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ERp44 C160S/C212S mutants regulate IP$_3$R$_1$ channel activity

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ABSTRACT

Previous studies have indicated that ERp44 inhibits inositol 1,4,5-trisphosphate (IP$_3$)-induced Ca^{2+} release (IICR) via IP$_3$R$_1$, but the mechanism remains largely unexplored. Using extracellular ATP to induce intracellular calcium transient as an IICR model, Ca^{2+} image, pull down assay, and Western blotting experiments were carried out in the present study. We found that extracellular ATP induced calcium transient via IP$_3$Rs (IICR) and the IICR were markedly decreased in ERp44 overexpressed Hela cells. The inhibitory effect of C160S/C212S but not C29S/T396A/ΔT(331–377) mutants of ERp44 on IICR were significantly decreased compared with ERp44. However, the binding capacity of ERp44 to L3V domain of IP$_3$R$_1$ (1L3V) was enhanced by ERp44 C160S/C212S mutation. Taken together, these results suggest that the mutants of ERp44, C160/C212, can more tightly bind to IP$_3$R$_1$ but exhibit a weak inhibition of IP$_3$R$_1$ channel activity in Hela cells.

KEYWORDS   ERp44, mutants, ATP, inositol 1,4,5-trisphosphate (IP$_3$) receptors, calcium transient

INTRODUCTION

Ca^{2+} signaling plays a pivotal role in the regulation of numerous cellular functions (Berridge et al., 2003). Two main sources lead to Ca^{2+} influxes in the cytosol: the plasma membrane and the endoplasmic reticulum (ER). There are two major types of intracellular calcium release channels or receptors, rynaodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP$_3$Rs), located on sarcoplasmic reticulum (SR) or ER and nuclear membrane. Upon binding to IP$_3$, IP$_3$Rs release Ca^{2+} to integrate signals from numerous small molecules and proteins and modulate diverse cellular functions (Patterson et al., 2004).

Three IP$_3$R isoforms are known, IP$_3$R$_1$, IP$_3$R$_2$ and IP$_3$R$_3$, with different tissue distribution (Berridge et al., 2000). A striking feature of IP$_3$Rs is the presence of a large cytoplasmic region with an IP$_3$ binding pocket close to the N terminus (Tu et al., 2005). The channel domain contains six transmembrane domains, and as a result there are three "loops" that reside in the ER lumen (Higo et al., 2005). The events occurring in the luminal domains of IP$_3$Rs have attracted great concern, and some important progress has been made over the past several years (Anelli et al., 2003; Boehning et al., 2003; Marciniak et al., 2004; Higo et al., 2005; Li et al., 2009; Cortini and Sitia, 2010).

ERp44 is firstly characterized as an ER resident protein functioning in protein folding, thiol-mediated retention (Anelli et al., 2002, 2003), secreted molecules polymerizing (Alloza et al., 2006; Wang et al., 2008), and identified as the first regulatory protein of IP$_3$Rs from the ER lumen side. It has been reported that ERp44 affects calcium transient (Higo et al., 2005; Pan et al., 2011) and gene transcription (Pan et al., 2011) by binding to and inhibiting IP$_3$ receptor subtype 1 (IP$_3$R$_1$). Capable of interacting with both Ero1α and IP$_3$R$_1$, ERp44 may function as a signal integrator to link redox and Ca^{2+} signaling (Li et al., 2009; Cortini and Sitia, 2010; Higo et al., 2010). However, the mechanism by which ERp44 and its mutants regulate IP$_3$R activity and Ca^{2+} release remains unknown.

It has been reported that only IP$_3$Rs are expressed in Hela cells (Hattori et al., 2004; Higo et al., 2005). Extracellular ATP induces an intracellular Ca^{2+} transient by activating

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phospholipase C (PLC)-associated P2Y purinergic receptors and producing inositol 1,4,5-trisphosphate (IP$_3$) which leads to Ca^{2+} release from intracellular stores (Berridge,1993; Gerasimenko and Gerasimenko, 2004; Chen et al., 2009). P2Y receptors belong to the large group of G-protein-coupled receptors including at least five distinct subtypes of P2Y receptors, which play an important role in Ca^{2+} signaling in response to extracellular ATP (Sabała et al., 2001; Gerasi-menko and Gerasimenko, 2004; Fischer et al., 2009).

