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The molecular interaction of heart LIM protein (HLP) with RyR2 and caveolin-3 is essential for Ca^{2+}-induced Ca^{2+} release in the heart

Dong Woo Song [a], Kyung-Eun Lee [b], Jae Yong Ryu [a], Hyesung Jeon [c, 1], Do Han Kim [a, *]

[a] School of Life Sciences and Systems Biology Research Center, Gwangju Institute of Science and Technology (GIST), Gwangju, South Korea
[b] Advanced Analysis Center, Korea Institute of Science and Technology, Seoul, South Korea
[c] Center for Theragnosis, Biomedical Research Institute, Korea Institute of Science and Technology, Seoul, South Korea

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ABSTRACT

The heart LIM protein (HLP) is a LIM-only protein family member that mediates protein–protein interactions. To date, no studies have yet been conducted regarding its function in the heart. In the present study, we have identified that HLP binds the cytosolic region of RyR2 in the heart using a bacterial two-hybrid system, LC-MS/MS, co-immunoprecipitation, and GST-pull down assays. Microscopy revealed that HLP forms a triple complex with RyR2 and caveolin-3. siRNA and adenovirus-mediated KD of HLP decreased the electrically evoked Ca^{2+} release from the sarcoplasmic reticulum without directly affecting SERCA2 and RyR2 activities. Collectively, the HLP-RyR2 interaction in the cell surface caveolae region may be essential for efficient excitation-contraction coupling in the heart.

1. Introduction

RyR2 is a cation-selective receptor channel located in the cardiac sarcoplasmic reticulum (SR) responsible for Ca^{2+} release from the SR [1]. In the mammalian heart, excitation-contraction (E-C) coupling is initiated by Ca^{2+}-induced Ca^{2+} release (CICR) from the SR in response to L-type Ca^{2+} channel depolarization [2]. CICR is triggered not only in the dyadic space between a well-developed transverse-tubules and junctional SR, but also around the periphery of the cells [3]. According to previous reports, caveolin-3-associated DHPR in caveolae, the specialized microdomain of sarcolemma, may be essential for CICR regulation [4,5].

The cysteine-rich protein (Crip) family is characterized by the presence of two conserved LIM domains, which are zinc finger-like motifs [6]. Structural evidence with the aa sequence indicates that the LIM domain is composed of a cysteine-rich region and forms a distinct structural module for protein–protein interactions [6,7]. Three members of the Crip family, Crip1, Crip2/heart LIM protein (HLP), and Crip3/thymus LIM protein (TLP) have been identified [8–10]. HLP is a protein of 208 aa and its expression is abundant in the adult heart, as well as in the brain and lung [9,11,12]. Several binding partners of HLP have been previously identified in various tissues [11,13–16]. Ham et al. found that the cytosolic protein phosphatase, PTP-BL, interacts with HLP through its fourth PDZ domain, and both proteins partially co-localized at the apical sides of epithelial cells [11]. In a yeast-two hybrid screen using a cDNA library of rat intestinal longitudinal muscle [13] or spinal cord [14], HLP has been identified as a binding protein of the cGMP-dependent protein kinase Iβ (cGKIβ). Another study showed that both cGKIα and cGKIβ interact with the third zinc finger motif of HLP in a phosphorylation-dependent manner [15]. Recently, the functional importance of an interaction between HLP and nuclear factor-κB (NF-κB) in cancer development has also been suggested [16]. However, despite its high expression in the heart, its biological roles have not been reported.

In the present study, we identified the novel molecular interaction of HLP and RyR2. In addition, we found that HLP interacts with caveolin-3 in the caveolae region, and disruption of the

Abbreviations: aa, amino acids; BSA, bovine serum albumin; cGMP, cyclic guanosine monophosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CSQ, calsequestrin; DHPR, dihydropyridine receptor; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione *S*-transferase; HLP, heart LIM protein; KD, knock down; LIM, Lin11, Isl-1 & Mec-3; NP-40, nonidet P-40 (octylphenoxypolyethoxyethanol); PBS, phosphate buffered saline; PTP-BL, protein tyrosine phosphatase basophil-like; RT, room temperature; RyR2, ryanodine receptor type 2; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SERCA, sarco/endoplasmic reticulum calcium ATPase; shRNA, short hairpin ribonucleic acid; WH, whole homogenates.

* Corresponding author. School of Life Sciences, GIST, 1 Oryong-dong, Buk-gu, Gwangju 500-712, South Korea.
E-mail address: dhkim@gist.ac.kr (D.H. Kim).
[1] Current address: Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.

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interaction between HLP and RyR2 by HLP-KD could inhibit CICR. Our study provides novel evidence suggesting that HLP plays an important role in the functional coupling between caveolin-3 on the surface membrane and RyR2 in the SR by providing the proper microdomain for E-C coupling.

