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p32, a novel binding partner of Mcl-1, positively regulates mitochondrial Ca^{2+} uptake and apoptosis

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ABSTRACT

Mcl-1 is a major anti-apoptotic Bcl-2 family protein. It is well known that Mcl-1 can interact with certain pro-apoptotic Bcl-2 family proteins in normal cells to neutralize their pro-apoptotic functions, thus prevent apoptosis. In addition, it was recently found that Mcl-1 can also inhibit mitochondrial calcium uptake. The detailed mechanism, however, is still not clear. Based on Yeast Two-Hybrid screening and co-immunoprecipitation, we identified a mitochondrial protein p32 (C1qbp) as a novel binding partner of Mcl-1. We found that p32 had a number of interesting properties: (1) p32 can positively regulate UV-induced apoptosis in HeLa cells. (2) Over-expressing p32 could significantly promote mitochondrial calcium uptake, while silencing p32 by siRNA suppressed it. (3) In p32 knockdown cells, Ruthenium Red treatment (an inhibitor of mitochondrial calcium uniporter) showed no further suppressive effect on mitochondrial calcium uptake. In addition, in Ruthenium Red treated cells, Mcl-1 also failed to suppress mitochondrial calcium uptake. Taken together, our findings suggest that p32 is part of the putative mitochondrial uniporter that facilitates mitochondrial calcium uptake. By binding to p32, Mcl-1 can interfere with the uniporter function, thus inhibit the mitochondrial Ca^{2+} uploading. This may provide a novel mechanism to explain the anti-apoptotic function of Mcl-1.

1. Introduction

Apoptosis [1], also called programmed cell death, is a critical self-suicide process in many biological events, such as ischemia, neurodegeneration, autoimmunity and viral infections [2]. The signaling pathways of apoptosis are very complicated. The mitochondria dependent pathway is shown to be the most important one [3]. Many apoptotic stimuli can trigger mitochondria to release some inter-membrane proteins (*e.g.* cytochrome c and Smac) into the cytosol to activate the downstream apoptotic pathways (including the caspase cascade) [4]. This triggering process, to a large extent, is mediated by proteins belonging to the Bcl-2 family proteins. The Bcl-2 family proteins can be subdivided into pro-apoptotic proteins, which include multi-domain proteins (*e.g.* Bax and Bak), the 'BH3 domain only' proteins (*e.g.* Bid, Bim, Bad, Puma, *etc.*), and anti-apoptotic proteins (*e.g.* Mcl-1, Bcl-2 and Bcl-xL). Mcl-1 is one of the major anti-apoptotic proteins. It has been reported that Mcl-1 can bind to and inactivate some pro-apoptotic proteins, such as Bax, Bak, Bim, Bad and Bok at the resting stage [5]. In addition, Mcl-1 can inhibit mitochondrial calcium signal [6]. Since it has been demonstrated that Ca^{2+} mobilization from ER to mitochondria can play a positive role in the apoptotic signaling pathway [7], the inhibition of Mcl-1 on mitochondrial calcium uptake may provide an additional interpretation for the anti-apoptotic function of Mcl-1. The detailed mechanism for this inhibitory function of Mcl-1, however, is poorly understood at present.

In this study, we conducted a series of experiments to examine the two questions: (1) Are there any novel binding partners of Mcl-1 which is involved in regulating apoptosis? (2) Are these novel binding partners of Mcl-1 participated to regulate mitochondrial calcium uploading? We firstly applied the Y2H assay to fish out p32 as a novel binding partner of Mcl-1 and confirmed their interaction using co-immunoprecipitation. We also examined the effect of p32 and Mcl-1 on mitochondrial calcium uptake using live cell

Abbreviations: Co-IP, co-immunoprecipitation; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; RuRed, Ruthenium Red; YFP, yellow fluorescent protein; Y2H, Yeast Two Hybrid.

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imaging. Finally, we showed that the regulation of Mcl-1 and p32 on mitochondrial Ca^{2+} was related to the mitochondrial calcium uniporter. Our data indicates that p32 (C1qbp) is a novel binding partner and antagonist of Mcl-1, which can positively regulate apoptosis and mitochondrial calcium uptake.

2. Materials and methods

2.1. Chemicals and antibodies

Mowiol 4-88 was from Calbiochem (San Diego, CA). Anti-p32 goat monoclonal, anti-cdc2 mouse monoclonal, anti-Mcl-1 rabbit polyclonal and anti-GFP monoclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GFP polyclonal antibody was purchased from Molecular Probes. Anti-β-tubulin mouse monoclonal antibody was from Sigma–Aldrich, Inc. Rhod-2-AM and MitoTracker Red CMXRos were from Molecular Probes. Adenosine 5′-triphosphate (ATP) disodium salt and sodium borohybride (NaBH4) were from Sigma–Aldrich, Inc. Disuccinimidyl suberate (DSS) and Dithio-bismaleimidoethane (DTME) were from Pierce, Thermo Fisher Scientific Inc (Rockford, IL). Tween 20 was from USB Corp.

