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SLP-2 interacts with prohibitins in the mitochondrial inner membrane and contributes to their stability

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Abstract

Stomatin is a member of a large family of proteins including prohibitins, HflK/C, flotillins, mechanoreceptors and plant defense proteins, that are thought to play a role in protein turnover. Using different proteomic approaches, we and others have identified SLP-2, a member of the stomatin gene family, as a component of the mitochondria. In this study, we show that SLP-2 is strongly associated with the mitochondrial inner membrane and that it interacts with prohibitins. Depleting HeLa cells of SLP-2 lead to increased proteolysis of prohibitins and of subunits of the respiratory chain complexes I and IV. Further supporting the role of SLP-2 in regulating the stability of specific mitochondrial proteins, we found that SLP-2 is up-regulated under conditions of mitochondrial stress leading to increased protein turnover. These data indicate that SLP-2 plays a role in regulating the stability of mitochondrial proteins including prohibitins and subunits of respiratory chain complexes.

Keywords: Mitochondria; Stomatin; Prohibitin; Chaperone; AAA protease; Protein turnover

1. Introduction

Stomatin is a plasma membrane protein that was first identified in human erythrocytes [1]. Homologues of this protein have been found in a wide range of cell types and organisms such as zebrafish, *Drosophila*, *Caenorhabditis elegans*, plants and bacteria. Stomatin is the founding member of a family of proteins which includes SLP-1, 2 and 3 in mammals. The homology between members of this family mainly resides in the stomatin domain, which is composed of (L/I/V) (K/R) repeats. In addition, due to the presence of a band 7 motif, stomatin has been predicted to belong to the SPFH family which comprises stomatins, prohibitins, flotillins and HflK/C bacterial proteases [2]. Finally, SLP-1 and SLP-3 also share an N-terminal transmembrane domain with stomatin, a feature absent from SLP-2.

The function of stomatin and SLPs is still unclear. Nevertheless, since they belong to a superfamily of proteins involved in protein turnover, stomatins have been suggested to fulfill a similar function [2,3].

Recently, in an effort to establish the proteome of the mitochondrial inner membrane (MIM), we have identified SLP-2 as a component associated with the MIM [4]. The same protein was also found to be a component of human and plant mitochondria in recent proteomic surveys [5–9]. Here, we have confirmed that SLP-2 is a protein attached to the MIM and we further investigated its function in the mitochondria. Our results show that SLP-2 interacts with prohibitins and that depleting HeLa cells of SLP-2 are accompanied by a decrease in the protein levels of prohibitins and those of subunits of complexes I and IV due to an enhanced proteolysis by metalloproteases. Furthermore we found that SLP-2 is up-regulated under conditions of mitochondrial stress leading to increased protein turnover. These data suggest that SLP-2 plays a role in regulating the stability of prohibitins and subunits of respiratory chain complexes.

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2. Materials and methods

2.1. Cell culture and reagents

HeLa, 293T, MEF and Cos-7 cells were cultured in DMEM+10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine and maintained in 5% CO_2 at 37 °C. Stress assays were performed by culturing cells in medium containing 50 μg/ml or 100 μg/ml of chloramphenicol (Fluka) for 96 h. Etoposide (100 μM) was added to the cells for 1 h after 72 h of transfection and the cells were further cultured in fresh medium for 24 h. Cycloheximide (Sigma) treatment of HeLa cells was performed at 30 μg/ml for 0, 6 or 9 h and phenanthroline (Sigma) was used at 5 mM for 30 min.

2.2. DNA cloning

All the constructs in this study were generated by standard PCR cloning procedures using a mouse liver marathon-ready cDNA library (Clontech). The amplified sequences were C-terminally fused to HA (YPYDVPDYA) tags by PCR and cloned into the pCI mammalian expression vector (Promega). The first 150 nucleotides of SLP-2 (1–50 first amino acids) were amplified by PCR and cloned into the pEGFP-N1 vector (Clontech). All the constructs were sequenced to verify the fidelity of the cDNA sequences obtained.

2.3. Immunocytochemistry

For detection of endogenous SLP-2, the cells were fixed with methanol and the antibody to SLP-2 used at a 1:100 dilution.

2.4. Isolation of mitochondria from HeLa cells

HeLa cells were seeded on 14 cm dish at 80% of confluency for 1 h before transfection with 25 μg of DNA using the classical Ca/phosphate method. After 48 h of expression, HeLa cells were treated as described by Eskes et al. [10] to prepare mitochondrial and cytosolic fractions.

2.5. Preparation of the different subcompartments of mouse liver mitochondria

Mouse liver mitochondria, mitoplasts, MIM and MOM were purified as described by Da Cruz et al. [4].

2.6. Mitoplasts preparation and proteinase K assay

100 μg of the mitochondrial fraction from HeLa cells was incubated with 2 mg/ml of digitonin (Serva) in a final volume of 100 μl of MB buffer for 20 min on ice. The solubilised fractions were then centrifuged at 10,000 *g* for 10 min at 4 °C. The pellet was washed with 1 ml of MB buffer and centrifuged again in the same conditions. The resulting pellet contains the mitoplast fraction.

Mitoplasts isolated from untransfected HeLa cells were resuspended at 0.5 mg/ml in MB buffer in the presence of 0.25 μg/ml of proteinase K (Invitrogen) for 30 min on ice. The digestion was stopped after addition of 1 mM PMSF. The digested fraction was subsequently centrifuged at 10,000 *g* for 5 min at 4 °C. The pellet was further lysed in 25 μl of 1× SDS loading buffer and analyzed by Western blotting.

