-terminal of split luciferase (**Fig. 3A**). Also, the three-dimensional structure of the LAIREΔS, including the location of the different domains, is shown as a model of studied constructs in **Figure 3B**. To test *in vitro* split luciferase complementation assay, these constructs were transiently transfected in the HEK293T cells,

incubated for 48 h, and thereafter the lysed cells were harvested to monitor protein expression.

To determine the kinetics of ligand-induced FLuc reassembly, the lysate from HEK293T cells expressing the fusion proteins was assayed after exposure to IP3 (25 μM) (**Fig. 4**). As shown in **Figure 4**, the maximum luciferase activity due to complementation of luciferase fragments was obtained from the cells that had been transfected with the Nluc- IBC-Cluc in which IBC is connected to luciferase fragments
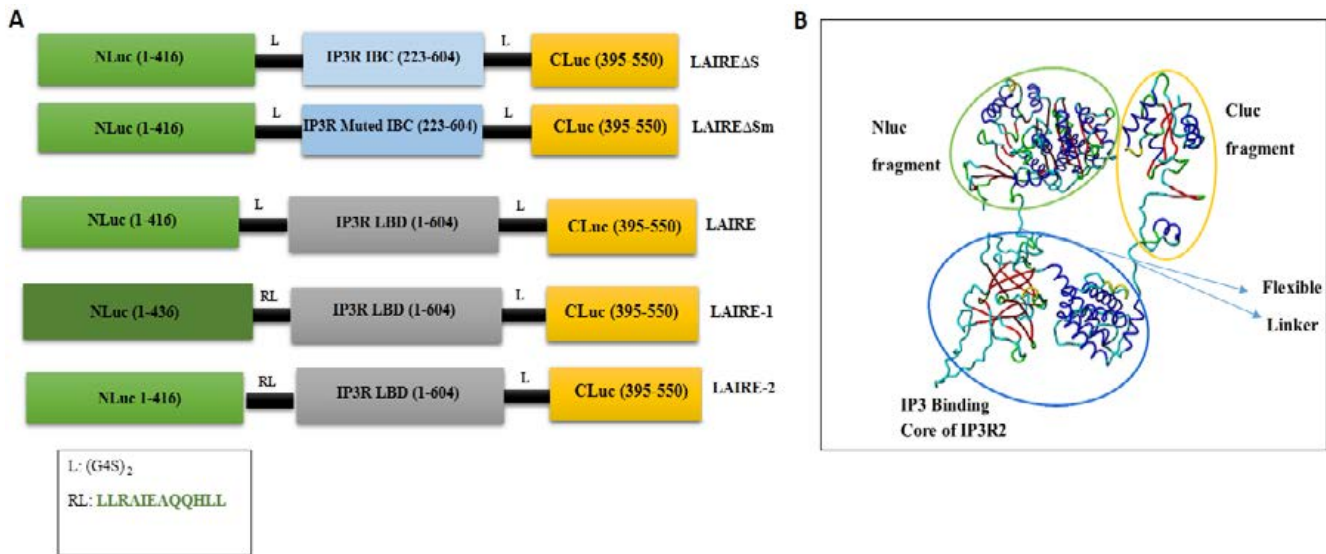

Figure 3. A) Construction and expression of split biosensors. Schematic representations of the domain structures of biosensor variants. Complementation reporter constructs consist of the ligand- or IP3-binding domain of human IP3R type 2 (1-604, 223-604 amino acids) fused via rigid and flexible linkers (RL, L. respectively) to N- and C-domain of luciferase on each site. Arginine and Lysine to alanine mutations were introduced in the IP3-binding residues at positions 503 and 507, respectively. B) Ribbon representation of LAIREΔS as a model of studied constructs.
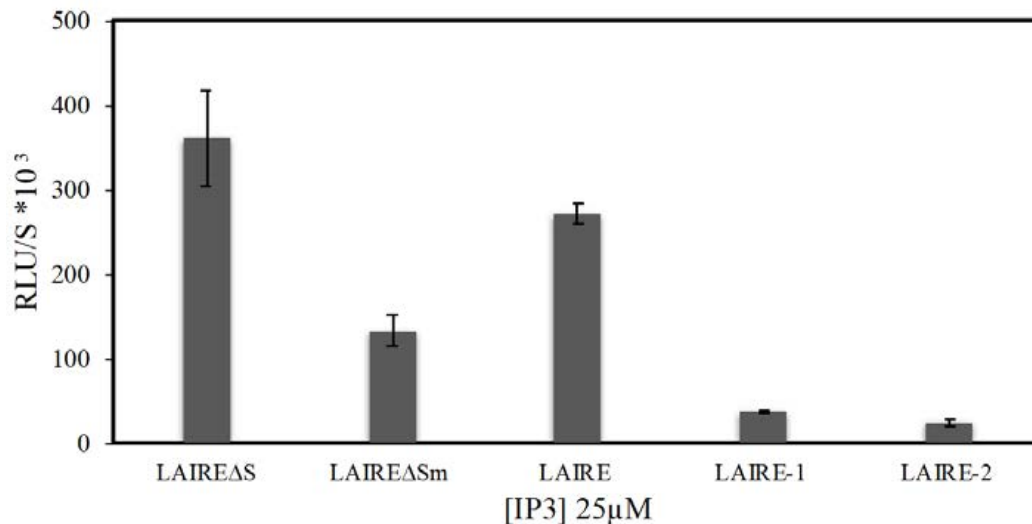

Figure 4. Effect of 25μM IP3 on the luminescence intensity of the intra-molecular sensors in HEK 293T cells expressing these split luciferase InsP3 biosensors.

