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Mitochondrial proteases act on STARD3 to activate progesterone synthesis in human syncytiotrophoblast

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ABSTRACT

Background: STARD1 transports cholesterol into mitochondria of acutely regulated steroidogenic tissue. It has been suggested that STARD3 transports cholesterol in the human placenta, which does not express STARD1. STARD1 is proteolytically activated into a 30-kDa protein. However, the role of proteases in STARD3 modification in the human placenta has not been studied.
Methods: Progesterone determination and Western blot using anti-STARD3 antibodies showed that mitochondrial proteases cleave STARD3 into a 28-kDa fragment that stimulates progesterone synthesis in isolated syncytiotrophoblast mitochondria. Protease inhibitors decrease STARD3 transformation and steroidogenesis.
Results: STARD3 remained tightly bound to isolated syncytiotrophoblast mitochondria. Simultaneous to the increase in progesterone synthesis, STARD3 was proteolytically processed into four proteins, of which a 28-kDa protein was the most abundant. This protein stimulated mitochondrial progesterone production similarly to truncated-STARD3. Maximum levels of protease activity were observed at pH 7.5 and were sensitive to 1,10-phenanthroline, which inhibited steroidogenesis and STARD3 proteolytic cleavage. Addition of 22(*R*)-hydroxycholesterol increased progesterone synthesis, even in the presence of 1,10-phenanthroline, suggesting that proteolytic products might be involved in mitochondrial cholesterol transport.
Conclusion: Metalloproteases from human placental mitochondria are involved in steroidogenesis through the proteolytic activation of STARD3. 1,10-Phenanthroline inhibits STARD3 proteolytic cleavage. The 28-kDa protein and the amino terminal truncated-STARD3 stimulate steroidogenesis in a comparable rate, suggesting that both proteins share similar properties, probably the START domain that is involved in cholesterol binding.
General significance: Mitochondrial proteases are involved in syncytiotrophoblast-cell steroidogenesis regulation. Understanding STARD3 activation and its role in progesterone synthesis is crucial to getting insight into its action mechanism in healthy and diseased syncytiotrophoblast cells.

1. Introduction

Mitochondria carry out cellular respiration and ATP synthesis to supply the energy requirements of aerobic cells. Mitochondria are essential for the synthesis of a number of important biological compounds such as lipids, heme, amino acids, nucleotides and steroid hormones. The physiological function and homeostasis of mitochondria entail selective proteolysis in which various specific mitochondrial proteases, including processing peptidases, ATP-dependent proteases, and oligopeptidases are involved [1].

Steroid hormones are synthesized from cholesterol, a substrate for mitochondria of specialized cells of the adrenal cortex, gonads and placenta. The steroidogenic acute regulatory protein (StAR; STARD1) [2–6], a nuclear-encoded mitochondrial protein expressed upon stimulation of steroidogenic tissues by their respective trophic hormones [7–9], promotes cholesterol supply to mitochondria from acutely regulated steroidogenic tissue. It has been suggested that STARD3 (or MLN64), a member of the START domain family, is the protein responsible for transporting cholesterol in the human placenta [10], a steroidogenic tissue which does not show acute regulation of steroidogenesis nor expresses STARD1 [11].

The amino acid sequence of the STARD3 carboxy-terminal region sequence is similar to that of STARD1 [12]. While full-length STARD3 has minimal STARD1-like activity, the 234 amino-terminal residue deletion (N-218 STARD3) results in a protein with substantial STARD1-like activity in transfected cells [10]. Like N-62 STARD1 (a StAR protein with a deletion of 62 amino-acids in its amino-

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terminal region), N-218 STARD3 lacks a mitochondrial leader sequence that prevents it from entering the mitochondrion, and apparently exerts its function in the outer mitochondrial membrane. Alpy et al. [13] identified the full-length STARD3 to be associated with late endosomes, which constitute its sole cellular location reported to date.

STARD1 and STARD3 transfer cholesterol from the outer to the inner mitochondrial membrane, where the cholesterol side chain cleavage enzyme, cytochrome P450 (P450scc, CYP11A1; EC 1.14.15.6), converts it to the first product in steroid hormone synthesis: pregnenolone [14–16]. Hypothetical mechanisms suggest that STARD1 activates cholesterol transfer by virtue of its association with a macromolecular complex that consists of outer membrane proteins such as the mitochondrial membrane translocator protein (TSPO), and the TSPO-associated protein PAP7, that bind and lead the regulatory subunit RIα of the cAMP-dependent protein kinase (PKARIα) towards mitochondria [17]. However, it has been recently described that, in knockout mice with a specific TSPO deletion, gametogenesis, reproduction, histological structure, and steroidogenesis of Leydig cells are not affected. This suggests that the presence of TSPO is not crucial for steroidogenesis [18]. Previous data from our laboratory have also demonstrated that TSPO (earlier named PBR) is absent from the human placenta [19]. In human placenta mitochondria, STARD3 is associated with steroidogenic contact sites and HSP60, resulting in an increase in progesterone synthesis [20,21].

STARD1 is synthesized in the cytosol as a 37-kDa pre-protein carrying an amino terminal targeting sequence that directs its import into mitochondria, where it is proteolytically processed to a mature 30-kDa protein [2,22–24]. Although the important role of STARD1 proteolysis during steroid hormone synthesis by acutely regulated steroidogenic tissue has been described, the role of proteases in the modification of STARD3 in the human placenta has not been studied.

In this work, the participation of mitochondrial proteases in the modification of STARD3 in progesterone synthesis in human syncytiotrophoblast mitochondria was determined. Although the role of STARD3 in the human placenta remains to be elucidated, its proteolysis from a 55-kDa protein into lower molecular weight proteins appears to be essential for placental steroidogenesis.

2. Materials and methods

2.1. Isolation of human syncytiotrophoblast mitochondria

Full term human placentas were collected immediately after normal delivery. Mitochondria were prepared as previously described [25]. Briefly, placental cotyledons were placed in ice-cold 250 mM sucrose, 1 mM EDTA, and 10 mM Tris, pH 7.4. The suspension was homogenized with a Polytron (Brinkmann Instruments, Westbury, NY, USA) at 3000 rev/min for 1 min for two cycles with a one minute interval. The whole process was carried out at 4 °C. The pH of the homogenate was adjusted to pH 7.4 with Tris and centrifuged at 1500 *g* for 15 min. The supernatant was recovered and centrifuged at 4000 *g* to obtain a pellet of cytotrophoblast mitochondria (heavy mitochondria). The supernatant was centrifuged again at 16,000 *g* for 15 min and the pellet containing the syncytiotrophoblast mitochondria (light mitochondria) was suspended in the same solution and centrifuged at 1500 *g* for 10 min to remove the remaining erythrocytes. Then, the mitochondrial pellet was obtained by centrifugation of the last supernatant at 12,000 *g* for 10 min. To purify mitochondria of syncytiotrophoblast, the enriched mitochondrial suspension was loaded onto a 35% sucrose solution (25 ml) and centrifuged at 15,000 *g* for 45 min at 4 °C. The mitochondrial fraction was collected, suspended in 250 mM sucrose, 1 mM EDTA, and 10 mM Tris (pH 7.4) and centrifuged at 16,000 *g* for 15 min at 4 °C. The resulting mitochondrial pellet was suspended in this buffer and stored at 4 °C. Protein concentration was measured as reported by Refs. [26,27].

2.2. Mitochondrial oxygen consumption

Oxygen uptake was estimated polarographically using a Clark type electrode in a mixture containing 250 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM EDTA, 10 mM succinate, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.2% bovine serum albumin and 1 mg/ml of syncytiotrophoblast mitochondrial protein [28]. Temperature was maintained at 37 °C and oxygen consumption was stimulated by the addition of 300–500 nmol ADP (state 3 of respiration). Respiratory control was defined as oxygen uptake rate of state 3/oxygen uptake rate of state 4 (state 4 started when all ADP was converted into ATP, and respiration slowed down) [29].