2. Materials and methods

2.1. Isolation of rat adult and neonatal cells

All animal procedures were approved by the Gwangju Institute of Science and Technology Animal Care and Use Committee. Neonatal cardiomyocytes from 1 to 3 days old Sprague–Dawley rats were isolated using the neonatal cardiomyocyte isolation system (Worthington Biochemical) as described previously [17]. Adult cardiomyocytes from 10- to 14-weeks-old male Sprague–Dawley rats (240–260 g) were enzymatically isolated using the Langendorff system as described previously [18].

2.2. In vitro translation and binding assay

[^{35}S]-Methionine-labeled RyR2 bait (RB) was synthesized in the presence of [^{35}S]-methionine (Amersham Pharmacia Biotech) according to the manual of the TNT Quick Coupled Tanscription/Translation system (Promega). GST and GST-HLP immobilized on Glutathione-Sepharose beads were mixed with the *in vitro* translated proteins in the binding buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.2% NP-40, 1 mM phenylmethylsulfonyl fluoride, and the protease inhibitor cocktail for overnight at 4 °C. After seven washes with the binding buffer, the bound proteins were analyzed by SDS-PAGE and autoradiography.

2.3. Co-immunoprecipitation and GST-pull down assay

Co-immunoprecipitation and GST-pull down assays using heart homogenates were performed as described previously [19].

2.4. Immunofluorescence microscopy

After fixation with 4% paraformaldehyde and permeabilization with 0.1% Triton X-100, HL-1 cells or adult rat ventricular cardiomyocytes were treated with the proper primary antibodies in 3% BSA/PBS at 4 °C for overnight. After washing with PBS, cells were incubated with appropriate FITC-, Texas Red- or Alexa Fluor-conjugated secondary antibodies at 37 °C for 1 h. Cells were washed with PBS and mounted on a clean slide. The prepared slides were examined with an LSM 700 confocal laser scanning microscope (Carl Zeiss).

2.5. Electron microscopy

The mouse atrium and rat adult ventricular myocytes were fixed with 2.5% glutaraldehyde dissolved in 0.1 M cacodylate buffer overnight at 4 °C and were subjected to electron microscopic experiments as previously described, with slight modifications [20]. The 25 nm-gold conjugated anti-chicken goat antibody and the 10 nm-gold conjugated anti-mouse goat antibody were used for detection of HLP and RyR2, respectively.

2.6. Ca^{2+} transient and caffeine-induced Ca^{2+} release measurement

HL-1 cells and rat neonatal ventricular myocytes grown on 18 mm cover slips were loaded with 2 μM fura2-AM (Molecular Probes) in the Tyrode solution (10 mM HEPES-NaOH. pH 7.4, 135 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) at 37 °C for 30 min. After washing 3 times with fura2-AM-free Tyrode solution, cells were placed in a circulating chamber mounted on the stage of an inverted microscope (Nikon Eclipse TE-100F) and superfused with the Tyrode solution. The cells were field stimulated at a frequency of 1 Hz (40 V), and a dual-beam excitation spectrofluorometer setup (IonOptix) was used to record fluorescence emission (505 nm) elicited from exciting wavelengths of 340 nm and 380 nm. Several parameters including Ca^{2+} transient amplitude, baseline, time to reach 50% of peak and time to reach 50% of baseline were obtained and analyzed using the analysis program, Ion Wizard software (IonOptix). Total SR Ca^{2+} content was measured by empting the SR with application of 40 mM caffeine in Ca^{2+} and Na^{+}-free Tyrode solution. The amplitude of caffeine-induced Ca^{2+} release was used as an index of SR Ca^{2+} content.

2.7. Statistics

Statistical significance was estimated by unpaired Student's *t*-test for comparison of two groups. n = Number of independent experiments. Values are expressed as means ± SEM, and of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Interaction of HLP with RyR2

We used a bacterial two-hybrid screen with the cytosolic region of RyR2 (1353–1851 aa) as bait, and we identified several positive clones as candidate binding partners. Among them, voltage-dependent anion channel 2 (VDAC2) has been recently identified as a RyR2 binding partner [20]. HLP was also identified as a binding partner of RyR2, and so we performed an *in vitro* binding assay using ^{35}S-labeled RyR2-bait (RB) and GST-HLP. Specific binding was observed when ^{35}S-labeled RB was incubated with GST-HLP, but not with GST alone (Fig. 1A). In a co-immunoprecipitation assay, a mouse monoclonal RyR2 antibody precipitated endogenous HLP (Fig. 1B), whereas mouse IgG alone failed to do so. This molecular association was also verified in reciprocal experiments in which endogenous HLP and RyR2 were co-immunoprecipitated by anti-HLP serum but not by normal rabbit serum (Fig. 1C). The interaction did not show any significant differences upon the addition of 600 nM Ca^{2+} (Fig. 1B, C).