2.7. Gel filtration chromatography

500 μg of mitochondria isolated from untransfected HeLa cells were solubilised in 25 mM Hepes, 300 mM NaCl, 1% Triton X-100 and protease inhibitors (Roche) for 2 h on ice. The solubilised mitochondria were further centrifuged at 100,000 *g* for 30 min. The supernatant was injected into a Superose 6 column connected to a FPLC system. 2 ml fractions were collected and TCA precipitated.

2.8. Immunoprecipitation

500 μg of mitochondria isolated from untransfected or transfected HeLa cells with pCI, SLP2-HA were solubilised with Otter buffer (10 mM Hepes, 143 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4) supplemented with 1% of Nonidet P-40 and protease inhibitors (Roche) for 2 h on ice. The solubilised mitochondria were further centrifuged at 100,000 *g* for 30 min. The supernatant was incubated with 10 μg of anti-HA, anti-SLP-2 or anti-prohibitin-1 antibody on rotation for 16 h at 4 °C. Then, 30 μl of protein A-Sepharose beads (Amersham) were added and incubated on rotation for 4 h at 4 °C. Protein A-Sepharose was recovered by centrifugation and washed four times with the Otter buffer. The washed Sepharose was suspended in 1× loading buffer analyzed by Western blotting or silver nitrate staining.

2.9. Cross-linking

2 mg of mitochondria isolated from HeLa cells were suspended in MB buffer (supplemented with proteinase inhibitors) at 2 mg/ml and incubated with 1 mM DSP for 30 min at RT. The reaction is further quenched by adding 50 mM Tris pH 8 for 30 min at RT. The cross-linked mitochondria were spun at 10,000 *g* for 10 min and the pellet was solubilised with RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, supplemented with protease inhibitors) for 16 h at 4 °C. The sample was further centrifuged at 10,000 *g* for 10 min and the supernatant was subsequently incubated with the anti-SLP-2 antibody to immunoprecipitate the endogenous SLP-2 cross-linked complex as described above. However, the washes were done with the RIPA buffer. Finally the washed Sepharose beads were incubated with 1× loading buffer containing 5% β-mercaptoethanol for 5 min at 100 °C in order to de-cross-link the immunoprecipitated fraction.

2.10. Antibodies

Anti SLP-2 antibody was produced by immunizing rabbits with synthetic peptide with a sequence CRKRATVLESEGTRES. The monoclonal anti-prohibitin-1 antibody was from Neomarkers, the anti-HSP70 from ABR, the anti-COX4 from Molecular Probes, the anti-TIM23 and anti-TIM44 from BD Biosciences, the anti-HA from Covance and the anti-GAPDH from Abcam. The polyclonal antibodies were the anti-fumarase from Nordic Immunology, the anti SMAC-Diablo from ProSci, anti-prohibitin-2 from Upstate and the anti-hFis1 from Apotech. The goat anti-VDAC (N-18) was from Santa Cruz. The anti-complex I (ND6), anti-complex II and anti-complex III (core 2) antibodies were kindly provided by R. Rossignol (University of Bordeaux).

2.11. Protein digestion and liquid chromatography–mass spectrometry (LC–MS/MS)

The protein spots were excised with a pipette tip from the gel and transferred to 96-well plates. In-gel proteolytic cleavage was performed automatically in the robotic workstation Investigator ProGest (Perkin Elmer Life Sciences). The digestion protocol included washing of the gel pieces with ammonium bicarbonate, cysteine reduction with DTT and alkylation by iodoacetamide followed by overnight digestion with trypsin (Promega) and extraction of the resulting peptides in 30% (v/v) acetonitrile, 2% (v/v) formic acid. Peptide mixtures were concentrated and resuspended in 10 μl of 0.5% formic acid before LC–MS/MS analysis. Liquid chromatography–tandem mass spectrometry was performed on a SCIEX QSTAR PULSAR (Concord, Ontario, Canada) hybrid quadrupole-time of flight instrument equipped with a nanoelectrospray source and interfaced to an LC-Packings Ultimate HPLC system. Peptides were separated on a PepMap reversed-phase capillary C18 (75 μm ID × 15 cm) column at a flow rate of 200 nl/min with a 52-min gradient of acetonitrile (0–40%). The instrument controlling Analyst software was used to perform peak detection and automatically select peptides with charge states 2+ to 4+ for collision-induced fragmentation. The resulting sets of CID spectra were bundled and used to search with the MASCOT software a composite protein sequence database formed by merging SWISSPROT and TrEMBL.

2.12. Cell lysis

Whole cells were lysed for 20 min on ice in lysis buffer (10 mM Hepes, 300 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1% Nonidet P-40, 0.1% SDS, pH 7.4) supplemented with protease inhibitors (Roche). The lysate was centrifuged at 10,000 *g* for 10 min. 15 μg of total protein was loaded per lane of SDS/PAGE.