2.3. Mitochondrial enzyme activity determination

Activities of complex I (NADH:DCPIP oxidoreductase) and complex II (succinate:DCPIP oxidoreductase) were determined spectrophotometrically at 600 nm by following the reduction of the artificial electron acceptor 2,6-dichlorophenol-indophenol (DCPIP; 50 μM; $_{DCPIP}$ = 21 mM^{-1} cm^{-1}). Mitochondria were permeabilized with 0.01% Triton X-100 and incubated in 30 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 120 mM KCl, pH 7.4, and either 500 μM NADH (complex I) or 2 mM succinate (complex II). Complex II was activated by pre-incubation in the presence of 0.2 mM phenazinemethosulfonate (PMS) for 10 min at 25 °C [30,31]. The protein concentration of syncytiotrophoblast mitochondria was 50 μg/ml and the reaction was started by the addition of NADH or succinate. ATP synthesis by complex V was measured at 37 °C using an assay coupled to the reduction of $NADP^+$ ($_{340\ nm}$ = 6.2 mM^{-1} cm^{-1}). The reaction mixture contained 0.5 mM $NADP^+$, 1 mM ADP, 6 units/ml glucose-6-phosphate dehydrogenase, 16 units/ml hexokinase, 10 mM succinate, 100 μM P^1,P^5-di(adenosine-5′) pentaphosphate-ammonium, 10 mM glucose, 150 mM sucrose, 5 mM $MgCl_2$, 20 mM Tris/HCl, and 20 mM KH_2PO_4, at pH 7.5. ATP synthesis was started by the addition of syncytiotrophoblast mitochondria (50 μg/ml). The values reported were obtained by subtracting the rate of ATP synthesis in the presence of oligomycin (5 μg/mg mitochondrial protein) from the amount of ATP synthesis under control conditions [32].

2.4. Mitochondrial progesterone synthesis

Progesterone synthesis was determined at 37 °C as previously reported [28] in a medium (P4M) containing 120 mM KCl, 10 mM MOPS, 0.5 mM EGTA, 10 mM isocitrate, and 5 mM KH_2PO_4, pH 7.4 in a final volume of 500 μl with 1 mg/ml of syncytiotrophoblast mitochondrial protein. Where indicated, 25 μM 22(*R*)-hydroxycholesterol was added to verify cytochrome P450scc, adrenodoxin, adrenodoxin reductase and 3β-hydroxysteroid dehydrogenase activities [33]. After incubation, the reaction was stopped with 75 μl methanol and progesterone was determined using a radioimmunoassay kit (Diagnostic Systems Laboratories, Inc. Webster, Texas, USA), following the manufacturer's instructions. The concentration of progesterone at time zero was subtracted from the amount of progesterone at different times and this net progesterone synthesis was reported. Alternatively, syncytiotrophoblast mitochondria were incubated for 20 min at 37 °C in P4M and centrifuged at 14,000 *g* in an Eppendorf 5415R refrigerated centrifuge for 15 min at 4 °C. The mitochondrial pellet and the supernatant were separated. The supernatant was concentrated in an Amicon Ultra Centrifugal Filter system (10K) and either processed for SDS-PAGE and Western blot analysis using anti-MLN64 antibodies (*vide infra*) or used to stimulate mitochondrial progesterone synthesis.

2.5. Effect of protease inhibitors in proteolytic STARD3 cleavage and mitochondrial progesterone synthesis

The effect of protease inhibitors in STARD3 cleavage was assessed by incubating mitochondria in PM4 medium with either one of the following: the inhibitor mixture from Sigma (cat. P8215), PMSF (1 mM), EGTA (5 mM), EDTA (5 mM), or 1,10-phenanthroline (9 mM), for 20 min at 37 °C. Afterwards, mitochondrial proteins were processed for SDS-PAGE and Western blot analysis against the STARD3 protein (*vide infra*). To verify the effects of STARD3 on progesterone synthesis, 2 μM of purified N-218 STARD3 was added to mitochondria incubated in the P4M medium.

2.6. In-gel protease activity assays

SDS-polyacrylamide (8%) gels were co-polymerized with porcine gelatin (1 mg/ml) and loaded with syncytiotrophoblast mitochondria (50 μg per well) in non-denaturing loading buffer with or without β-mercaptoethanol. Electrophoresis was performed under constant current (10 mA per gel) for 6 h at 4 °C. Gels were washed in 2.5% Triton X-100 for 30 min to eliminate SDS remnants and incubated overnight at 37 °C in an activation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM $CaCl_2$, and 0.02% NaN_3). The buffers used to determine pH-dependence activity were: sodium acetate (from pH 4 to 5.3), MES (from pH 5.3 to 7), Tris (from pH 7 to 8.8) and AMPSO (pH 8.8). Gels were stained with 0.2% Coomassie® brilliant blue R-250 (Sigma). MMP2 and MMP9, constitutively secreted by U937 (ATCC; Rockville, MD) (a promyelocyte cell line) were used as activity standard markers. Protease activity correlated with the unstained band and the densitometric analysis was performed with the Image Analysis software version 1.0 (Thermo Fisher Scientific Inc.). The intensities of activity were measured by peak integration after densitometric analysis.

2.7. SDS gel electrophoresis and Western blot analysis

Syncytiotrophoblast mitochondrial proteins (50 μg per well) were separated by SDS-PAGE according to Laemmli [34] in a 10% polyacrylamide gel under denaturing conditions. After the run, proteins were stained with either Coomassie® brilliant blue R-250 or silver, using a commercial kit (Bio-Rad) (see Fig. 2D). Alternatively, proteins were electrotransferred to a PVDF membrane (Immobilon P; Millipore, Bedford, MA) in a semi-dry electroblotting system (Bio-Rad) at 25 V for 50 min. Membranes were blocked in 500 mM NaCl, 0.05% Tween-20, and 20 mM Tris–base, pH 7.5 (TTBS buffer), containing 5% blotting grade blocker non-fat dry milk (Bio-Rad). Then, membranes were incubated with anti-MLN64 polyclonal antibodies (1:1000). Immunoreactive bands were visualized with the Enhanced ChemiLuminescence assay (Amersham Life Science, Inc.), according to the manufacturer's instructions, using horseradish peroxidase-conjugated goat antimouse IgG (Pierce) at a dilution ratio of 1:35,000, and densitometric analyses were performed with the Image Analysis software version 1.0 (Thermo Fisher Scientific Inc.). The intensities of proteins were measured by peak integration after densitometric analysis.

The presence of endosome marker proteins, Niemann–Pick type C1 protein (NPC1) and Rab5 was assayed [35,36] by Western blot, as described above. The dilution ratio of either anti-NPC1 or anti-Rab5 antibodies was 1:2000. After being washed, the blots were incubated with the corresponding secondary antibodies. Protein–antibody complexes were visualized as described above.

2.8. Production of the N-218 STARD3 protein

The recombinant N-218 STARD3 protein was produced in BL21 *Escherichia coli* expressing human STARD3-START (amino acids 218–445; N-218 STARD3) [10] as previously described [37]. The expressed protein contained a His_6-tag at the C-terminus. Bacteria were cultivated in LB medium containing 25 μg/ml ampicillin. For protein expression, 400 ml of growth medium (with antibiotic) was inoculated with 1 ml of BL21 overnight culture. The culture was incubated at 37 °C with constant shaking until an optical density of 0.5–1.0 at 600 nm was reached. Expression was induced by the addition of 0.5 M isopropyl-β-D-thiogalactopyranoside. After 4.5 h bacteria were pelleted.

The resulting pellet was suspended in ice in 10 ml of the following buffer: 300 mM NaCl, 50 mM NaH_2PO_4, 20 mM Tris–HCl (pH 7.4), and 10 mM β-mercaptoethanol. Bacteria were sonicated in ice (15 pulses of 1 s, three times at maximum output level), using a MSE Soniprep (UK) model 150. The suspension was centrifuged at 4 °C for 30 min at 20,000 *g*. The supernatant was incubated with 500 μl of Ni^{2+}–nitrilotriacetic acid–agarose matrix (Qiagen, Hilden, Germany). The mixture was incubated with constant rotation at 4 °C overnight. The matrix was placed in a column and washed with 20 ml of the following buffer: 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0), and 20 mM imidazole. To avoid aggregation of N-218 MLN64, the elution buffer was supplemented with 40% (w/v) glycerol. The eluted proteins were dialyzed (molecular mass cutoff: 12-kDa; Sigma) against the following buffer: 150 mM NaCl, 50 mM KCl, 50 mM Tris (pH 7.4), 10 mM dithiothreitol, and 40% (w/v) glycerol [37].