2.2. Plasmid and vector-based siRNA construction

The human Mcl-1 gene was kindly provided by Dr. Steven W. Edwards from University of Liverpool [5]. Mcl-1 was amplified by primers: Forward 5′-CCGGAATTCCGATGTTTGGCCTCAAAAGAA ACG-3′ and Reverse 5′-CGCG-GATCCCGCTATCTTATTAGATATGCC AAAC-3′. Then amplified Mcl-1 was cloned into pEGFP-C3 vector (Clontech, CA) by EcoRI and BamHI (Roche). The human p32 gene construct pYW59 was kindly provided by Dr. S. Diane Hayward [8]. The p32 was cloned into pEYFP-N1 and pDsRed-N1 vectors (Clontech, CA) by primers: Forward 5′-CCGCTCGAGATGCTGCC TCTGCTGCGCTG-3′ and Reverse 5′-GGAATTCCCTGGCTCT-TGACAA AACTCTTG-3′.

Vector-based siRNA was carried out on the basis of the pSilencer™ 3.1-H1 hygro Kit (Ambion). The sequences of siRNA against Mcl-1 and p32 were: 5′-CGGGATCCA-AGTATCACAGACGTTCTCTT CAAGAGAGAGAACGTCTGTGATACTTTTTTT-TGGAAAAGCTTGGG-3′ and 5′-CGGGATCCAATGGGACAGAAGCGAAATTT-CAAGAGAATTTCGC TTCTGTCCCATTTTTTTTGGAAAAGCTTGGG-3′.

2.3. Cell culture and transfection

HeLa cells were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37 °C. The fusion genes and siRNAs were transfected into cell by LipofectamineTM 2000 (invitrogen) or electroporation [9].

2.4. Yeast Two-Hybrid

The Y2H screen was performed with the MATCHMAKER Two-Hybrid system III (Clontech). Mcl-1 was cloned into the plasmid pGBT9. Genes encoding the interacting proteins were screened from the positive clones using the standard Y2H method [10].

2.5. Immunofluorescence staining and Western blotting

The immunofluorescence staining and Western blotting were performed according to previous protocol [9].

2.6. Cross-linking

The thiol-specific cross-linker DTME (Dithio-bismaleimidoethane) was dissolved in DMSO at 12.4 mg/ml (40 mM) just before use. Then it was diluted to 0.5 mM in 1× PBS and applied into cell culture dishes. Incubate the cells with mild shaking for 1 h. Then aspirate the crosslinker and collect the cell. The cell pellet could be subjected to Western blotting analysis or co-immunoprecipitation. DSS was dissolved in DMSO at 7.4 mg/ml (20 mM) just before use. Then it was diluted to 2 mM in 1× PBS and applied to crosslinking anti-GFP antibody with dynabeads protein G. To stop the reaction, incubate the beads with 100 mM Tris·HCl (pH 7.5) buffer.

2.7. Co-immunoprecipitation assay

The co-immunoprecipitation assay was performed as manufacture's protocol. Briefly, Dynabeads® Protein G (Invitrogen) was washed with 500 μl Citrate–Phosphate buffer (24.5 mM Citric Acid and 51.7 mM Dibasic Sodium Phosphate dehydrate, pH 5.0) twice. The supernatant was removed after placing the tubes on the magnet for 2 min. Add 2 μl anti-GFP antibody to 100 ul beads resuspended in Citrate–Phosphate buffer and incubate the tube with mild vortex at room temperature for 1–2 h. Discard supernatant, wash with 1 ml 1× PBS twice and covalently crosslink beads with antibody by freshly prepared 2 mM DSS with mild vortex at room temperature for 1 h. Then quench the crosslinking reaction by 100 mM Tris·HCl buffer (pH 7.5) with mild vortex at room temperature for 30 min and repeat once. Wash beads with 1 ml 1× PBS containing 0.05% tween 20 three times and elute the unbound antibody from beads by 100 μl of 0.1 M Citrate buffer (pH 2.2) twice. Neutralize the acidic beads with 1 ml 1× PBS containing 0.05% tween 20, remove the supernatant on the magnet and repeat twice. To pull down GFP fusion proteins from cell lysate, incubate the cell lysate with anti-GFP antibody coupled Dynabeads® Protein G for overnight at 4 °C on a rotator. Then centrifuge at 14000 rpm for 30 s, carefully remove supernatant completely and wash the beads 3 times with 1 ml 1× PBS containing 0.05% tween 20. After the last wash, remove the supernatant and incubate the beads for 2 min within 30 μl of 0.1 M Citrate buffer (pH 2.2). Transfer the supernatant to fresh tubes and the elution could be applied to SDS–PAGE.