2.13. RNA interference

shRNA targeting the SLP-2 transcript was synthesized in vivo using the recently developed pRETRO vector (generously provided by Dr. Agami) and previously described in [11,12]. Nucleotides 697–715 of the SLP-2 transcript were chosen as targets for RNA interference. To generate pRETRO SLP-2 shRNA, the pRETRO vector was digested with BglII and HindIII and the annealed oligos (5′ GatccccGGCTGAACAGATAAATCAGttcaagagaCTGATTTATCTGTTCAGCCtttttggaaa3′) and (5′AgcttttccaaaaaGGCTGAACAGATAAATCAGtctcttgaaCTGATTTATCTGTTCAGCCggg3′) were ligated into the vectors. Similarly, as a control, we generated a pRETRO Luc shRNA, which targets the Luciferase transcript.

For transient transfections, HeLa cells were plated on 10 cm dishes and transfected with 20 μg of the appropriate pRETRO constructs using Ca/phosphate method. 24 h after transfection, puromycin (Calbiochem) was added at 3 μg/ml and the cells were incubated for a further 24 h. After puromycin incubation the cells were washed and left to recover for a further 72 h.

For infection of HeLa or MEF cells, the retroviral supernatants were produced by transfecting 293T cells with 20 μg of pRETRO shRNA vector, 15 μg of pCMVgag vector (Packaging plasmid) and 5 μg of pMD2G vector (Envelope plasmid) by calcium-phosphate method on a 10 cm dish. 24 h post-transfection, the tissue culture medium was centrifuged at 1000 *g* for 10 min at 4 °C and filtered through a 0.45 μm filter. The viral supernatant was used to infect HeLa cells for at least 6 h and allowed to recover for 12 h with fresh medium. The infected cells were selected with puromycin 2 μg/ml for 48 h.

2.14. RNA extraction and Q-PCR

Real-time quantitative RT-PCR (Q-PCR) was performed to determine the complex III core 2 and complex IV subunit 4 mRNA levels in cells transfected either with Ctrl or SLP-2 shRNA after 120 h of transfection. Total RNA from cells transfected with Ctrl shRNA or SLP-2 shRNA was isolated with Trizol (Invitrogen) and prepared for reverse transcription according to manufacturer's instructions. Q-PCR was performed using the iQSYBR Green supermix (Bio-Rad) with the iCycler iQ detection system according to manufacturer's instructions. Elongation translation factor 1 (EEF1A1), TATA box-binding protein (TBP) and the transferrin receptor (TFRC) genes were also measured as endogenous references across all experimental conditions. The fold changes in complex III or complex IV mRNAs expression were calculated using a previously published formula [13].

3. Results

3.1. SLP-2 is a mitochondrial inner membrane protein

In an earlier study, we identified SLP-2 as a protein attached to the MIM [4]. In order to confirm the mitochondrial localization of SLP-2 we generated a rabbit polyclonal antibody against SLP-2. In HeLa cell lysates, this antibody recognizes a single band migrating at the expected molecular weight of SLP-2 (38 kDa; Fig. 1A). This band was not detected in HeLa cells depleted of SLP-2 by RNA interference, thus confirming the specificity of the antibody (Fig. 1A). Western blotting analysis of subcellular fractions of HeLa cells revealed that SLP-2 is present in the mitochondrial enriched fraction (Fig. 1B). This localization was confirmed by immunodetection of endogenous SLP-2 (Fig. 1C) and by detection of exogenous SLP-2 fused at its C-terminus with an HA tag (Fig. 1C) which both co-localized with specific mitochondrial markers (Fig. 1C). The targeting of several proteins of the MIM requires an N-terminal mitochondrial localization signal (MLS) [14]. Deletion of the first 50 amino acid sequence of the N-terminus of SLP-2, which according to the MITOPROT database contains a mitochondrial signal peptide, abrogated the mitochondrial targeting and led to the diffuse cytosolic localization of the mutant protein (1–50Δ) SLP2-HA (Fig. 1C). To further confirm that this region contains the MLS, the first 50 amino acid sequence of the N-terminus of SLP-2 was fused to a GFP protein and its localization was assessed. As shown in Fig. 1C, this GFP fusion protein (SLP2 (1–50)-GFP) co-localized with Mitotracker Red, indicating that the first 50 amino acid sequence of SLP-2 contains a MLS. Altogether, these data demonstrate that SLP-2 localizes to the mitochondria.

To validate the association of SLP-2 with MIM inferred from our proteomic study [4], we further analyzed the submitochondrial localization of SLP-2. The results shown in Fig. 1D indicated that SLP-2 is highly enriched in purified MIM and is resistant to alkali and proteinase K treatments in mitochondria (Fig. 1E and data not shown). To further assess the topology of SLP-2 regarding the MIM we studied the accessibility of SLP-2 to proteinase K (PK) by treating mitoplasts, obtained from mitochondria isolated from HeLa cells, with PK. As shown in Fig. 1F, treatment of the mitoplasts with PK led to a complete degradation of MIM proteins facing the IMS such as TIM23, while TIM44 (a MIM protein facing the matrix) and HSP70 (a matrix protein) were unaffected. SLP-2 was only partially proteolysed as evidenced by the appearance of a lower molecular weight SLP-2 band. Altogether, these data suggest that SLP-2 is attached to the MIM, facing the IMS, in such a manner that its accessibility to proteases is limited.