In the present study, using confocal microscopy, GST-pull down assay, and Western blotting, we have investigated the role and mechanism of ERp44 and its mutants in regulating IP$_3$R channel activity and Ca^{2+} transients in Hela cells, and demonstrated, for the first time, that mutation of ERp44 C160S/C212S enhances its interaction with IP$_3$R$_1$ but decreases its inhibitory effect on IP$_3$R$_1$ channel activity and IP$_3$-induced Ca^{2+} release (IICR).

RESULTS

C160S/C212S but not C29S/T396A/ΔT(331–377) mutants of ERp44 inhibit IP$_3$Rs activity

It has been reported that ERp44 interacts with IP$_3$R$_1$, inhibiting its Ca^{2+} channel activity in Hela cells (Higo et al., 2005; Pan et al., 2011). Here we examined this in our experimental system to see if it did the same as reported. To explore the role of extracellular ATP and ERp44 in Ca^{2+} transient, pCMV-IRES-RED (control) (Fig.1Aa and 1B) and pCMV-ERp44-IRES-RED (ERp44 over-expressed) (Fig. 1Ab and 1C) were constructed and transfected into Hela cells.

Consistent with previous studies (Higo et al., 2005; Pan et al., 2011), ATP (10 μmol/L) typically caused a huge Ca^{2+} transient in the control and ERp44 over-expressed cells (Fig. 2A). The amplitude and half time decay of Ca^{2+} transient