2.9. Tandem mass spectrometry (LC/ESI–MS/MS)

The protein band (indicated as 28-kDa in Fig. 2B) was cut off from the Coomassie® brilliant blue R-250-stained SDS-PAGE gels and sent to the Proteomics Core Facility at the University of Arizona, USA, to determine its identity.

Outer mitochondrial membranes were isolated as reported by Uribe et al. [20]. Briefly, 20–25 mg of mitochondrial protein was incubated at 4 °C with 10 mM H_3PO_4 and adjusted to pH 7.3 with Tris base in the presence of protease inhibitor cocktail (Complete, Roche). After incubation, sucrose was added to attain a final concentration of 0.38 M. The resulting mixture was incubated for 20 more minutes at 4 °C and then centrifuged at 12,500 *g* for 10 min. The supernatant containing the outer mitochondrial membranes was centrifuged at 137,000 *g* for 1 h to recover the membrane fraction. The resulting mitochondrial outer membrane was incubated in 100 mM ammonium bicarbonate (pH = 7.8) for 30 min, centrifuged at 100,000 *g* at 4 °C and sent to the Proteomics Core Facility at the University of Arizona, USA.

2.10. Statistical analyses

Statistical analyses (one- and two-way analyses of variance, ANOVA) of the data were performed using Sigma Stat software, version 3.5. When necessary, nonlinear regression of the data to a single exponential decay equation was performed in Sigma Plot software, version 10.0.

2.11. Materials

Analytical grade reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), E. Merck (Darmstadt, Germany), and Bio-Rad (Hercules, CA, USA).

3. Results

3.1. Functional state of syncytiotrophoblast mitochondria

To determine the functional integrity of isolated syncytiotrophoblast mitochondria, respiratory rates and respiratory controls were calculated from oxygen uptake traces using succinate as substrate (Table 1). Oxygen uptake in state 3 and state 4 was 135 ± 28 ng atom of oxygen/min·mg protein, and 21 ± 8 ng atom of oxygen/min·mg protein, respectively. The respiratory control value was 6.7 ± 2 while ATP synthesis rate of complex V was 160 ± 12 nmol/min·mg protein. Addition of 2,4-dinitrophenol to energized mitochondria increased the permeability of the

Table 1
Bioenergetics and steroidogenic parameters of syncytiotrophoblast mitochondria.

Complexes activities[a]	
Complex I	113 ± 20 μmol/min·mg
Complex II	14 ± 4 μmol/min·mg
Complex V	160 ± 12 nmol/min·mg
Oxygen uptake	
State 3[b]	135 ± 28 ng atom of oxygen/min·mg protein
State 4[c]	21 ± 8 ng atom of oxygen/min·mg protein
Respiratory control[d]	6.7 ± 2
Progesterone synthesis[e]	
Control	30.6 ± 1.3 ng progesterone/min·mg
+22(*R*)-hydroxycholesterol	85 ± 6.0 ng progesterone/min·mg

[a] Specific activities from complexes I and II were measured spectrophotometrically in sonicated mitochondria: complex I, NADH:DCPIP oxidoreductase and complex II, succinate:DCPIP oxidoreductase. Complex II activity was stimulated as described in Materials and methods section. Specific complex V activity was determined in intact mitochondria as ATP synthesis. Values shows are the mean ± S.D. (n = 7 independent determinations from different placental tissue).

[b] Defined as oxygen consumption stimulated by ADP added in presence of succinate as substrate. Values are the mean ± S.D. (n = 25 independent determinations from different placental tissue).

[c] Defined as oxygen consumption reduction due to all ADP added was converted to ATP. Values are the mean ± S.D. (n = 25 independent determinations from different placental tissue).

[d] Respiratory control = oxygen uptake rate of state 3/oxygen uptake rate of state 4. Values are the mean ± S.D. (n = 25 independent determinations from different placental tissue).

[e] Progesterone synthesis was determined as described in Materials and methods section. 22(*R*)-hydroxycholesterol was used to verify cytochrome P450scc, adrenodoxin, adrenodoxin reductase and 3β-hydroxysteroid dehydrogenase activities [30]. Values here are the mean ± S.D. from eight determinations from eight different placental tissues.

coupling membrane to protons, and induced maximum respiratory rate (205 ± 33 ng atom of oxygen/min·mg protein); no oligomycin-sensitive-ATP synthesis occurred. These data indicate functional coupling of mitochondrial respiration and ATP synthesis in syncytiotrophoblast mitochondrial preparations. Activities of 113 ± 20 μmol/min·mg protein for NADH:DCPIP oxidoreductase (complex I), and 14 ± 4 μmol/min·mg protein for succinate:DCPIP oxidoreductase (complex II) were also assessed (Table 1).

Endosome marker proteins, NPC1 and Rab5 [35,36], were not identified by Western blot in isolated syncytiotrophoblast mitochondria. Taken together, these results indicate the presence of functional mitochondria isolated from syncytiotrophoblast cells, capable of increasing oxygen consumption and of synthesizing ATP upon the addition of ADP.

Human syncytiotrophoblast mitochondria are steroidogenic organelles that synthesize progesterone due to the presence of 3β-hydroxysteroid dehydrogenase in their inner membrane [25,38]. The rate of progesterone synthesis was 30.6 ± 1.3 ng progesterone/min·mg protein and was not modified when exogenous cholesterol was added (data not shown). Furthermore, previously reported data indicated that placental mitochondria have enough cholesterol [39] so addition of exogenous cholesterol is not necessary to stimulate progesterone synthesis. The addition of 22(*R*)-hydroxycholesterol — a soluble substrate used to verify cytochrome P450scc, adrenodoxin, adrenodoxin reductase and 3β-hydroxysteroid dehydrogenase activities [33], increased steroidogenic activity to 85 ± 6.0 ng progesterone/min·mg protein (Table 1 and Fig. 5A). These results are in agreement with the specialized role of syncytiotrophoblastic tissue [25] and evidence that isolated mitochondria, as used in this work, retain their physiological function.

3.2. Syncytiotrophoblast mitochondrial protease activity

The experiments performed in the present study were designed to assay the role of syncytiotrophoblast mitochondrial proteases in progesterone synthesis. The first approach was to detect the in-gel protease activities and its pH dependence (Fig. 1A). Densitometric analysis of in-gel protease activity showed that it was null at low pH (pH = 4.3), while an increase was observed starting at pH = 5.3 (Fig. 1A). Since the identity of the proteases in syncytiotrophoblast mitochondria is unknown (but an effort to elucidate it is in progress in our lab), the intensity of each activity band was pooled to obtain the total activity of proteases from each pH value (Fig. 1B). Total protease activity was maximum at pH 7.5 (Fig. 1B) and five different protease activity bands were observed (Fig. 1A). Interestingly, the band with the lowest molecular weight (marked as E) was only detected at pH = 7.5 (Fig. 1A). Mitochondrial protease activity was compared with MMP9 and MMP2 activity constitutively secreted by U937, used as protease activity standards (Fig. 1C). The proteolytic activity was sensitive to 1,10-phenanthroline (Fig. 1D) or β-mercaptoethanol (data not shown), suggesting that syncytiotrophoblast mitochondria contain metalloproteases.