3.2. SLP-2 can be immunoprecipitated with prohibitins

In an effort to elucidate the function of SLP-2, we searched for SLP-2 interacting proteins. First we assessed whether SLP-2 was present in a high molecular weight complex using gel filtration chromatography. This showed that endogenous SLP-2 or over-expressed SLP2-HA was present in a fraction corresponding to a molecular weight of 250 kDa (Supplementary Fig. 1A and data not shown), suggesting that SLP-2 is present in a multi-protein complex. To identify the components of this complex, SLP2-HA was immunoprecipitated from a mitochondrial fraction isolated from HeLa cells over-expressing SLP2-HA. Using an anti-HA antibody, SLP2-HA was immunoprecipitated as a doublet. Of note, mass spectrometric analysis respectively identified the upper and lower bands as the full length and an N-terminally cleaved form of SLP-2 lacking the predicted mitochondrial signal sequence (Supplementary Fig. 1B and data not shown). Silver nitrate staining analysis of the immunoprecipitated fraction revealed the presence of several other bands in addition to those corresponding to SLP-2 (Fig. 2A). Two of these bands were identified as prohibitins 1 and 2 by LC/MS/MS, and their identity was confirmed by Western blotting using anti-prohibitin-1 and anti-prohibitin-2 antibodies (Fig. 2B). Ectopically expressed SLP-2 and endogenous prohibitin-1 could also be co-precipitated using an anti-prohibitin-1 antibody (Fig. 2C). To avoid possible artifacts due to over-expression of proteins, we tested whether endogenous SLP-2 and prohibitin-1 also interact. As shown in Fig. 2D, prohibitin-1 was immunoprecipitated using an anti-SLP-2 antibody but in small quantities compared to the protein input. We hypothesized that this was due to a weak interaction between

the two proteins resulting from the high amount of detergent (1% NP40) necessary to extract these proteins. Therefore, to have a better estimate of the level of interaction between these proteins and to eliminate the possibility that the two proteins might interact following mitochondrial membrane extraction, mitochondria isolated from HeLa cells were treated with the cell permeable cross-linker DSP and SLP-2 was immunoprecipitated. As shown in Fig. 2E, higher amounts of prohibitin-1 were co-precipitated with SLP-2. Furthermore, Western blotting analysis of the mitochondrial fractions obtained by gel filtration chromatography (Supplementary Fig. 1A) revealed that prohibitin-1 and prohibitin-2 are present in the 250 kDa complex, as SLP-2. Altogether these results strongly