2.8. Mitochondrial Ca^{2+} measurement and living cell imaging system

The mitochondrial Ca^{2+} measurement was performed according to previous protocol [7]. Briefly, 5 μM dihydroRhod-2-AM was loaded into HeLa cell in 37 °C for 30 min and cells were washed by Krebs–Ringer bicarbonate buffer three times. Cells then are ready for observation under confocal fluorescent microscope.

3. Results

3.1. P32 is a novel binding partner of Mcl-1

In order to identity the novel binding partners of Mcl-1, we applied the Yeast Two-Hybrid (Y2H) method in this study. The Mcl-1 gene was cloned into the plasmid pGBT9 so that the Mcl-1 protein was fused with the DNA-binding domain of GAL4, which serves as the bait. A human cDNA library cloned into pCAT2 was co-transformed into yeast strain Y190. Genes encoding the potential interacting proteins were screened from the positive clones using the standard Y2H method [10]. As a result, p32 was identified as a potential binding partner of Mcl-1 (data not shown). Then, we performed the co-immunoprecipitation assay to verify the interaction between Mcl-1 and p32 *in vitro*. We over-expressed GFP, GFP-Mcl-1 or p32-YFP in HeLa cells and pulled down GFP or GFP fusion protein by anti-GFP antibody-conjugated dynabeads Protein G. As shown in Fig. 1A, the GFP, GFP-Mcl-1 or P32-YFP protein, which has the molecular weight of 27, 67 or 59 kDa respectively, was detected in the elution after immunoprecipitation.

When immunoblotting with anti-p32 antibody, we found that a endogenous p32 was pulled down with GFP-Mcl-1 and p32-YFP but not GFP, which suggested that Mcl-1 can indeed bind to p32 and p32 can form homo-oligomer.

3.2. p32 colocalizes with Mcl-1 at mitochondria

The subcellular localization of p32 is still controversial. Some have reported p32 to be present predominantly in the mitochondria [11,12], others have found it was distributed in the cytoplasm [13], cell surface [14], and nucleus [13,15]. Furthermore, the distribution of p32 may change under certain treatment. During adenovirus infection, p32 translocated from mitochondria to the nucleus accompanied with a viral core protein [13]. Upon LMB (a specific inhibitor of CRM-dependent nuclear export) treatment, mitochondrial protein P32 could accumulate in nucleus [16].

To examine the subcellular distribution of p32, we examined the localization of p32-YFP fusion protein in HeLa cells. We overex-