To locate the HLP binding site in the cytoplasmic region of RyR2, four GST-tagged sub-domains of RB were recombinantly expressed (Fig. 1D, E) and subjected to GST-pull down assays. HLP was found to interact with the third sub-domain of RB (RB-C) but not with the GST-fusion proteins of the other sub-domains (Fig. 1F), suggesting that RyR2 interacts with HLP through its cytosolic region encompassing aa 1580–1741. This region contains one region that is rich in acidic aa (1635–1650) which may interact electrostatically with the basic amino acid rich regions of HLP (e.g. aa 23–37, 97–112, and 128–138).

3.2. Co-localization of HLP and RyR2 in cardiomyocytes

We further studied the physical interaction between HLP and RyR2 by using microscopy. RyR2 expression was evenly distributed throughout the cytoplasm of cardiomyocytes and was arranged in a regular cross-striated pattern, in accordance with its junctional SR localization (Fig. 2A, B). However, comparison of the z-stack images taken from the bottom and middle slices of stained cells revealed that HLP is mainly expressed near the membrane (Fig. 2A). The dominant localization of HLP, adjacent to the cell boundary, was evident in the images of sections taken through the Y–Z axis (Fig. 2A and B, right lower panel), suggesting that the functional

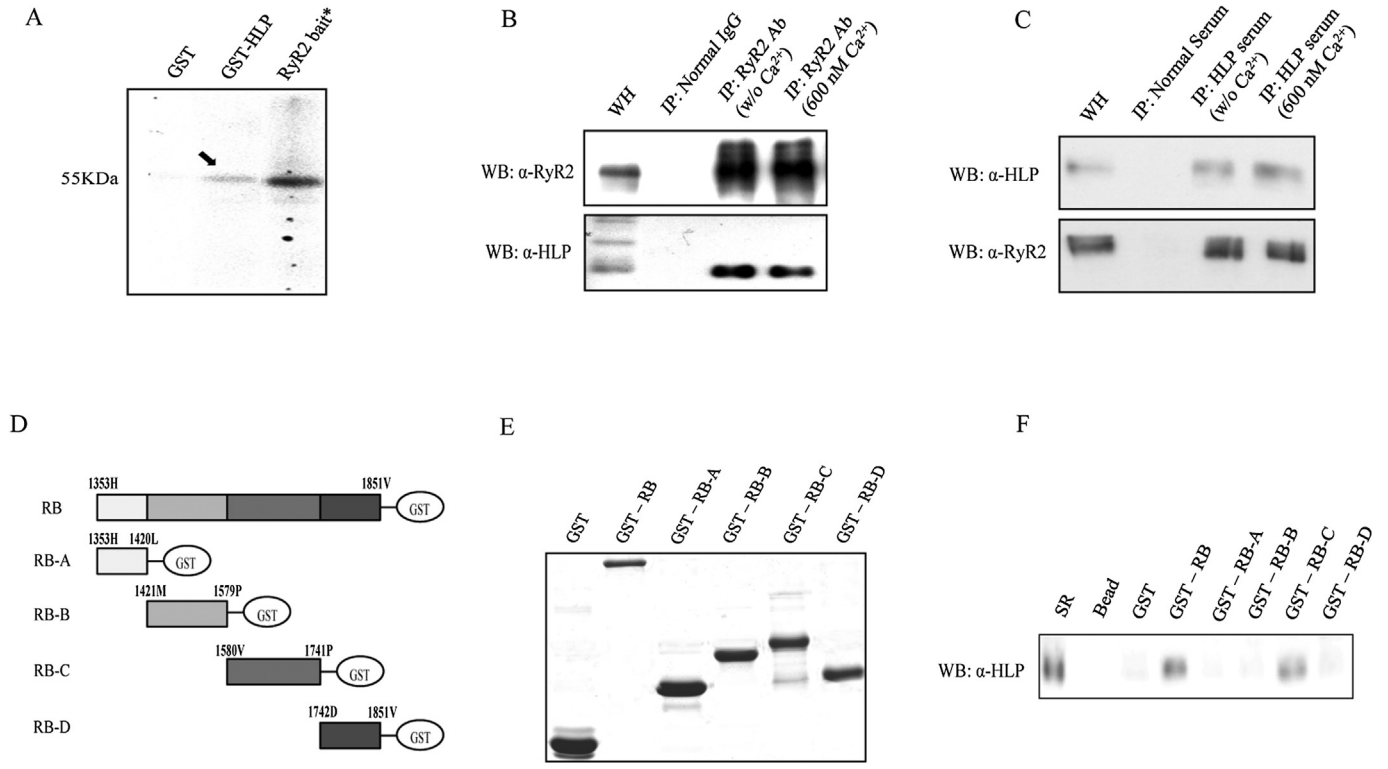

Fig. 1. Association of HLP with RyR2. (A) *In vitro* translated- ^{35}S-labeled RyR2-bait (RB) was incubated with GST-HLP or GST alone and the association of the two proteins was analyzed by SDS-PAGE and autoradiography. (B, C) The proteins immunoprecipitated with anti-RyR2 antibody (B) or with anti-HLP serum (C) were probed with anti-RyR2 and anti-HLP antibodies in the absence or presence of 600 nM Ca^{2+}. WH: 50 μg mouse WH. (D) A schematic diagram representing the deletion mutants of GST-RyR2. (E) Coomassie blue-stained gel of purified GST-fusion proteins separated by SDS-PAGE. The fusion proteins include GST alone, GST-RB (~71), GST-RB-A (~33), GST-RB-B (~38), GST-RB-C (~41) and GST-RB-D (~35 kDa). (F) GST-pull down proteins with each of the GST-constructs shown above were immunoblotted with anti-HLP antibody. SR input: 50 μg.