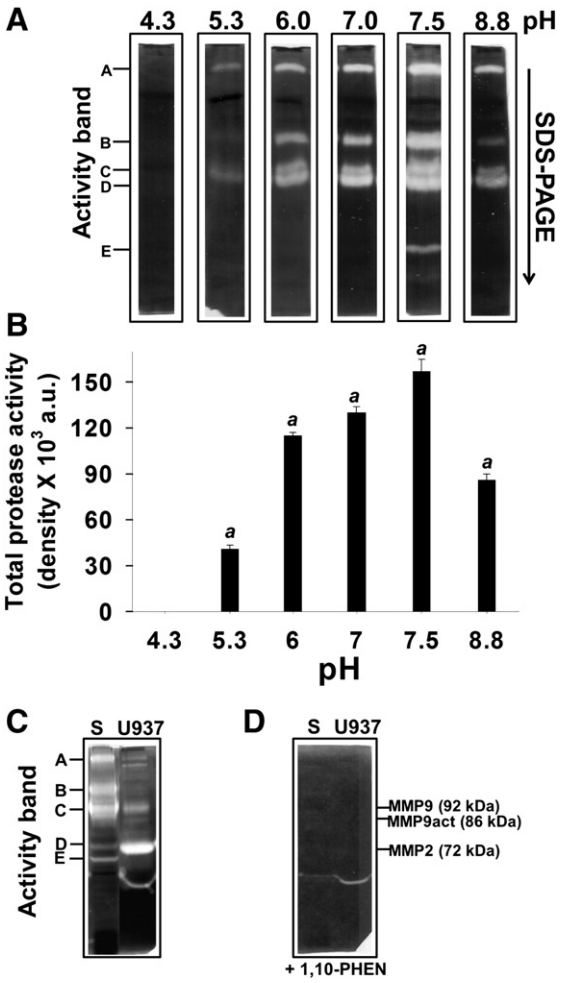
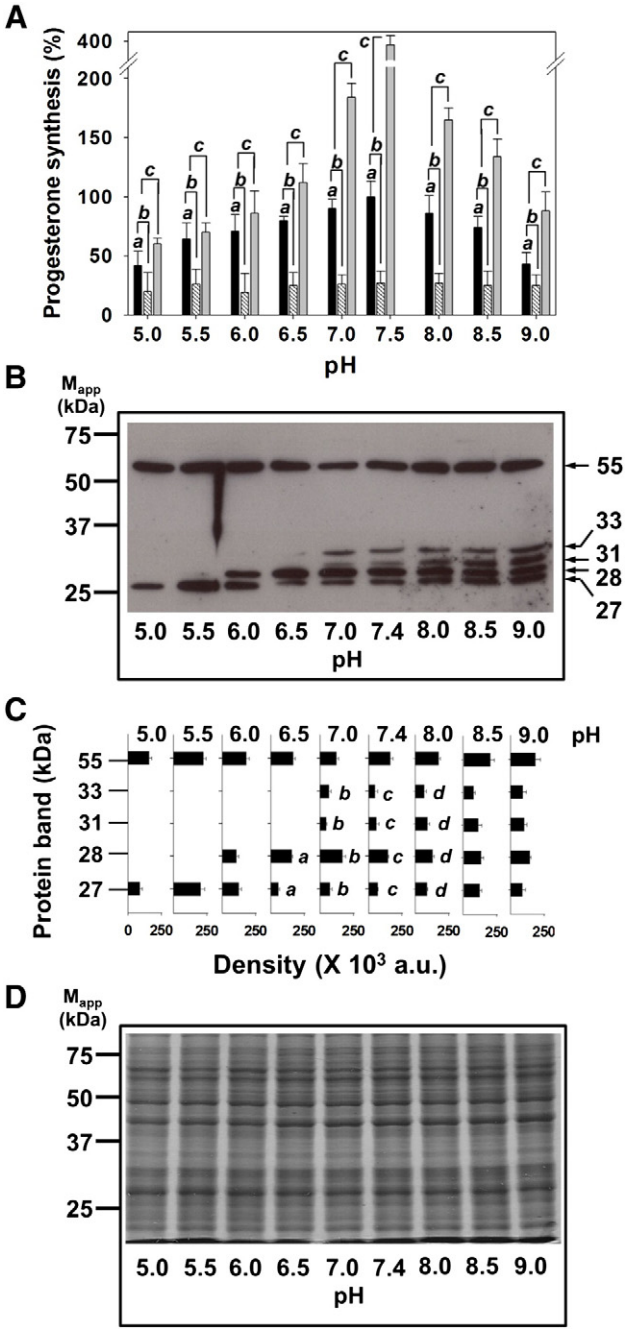


Fig. 1. In-gel protease activity from syncytiotrophoblast mitochondria. A) pH dependence of protease activity. Buffer used: sodium acetate (from pH 4 to 5.3), MES (from pH 5.3 to 7), Tris (from pH 7 to 8.8) and AMPSO (pH 8.8), and continued with the protocol described in the Syncytiotrophoblast mitochondrial protease activity section. B) Densitometric analysis from in-gel protease activity shown in A. The density of each band of protease activity was defined as band-intensity/area and the total protease activity shown is the sum of protease activity in each band. A significant increase in the intensity of total protease activity was observed at pH = 7.5, as compared to protease-activity at alkaline or acid pH values. The one-way ANOVA test indicates that these differences are statistically significant (indicated as *a*) ($p < 0.001$, n = 4, from four different placental tissues) (all Pairwise Multiple Comparison Procedures were performed with the Tukey test). Results are presented as the mean ± S.D.; a.u. = arbitrary units. C) Mitochondrial protease activity was determined at pH = 7.4 and compared to MMP2 and MMP9 proteases constitutively secreted by U937 used as protease activity standard (ATCC; Rockville, MD). S means protease activity from syncytiotrophoblast mitochondria. D) Inhibition of protease activity by 1,10-phenanthroline (9 mM) added to protease incubation mixture (+1,10-PHEN). Gels were stained with Coomassie® brilliant blue R-250 and protease activity correlated with the unstained band.

3.3. Syncytiotrophoblast mitochondrial protease and progesterone synthesis

In steroidogenic tissues acutely regulated, STARD1 promotes cholesterol transfer from the outer to the inner mitochondrial membrane. Once in the mitochondrion, steroidogenesis increases and STARD1 is proteolytically cleaved. Since the human placenta expresses STARD3 instead of STARD1 [10,21] we investigated the possible relationship among protease activity, STARD3 proteolytic cleavage, and progesterone synthesis in syncytiotrophoblast mitochondria (Fig. 2). It is important to mention that STARD3 remains tightly bound to isolated syncytiotrophoblast mitochondria (Fig. 2B and [21]), although it was described as an endosome protein [13]. The endosome markers Rab5 and NPC1 were not detected in the syncytiotrophoblast mitochondrial fraction (data not shown), in accordance with previous reports [21].



Since protease activity was dependent on pH, progesterone synthesis and STARD3 proteolysis were analyzed at different pH values. The synthesis of progesterone increased from pH = 5 to 7.4 (Fig. 2A, black bars), but a decrease was observed at pH = 9. This decrease might be due to the steroidogenic machinery being affected by the alkaline conditions. The effect of pH on cytochrome P450scc and 3β-hydroxysteroid dehydrogenase activities was verified by the addition of 22(*R*)-hydroxycholesterol to mitochondria incubated under different pH conditions (Fig. 2A, gray bars). As anticipated, progesterone production was increased by 22(*R*)-hydroxycholesterol at different pH values, with maximum levels of production detected at pH = 7.4, confirming the inhibitory effect of acid or alkaline pH on cytochrome P450scc and 3β-hydroxysteroid dehydrogenase. Concomitant with the increase in progesterone synthesis at pH = 7.4, Western blot assays for STARD3 revealed that the 55-kDa protein was proteolytically processed into four proteins released from mitochondria with molecular weights of 27, 28, 31, and 33-kDa (Fig. 2B). Densitometric analysis showed that the relative signal of the STARD3 55-kDa protein was similar at every pH value tested. However, the relative intensity of the proteolytic products increased as pH became alkaline (Fig. 2B and C). The initial proteolytic product was the 27-kDa protein, although the signal of the 28-kDa protein appeared at pH = 6.0. The signal increased and the 28-kDa protein was shown to be the main proteolytic product throughout the pH range tested (Fig. 2C). STARD3 cleavage matched the increase of progesterone synthesis in the physiologic pH range. Although the actual 28-kDa/STARD3 stoichiometry could not be estimated by densitometric analysis, the results are consistent with the hypothesis that STARD3 must be proteolytically processed into a smaller protein that promotes progesterone synthesis in the human placenta [40].