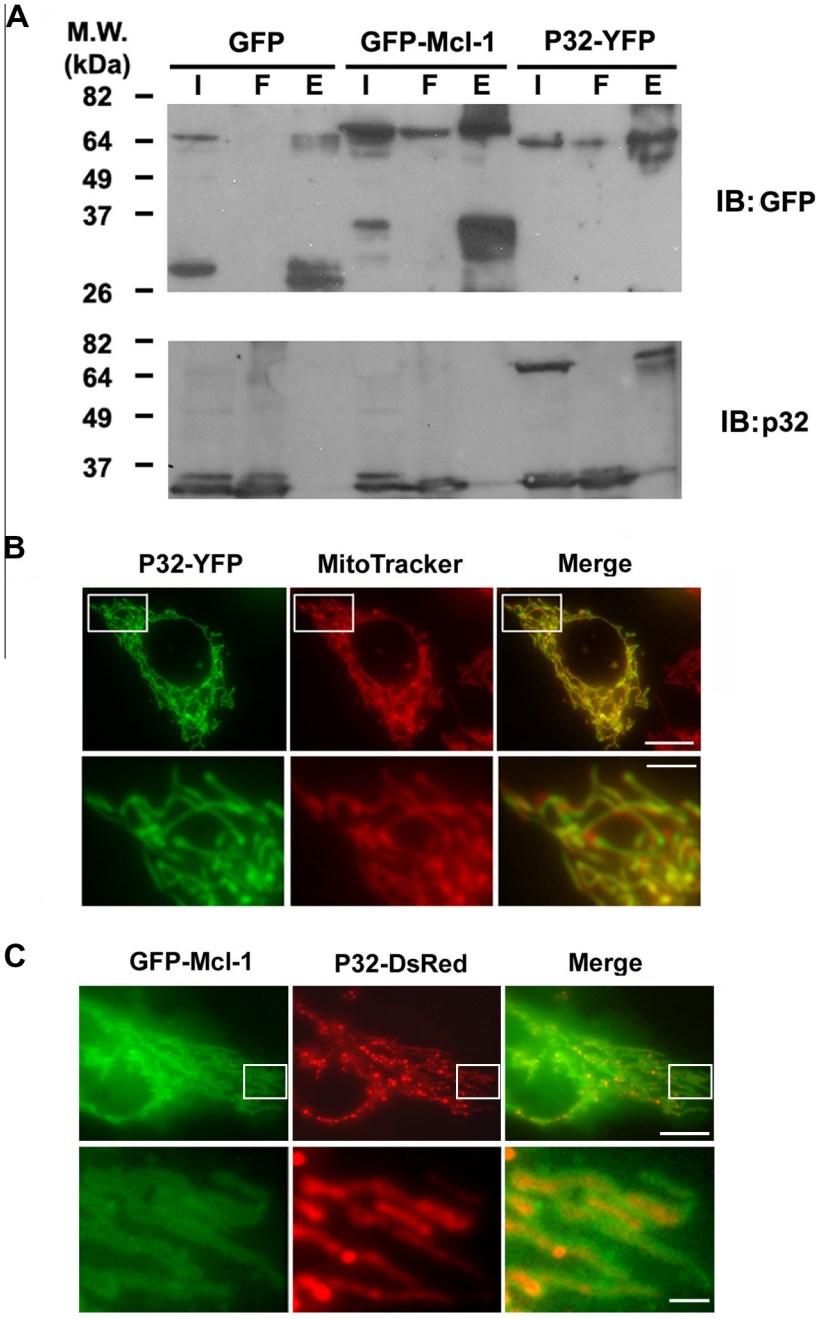

Fig. 1. p32 is a binding partner of Mcl-1. (A) Specific interaction between p32 and Mcl-1 *in vitro*. Cells were transfected with GFP, GFP-Mcl-1 or p32-YFP by Lipofectamine 2000. After 24 h of transfection, the GFP, GFP-Mcl-1 or p32-YFP protein was pulled down by Dynabeads Proteins G conjugated with anti-GFP antibody. The samples were then subjected to Western blotting analysis by anti GFP or p32 antibody. I: input before Co-IP; F: unbound flow-through after Co-IP; E: elution after Co-IP. (B) Fluorescent images of p32-YFP (green) and MitoTracker-Red CMXRos in HeLa cells. Cells were transfected with p32-YFP by Lipofectamine 2000. After 24 h of transfection, cells were stained with 0.1 μM MitoTracker Red CMXRos in culture medium for 5 min and washed with 1× PBS. The fluorescence of p32-YFP and MitoTracker Red was recorded by fluorescent microscope. The color combined image was shown as "merge". The region marked with rectangle was enlarged and shown at lower panel. Scale bar: 10 μm (upper panel) and 3 μm (lower panel). (C) Fluorescent images of GFP-Mcl-1 (green) and p32-DsRed (red) in HeLa cells. The region marked with rectangle was enlarged and shown at lower panel. Scale bar: 10 μm (upper panel) and 2 μm (lower panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pressed P32-YFP for 24 h and stained mitochondria with MitoTracker Red CMXRos. As shown in Fig. 1B, the p32-YFP perfectly colocalized with the red fluorescence of MitoTracker Red. This result suggested that the p32-YFP fusion protein is localized at mitochondria exclusively. We also performed immunofluorescent staining by using anti-p32 antibody and found that the distribution of endogenous p32 showed a filamentous pattern, indicating that p32 localized at mitochondria (data not shown). Then we examined whether p32 co-localized with Mcl-1. We performed double transfections of both GFP-Mcl-1 and p32-DsRed in HeLa cells. As shown in Fig. 1C, GFP-Mcl-1 showed a hollow structure which was surrounding p32-DsRed under high magnification. Our data indicated that GFP-Mcl-1 might localize to the outer membrane of mitochondria while p32 was in the matrix of mitochondria.

3.3. p32 is pro-apoptotic but does not affect the stability of Mcl-1

After examining the subcellular localization of p32, we then investigated the apoptotic property of p32 in HeLa cells. As shown by cell counting analysis in Fig. 2A, the cells over-expressing p32-YFP showed a slightly higher cell death rate than YFP-transfected control group after UV irradiation. On the other hand, knockdown of p32 could reduce UV-induced apoptosis in HeLa cells compared to pSilencer control (Fig. 2B). The efficacy of vector-based pSilencer-p32 was shown in Fig. 2D. Therefore, p32 is a pro-apoptotic protein in UV-induced apoptosis in HeLa cell.

Because the degradation of Mcl-1 was reported to be the prerequisite of apoptotic initiation [17], we then examined whether the pro-apoptotic function of p32 is through destabilizing Mcl-1. We manipulated the expression level of p32 and examined the protein level of endogenous Mcl-1. As shown in Fig. 2C and D, the Mcl-1 protein level was not significantly altered after overexpressing p32-YFP or knockdown of p32. In summary, p32 does not regulate the stability of Mcl-1, therefore other mechanisms for the pro-apoptotic function of p32 need to be explored.

3.4. Mcl-1 and p32 regulate mitochondrial calcium uptake

As shown in Fig. 1A, a proportion of endogenous p32 could be co-immunoprecipitated with P32-YFP, suggesting that p32 can form homo-oligomer. This partially supports the prediction that p32 might form a size-controllable trimer functioning as a mitochondrial cation channel [18]. Moreover, it has been already demonstrated that mitochondrial calcium uptake can play a positive role in the apoptotic signaling pathway [7]. Therefore, we examined whether the Mcl-1/p32 interaction played a role in regulating mitochondrial calcium uptake.