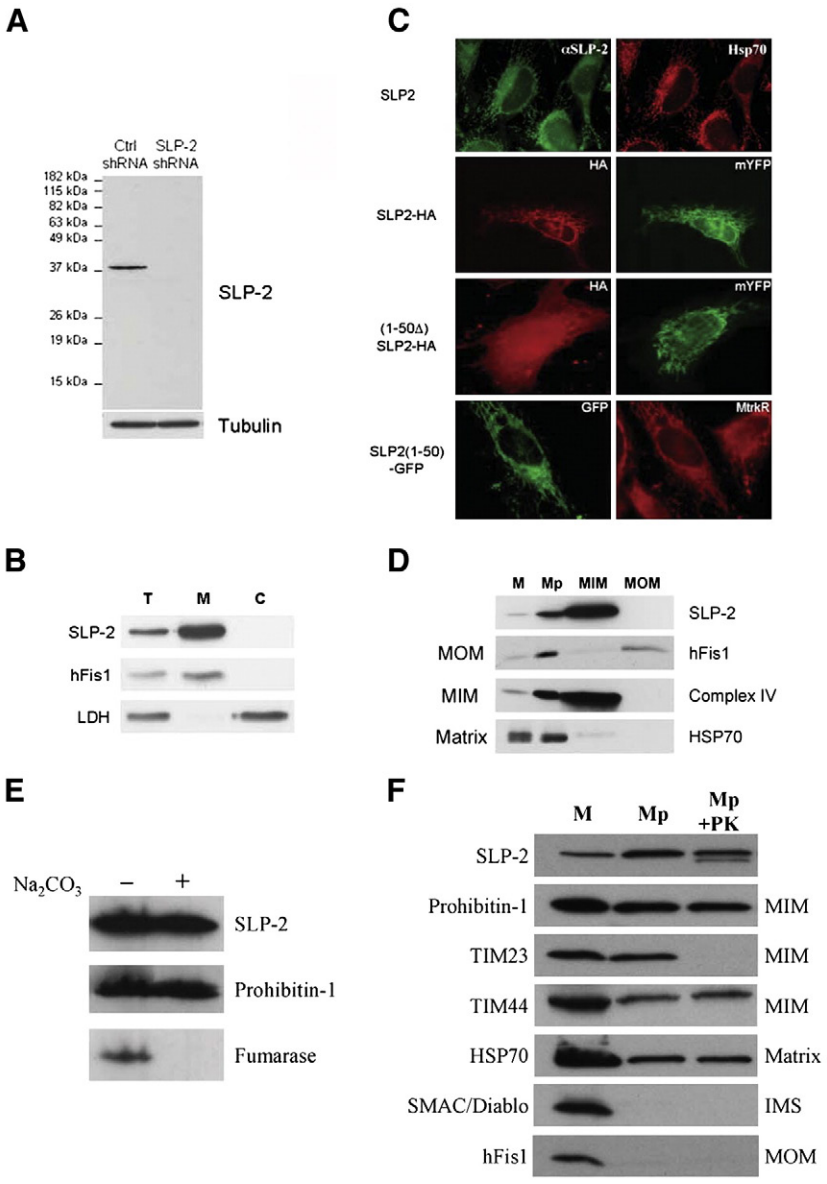

Fig. 1. SLP-2 is a mitochondrial inner membrane protein. A) HeLa cells were transfected with the pRETRO Luc shRNA (Ctrl shRNA) or the pRETRO SLP-2 shRNA (SLP-2 shRNA) for 120 h as described in Materials and methods. An equal amount of proteins (20 μg) from the respective cell lysates was analyzed by Western blotting using the anti-SLP-2 antibody. Tubulin was used as a loading control. B) HeLa cells were fractionated as outlined in Materials and methods. (T) Total cell extract, (M) mitochondrial fraction and (C) cytosolic fraction were analyzed by Western blotting using the hFis1 and lactate dehydrogenase (LDH) as markers of the mitochondria and the cytosol respectively. C) Immunocytochemistry of endogenous SLP-2 using a rabbit antibody raised against SLP-2 and of C-terminally (SLP2-HA) HA tagged SLP-2 fusion protein or SLP-2 lacking the first 50 amino acids fused to HA tag at the C-terminus ((1–50Δ)SLP2-HA) using an anti-HA monoclonal antibody. The N-terminally first 50 amino acids of SLP-2 fused to a GFP protein at the C-terminus (SLP2(1–50)-GFP) was transfected into HeLa and 24 h later its localization was assessed and compared with the Mitotracker Red (MtrkR) staining. D) Purified mitochondria (M), mitoplasts (Mp), mitochondrial inner and outer membranes (MIM and MOM respectively) from mouse liver were prepared as described in Materials and methods. An equal amount of proteins (25 μg) was analyzed by Western blotting using the indicated antibodies. E) Mitochondria isolated from HeLa cells were treated with sodium carbonate (see Materials and methods) and analyzed by SDS-PAGE and Western blotting using the indicated antibodies. F) Mitochondria isolated from HeLa cells were treated with 2 mg/ml of digitonin to obtain mitoplasts which were further treated with 0.25 μg/ml of proteinase K for 30 min on ice. The levels of endogenous SLP-2 protein were assessed by SDS-PAGE and Western blotting. hFis1 was taken as a marker for the mitochondrial outer membrane; SMAC-Diablo as the intermembrane space marker; prohibitin-1, TIM23 and TIM44 as the mitochondrial inner membrane markers and HSP70 as the mitochondrial matrix marker.

suggest that SLP-2 and prohibitins that interact *in situ* in the MIM are part of the same protein complex.

3.3. SLP-2 is required for the stability of prohibitins and subunits of respiratory complexes I and IV

Mitochondrial inner membrane proteins such as prohibitins have previously been shown to assist the proper folding and assembly of respiratory complexes [15–19]. Since it has been proposed that stomatins are involved in regulating protein stability [2,3], we examined whether the stability of mitochondrial proteins was altered in HeLa cells depleted of SLP-2 by RNA interference. As shown in Fig. 3A and Supplementary Fig. 2A, the protein levels of ND6 (a subunit of complex I), COX4 (complex IV), prohibitin-1 and prohibitin-2 were decreased in SLP-2 depleted cells (33.9±12.5%, 30.2±16.1%, 38.3±9.2% and 31.3±13.6%;