Since Western blot results suggested that STARD3 was processed into a protein belonging to a family of low molecular weight proteins (Fig. 2B), it was important to define its identity. MS/MS analysis of the 28-kDa protein showed that it contains the START-domain sequence (Fig. 3). Moreover, the 28-kDa protein shared 68% of protein identity and 72% of protein similitude to the human STARD3 (Fig. 3). These results confirm the identity of the 28-kDa protein as STARD3, and therefore will be indicated as STARD3-28 kDa from now on. Interestingly, the size of STARD3-28 kDa is approximate to that of the domain of N-218

Fig. 2. Proteolytic cleavage of STARD3 and progesterone synthesis in syncytiotrophoblast mitochondria. A) pH dependence of progesterone synthesis in the presence (dashed bars) or absence (black bars) of 9 mM 1,10-phenanthroline. The progesterone synthesis medium described in the Materials and methods section was supplemented with the following buffer: sodium acetate (from pH 4 to 5.3), MES (from pH 5.3 to 7), Tris (from pH 7 to 8.8) and AMPSO (pH 8.8), and the results are expressed as the mean ± S.D. of at least five separate experiments, with five different placental tissues. The one-way ANOVA analysis of control conditions (black bars) showed a significant increase in progesterone (P4) synthesis at pH = 7.4 when compared to P4 production at lower or higher values of pH (indicated by *a*). The difference is greater than expected by chance and there is a statistically significant difference ($p \leq 0.001$, $n = 8$) (all Pairwise Multiple Comparison Procedures were performed with the Tukey test). 100% of progesterone synthesis taken at pH 7.4 was considered as the maximum with a value of 30 ± 1.3 ng P4/mg/min. The two way ANOVA analysis showed a statistically significant difference between the treatment groups when 1,10-phenantroline was present (dashed bars) or absent (black bars) (indicated as *b*) ($p \leq 0.005$, $n = 16$) (all Pairwise Multiple Comparison Procedures was performed with the Holm–Sidak method). The addition of 22(*R*)-hydroxycholesterol to mitochondria increased P4 synthesis (gray bars). A two-way ANOVA analysis showed a statistically significant difference between control conditions and 22(*R*)-hydroxycholesterol addition (indicated as *c*) ($p \leq 0.001$, $n = 5$) (all Pairwise Multiple Comparison Procedures were performed with the Holm–Sidak method). B) Western blot against STARD3 protein. Mitochondria were incubated in P4M medium as described in the Materials and methods section, and after 20 min of incubation at 37 °C mitochondrial proteins were resolved in SDS-PAGE, and processed for Western blot or stained with Coomassie® brilliant blue R-125 (D). C) Densitometric analysis from Western blot shown in B. The one-way ANOVA of each band intensity at different pH values showed a significant increase in the 28-kDa protein band intensity at pH = 6.5 (indicated by *a*), 7.0 (indicated by *b*), 7.4 (indicated by *c*), and 8.0 (indicated by *d*), when compared to the other STARD3 proteolytic products (*i.e.* 27, 31 and 33-kDa proteins) ($p \leq 0.001$, $n = 4$, from four different placental tissues) (all Pairwise Multiple Comparison Procedures were performed with the Tukey test), whereas the 55-kDa protein (STARD3 full length protein) intensity was similar at all pH values tested. The density of each band was defined as band-intensity/area. a.u. = arbitrary units.

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STARD3          MSKLPRELTRDLERSLPAVASLGSSLSHSQSLSSHLLPPPEKRRAISDVRRTFCLFVTFD 60
STARD3-28kDa    MSKLPRELTRDLERSLPAVASLGSSLSHSQSLSSHLLPPPEKRRAISDVRRTFCLFVTFD 60
                ************************************************************

STARD3          LLFISLLWIIELNTNTGIRKNLEQEIIQYNFKTSFFDIFVLAFFRFSGLLLGYAVLRLRH 120
STARD3-28kDa    LLFISLLWIIELN-------------------------VLAFFRFSGLLLGYAVLRLRH 94
                *************                           ********************

STARD3          WW-------VIALLSKG--------AFGYLLPIVSFVLAWLETW---FLDFKVLPQEAEE 162
STARD3-28kDa    WWVIAVTTLVSSAFLIVKVILSELLSKGAFGYLLPIVSFVLAWLETWFLDFKVLPQEAEE 154
                **       * : :           : * :    . *   *      *************

STARD3          ER----WYLAAQVAVARGPLLFSGALSEGQFYSPPESFAGSDNESDEEVAGKKSFSAQER 218
STARD3-28kDa    ER----WYLAAQVAVARGPLLFSGALSEGQFYSPPESFAGSDNESDEEVAGKKSFSAQER 210
                **    ************************************************

                ⬇
STARD3          EYIRQGKEATAVVDQILAQEENWKFEKNNEYGDTVYTIEVPFHGKTFILKTFLPCPAELV 278
STARD3-28kDa    EYIRQGKEATAVVDQILAQEENWKFEKNNEYGDTVYTIEVPFHGKTFILKTFLPCPAELV 270
                ************************************************************

STARD3          YQEVILQPERMVLWNKTVTACQILQRVEDNTLISYDVSAGAAGGVVSPRDFVNVRRIERR 338
STARD3-28kDa    YQEVILQPERMVLWNKTVTACQILQRVEDNTLISYDVSAGAAGGVVSPRDFVNVRRIERR 330
                ************************************************************

STARD3          RDRYLSSGIATSHSAKPPTHKYVRGENGPGGFIVLKSASNPRVCTFVWILNTDLKG---- 394
STARD3-28kDa    RDRYLSSGIATSHSAKPPTHKYVRGENGPGGFIVLKSASNPRVCTFVWILNTDLKVGCWG 390
                ************************************************

STARD3          ----RLPRYLIHQSLAATMFEFAFHLRQRISELG-------------------ARA---- 427
STARD3-28kDa    AARAACPGTSSTRASRPPCLNLPFTCDSASASWGPGRDCAPSHPAGQGPVATTSRARKGA 450
                      *     ::  .. :::.*   .  :. *                  :**

STARD3          -------------------------
STARD3-28kDa    SWARTAHMGPGPRLSPSTEPRSAWS 475
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Fig. 3. Identification of the 28-kDa protein produced during steroidogenesis from human syncytiotrophoblast mitochondria as STARD3. The MS/MS analysis of the 28-kDa protein produced three peptides whose sequences are indicated in the boxes. The coverage of the 28-kDa protein was 16.3% and its identity was defined as STARD3 protein (ID J3QLM1_HUMAN from UNIPROT KB/TrEMBL). Sequences from whole human placenta STARD3 (indicated as STARD3-28 kDa) and STARD3 (NP_001159410 from NCBI) were aligned and showed an identity of 68% and 72% similitude (Clustal W program). START domain is underlined with a solid line; STARD3-28 kDa sequence is underlined with a dashed line. The arrow indicates the first amino acid in the N218-STARD3 reported by Ref. [40].

STARD3 (Fig. 3) reported by Ref. [40], which might have a role in cholesterol flux from the outer to the inner mitochondrial membrane. However, no mitochondrial targeting presequence was observed.

To verify the participation of proteases in STARD3 cleavage during progesterone synthesis, the effect of 1,10-phenanthroline, a metalloprotease inhibitor, on steroidogenesis was studied (Fig. 4). The time course of progesterone synthesis showed that 1,10-phenanthroline inhibits steroidogenesis (Fig. 4A). Indeed, progesterone synthesis was inhibited by 1,10-phenanthroline depending on its concentration and was totally abolished at 9 mM (Fig. 4B). Additionally, the 1,10-phenanthroline effect on progesterone synthesis at different pH values was explored (Fig. 2A, dashed bars). Once again, the protease inhibitor abolished mitochondrial steroidogenesis at every pH value, suggesting protease participation in progesterone synthesis. Simultaneously, the proteolytic cleavage of STARD3 was abolished by 1,10-phenanthroline (Fig. 4C). Other protease inhibitors like the protease inhibitor cocktail (Sigma), PMSF, EGTA, or EDTA (data not shown) prevented STARD3 cleavage into low molecular weight proteins (Fig. 4C).