To investigate the putative role of Mcl-1 and p32 in mitochondrial calcium uptake, we either over-expressed or silenced Mcl-1 or p32 in HeLa cells, used ATP to trigger calcium release from ER, and used Rhod-2 to measure mitochondrial Ca^{2+} elevation in response to ATP. As shown in Fig. 3A, compared with the YFP control group, the GFP-Mcl-1 transfected cells showed decrease mitochondrial Ca^{2+} elevation. On the other hand, the p32-YFP transfected group showed significant increase in mitochondrial Ca^{2+} elevation triggered by ATP, suggesting that Mcl-1 can suppress mitochondria calcium uptake while p32 can promote mitochondrial Ca^{2+} uptake. We then examined the effect of knockdown of Mcl-1 or p32 on mitochondrial Ca^{2+} signal. Compared to the control, knockdown of Mcl-1 did not affect mitochondrial Ca^{2+} elevation, while knockdown of p32 only had one-third of the mitochondrial Ca^{2+} uptake in response to ATP treatment, suggesting that p32 plays a critical role in promoting mitochondrial calcium uptake (Fig. 3B). Taken together, Mcl-1 functions as a negative regulator while p32 acts as a critical positive regulator of mitochondria Ca^{2+} elevation.

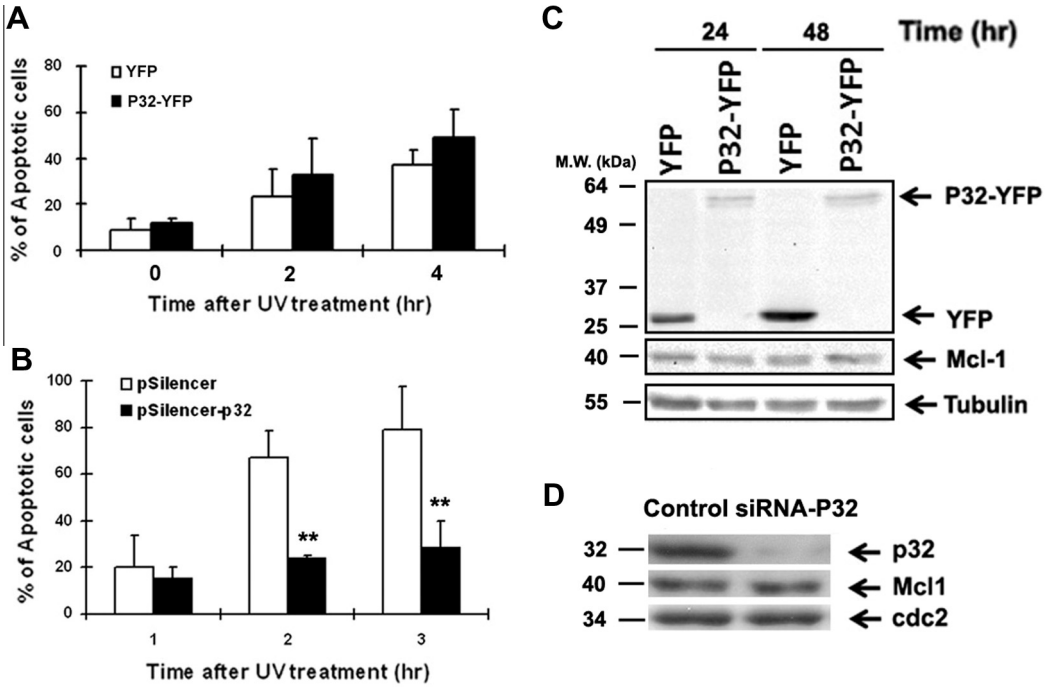

Fig. 2. p32 is a pro-apoptotic protein but does not affect the stability of Mcl-1. (A) The proportions of apoptotic cells in UV-induced apoptosis in YFP or p32-YFP transfected HeLa cells. Cells were transfected with either YFP or p32-YFP and treated with UV irradiation after 24 h of expression. The results were averaged from three independent experiments. (B) The proportions of apoptotic cells in UV-induced apoptosis in pSilencer or siRNA-p32 transfected HeLa cells. Cells were transfected with either pSilencer or siRNA-p32 and treated with UV irradiation after 36 h of expression. The results were averaged from three independent experiments. $^{**}p < 0.01$, *t*-test. (C) The protein level of Mcl-1 in either overexpressing (YFP or p32-YFP, upper panel) or silencing (pSilencer or siRNA-p32, lower panel) p32 in HeLa cells. YFP or p32-YFP transfected cells were subjected to Western blotting analysis after 24 or 48 h of expression. pSilencer or siRNA-p32 transfected cells were subjected to Western blotting analysis after 36 h of expression.