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using flexible linkers. Mutation of IP3 binding residues (residues R404A and K408A) brought about with loss of luciferase activity to 37% compared to LAIREΔS construct presumably through loss of sensitivity to IP3 in LAIREΔSm. On the other hand, in comparison with fusion protein with the same size, LAIREΔS has a smaller radius of gyration and is more tightly packed than LAIREΔSm. It may be argued that two fragments of luciferase are closer together in LAIREΔS construct and thus show a higher activity level in the presence of the ligand. As indicated in Fig. 3B mutation of IP3 binding residues brought about with loss of luciferase activity in cell lysates which implicates in the application of luciferase complementary assay in the interpretation of conformational change in the IP3 receptor. It seems that the flexible linker leads to a high background activity even for LAIREΔSm.

5. Discussion

Split luciferase complementary assay has been used as an approved procedure to monitor protein-protein interaction (12). The procedure can be used to assay the effect of stabilizing and disrupting compounds on protein-protein interaction. However, the procedure was implemented in the binding of IP3 to its receptor *in vitro* (13). In order to see the effect of linker flexibility and real conformational change on this interaction, a set of modeling and experimental design are used. To this purpose, we used ligand binding domain of the human IP3R2(1-604 and 224-604 amino acids for LBD and IBC respectively) that consists of all specific binding sites of IP3 that undergo conformational changes upon IP3 binding. The Nluc fragments of firefly luciferase are fused to the N-terminus of LBD/IBC and its CLuc is attached to the C-terminus of LBD/IBC (NLuc–LBD/IBC–CLuc). A flexible and helical forming peptide linkers were inserted between luciferase fragments and the IP3 binding core to assist luciferase fragments complementation.

As mentioned before, the N-terminal 225 amino acids (named suppressor domain) in IP3Rs have been mapped to inhibit IP3 binding to the IP3 binding core (7). As indicated in Fig. 4, the presence of the suppressor domain with IP3 binding core (LAIRE construct) decreased luciferase complementation to almost 64%. Thus, it is probable that the inhibitory effect of the suppressor domain in LAIRE construct on IP3 binding can affect bioluminescence recovery and decrease its intensity compared LAIREΔS construct. This our result is in contrast to that observed in previously FRET-based InsP3 Sensor. In this study, the

LIBRAΔN, which lacks N-terminal suppressor region, shows no IP3-dependent changes in fluorescence (9). One of the key issues in the construction of functional fusion proteins is a selection of the proper linker sequence (25). Besides the choice of the proper component proteins for a design of fusion proteins, the selection of a suitable linker to link the protein domains together can be complicated (26). Although flexible linkers are useful for connecting functional domains and allowing certain degree of movements, a lack of rigidity in these linker can be a limitation (27). For instant, the insertion of a flexible linker $(Gly)_4Ser$ between protein G and Vargula luciferase did not result in the recovery of immunoglobulin binding ability of the chimeric protein (28).

Then, we decided to study the effect of the α-helical linker on the reconstitution of luciferase fragments and light generation. The result indicated that the helical linker (in LAIRE-1 and LAIRE-2 constructs) compared to the flexible linker (in LAIRE construct) induced significantly lower luciferase activity when inserted between Nluc and ligand binding domain, suggesting that helical linkers far apart two fragments of luciferase thereby hinder luciferase complementation.

It seems that amino acid differences of the dissection sites can affect the reconstitution efficiency of the luciferase fragments. Results showed the N-terminal fragment of amino acids, 1-436(LAIRE-1), complement the C-terminal fragment of 395-550 amino acids, causing a sufficient recovery of bioluminescence compared to the N-terminal fragment of amino acids, 1-416(LAIRE-2). A similar result was found in the modeling of bound IP3 to luciferase fragments in which insertion of rigid linkers brought about with subtle increase of radius of gyration based on luciferase fragments complementation. The higher radius of gyration shows an increase of the distance between luciferase fragments and lower luciferase activity as indicated in **Figure 3B**.

In general, the increased amount of luciferase activity in the LAIRE and LAIREΔS constructs means that the Nluc and Cluc domains are closer together than those in the other constructs. Also, the flexible linker brings two inactive fragments of luciferase together relative to the rigid linker and leads to complementation of luciferase activity, which is detected using luciferin. However, this conformational flexibility in linker increases background to noise ratio and attenuates fold induction. It seems finding the optimal peptide linkers in length and composition for the construction of fusion proteins is often a process of trial and error.

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6. Conclusion

In this research, we tried to introduce the helix-forming peptide to IP3R biosensor as a linker to make a comparison between flexible and rigid linker. To study these, the rational design of a rigid linker was done using a ProDA database. It seems that the ligand binding properties of IP3 binding core make it more suitable for the design of biosensor than the ligand binding domain. In these biosensors, conformational changes in the IP3 binding domain are translated into bioluminescence activity of luciferase, which could be imaged using luciferin. The designed constructs were experimentally analyzed using Fluc complementary assay in order to evaluate the ability of designed linker for reconstitution efficiency of the luciferase fragments. On the other hand, the choice of proper length and structure of the linkers could control the distance between luciferase fragments and ultimately improve the bioactivity of luciferase moieties.

Acknowledgments

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