3.4. STARD3 cleavage participates in the placental steroidogenesis

Although the relationship between protease activity, STARD3 cleavage, and progesterone synthesis had been demonstrated, it was necessary to determine the potential role of proteolytically-cleaved STARD3. 22(*R*)-hydroxycholesterol – a cholesterol analog that reaches P450scc independently of the mitochondrial transport system used by cholesterol – was added to syncytiotrophoblast mitochondria to stimulate progesterone synthesis when the proteolytic cleavage of STARD3 into the STARD3-28 kDa protein was inhibited by 1,10-phenanthroline (Fig. 5A). 22(*R*)-hydroxycholesterol produced a three-fold increase in progesterone synthesis, even in the presence of 1,10-phenanthroline (Fig. 5A), which inhibited the formation of the STARD3-28 kDa protein (Fig. 4C), suggesting the following implications: 1) P450scc and all the enzymes involved in progesterone synthesis are functional during protease inhibition, and 2) protease activity might be involved in the transport of cholesterol, *i.e.* the STARD3-28 kDa protein is obtained from STARD3 proteolytic cleavage. When purified the N218-STARD3 protein was added to syncytiotrophoblast mitochondria (Fig. 4C, last lane) and an increase in progesterone synthesis was observed (Fig. 5B). This result suggests that STARD3 proteolysis is an important step in the human placenta progesterone synthesis.

In an attempt to determine the sub-mitochondrial site where the proteolytic cleavage of STARD3 occurs, syncytiotrophoblast mitochondria were centrifuged during progesterone synthesis. The supernatant, where proteins not bound to mitochondria were released, was recovered. This procedure allowed isolating the low molecular weight proteins derived from STARD3 proteolysis (Fig. 6A). It is important to mention that mitochondria showed a respiratory control of 3.2 ± 1.1 after centrifugation, which confirms that the inner membrane remained intact. This suggests that the proteolytic cleavage of STARD3 could take place in the intermembrane space and that proteins are released through the outer membrane as a consequence of centrifugation (Fig. 6A). The subsequent hypothesis is that STARD3 is cleaved in the intermembrane space where its proteolytic products (mainly STARD3-28 kDa) are involved in

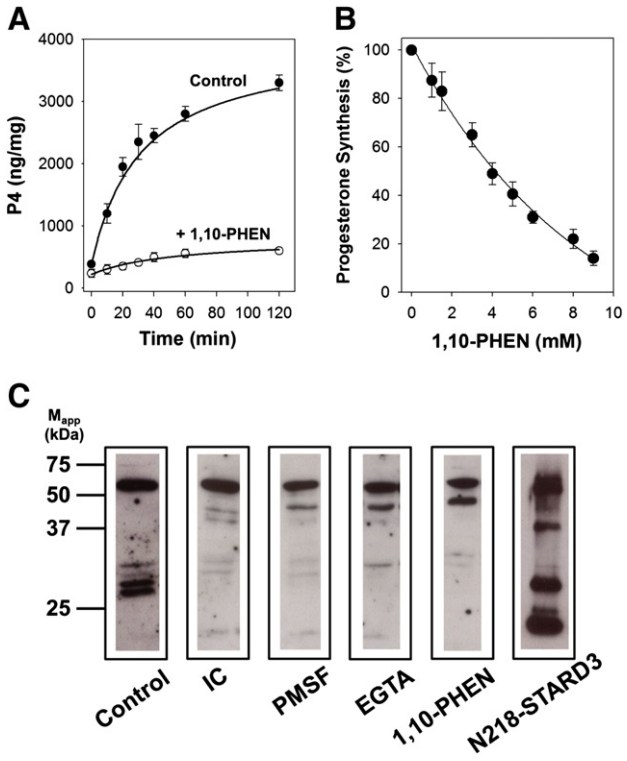

Fig. 4. Effect of 1,10-phenanthroline on mitochondrial progesterone synthesis and STARD3 cleavage. A) Time course of mitochondrial progesterone synthesis in the presence (○) or absence (●) of 9 mM 1,10-phenanthroline. Mitochondria were incubated in P4M medium at 37 °C and at the indicated times, an aliquot was removed and progesterone determined as described in the Materials and methods section. Results are presented as the mean ± S.D. of three separate experiments performed with three different placental tissues. B) Inhibition of progesterone synthesis by 1,10-phenanthroline (1,10-PHEN). The inhibition follows an exponential decay and the percentage of inhibition was calculated as the ratio between progesterone synthesis at the indicated 1,10-phenanthroline concentration against control conditions. The data were fitted to the equation $f = y_0 + a \cdot \exp^{(-b \cdot x)}$ using the Sigma Plot software, where $y_0 = -32.6 \pm 7.5$; $a = 143.72 \pm 16.4$; $b = 0.118 \pm 0.025$; $R = 0.9998$. C) Immunodetection of STARD3 protein during mitochondrial progesterone synthesis. Mitochondria were incubated in P4M for 20 min at 37 °C in the presence of an inhibitor cocktail from Sigma (cat. P8215), or PMSF (1 mM), or EGTA (5 mM), or 1,10-phenanthroline (9 mM) and then processed for Western blot analysis. Additionally, purified N-218 STARD3 (85 μM) was added to mitochondria.

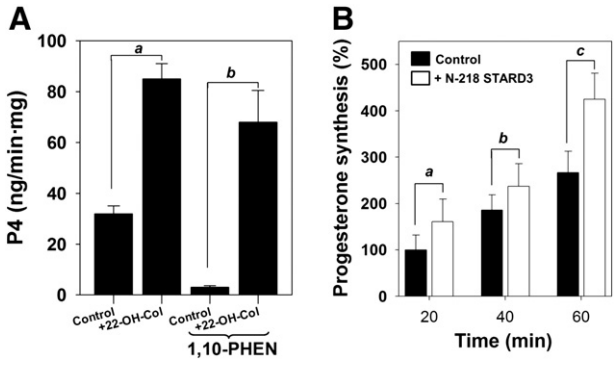

Fig. 5. Effect of 22(*R*)-hydroxycholesterol and purified N-218 STARD3 protein on progesterone synthesis. A) Syncytiotrophoblast mitochondria were incubated in P4M mixture for progesterone (P4) synthesis and 25 μM 22(*R*)-hydroxycholesterol was added. P4 was determined as described in the Materials and methods section. 1,10-Phenanthroline (1,10-PHEN) concentration was 9 mM. Results are presented as the mean ± S.D. of five separate experiments performed with five different placental tissues. The one-way ANOVA analysis showed a significant increase of P4 synthesis in the presence of 22(*R*)-hydroxycholesterol against control conditions (indicated as *a*) ($p \leq 0.0001$, $n = 4$, from four different placental tissues), or against 1,10-PHEN addition (indicated as *b*) ($p \leq 0.0001$, $n = 4$, from four different placental tissues) (all Pairwise Multiple Comparison Procedures were performed with the Tukey test). B) Time course of mitochondrial progesterone synthesis with (□) or without (■) N-218 STARD3 protein (85 μM). N-218 STARD3 protein was overexpressed and purified as described in the Materials and methods section. Results are presented as the mean ± S.D. of seven separate experiments performed with seven different placental tissues. The one-way ANOVA analysis showed a statistically significant difference in P4 production, which increased in the presence of N-218 STARD3 protein as compared to control conditions (indicated as *a* for 20 min; *b* for 40 min; *c* for 60 min) ($p \leq 0.001$, $n = 4$, from four different placental tissues) (all Pairwise Multiple Comparison Procedures were performed with the Tukey test).

cholesterol efflux between mitochondrial membranes. The high concentration of progesterone bound to a protein fraction that was released from the mitochondria (Fig. 6B, white bars) evidences the interaction of these peptides with steroid molecules. Denaturation of these low molecular weight proteins with cold methanol releases progesterone (Fig. 6B, black bars and Ref. [21]).

To verify the effect of the STARD3-28 kDa protein in progesterone synthesis, mitochondrial protein supernatant was collected and concentrated (as described in the Materials and methods section) and added to fresh and intact syncytiotrophoblast mitochondria (Fig. 6C, close circles), and progesterone synthesis was determined. Simultaneously, another set of fresh mitochondria was incubated with isolated N-218 STARD3 as control (Fig. 6C, open circles). The addition of the released protein fraction (*i.e.* STARD3-28 kDa protein) to fresh mitochondria induced an increase in progesterone synthesis just as N-218 STARD3 does, suggesting a similar role for both proteins.