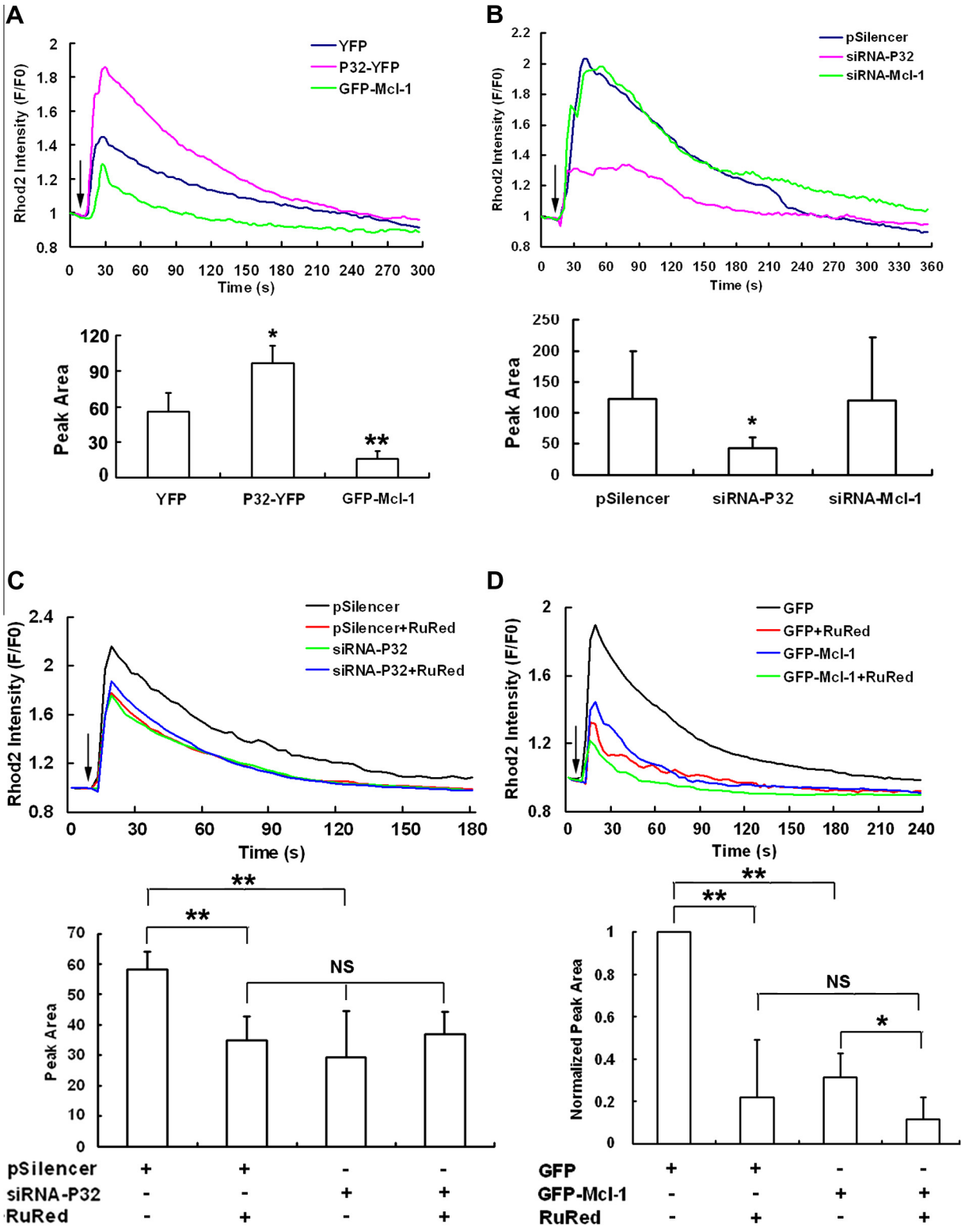

Fig. 3. Mcl-1 and p32 regulate mitochondrial Ca^{2+} uptake triggered by ATP. (A) The mitochondrial Ca^{2+} elevation in YFP, p32-YFP or GFP-Mcl-1 transfected HeLa cells triggered by 10 μM ATP and monitored by Rhod2. The peak areas were summarized at the lower panel. The results were averaged from 100 to 300 cells in seven independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$. (B) The mitochondrial calcium elevations in pSilencer, siRNA-p32 or siRNA-Mcl-1 transfected HeLa cells which were triggered by 10 μM ATP and monitored by Rhod2. The peak areas were summarized at the lower panel. The results were averaged from 100 to 200 cells in six independent experiments. $^{*}p < 0.05$. (C, D) Effects of RuRed on mitochondrial Ca^{2+} elevation in pSilencer/siRNA-p32 (C) or GFP/GFP-Mcl-1 (D) transfected HeLa cells. Mitochondrial Ca^{2+} elevation was triggered by 10 μM ATP and monitored by Rhod2. Cells were treated with 2 μM RuRed for 2 h before applying ATP. Data were averaged from four independent experiments. NS: not significant; $^{*}p < 0.05$; $^{**}p < 0.01$. All statistic analyses were student *t*-test.