coupling between HLP and RyR2 is spatially restricted to the surface of the cardiomyocytes.

In HL-1 cells [21], RyR2 was preferentially localized at the SR (Supplementary Fig. S1A). In addition, the majority of endogenous HLP-positive stains were apparently co-localized with RyR2 stains (Fig. 2C). This implies that HLP is closely associated with intracellular RyR2 near the SR membrane in HL-1 cells. Further electron micrographic results also revealed the SR-localization of RyR2 labeled with small gold particles (10 nm) (Fig. 2D, left upper panel). Moreover, HLP labeled with large gold particles (25 nm) was also found near the SR (Fig. 2D, left lower panel) and closely localized with RyR2 (Fig. 2D, right panel).

3.3. Interaction of HLP with caveolin-3

To investigate a possible adaptor function of HLP in the heart, the interaction of HLP with another protein, caveolin-3, was tested using co-immunoprecipitation assays. Caveolin-3 is the main component of cardiac caveolae, and the functional relevance of caveolae for RyR2-mediated Ca^{2+} release has been previously demonstrated in neonatal and adult ventricular cardiomyocytes [4,22]. Interestingly, HLP-specific and caveolin-3-specific antibodies co-precipitated caveolin-3 and HLP, respectively, indicating the molecular association of the two proteins (Fig. 3A and B). To further confirm this interaction, we cotransfected *myc*-HLP and Flag-caveolin-3 into HEK293 cells and performed immunoprecipitation. Expression of the tagged proteins was observed in the transfected cells (Fig. 3C), whereas non-transfected cells did not show any detectable HLP or caveolin-3 (data not shown), suggesting that the two proteins are not expressed in the non-muscle HEK293 cells. Importantly, Flag-caveolin-3 was detected in cell lysates immunoprecipitated with the anti-*myc* antibody (Fig. 3C). This result indicates that HLP directly interacts with caveolin-3 without assistance from other muscle proteins. This molecular interaction was further supported by the electron microscopic localization of HLP in caveolae-like invaginated membranes (Supplementary Fig. S1B). Subsequent confocal microscopic analyses revealed that co-localization of HLP and caveolin-3 was observed in the images obtained from the bottom plane of stained cells in the z direction (Fig. 3D), whereas the middle slices of the confocal z-stacks showed weak or no co-localization (Fig. 3E), suggesting that HLP and caveolin-3 are localized predominantly near the cell surface area.

3.4. Effect of HLP on Ca^{2+}-induced Ca^{2+} release

To examine the effect of HLP on RyR2 function, we performed KD experiments in cardiomyocytes by using HLP-specific siRNAs. Adenovirus-mediated transfection of HLP siRNA successfully decreased the protein level of HLP by greater than 70% without changing the levels of RyR2, DHPR, CSQ, and SERCA (Fig. 4A). Next, the intracellular Ca^{2+} transient was measured using electrical field stimulation at 1 Hz in fura2-loaded cardiomyocytes. Fig. 4B shows the representative Ca^{2+} transient traces of control and HLP knocked-down neonatal ventricular cells, and the summary of the results are shown in Fig. 4C–F. The results revealed that the Ca^{2+} transient amplitude significantly decreased in neonatal cardiomyocytes infected with Ad-siHLP (Ad-NC *vs.* Ad-siHLP:

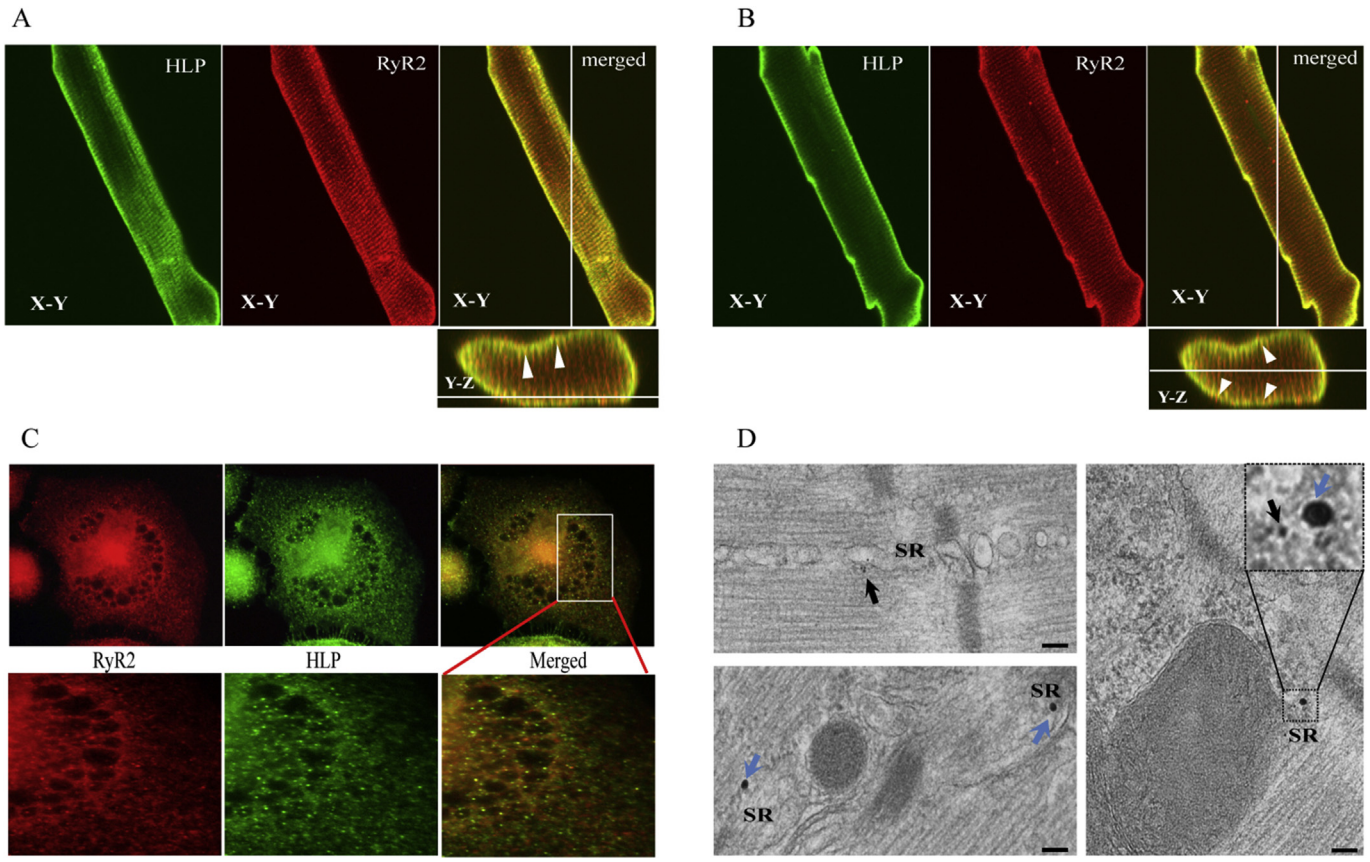

Fig. 2. Co-localization of HLP with RyR2 in cardiomyocytes. (A, B) Co-localization of HLP and RyR-2 in adult ventricular myocytes. The representative z-stack images of the bottom (A) or the middle slice (B) of the stained cells are shown. HLP molecules were stained first with anti-HLP antibody followed by Alexa-488-conjugated secondary antibody (green). RyR2 molecules were stained with anti-RyR2 antibody followed by Alexa-594-conjugated secondary antibody (red). The images for the X–Y plane were obtained, as the samples were sectioned virtually along the white horizontal line in the Y–Z plane. The image on the bottom right is for the Y–Z plane, as the samples were sectioned virtually along the white vertical line in the merged X–Y plane. The white arrow heads indicate highly co-localized regions. (C) HL-1 cells were stained with anti-RyR2 antibody and Texas-Red conjugated secondary antibody (red, left). HL-1 cells were also stained with anti-HLP antibody and FITC-conjugated secondary antibody (green, middle). The merged images are shown on the right. (D) Electron microscopic immunogold images of SR membranes in HL-1 cells are shown. The black arrow indicates the small gold particles (10 nm) associated with RyR2 molecules. The light blue arrows indicate the large gold particles (25 nm) associated with HLP. Double immunogold labeling revealed co-localization of HLP and RyR2 in HL-1 cells. Scale bars: 100 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.448 ± 0.019 *vs.* 0.392 ± 0.012; Fura ratio △340/380, n = 4) (Fig. 4D). The values of other parameters tested were not different between the two groups. This finding was also confirmed in HL-1 cells. HLP KD significantly decreased the Ca^{2+} transient amplitude in HL-1 cells, as shown in Supplementary Fig. S2C.