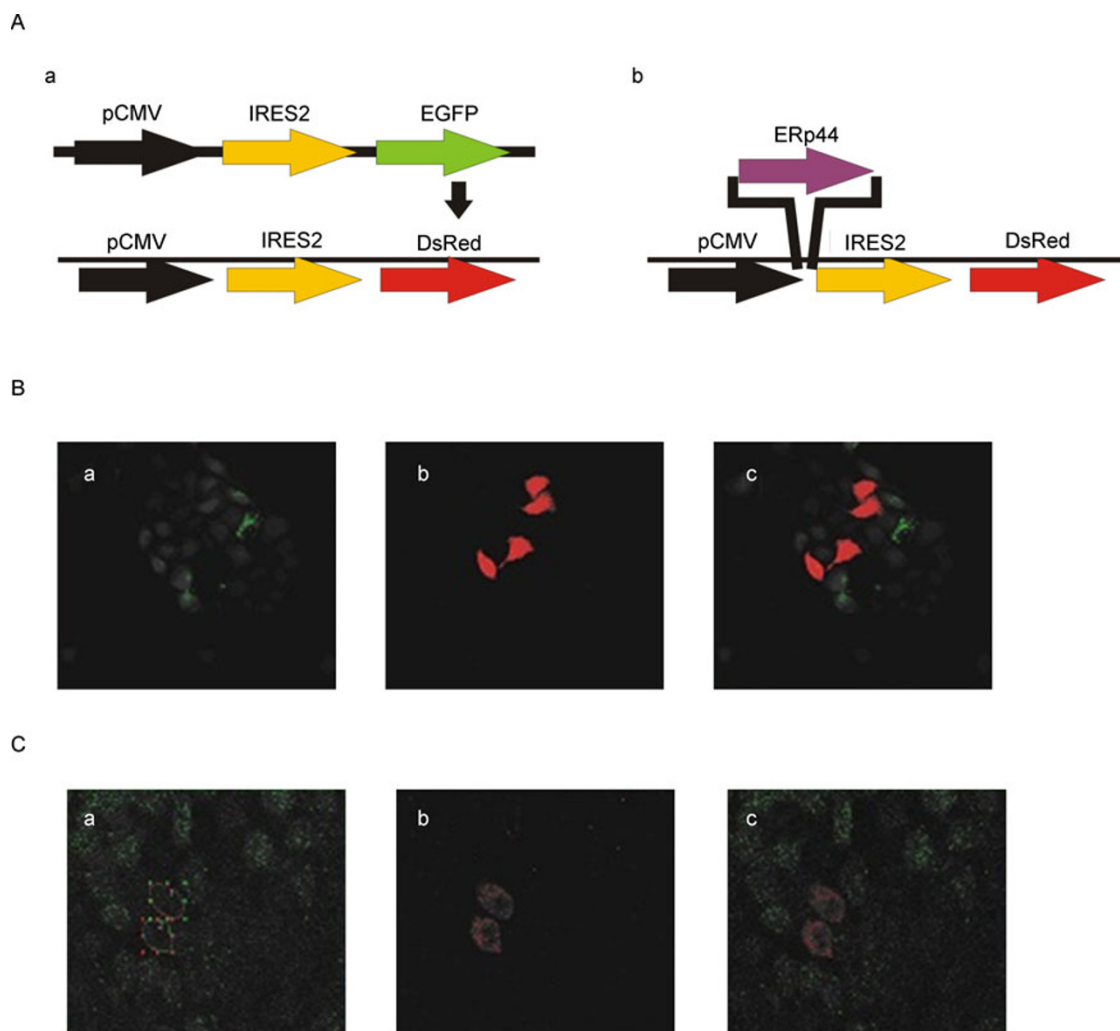
Figure 1. Construction and expression of the recombinant vectors. (A) DsRed tagged ERp44 (pCMV-ERp 44-IRES-DsRed) and negative control DsRed (pCMV-IRES-DsRed). (B and C) Cells transfected with pCMV-IRES-DsRed (B) and pCMV-ERp44-IRES-DsRed (C) were visulized by fluorescence microscopy. a, b, and c are representatives of light microscopy image (a), fluorescence microscopy image (b), and a, b merged images (c), respectively.
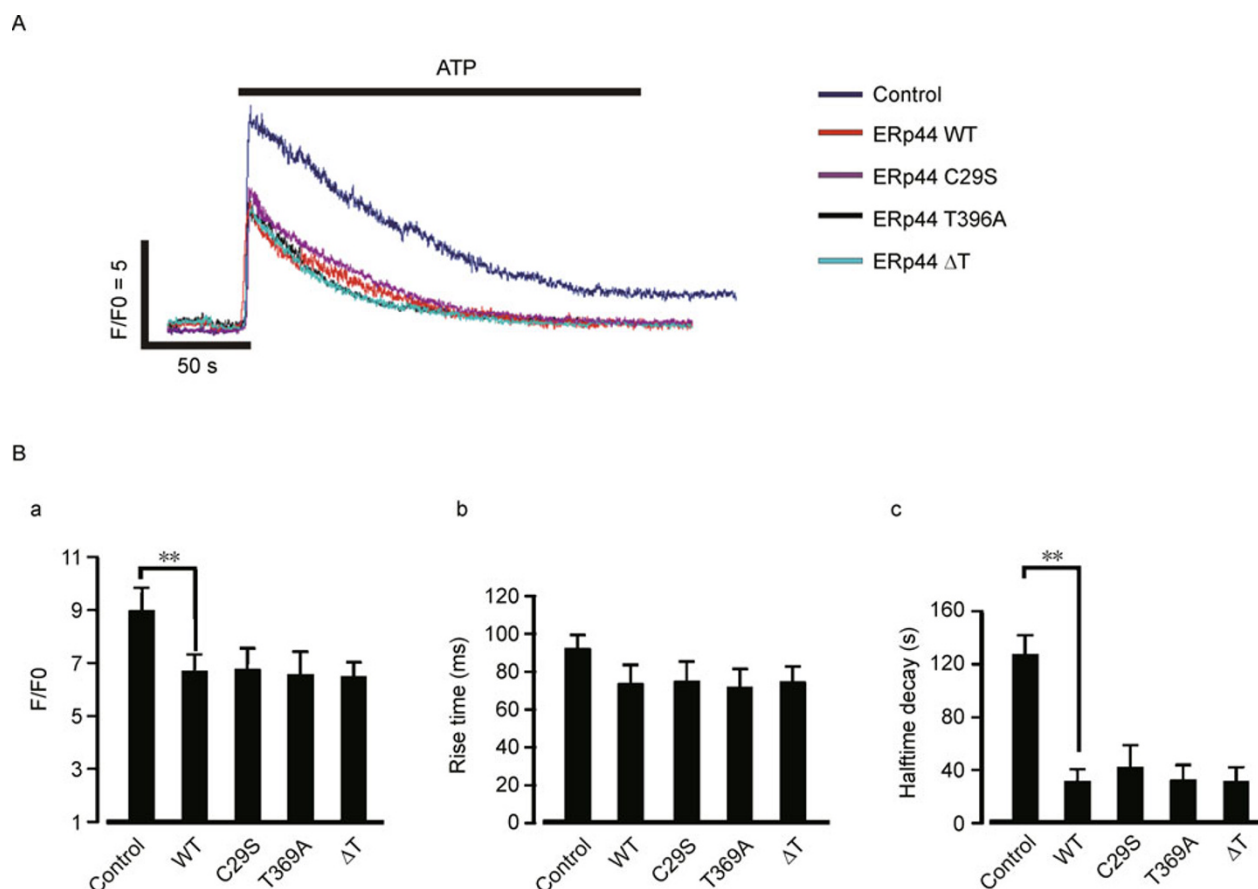
Figure 2. ERp44 C29S/T396A/ΔT(331–377) mutants does not change the effects of ERp44 on extracellular ATP-induced calcium transient. (A) Representative Ca^{2+} transients induced by ATP from linescan images recorded from Hela cells transfected with pCMV-IRES-DsRed cell (control), pCMV-ERp44-IRES-DsRed cell (ERp44) and pCMV-ERp44 C29S/T396A/ΔT(331–377)-IRES-DsRed (ERp44 C29S/T396A/ΔT). (B) Summary data of the extracellular ATP induced Ca^{2+} transients. The decrease of Ca^{2+} transients amplitude (a), rise time (b), and halftime decay (c) caused by ERp44 protein was not significantly affected by C29S/T396A point mutants and ΔT(331–377) deletion mutant (* $p < 0.05$ and ** $p < 0.001$), respectively.