3.5. p32 and Mcl-1 regulates mitochondrial Ca^{2+} uptake through mitochondrial calcium uniporter

The mitochondrial Ca^{2+} uptake was reported to be mediated by mitochondrial calcium uniporter [19]. Therefore, we examined whether p32 and Mcl-1 regulate mitochondrial Ca^{2+} through the mitochondrial calcium uniporter. We then applied Ruthenium Red (RuRed), an inhibitor of mitochondrial calcium uniporter and investigate the effects of Mcl-1 and p32 on mitochondrial Ca^{2+} elevation. We found that, in p32 knockdown cells, RuRed treatment showed almost the same mitochondrial Ca^{2+} elevation as no treated cells in response to ATP treatment (Fig. 3C). The result suggested that the inhibitory effect of silencing p32 eliminated the effect of RuRed on mitochondrial calcium uniporter. Next, we tested whether the inhibitory effect of Mcl-1 depends on the mitochondrial calcium uniporter. As shown in Fig. 3D, when treated with RuRed, GFP-Mcl-1 overexpressed cells showed similar mitochondrial Ca^{2+} elevation as the control group, suggesting that Mcl-1 does not have additional inhibitory effect when the mitochondrial calcium uniporter is suppressed. Taken together, our results suggest that the effects of p32 and Mcl-1 on mitochondrial calcium uptake depends on the mitochondrial calcium uniporter.

4. Discussions

In this study, we identified p32 (C1qbp, human complement component 1, q subcomponent binding protein) as a novel binding partner of Mcl-1 by Y2H screening. p32 was characterized as a component of human pre-mRNA splicing factor [15] and reported to be pro-apoptotic, since over-expression of p32 led to growth inhibition, cellular stress and subsequently apoptosis, and p32 was significantly upregulated during cisplatin-induced apoptosis