Because the size of CICR is affected by SR Ca^{2+} content [23], we next measured the caffeine-induced Ca^{2+} release. Rapid application of 40 mM caffeine produced transient cytosolic Ca^{2+} elevation caused by depletion of the SR (Supplementary Fig. S3A). The peak height of the Ad-siHLP group was similar to that observed in the Ad-NC group (Ad-NC *vs.* Ad-siHLP: 0.632 ± 0.053 *vs.* 0.614 ± 0.030; Fura ratio △340/380, n = 4), suggesting that the decreased CICR is not caused by SR loading (Supplementary Fig. S3B).

3.5. Effect of HLP on ryanodine binding to RyR2

[^{3}H]ryanodine binding assays are useful for examining RyR function [24] because ryanodine preferentially binds to open channels. Hyperbolic dependence of the amount of binding on the ligand concentration was observed on microsomes obtained from HL-1 cells transfected with the control and HLP siRNA (Supplementary Fig. S3C). The calculated K_d and B_{max} values for [^{3}H]ryanodine binding were similar between the two types of samples, suggesting that HLP does not modify the gating properties of RyR2.

4. Discussion

In the present study, we have examined the functional role of HLP in the heart by using various biochemical and genetic manipulation methods. The new findings generated from our study are as follows: (1) HLP directly interacts with the cytosolic acidic region of RyR2 as well as caveolin-3 (Figs. 1 and 2); (2) co-localization of HLP, RyR2, and caveolin-3 is spatially restricted to the cell surface of the cardiomyocytes (Figs. 2 and 3; Supplementary Fig. S1B); (3) KD of HLP impairs CICR without directly affecting SERCA2 and RyR2 (Fig. 3; Supplemental Fig. S2 and S3); (4) we propose that HLP is a linker protein between RyR2 and caveolin-3 that may play a pivotal role for functional coupling between caveolae and SR.

The molecular interaction between RyR2 and caveolin-3 has been studied previously [25,26]. Head et al. [25] demonstrated an association of RyR2 with caveolin-3 in adult ventricular cardiomyocytes by co-immunoprecipitation. The notion that the RyR2 and caveolin-3 interaction may be indirect and mediated through a linker protein was suggested by another group that could not detect direct binding between the proteins under their detergent