induced by extracellular ATP (IP_3-induced Ca^{2+} release, IICR) were significantly decreased to 6.20 ± 0.66 (ERp44 over-expressed cells) from 8.77 ± 0.98 (control cells, Fig. 2Ba), and to 32.0 ± 8.0 s (ERp44 over-expressed cells) from 128.3 ± 14.1 s (control cells, Fig. 2Bb), respectively.

Previous studies have indicated that ERp44 uses C29 to establish disulfide bonds with client proteins, hence mediating their retention (Anelli et al., 2003; Wang et al., 2008) and T396A/ΔT(331–377) mutants of ERp44 showed significant alteration in ERp44 activity (Wang et al., 2008). We then investigated the significance of ERp44 C29S/T396A/ΔT (331–377) mutants in terms of the inhibition of IP_3Rs by ERp44. The Ca^{2+} transient was examined in pCMV-IRES-RED, pCMV-ERp44-IRES-RED and pCMV-ERp44 C29S/T396A/ΔT(331–377) mutants-IRES-RED cells. As shown in Fig. 2, the amplitude and half time decay of IICR in the cells expressing ERp44 C29S mutants was indistinguishable from

that in the cells expressing ERp44 (6.35 ± 0.88 *vs* 6.20 ± 0.66 (Fig. 2A and 2Ba) and 39.3 ± 16.4 s *vs* WT 32.0 ± 8.0 s (Fig. 2A and 2Bc)). Similarly, ERp44 T396A/ΔT(331–377) mutants did not alter the inhibitory effect of ERp44 on IICR, suggesting that the residue pair C29/T396, as well as the region from 331 to 377, is not required for the inhibition of ERp44 on IICR (Fig. 2A, 2Ba and 2Bc).

ERp44 contains six cysteine residues (29, 63, 160, 212, 272 and 289). It has been reported that the short distance between Cys160 and Cys212 in domain b suggests a strong potential to form a covalent linkage and is likely to be present *in vivo* (Patterson et al., 2004). The disulphide bond of ERp44 may be important for inhibition of IP_3Rs activity by blocking free thiol groups in the L3V domain of IP_3Rs (Higo et al., 2005). Thus, we next examined if C160/C212 of ERp44 are important for their inhibition on IP_3Rs. The Ca^{2+} transient was examined in pCMV-IRES-RED, pCMV-ERp44-IRES-RED

and pCMV-ERp44 C160S/C212S mutants-IRES-RED cells. As shown in Fig. 3, the amplitude and half time decay of IICR were markedly increased to 8.30 ± 1.10 (cells expressing ERp44 C160S mutants) from 6.20 ± 0.66 (cells expressing ERp44; Fig. 3A and 3B) and to 93.7 ± 17.3 ms (cells expressing ERp44 C160S mutants) from 32.0 ± 8.0 ms (cells expressing ERp44, Fig. 3A and 3D), respectively. Similarly, ERp44 C212S mutants significantly increased the amplitude and half time decay of IICR from 6.20 ± 0.66 (cells expressing ERp44) to 7.96 ± 1.02 (cells expressing ERp44 C212S mutants) and from 32.0 ± 8.0 ms (cells expressing ERp44) to 73.7 ± 15.1 ms (cells expressing ERp44 C160S mutants), respectively. These results indicated that the inhibition of ERp44 on IICR was significantly decreased by C160S/C212S mutation of ERp44.