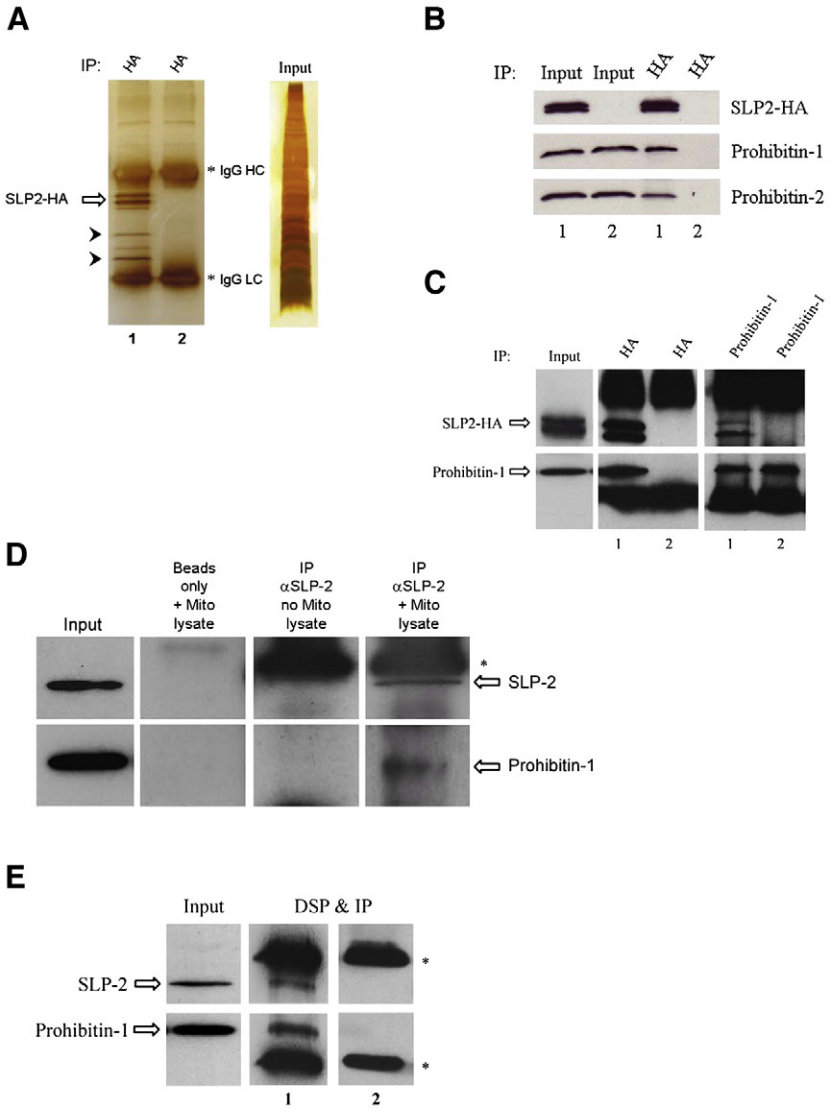

Fig. 2. SLP-2 interacts with prohibitins. A) Mitochondria isolated from HeLa cells transfected either with SLP2-HA (1) or pCI vector (2) were solubilised and SLP2-HA was immunoprecipitated with the anti-HA antibody. Immunoprecipitates were revealed by silver nitrate staining. IgG HC (heavy chain of the antibody) and IgG LC (light chain of the antibody) are indicated with an asterisk. The dark arrowhead corresponds to the bands that were analyzed by LC/MS/MS. B) Mitochondria isolated from HeLa cells transfected either with SLP2-HA (1) or the empty expression vector (2) were solubilised and SLP2-HA was immunoprecipitated with the anti-HA antibody. Immunoprecipitates were analyzed by Western blotting using the anti-HA, anti-prohibitin-1 and prohibitin-2 antibodies. The input lane corresponds to 10% of the mitochondrial fraction used for immunoprecipitation. C) Immunoprecipitation of the fractions described above was performed with the anti-HA or the anti-prohibitin-1 antibody. Immunoprecipitates were analyzed by Western blotting using the anti-HA and anti-prohibitin-1 antibodies. D) Endogenous SLP-2 was immunoprecipitated from mitochondria isolated from untransfected HeLa cells. The immunoprecipitates were analyzed by Western blotting using the anti-SLP-2 and anti-prohibitin-1 antibodies. The second lane corresponds to mitochondria isolated from untransfected HeLa cells and incubated only with the protein A-Sepharose beads and the third lane corresponds to the protein A-Sepharose beads incubated with the anti-SLP-2 antibody in the IP buffer only. The asterisk corresponds to the IgG heavy chain. E) Mitochondria isolated from HeLa cells transfected either with SLP2-HA (1) or pCI vector (2) were cross-linked with DSP and endogenous SLP-2 was immunoprecipitated with anti-SLP-2 antibody. The immunocomplexes were de-cross-linked as described in Materials and methods, before analysis by Western blotting using anti-SLP-2 and anti-prohibitin-1 antibodies. The asterisks correspond to the IgG heavy and light chains. Input lanes in panels C and D correspond to 10% of the mitochondrial fraction used for immunoprecipitation.

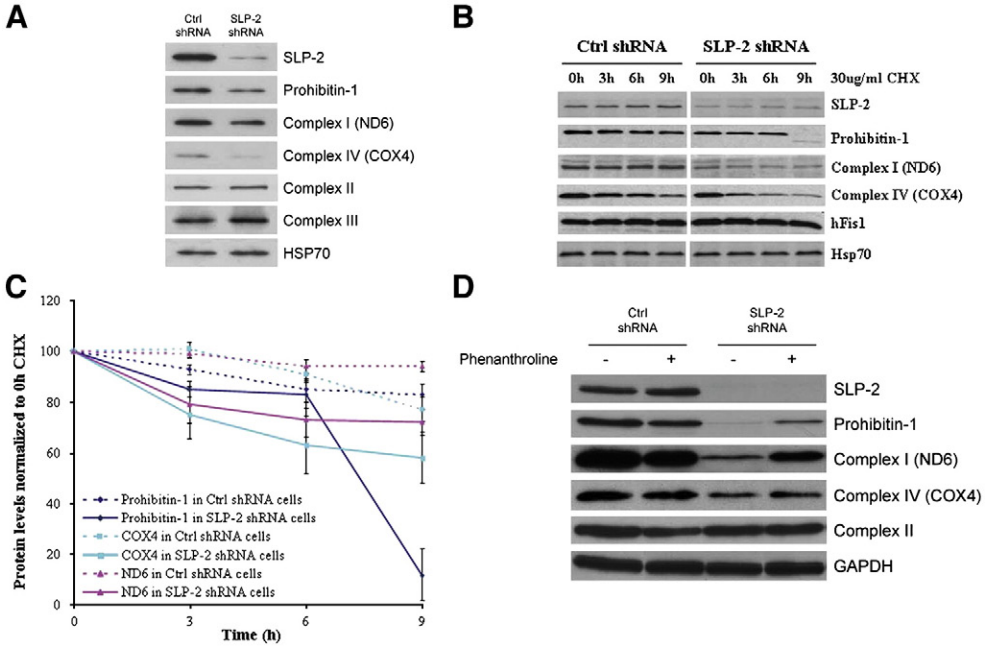

Fig. 3. SLP-2 is required to maintain protein levels of specific components of the respiratory chain as well as those of prohibitin-1. A) HeLa cells were infected with the Ctrl shRNA or SLP-2 shRNA as outlined in Materials and methods. Lysates of the respective infected cells were analyzed by Western blotting with the indicated antibodies. B) HeLa cells were transiently transfected with the Ctrl shRNA or SLP-2 shRNA as outlined in Materials and methods. After 96 h of transfection, the cells were treated with 30 μg/ml of cycloheximide (CHX) for 0, 3, 6, and 9 h. Lysates of the respective treated cells were analyzed by Western blotting with the indicated antibodies. C) The bands observed in the Western blot shown in panel B were quantified using ImageJ. The graph represents the values obtained with this quantification, normalized to the loading control (HSP70) and normalized to the values obtained at 0 h of cycloheximide treatment (CHX). D) Infected HeLa cells were treated with 5 mM phenanthroline for 30 min. The respective infected cell lysates were analyzed by Western blotting with the indicated antibodies.