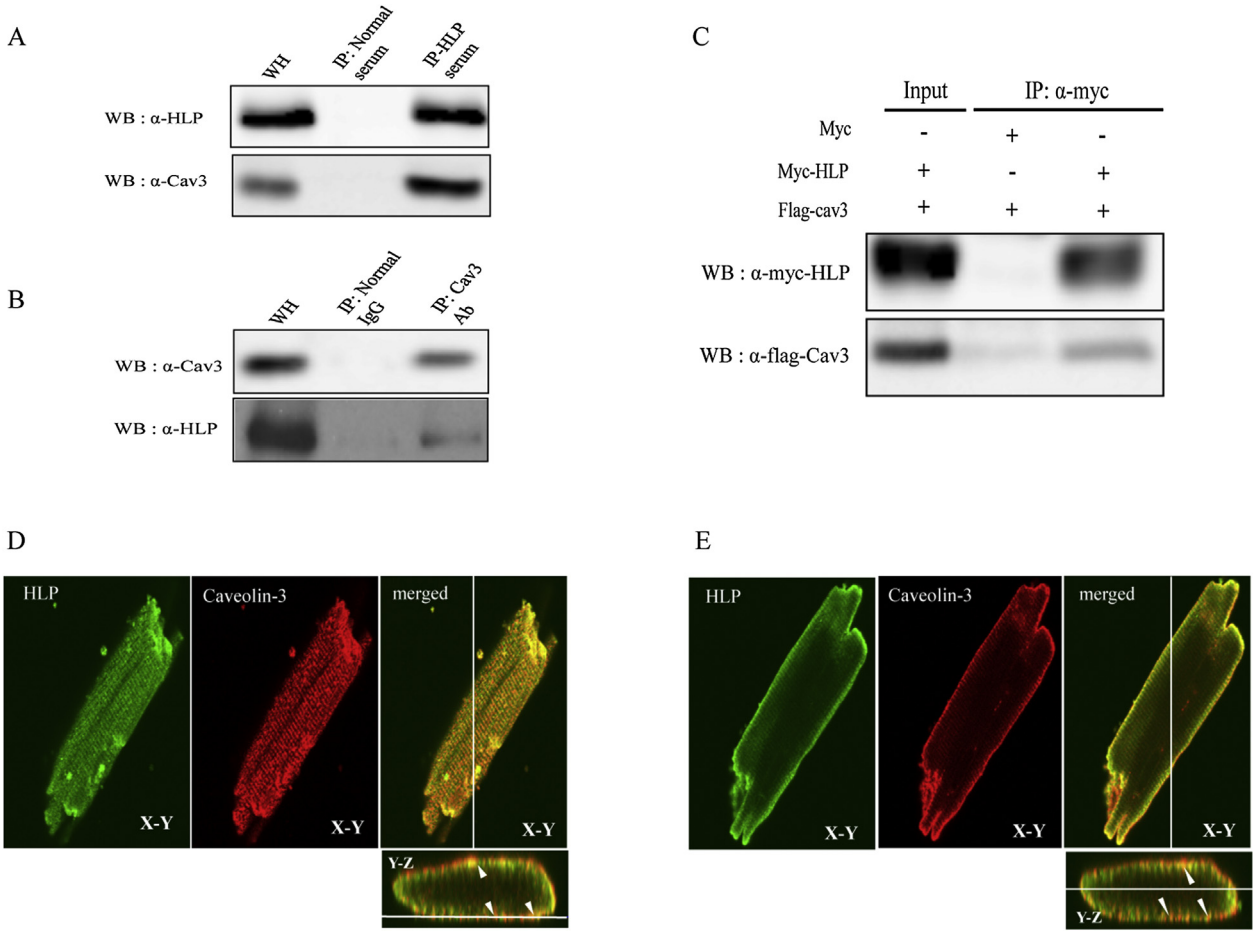

Fig. 3. Association of HLP with caveolin-3. (A, B) The immunoprecipitated proteins with anti-HLP serum (A) or with anti-caveolin-3 antibody (B) were probed with anti-HLP and anti-caveolin-3 antibodies. WH: 50 μg mouse WH. (C) HEK293 cells were co-transfected with *myc*-empty vector plus flag-caveolin-3 or *myc*-HLP plus flag-caveolin-3. Immunoprecipitation was performed with anti-*myc* antibody. The immunoprecipitated proteins were probed with anti-*myc* or anti-flag antibody. (D, E) The representative z-stack images of the bottom (D) or the middle slice (E) of the stained adult ventricular myocytes are shown. HLP molecules were stained with anti-HLP antibody followed by Alexa-488-conjugated secondary antibody (green). Caveolin-3 was stained with anti-caveolin-3 antibody followed by Alexa-594-conjugated secondary antibody (red). The images for the X–Y plane were obtained as the samples were sectioned virtually along the white horizontal line in the Y–Z plane. The image on the bottom right is for the Y–Z plane as the samples were sectioned virtually along the white vertical line in the merged X–Y plane. The white arrow heads indicate highly co-localized regions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solubilization conditions [26]. We also found no direct interaction between RyR2 and caveolin-3 (data not shown). Therefore, the present study supports the idea that HLP acts as a molecular linker to physically and functionally couple RyR2 and caveolin-3 in the heart. Interestingly, the direct interaction between RyR1 and caveolin-3 has been demonstrated in skeletal muscle [27], where endogenous HLP mRNA and protein are not detectably expressed [11,28]. Therefore, the tissue-specific mechanism for coupling caveolin-3 with RyR could depend on the expression level of the linker protein(s) and the RyR isoform type.

Our observation that HLP KD decreased the electrically evoked Ca^{2+} release (Fig. 4 and Supplementary Fig. S2) raises a question regarding the functional role of HLP in excitation-contraction coupling. Caveolae, which are located throughout the cell periphery, are known to be involved in CICR in various cardiac tissues [3,4]. Previously, both the molecular interaction and co-localization of DHPR with caveolin-3 at caveolae regions were demonstrated by immunoprecipitation and electron microscopic assays [5]. Caveolin-3-associated DHPR is likely a crucial component for CICR occurring in the caveolar microdomain. In light of our data revealing a direct interaction of HLP with caveolin-3 and RyR2 (Figs. 1 and 3) and the co-localization of HLP with RyR2 in caveolae regions (Figs. 2 and 3; Supplementary Fig. S1B), we propose that HLP is a protein that contributes to the molecular and functional coupling between caveolin-3-associated DHPR and RyR2. Thus, the loss of molecular connections in the protein complex may impair the efficiency of CICR as observed in HLP KD cardiomyocytes (Fig. 4).

Although caveolae are necessary for normal CICR, the SR Ca^{2+} load was not affected by the destruction of caveolae [4]. Similarly, Ca^{2+} loading, as determined by caffeine-induced Ca^{2+} release, was not changed by HLP KD (Supplementary Fig. S3), indicating that the HLP- or caveolin-3-mediated protein complex is not functionally related to SR Ca^{2+} uptake under basal conditions. Previously, it was suggested [4] that caveolae modulation of CICR is substantially dependent on the number of RyR2 activated by Ca^{2+} entry through caveolemmal DHPR, but not by the direct modification of single channel function. This is consistent with our observation that HLP did not change the properties of [^{3}H]ryanodine binding to RyR2 (Supplementary Fig. S3C).