ERp44 C160S/C212S mutants enhance the interaction between ERp44 and L3V domain of IP$_3$R1 (1L3V)

It has been reported that interaction between 1L3V and ERp44 modulates IP$_3$R$_1$ channel activity (Higo et al., 2005).
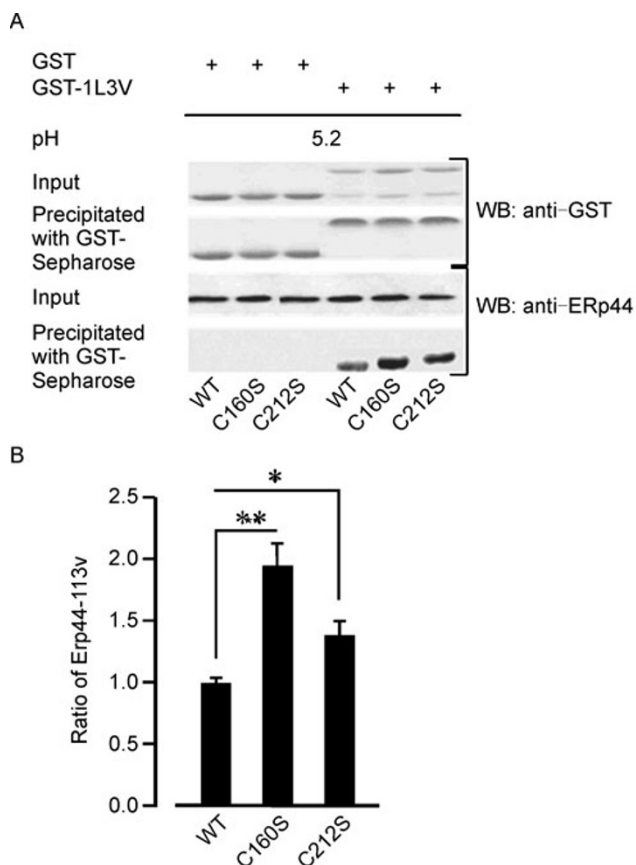
To clarify if the mutants of ERp44 share the same mechanism as ERp44, we next performed a series of pull down experiments to test whether ERp44 C160S/C212S mutants regulate IP$_3$R$_1$ channel activity by altering interaction between ERp44 and L3V domain of IP$_3$R1 (1L3V). Glutathione (GSH)-S-transferase (GST) was fused to 1L3V (Fig. 4A, lanes 4–6) and GST as negative control (Fig. 4A, lanes 1–3) for further pull down assay. Under acidic conditions (pH 5.2), the binding of ERp44 to lL3V was significantly increased by ERp44 C160S (Fig. 4A, lane 5) and ERp44 C212S mutants (Fig. 4A, lane 6) from ERp44 WT control (Fig. 4A, lane 4), respectively. These data suggest that the ERp44 C160S/C212S mutants, compared with ERp44, both exhibit an enhanced binding capacity to 1L3V.