$n=4$, respectively), while levels of HSP70, subunits of complexes II and III remained constant. No decrease in the mRNA levels of COX4 was observed (Supplementary Fig. 2B) indicating that the drop in the protein level of COX4 was due to a post-transcriptional event, possibly proteolysis.

To test whether the absence of SLP-2 affects the turnover of subunits of OXPHOS complexes as well as that of prohibitins, HeLa cells were treated with cycloheximide (CHX, an inhibitor of the synthesis of proteins encoded by nuclear genes) for 3, 6 and 9 h and the protein levels were monitored by Western blotting. As shown in Fig. 3B and C, the levels of ND6, COX4 and prohibitin-1 were significantly decreased in SLP-2 depleted cells after 9 h of CHX treatment (28±5.2%, 37±10.2% and 87±10.7%; $n=4$, respectively) compared to control cells (6±2.3%, 14±6.7% and 18±6.2%; $n=4$, respectively). These results were confirmed using SLP-2 depleted mouse embryonic fibroblasts (MEFs). In these cells, both prohibitin-1 and 2 levels were found to be significantly decreased 3 h after addition of cycloheximide (Supplementary Fig. 2C; 67% and 30% respectively) compared to control cells (90% and 97% respectively). These data suggest that cellular depletion of SLP-2 enhances the degradation of specific subunits of OXPHOS complexes as well as that of prohibitins.

In an effort to determine the proteolytic degradation process responsible for the increased turnover of these proteins in SLP-2 depleted cells, we tested several protease inhibitors, including caspase, proteasome and metalloprotease inhibitors. Only phenanthroline, a metalloprotease inhibitor, was found to significantly prevent the decrease in the level of these proteins (Fig. 3D and data not shown). This suggests that metalloproteases, possibly AAA proteases, are responsible for the degradation of ND6, COX4 and prohibitin-1 in SLP-2 depleted cells.

3.4. Increased levels of SLP-2 in stressed mitochondria

Since it has been shown that under conditions of increased mitochondrial protein turnover the levels of proteins involved in the regulation of protein stability (e.g. prohibitin-1, Cpn60 [20,21]) increase, we examined the levels of SLP-2 under conditions of mitochondrial stress such as those generated by cell exposure to chloramphenicol (CAP), an inhibitor of the synthesis of proteins encoded by the mitochondrial genome [22]. Here, we found that the levels of SLP-2 were significantly increased in HeLa cells (Fig. 4A; 192%±11.3%; $n=5$) and Cos-7 cells (Supplementary Fig. 3; 156%±11.3%; $n=3$) treated with CAP. Furthermore, in agreement with previous studies [20], prohibitin-1 was up-regulated under the same conditions. Interestingly, levels of HSP70, a known mitochondrial chaperone, remained constant under these conditions, indicating that treating cells with CAP leads to the up-regulation of specific mitochondrial stress response proteins. Of note, levels of mitochondrial encoded proteins were significantly decreased (33%±4.2%; $n=4$, data not shown), thus validating CAP activity. In order to determine if the increase in the levels of SLP-2 and prohibitin-1 was restricted to mitochondrial specific stresses, HeLa cells were exposed to etoposide, a DNA damaging agent, and the levels of SLP-2 and prohibitin-1 expression were determined. As shown in Fig. 4A, this treatment did

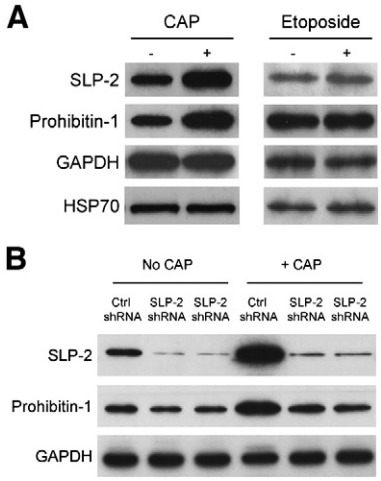

Fig. 4. SLP-2 and prohibitin-1 are both up-regulated during chloramphenicol induced stress and the up-regulation of prohibitin-1 is SLP-2 dependent. A) HeLa cells were treated with 100 μg/ml chloramphenicol (CAP) for 96 h and 100 μM etoposide for 1 h. The levels of SLP-2 and prohibitin-1 were assessed by Western blotting analysis of the cell lysates. GAPDH and HSP70 were used as a loading control. B) Infected HeLa cells were described in Fig. 3A and were treated with 100 μg/ml chloramphenicol (CAP). 96 h after CAP treatment, the respective infected cell lysates were analyzed by Western blotting with the indicated antibodies.

not induce an up-regulation of the protein levels of SLP-2 or prohibitin-1, suggesting that SLP-2 and prohibitin-1 might be up-regulated specifically in response to mitochondrial stress.