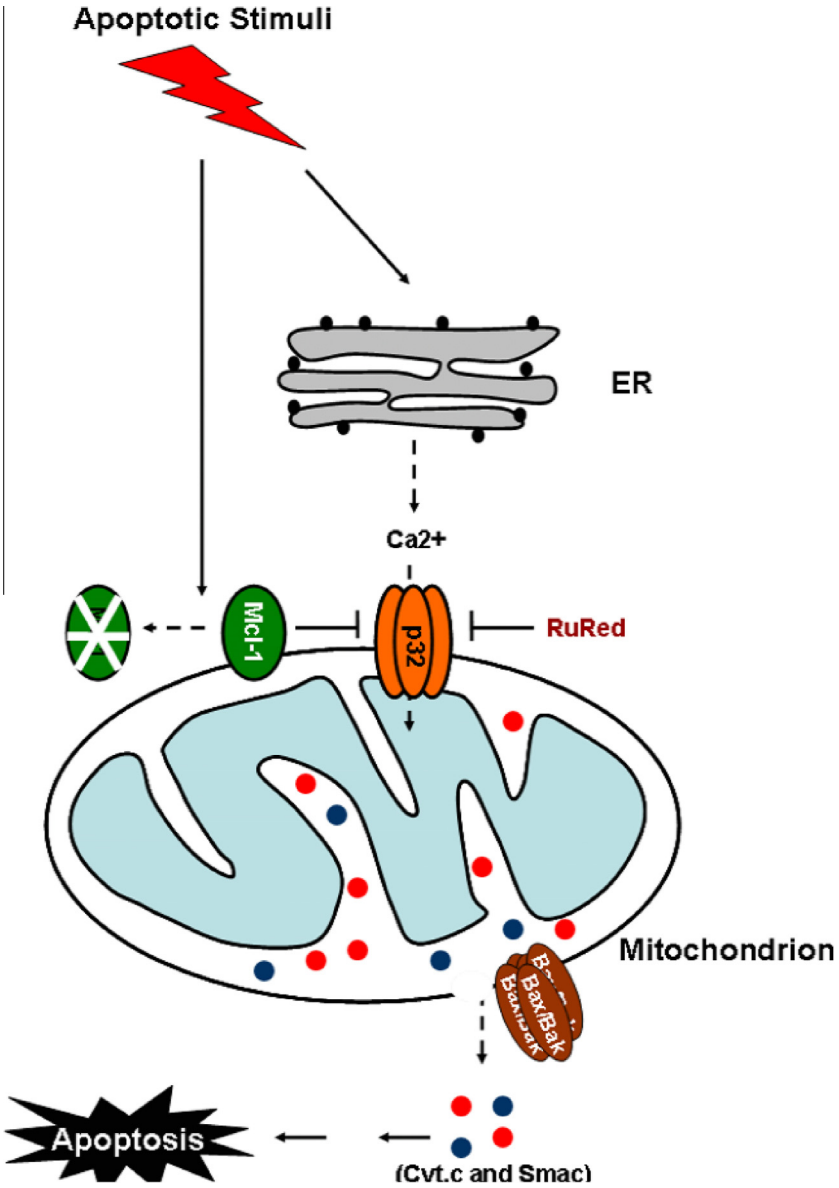

Fig. 4. The hypothetical model of the regulation of Mcl-1 and p32 on mitochondrial Ca^{2+} upload. The homo-trimer of p32 could be part of mitochondrial calcium uniporter, which controls the calcium uptake of mitochondria. In non-apoptotic cells, Mcl-1 can bind to p32 and inactivate its uniporter property. Upon apoptotic stimuli, the Mcl-1 protein is rapidly eliminated and the uniporter is activated, thus leading to uptake of calcium ions which are released from ER. The upload of mitochondrial calcium as well as the oligomerization of Bax/Bak will cause the mitochondrial outer membrane permeabilization (MOMP). MOMP will subsequently cause release of apoptotic factors (*e.g.* as cyt.c and Smac) and the process of apoptosis.

in HeLa cells [20]. Moreover, p32 could bind to HrK, a BH3-only protein, to facilitate apoptotic progression [21]. Results of our cell counting analysis also confirmed that p32 is a pro-apoptotic protein because knockdown of p32 delayed apoptotic progression (Fig. 2B).

To investigate the mechanism on how p32 regulates apoptosis, we first ruled out the possibility that p32 affected the stability of Mcl-1. The recent finding that Mcl-1 can inhibit mitochondrial calcium signaling induced by either Ca^{2+} agonists or apoptotic stimuli suggests a novel mechanism for Mcl-1 to promote cell survival [6]. It was known previously that mitochondrial calcium uploading is related to apoptotic progression [7]. Moreover, mitochondrial calcium uptake in response to apoptotic stimuli was proposed to be mediated by the uniporter. The uniporter allows Ca^{2+} ions to be transported passively into mitochondria without direct coupling to ATP hydrolysis or transporting other ions. However, the characteristics and composition of the uniporter are still not clear. The crystal structure study revealed that p32 can form a doughnut-shaped homotrimer with a sizable central pore, indicating that p32 may regulate the intra-mitochondrial concentration of divalent cations such as Ca^{2+} [18]. We also demonstrated that p32 can act as a positive regulator of mitochondria calcium uptake, since over-expression of P32-YFP could significantly promote while knockdown of p32 by siRNA could reduce mitochondrial calcium uptake triggered by ATP (Fig. 3A & B). Furthermore, the regulatory role of p32 on mitochondrial Ca^{2+} uptake appeared to be dependent on the uniporter. Therefore, it is possible that p32 could be part of the uniporter. Furthermore, we have demonstrated that: (1) Mcl-1 functions as a negative regulator because overexpression of Mcl-1 could significantly reduce mitochondrial Ca^{2+} uptake triggered by ATP. (2) The inhibitory effect of Mcl-1 appears to depend on the mitochondrial calcium uniporter. Based on these findings, we hypothesize that Mcl-1 may prevent apoptotic progression by suppressing mitochondrial calcium uptake through inhibiting p32, which might function as part of the mitochondrial uniporter (see Fig. 4).

p32 showed the strong interaction with Mcl-1 since positive colonies containing the p32 gene were repeatedly fished out in Yeast Two-Hybrid Screening. However, our Co-IP result demonstrated that only a small portion of p32 could interact with Mcl-1. Moreover, our imaging results showed that p32 was mainly localized in the matrix of mitochondria, while Mcl-1 was localized at the mitochondria outer membrane as well as cytoplasm. It seems that the interaction of p32/Mcl-1 may be restricted to the conjunctions of the outer and inner membranes of mitochondria. Regarding the capability of pore-formation, p32 may be a calcium uniporter crossing the outer and inner membranes of mitochondria and Mcl-1 binds and inhibits p32 at the outer membrane of mitochondria in healthy cells. It is also possible that, besides Mcl-1, other anti- or pro-apoptotic proteins could be binding partners of p32. Further investigations are required to sort out the above speculations.

When investigating the roles of Mcl-1 and p32 in mitochondrial calcium uptake, we mainly applied ATP to trigger mitochondrial calcium elevation. ATP can activate the G-protein coupled receptor at the plasma membrane, which results in the activation of phospholipase C-β. The elevation of mitochondrial Ca^{2+} is an immediate response upon the treatment of ATP, which allow us to examine the roles of Mcl-1 and p32 in regulating mitochondrial Ca^{2+} in a relative short time period. The IP3 receptor also plays a positive role in ER calcium release as well as apoptotic progression during UV-induced apoptosis [22], so the conclusions achieved from ATP-triggered mitochondrial calcium uptake may be able to apply to UV triggered mitochondrial calcium uptake. However, ATP cannot induce apoptosis in HeLa cells so it requires further investigations to confirm the regulatory roles of Mcl-1 and p32 in regulating $[Ca^{2+}]$mito triggered by apoptotic stimuli, such as UV and TNFα.

Conflict of interest

The authors declare no conflict of interest.

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