4. Discussion

The physiological functions of human syncytiotrophoblast are crucial for the maintenance of pregnancy. Our group is interested in the study of the molecular mechanisms involved in the synthesis of progesterone by the human placenta. The study described here sought to reveal the role of mitochondrial proteases regarding STARD3 during progesterone synthesis in mitochondria from syncytiotrophoblast cells.

It has been put forward that the STARD3 protein, similarly to STARD1, transfers cholesterol from the outer to the inner mitochondrial membrane [40]. Although the molecular mechanism of STARD1 activity is still unknown, two models have been proposed. In the first one, STARD1 transfers cholesterol during its import into mitochondria. In the matrix, proteases degrade STARD1 to prevent its accumulation and subsequent mitochondrial damage [41–43]. Thus, it has been proposed that STARD1 import into the mitochondrial matrix serves as an off-switch for STARD1 activity. In the second model, mitochondrial import is not required for STARD1 activity, since N-terminally truncated STARD1 proteins retain full activity and it was not imported into mitochondria [4,6,22,44].

Despite the important role of STARD1, some steroidogenic tissues do not express it, as the human placenta. In this regard, elucidating the function of STARD3 is of considerable interest since it might promote steroidogenesis in tissues that do not express STARD1. Because the STARD1 role in steroidogenesis is associated with mitochondrial protease activity, in the present work we determined both, protease activity in syncytiotrophoblast mitochondria and proteolytic activation of STARD3.

Protease activity in syncytiotrophoblast mitochondria was associated with five different bands with an optimal pH = 7.5, and was sensitive to 1,10-phenanthroline, EGTA, EDTA, PMSF and β-mercaptoethanol, which suggests the presence of metalloproteases (Figs. 1, 2B and 4C).

Western blotting of isolated human syncytiotrophoblast mitochondria revealed the presence of apparently full-length (55-kDa) STARD3 and various proteolytic products (27, 28, 31, and 33-kDa) (Fig. 2B). Although STARD3 has been reported to be associated with endosomes [13], STARD3-antibodies recognized a protein of approximately 54 kDa in the isolated syncytiotrophoblast mitochondria, where Rab5 and NPC1 were not detected. This result is in accordance with Ref. [21]. A possible explanation is that some endosomes could still be present in the pellet that comprises isolated mitochondria. Also, this result could indicate a close

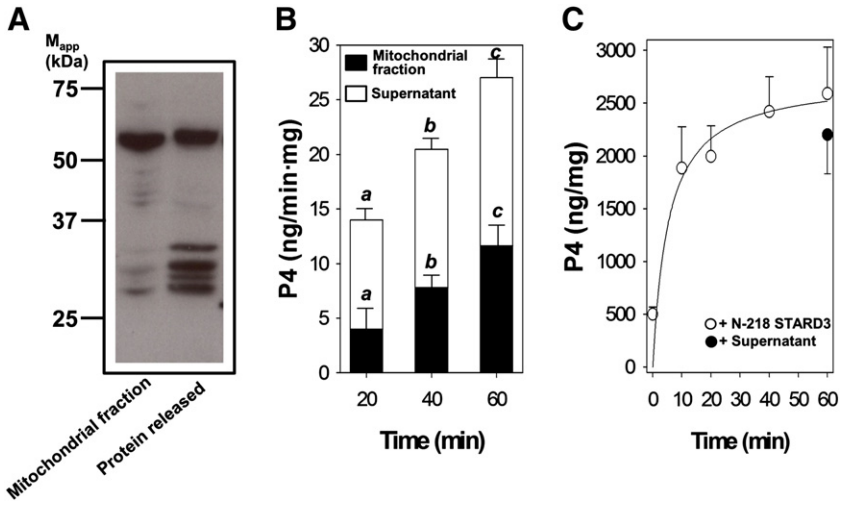

Fig. 6. Release of proteolytically cleaved STARD3 from syncytiotrophoblast mitochondria during progesterone synthesis. Syncytiotrophoblast mitochondria were incubated in P4M medium for 20 min at 37 °C as described in the Materials and methods section, and then mitochondria were centrifuged and the mitochondrial pellet and supernatant were separated and analyzed by Western blot against STARD3 (A); progesterone content was also determined (B). Where indicated, (■) corresponds to mitochondrial pellet and (□) corresponds to protein released to supernatant. A one-way ANOVA test indicated that the differences in progesterone content between mitochondrial pellet and supernatant are statistically significant (statistical significance indicated as *a* for 20 min; *b* for 40 min; *c* for 60 min) ($p < 0.001$, $n = 7$, from seven different placental tissues) (all Pairwise Multiple Comparison Procedures were performed with the Tukey test). Results are presented as the mean, and error bars indicate S.D. of seven separate experiments. The effect of isolated N-218 STARD3 (○) or mitochondrial supernatant (●) on progesterone synthesis is shown in (C). Protein released from mitochondria during progesterone synthesis was collected, pooled and concentrated in an Amicon Ultra Centrifugal Filter (10K) and added to fresh syncytiotrophoblast mitochondria to determine its effect on progesterone synthesis. Simultaneously, another set of mitochondria were incubated with N-218 STARD3 protein (85 μM), and progesterone synthesis was determined. No significant differences were observed between protein released from mitochondria and N-218 STARD3 in progesterone synthesis stimulation. The total progesterone content of the mitochondrial supernatant protein was 900 ng of progesterone/mg of protein/60 min, and it was subtracted from the values showed in the graph.

relationship between mitochondria and endosomes during placental steroidogenesis. Nevertheless this hypothesis should be further investigated.

The fact that inner-membrane-impermeable 1,10-phenanthroline, EDTA and EGTA inhibited STARD3 proteolytic cleavage (Fig. 4C) suggests that metalloproteases could be located in the intermembrane space (IMS), *i.e.* the YME1L, an *i*-AAA protease that exerts its activity on the IMS side of the inner membrane of mitochondria (Fig. 7) [45, 46], or in the cytoplasmic side of the outer membrane. Proteolytic