Since we have shown that SLP-2 is required to maintain normal prohibitin-1 levels and that prohibitin-1 increases in response to mitochondrial stress, we tested whether the increase in prohibitin-1 levels after CAP treatment was SLP-2 dependent. HeLa cells depleted of SLP-2 were treated with CAP and the levels of prohibitin-1 were assessed by Western blotting. As shown in Fig. 4B, the increase in the protein levels of prohibitin-1 upon CAP treatment was significantly reduced in SLP-2 depleted cells (238%±16.2% in control cells compared to 147%±7.4% in SLP-s depleted cells, $n=3$). This further indicates that SLP-2 is involved in promoting the stability of prohibitin-1.

4. Discussion

Stomatins and stomatin like proteins such as SLP-2 are members of a large family of proteins which also contains prohibitins, HflK/C, flotillins, mechanoreceptors and plant defense proteins [2,3]. Although it has been suggested that stomatins share a common molecular function with other members of this superfamily, namely in the regulation of protein turnover, no experimental data has confirmed this hypothesis. Here, we show that SLP-2 is a MIM protein that interacts with prohibitins and depleting SLP-2 in HeLa cells and MEFs leads to an increased proteolysis (possibly mediated by metalloproteases) of prohibitins and of subunits of complexes I and IV. Further supporting the role of SLP-2 in regulating the stability of specific mitochondrial proteins, we found that SLP-2 is up-regulated under conditions of mitochondrial stress leading to increased protein turnover.

It has previously been reported that SLP-2 is localized to the plasma membrane of mature human erythrocytes [23]. However, several proteomic studies of mitochondria obtained from various cell types and different species have also identified SLP-2 as a mitochondrial component [5–9]. In this study, we further provide biochemical and immunocytochemical proofs that SLP-2 is strongly associated with the MIM. Our finding is in accordance with recent data published during the preparation of this manuscript [24]. The localization of SLP-2 in the plasma membrane of erythrocytes may be cell specific, and may result from the absence of mitochondria in those cells. Another mitochondrial protein has been shown to have such a dual localization, albeit in the same cell type: the mitochondrial β-chain ATP synthase which is found both at the mitochondria and at the plasma membrane [25].

Interestingly, unlike other members of the stomatin family, SLP-2 does not contain any putative transmembrane domain. Nevertheless, we found that SLP-2 is highly resistant to alkali and PK treatments. We believe that these properties of the protein could be due to SLP-2 being part of a high molecular weight complex together with prohibitins (Supplementary Fig. 1A, C and Fig. 2) in which the protein could be "buried", thus making it less accessible to PK. Since prohibitins are integral MIM proteins this would also explain the resistance of SLP-2 to alkali treatment. An additional, non-mutually exclusive explanation could be that SLP-2 is associated with lipid microdomains that would resemble raft structures. This hypothesis is based on the findings that members of the stomatin family are associated to rafts and serve as markers for the lipid microdomains [26].

Prohibitins are involved in diverse cellular processes such as proliferation, aging and the assembly of mitochondrial respiratory complexes [27,28]. In yeast, they have been shown to interact with two subunits of complex IV [16], and in mammals, with components of complex I [9,29]. Consistently, prohibitins have been shown to be required for stabilizing subunits of mitochondrial respiratory chain complexes and controlling their assembly [16,18,19]. Here we show that protein levels of prohibitin-1 and prohibitin-2 are significantly decreased in SLP-2 depleted cells, which suggests that SLP-2 may regulate the stability of prohibitins through a direct or indirect interaction with this protein. It had been previously reported that following mitochondrial stress induced by chloramphenicol (CAP), the levels of prohibitins increase significantly [20]. Here we confirm this result and show that SLP-2 is required for the increase in prohibitin-1 levels in cells cultured with CAP. This result further supports the findings that the stability of prohibitin-1 relies on SLP-2 directly or indirectly. In addition to a drop in prohibitins levels, a decrease in the levels of subunits of complexes I and IV of the respiratory chain was also observed in SLP-2 depleted cells, thus suggesting that SLP-2 is also required for the stability of other mitochondrial proteins such as subunits of the OXPHOS complexes. Given the role of prohibitins in the stability of various respiratory chain subunits in yeast [16,18], it is possible that the decrease in the levels of these proteins in SLP-2 depleted cells is the direct consequence of the loss of prohibitins. Cellular depletion of SLP-2 causes an accelerated degradation of prohibitins and subunits of complexes I and IV that could be dependent on AAA proteases. These findings suggest a regulatory function of the SLP-2/prohibitins complex during membrane protein turnover by the AAA proteases. SLP-2/

prohibitins could interfere with proteolysis by binding directly to substrate proteins or by interacting directly with AAA proteases. In support of the former hypothesis, it has been reported that the bacterial proteases, HflK and HflC, two members of the SPFH family, interact with the AAA protease FtsH in *Escherichia coli* [19,30]. Moreover, in the hyperthermophilic archaebacterium *Pyrococcus horikoshii*, a gene encoding a serine protease forms an operon with a gene encoding a stomatin homolog and the two proteins have been shown to work in the same pathway [31]. Altogether these data strongly suggest that SLP-2 could play a role in the control of proteolysis in the mitochondria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.02.006.

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