DISCUSSION

It has been commonly believed that ERp44 is an ER luminal protein and plays an important role in protein folding, thiol-mediated retention (Anelli et al., 2002, 2003) and secreted molecules polymerizing (Alloza et al., 2006; Fraldi et al.,
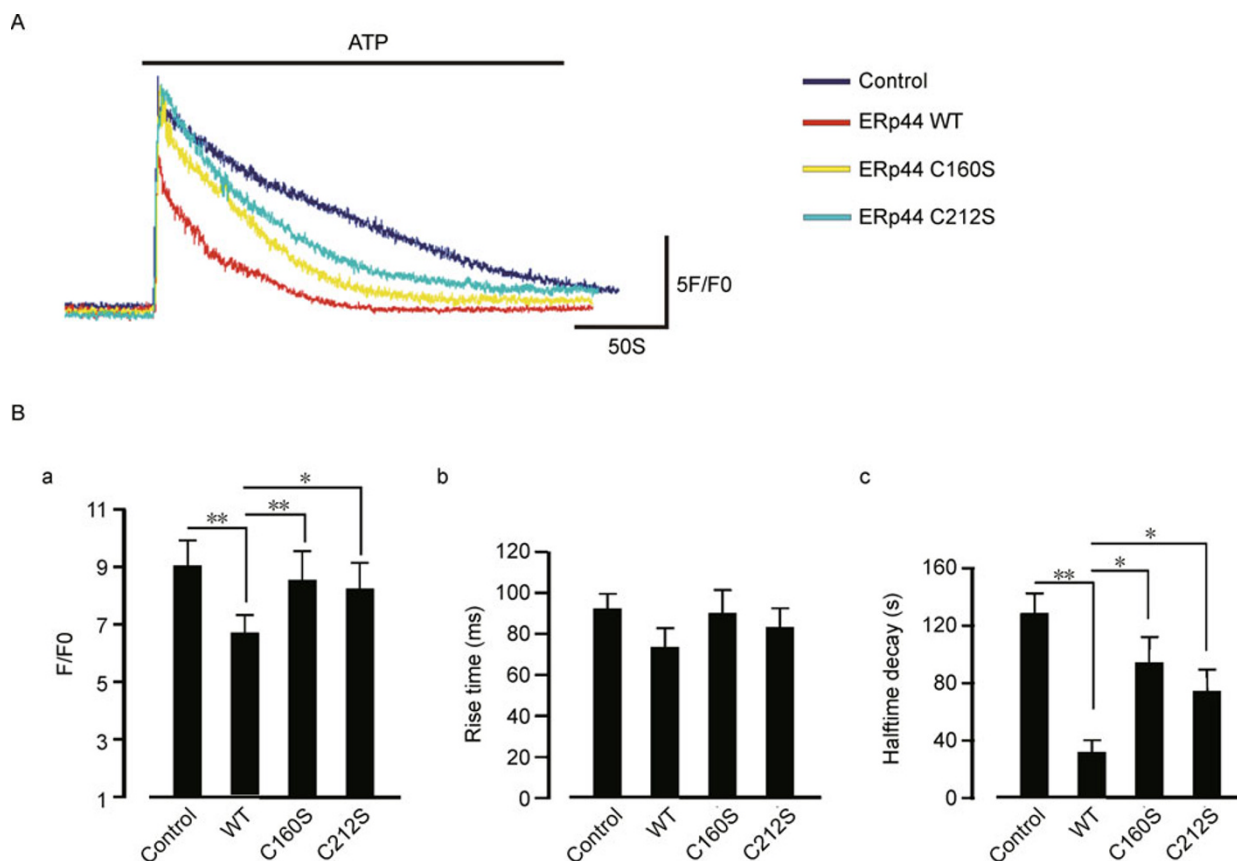
Figure 3. ERp44 C160S/C212S mutants decrease the effects of ERp44 on extracellular ATP-induced calcium transient. (A) Shows representative Ca^{2+} transients induced by ATP from linescan images recorded from Hela cells transfected with pCMV-IRES-DsRed cell (control), pCMV-ERp44-IRES-DsRed cell (ERp44) and pCMV-ERp44 C160S/C212S-IRES-DsRed (ERp44 C160S/C212S). (B) Summary data of the Ca^{2+} transients. The alteration of Ca^{2+} transients amplitude (a) and halftime decay (c) caused by ERp44 protein were significantly decreased by C160S and C212S point mutants. (* $p < 0.05$ and ** $p < 0.001$), respectively.

A

GST + + +

GST-1L3V   + + +

pH   5.2

Input

Precipitated
with GST-   WB: anti–GST
Sepharose

Input   WB: anti–ERp44

Precipitated
with GST-
Sepharose

WT C160S C212S WT C160S C212S

B

Figure 4. ERp44 C160S/C212S mutants enhance the binding of ERp44 to L3V domain of IP$_3$R$_1$ (1L3V). (A) Western blot analysis of the effects of ERp44 mutants on interaction of ERp44 and 1L3V by pull down assays. The binding of ERp44 to 1L3V were significantly enhanced by C160S (lane 5) and C212S (lane 6) point mutants. (B) Summary data of normalized ratio of ERp44/1L3V from pull down assays ($n = 5$, * $p < 0.05$ and ** $p < 0.001$), respectively.