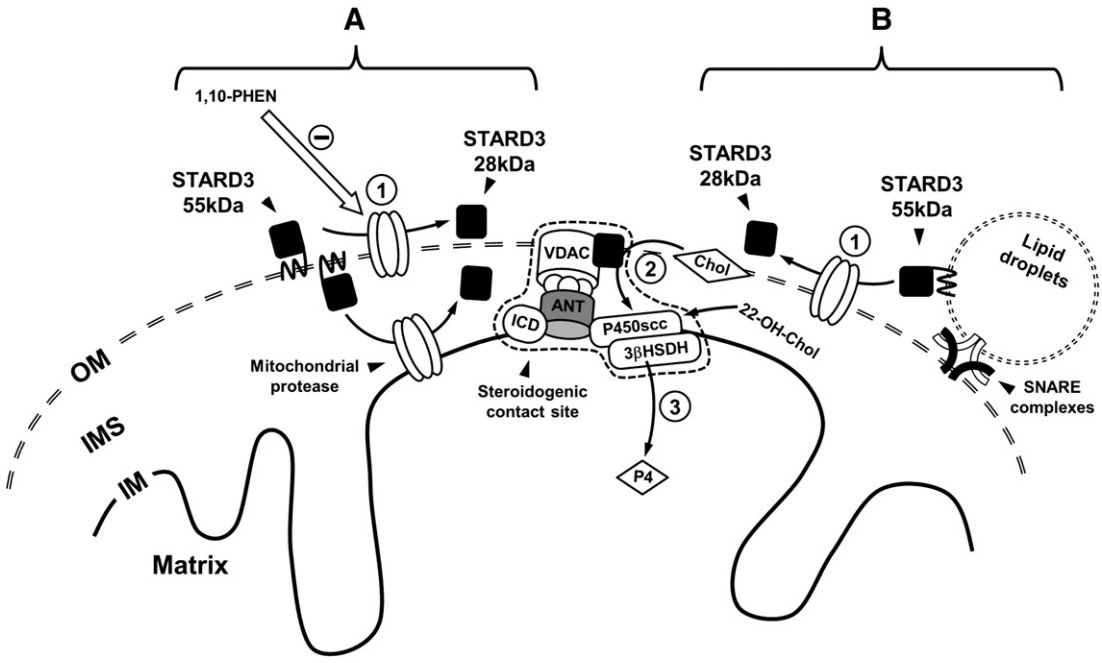

Fig. 7. Model proposed for the role of mitochondrial proteasomes in STARD3 cleavage and progesterone synthesis in human syncytiotrophoblast cells. Protease activities in the activation of MLN64 and the subsequent progesterone synthesis increase have been divided into three steps. Step 1: Proteolytic transformation of STARD3 from a 55-kDa into a 28-kDa protein by a protease that exerts its activity on the IMS side of the inner membrane of mitochondria or at the outer membrane (see text for details). This protease is sensitive to 1,10-phenanthroline, EGTA, or EDTA (shown as an empty arrow). Step 2: The STARD3-28 kDa protein, which has been shown to contain the cholesterol binding domain ([40] and the present work), would increase cholesterol flux from the outer into the inner mitochondrial membrane to reach the cytochrome P450scc machinery and increase progesterone production (Step 3). If protease activity is inhibited and no STARD3-28 kDa protein is produced, 22(*R*)-hydroxycholesterol might promote progesterone synthesis. STARD3 incorporation to mitochondria could occur without a classical mitochondrial targeting presequence (A) or through the association between mitochondria and lipid droplets *via* the SNARE complex (B). OM = outer mitochondrial membrane; IMS = intermembrane space; IM = inner mitochondrial membrane; steroidogenic contact site = marked with a dashed line; Chol = cholesterol; P450scc = cytochrome P450scc; 3βHSDH = 3β-hydroxysteroid dehydrogenase; 22-OH-Chol = 22(*R*)-hydroxycholesterol.

activation of STARD3 in the intermembrane space or in the outer membrane is also supported by the fact that STARD3 contains no mitochondrial leader sequence and hence does not enter the mitochondrial matrix. Instead its proteolytic products (*i.e.* STARD3-28 kDa protein) could be released from mitochondria by centrifugation without inner membrane damage (Fig. 6A). Although STARD3 lacks a classical mitochondrial targeting presequence, it is tightly joined to syncytiotrophoblast mitochondria and its role in steroidogenesis is demonstrated in the present work. It has been reported that many mitochondrial hydrophobic membrane proteins are synthesized without cleavable extensions [47]. These proteins typically contain several targeting signals that are distributed throughout the length of the protein [48]. This could be the case for STARD3. Once incorporated into mitochondrial membranes, STARD3 might be proteolytically activated and then associated with steroidogenic contact sites to promote cholesterol transport (Fig. 7A). Although this hypothesis needs to be elucidated, STARD3 intermembrane space location is important due to the possible role that has been suggested for the STARD3-28 kDa protein in cholesterol transfer between mitochondrial membranes [40].

Additionally, a model for the incorporation of STARD3 into mitochondria and its activation was put forward, based on the results from mass spectrometric analysis of mitochondrial outer membrane proteins (Fig. 7B). It has been reported that lipid droplets contain constituent proteins of the SNARE complexes [49–53], which include α-SNAP, Syntaxins and VAMP. Human syncytiotrophoblast mitochondria contained SNAP (P54920_SNAA_HUMAN), Syntaxin-3 (F8W9Y0_STX3_HUMAN), Syntaxin-7 (O15400-2_STX7_HUMAN), Syntaxin-12 (Q86Y82_STX12_HUMAN), Syntaxin-binding Protein-2 (STXBP-2, Q15833_E7EQD5_HUMAN), Syntaxin-binding Protein-3 (STXBP-3, O00186_STXB3_HUMAN), and VAMP-8 proteins (Q9BV40_VAMP8_HUMAN). It has been shown that the SNAP protein promotes interaction between lipid droplets and mitochondria [54], and that steroidogenic cells express SNARE proteins such as Syntaxin-17, SNAP-23, and SNAP-25 [55–59]. These observations strongly suggest that SNARE proteins might mediate cholesterol transport from lipid droplets to steroidogenic mitochondria, most likely by promoting the functional interaction between lipid droplets and mitochondria. In syncytiotrophoblast cells, the STARD3 protein might be incorporated into mitochondria from lipid droplets through SNARE complexes and proteolytically activated by mitochondrial proteases. Once STARD3-28 kDa has been produced, it might then be incorporated to steroidogenic contact sites [20] and promote cholesterol transport for progesterone synthesis (Fig. 7).

Steroidogenic contact sites are multiprotein complexes associated with cholesterol transport and steroidogenesis [20]. Several proteins like HSP60 might be involved, as suggested by Ref. [21]. Although TPSO is a protein apparently essential for several mitochondrial processes, its function does not seem to be crucial to the permeability of the transition pore [60], the steroidogenesis of Leydig cells [18] and the human placenta [19].

Nevertheless, it has been reported that the rate-determining step of placental progesterone synthesis is the electron supply to cytochrome P450scc from adrenodoxin reductase [61,62]. It has been demonstrated that in purified human syncytiotrophoblast mitochondria [63] or in the isolated steroidogenic contact sites [20], the addition of 22(*R*)-hydroxycholesterol increases progesterone synthesis compared with the control condition (*i.e.* mitochondria were incubated with isocitrate as oxidable substrate in a medium that promotes progesterone synthesis; see Refs. [20,21,28,32] and the Materials and methods section). This suggests that electron supply to P450scc might not be the limiting-step. The addition of 22(*R*)-hydroxycholesterol, a cholesterol analog that freely reaches cytochrome P450scc [33], rendered three-fold increases in progesterone production, even if proteases were inhibited with 1,10-phenanthroline (Fig. 5A) and no STARD3 proteolytic cleavage occurred (Fig. 4C). This evidences a specific effect of the protease inhibitor, as well as that the proteolytic products of STARD3 might be involved in cholesterol transport.

Mitochondrial metalloprotease activity, relative to alkaline pH; simultaneous proteolytic activation of STARD3; and an increase in progesterone synthesis, were observed (see Figs. 1, 2 and 4). Moreover, the proteolysis of 55-kDa STARD3 into a STARD3-28 kDa protein was associated with the maximal rate of progesterone synthesis observed (Fig. 2). It has been shown that the N-218 STARD3 protein (with similar size to STARD3-28 kDa) has substantial StAR-like activity in transfected cells [10]. In addition, Bose et al. [40] used a total protein homogenate from midterm human placenta to show that the presence of the full-length STARD3 protein and various proteolytic products, among which the 28-kDa peptide was predominant. However, purified syncytiotrophoblast mitochondria were used in this work to demonstrate that mitochondrial metalloproteases could be responsible for the proteolytic transformation of STARD3 into the STARD3-28 kDa protein, which could be involved in cholesterol transfer between mitochondrial membranes. Furthermore, the addition of the protein N-218 STARD3 or the STARD3-proteolytic-products (*i.e.* the STARD3-28 kDa protein which is released from mitochondria while progesterone synthesis occurs) to isolated syncytiotrophoblast mitochondria produced a similar enhancement of steroidogenesis (Fig. 6C), highlighting the role of STARD3 and its proteolytic product, the STARD3-28 kDa protein. Currently, transfection of HEK-293 cells with the whole progesterone synthesis machinery and the N-218 STARD3 protein is being performed in our laboratory to determine if STARD3 cleavage by proteases is the key step in progesterone synthesis. However, with all the results described so far, we propose that STARD3 is processed *in vivo* by metalloproteases from human syncytiotrophoblast mitochondria to the STARD3-28 kDa protein, a product similar to N-218 STARD3 that might promote steroidogenesis in a similar way to that described in the proposed model for the STARD1 protein (Fig. 7).

Although less is known about the roles of mitochondrial proteases in mammalian cells, it has been shown that loss-of-function mutations in human genes encoding mitochondrial proteases are often associated with clinical disorders [64–67]. Therefore, molecular and biochemical characterization of such proteolytic activities is of the utmost importance, as suggested by Ref. [68]. Finally, future research using STARD3 knockout mice [69] will be relevant to determine the specific cellular location of STARD3 and its role in reproduction.

The present study suggests that the STARD3 protein can be used as an authentic natural substrate to explore multiple mitochondrial proteases, to provide new insights into their mode of action in healthy and diseased steroidogenic cells, and to allow understanding of the different ways in which steroids are produced.

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