Although well-developed T-tubular structures are known to be a primary site of initiation of cardiac E-C coupling in adult ventricular tissue, it has been reported that caveolae disruption also decreased electrically evoked Ca^{2+} release in the tissue [22]. In addition,

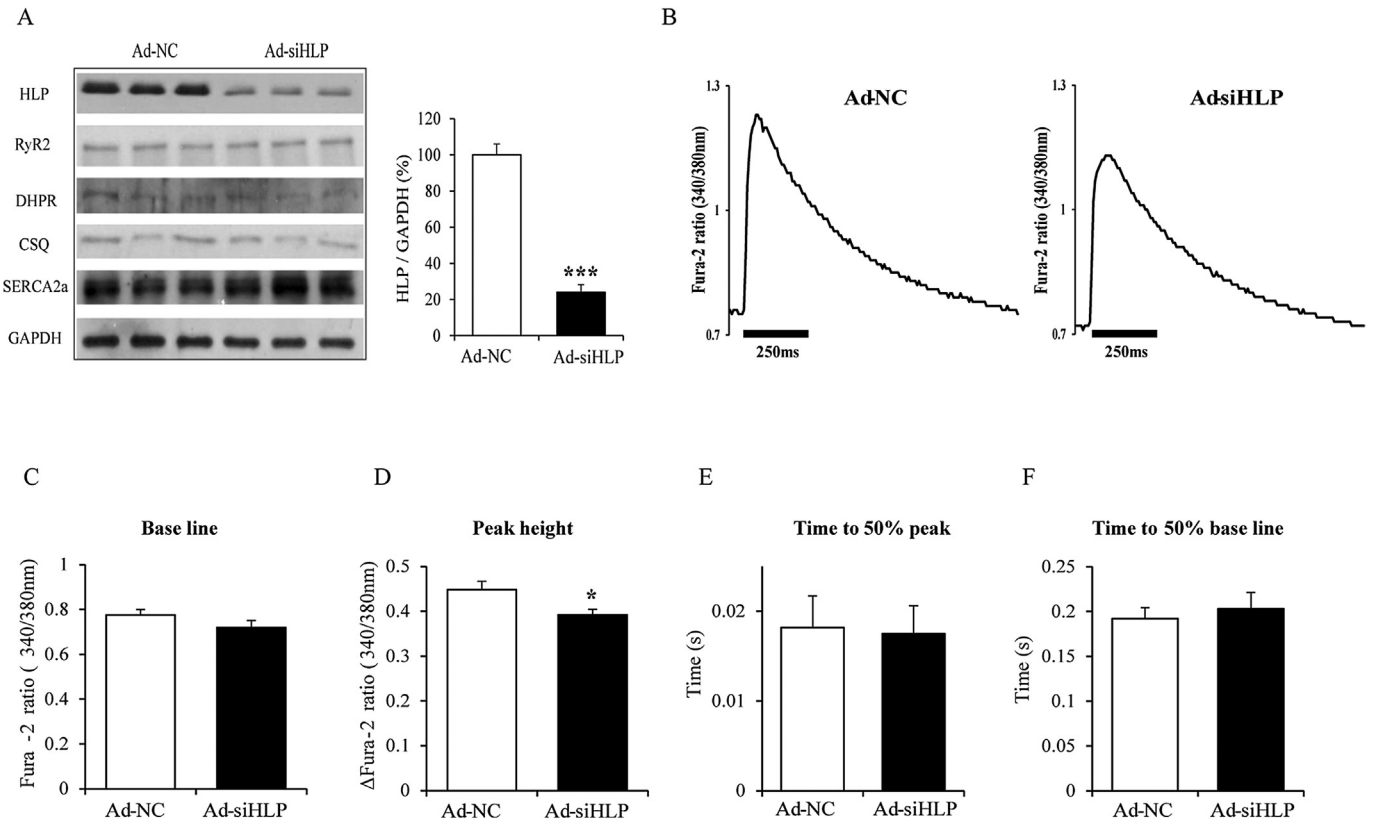

Fig. 4. Effects of HLP knock-down on field stimulation-induced Ca^{2+} release from SR. (A) Immunoblot results of HLP, GAPDH and the Ca^{2+}-handling proteins in neonatal ventricular cells infected with adenovirus delivering negative control siRNA (Ad-NC) or HLP siRNA (Ad-siHLP). (B) Representative traces of Ca^{2+} transients in Ad-NC and Ad-siHLP infected cells. Quantified basal level (C), peak amplitude (D), time to 50% of peak (E) and time to 50% of baseline of Ca^{2+} transients (F) in Ad-NC and Ad-HLP infected neonatal cardiomyocytes. Data show means ± SEM of four independent experiments (* statistically significant at $p < 0.05$ vs Ad-NC). Note that the peak height was significantly down-regulated by HLP KD.

caveolin-3-associated DHPR has been demonstrated in various cardiac tissues [5,26,29]. Therefore, it is possible that HLP could couple RyR2 and caveolin-3 to regulate CICR, regardless of the types and ages of the cardiac tissue, even though there must be some subtle differences.

In conclusion, we provide evidence that HLP is an important adaptor protein for interaction with RyR2 or caveolin-3 in the peripheral region of the heart. KD of HLP induced CICR impairment, which suggests a role for HLP in providing the microdomain for SR Ca^{2+} release.

Disclosures

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.045.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.045.

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