2008; Wang et al., 2008). Recent studies have shown that ERp44 is the first identified regulatory protein of IP$_3$R$_1$ function from the ER lumen side (Higo et al., 2005). As an ER luminal protein of the thioredoxin family, ERp44 directly interacts with the third luminal loop of IP$_3$R$_1$ and inhibits IP$_3$-induced Ca^{2+} release (IICR). The interaction of ERp44 with the third luminal loop of IP$_3$R$_1$ is dependent on pH, Ca^{2+} concentration, and redox state (Higo et al., 2005). However, the role and detailed mechanism of ERp44 regulating IICR have not been investigated thoroughly, yet.

In the present study, Hela cells were employed since only IP$_3$Rs are expressed in the cells (Hattori et al., 2004; Higo et al., 2005). We used 10 μmol/L ATP to induce Ca^{2+} release. This concentration of ATP was the minimum one that could induce functional and unsaturated calcium transient in our present study, which was also used by other groups (Higo et al., 2005). Using extracellular ATP to induce intracellular

calcium transient as an IICR model, consistent with previous studies (Higo et al., 2005; Pan et al., 2011) , we showed that IICR was significantly reduced by over-expression of ERp44, and was almost entirely abolished by cells pre-treated with 2-APB (Fig. 1).

The most intriguing feature of ERp44 is the presence of a C-terminal tail (residues 326–377), connecting domains a and b' in the crystal structure. Previous studies suggest a role of the ERp44 C-tail in regulating substrate binding and release, and ERp44 ΔT(331–377)/T396A mutants, which removes the C-tail, bound to Ero1α and Ig-μ more efficiently than ERp44 (Yamauchi et al., 2003; Wang et al., 2008). Next, ERp44 ΔT(331–377)/T396A mutants were used to examine the significance of removing of ERp44 C-tail in inhibition of IICR by ERp44. Our findings demonstrated that the inhibition of IICR by ERp44 was not altered by ERp44 T396A/ΔT (331–377) mutants (Fig. 2), suggesting that ERp44 C-tail is not required for the interaction between ERp44 and IP$_3$Rs. Although ERp44 interacts with substrates through mixed disulphide bonds (Otsu et al., 2006), hydrophobic interactions are important in aligning Cys29 to target cysteines in the client proteins (Wang et al., 2008). In the present study, we showed that the inhibition of IICR by ERp44 was not affected by ERp44 C29S (Fig. 2), indicating that hydrophobic interactions between Cys29 of ERp44 and IP$_3$R$_1$ is not involved in inhibition of IP$_3$R$_1$ by ERp44.

The oxidation state of cysteines 2496 and 2504 in L3V domain of IP$_3$R$_1$ plays a critical role in both interaction with and inhibition by ERp44, binding occurring when they are reduced (Higo et al., 2005). It has been reported that the distance between Cys160 and Cys212 in domain b of ERp44 is short, which suggests a strong potential to form a covalent linkage or intermolecular disulfide bond with IP$_3$R$_1$ (Wang et al., 2008). Next, ERp44 C160S/C212S mutants were used to test whether C160/C212 of ERp44 are important for its inhibition of IICR. Our findings demonstrated that the interaction between ERp44 and L3V domain of IP$_3$R$_1$ is enhanced by ERp44 C160S/C212S mutants (Fig. 4). A previous study shows that systematic cysteine mutations in ERp44 do not affect the binding, and amino acid residues 236–285 of ERp44 are necessary and sufficient for binding to 1L3V (Higo et al., 2005). The present study suggested that ERp44 C160S/C212S mutants affected the binding between ERp44 and 1L3V. Based on all of the evidences considered, we hypothesize that the binding between ERp44 C160S/C212S mutants and IP$_3$R$_1$ may be inner-molecular disulfide bond dependent, which is different from the previously report that the binding is intermolecular disulfide bond dependent (Higo et al., 2005; Kang et al., 2008; Higo et al., 2010).

Since ERp44 directly interacts with the L3V domain of IP$_3$R$_1$ and inhibits the channel activity, it is reasonable to speculate that ERp44 C160S/C212S mutants augment the inhibition of IICR by ERp44. Surprisingly, contrary to previous studies (Higo et al., 2005; Kang et al., 2008; Higo et al., 2010),

our experimental results show that ERp44 C160S/C212S mutants significantly decrease the inhibition of IICR by ERp44 (Fig. 3), suggesting that the intermolecular disulfide bond between ERp44 and IP$_3$R$_1$ is not involved in the inhibition of IICR by ERp44. Luminal-side regulation has been proposed to involve conformational changes of IP$_3$R that affect the channel activity (Thrower et al., 2000; Otsu et al., 2006). Another possibility is that the intermolecular disulfide bond causes conformational change and generates a new molecular surface of IP$_3$R$_1$ that can function as a novel binding site for other regulating proteins (Kang et al., 2008; Cortini and Sitia, 2010). Some luminal factors, such as the redox potential, Ca^{2+} (Higo et al., 2005; Kang et al., 2008) and Ero1α (Anelli et al., 2003; Li et al., 2009), have also been inferred to regulate IP$_3$R by affecting the binding of ERp44 to the L3-1 loop of IP$_3$R. Therefore, it is hard to exclude the possibility that the effect of luminal factors were altered by the formation of intermolecular disulfide bond between ERp44 and IP$_3$R. Hence, further experiments are needed to evaluate the precise mechanisms.

In summary, our study reveals that ERp44 C160S/C212S, possibly via inner-molecular disulfide bond with free thiol groups in the L3V domain of IP$_3$R$_1$, enhance the binding of ERp44 to IP$_3$R$_1$ and decrease the inhibition of IICR by ERp44 in Hela cells.

MATERIALS AND METHODS

Chemicals and reagents

High Dulbecco's modified Eagle's medium (H-DMEM), Trizol reagent and Lipofectamine 2000 were purchased from Invitrogen. Fluo-4 AM was from Molecular Probes, and M-MLV Reverse Transcriptase was from Promega. Antibodies were obtained from Cell Signaling or Milipore. Fetal bovine serum was obtained from GIBICO and other chemicals were purchased from Sigma.

Cell culture and cell transfection

Hela cell lines were obtained from ATCC (Manassas, VA, USA) and cultured in H-DMEM medium supplemented with 10% fetal bovine serum, 100 mg/L penicillin, and 100 mg/L streptomycin. The full-length of the ERp44's cDNA was kindly provided by Professor CC Wang (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). The expression vectors were constructed using the New England Biolabs's restriction enzymes and the details will be supplied on request. The cells for calcium images, Western blot, Co-IP and Pull down assay were transfected with pCMV-IRES-RED (control), pCMV-ERp44-IRES-RED (ERp44 WT), or pCMV-ERp44 Mutant-IRES-RED (ERp44 M).

Ca^{2+} image

After being transfected for 48 h, cells were incubated with 10 μmol/L Fluo-4 AM at room temperature for 10 min, and then placed onto the stage of an inverted microscope (SP5, Leica, Germany), using a Plan Apo 40× oil objective. Cells were perfused in a standard solution containing 150 mmol/L NaCl, 5.5 mmol/L KCl, 2 mmol/L CaCl$_2$, 1 mmol/L MgCl$_2$, 10 mmol/L HEPES and 3 mmol/L glucose, which was adjusted to pH 7.4 by NaOH. 488 nm and 543 nm were the excitation wave lengths for detection of Ca^{2+} and dsRED, respectively. The sequential scanning model was used in case of the crosstalk of the excitations. Linescans were obtained at an interval of 1.33 or 0.833 ms per line. Images were processed and analyzed using MATLAB 7.1 software (MathWorks).

GST-pull down assay

We used Glutathione Sepharose 4 Fast Flow beads (GE healthcare) to pull down GST and GST-1L3V-ERp44. 1 μmol/L GST-1L3V was incubated with 1.2 μmol/L ERp44 in 1 mL lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L DTT, 5 mmol/L EGTA) for 1 h at 4°C. The lysis buffer was then added to glutathione sepharose 4FF and rotated overhead for 1 h. Bound protein complexes were washed 4 times in lysis buffer and analyzed by subsequent Western blotting.

Western blotting

Total protein extracts were transferred onto PVDF membranes after separation by 12% SDS-PAGE. The membranes were blocked with 5% milk in TBST for 1 h, incubated for 2 h with primary antibodies, and then probed for 1 h with HRP-conjugated anti-rabbit IgG. After extensive wash with TBST, the target proteins were detected on the membranes by enhanced chemiluminescence.

Data analysis

SIGMAPLOT and ImageJ were used in statistical and imaging analyses. The groups were compared by one-way, repeated measures of ANOVA and significant differences between groups were determined by the Student's t test for pairwise comparisons. Results are expressed as mean ± SE where applicable. Only $p < 0.05$ is considered significant.

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