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REGULAR ARTICLE

Annexin A2 and S100A10 in the mammalian oviduct

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Abstract In many mammals, upon entry into the female reproductive tract, a subpopulation of sperm is stored in the oviduct forming a functional reservoir. In the oviducts of pig and cow, Annexin A2 (AnxA2) has been linked to the binding of sperm. This protein may exist as a monomer or bound to S100A10 and both forms are associated with different biological functions. S100A10 has not yet been reported in the oviduct. The objective of this work is to analyze for the presence of S100A10 in the oviduct and to advance the study of AnxA2 and S100A10 in this organ. This work shows the presence of both proteins, AnxA2 and S100A10, in the oviduct of human, pig, cow, cat, dog and rabbit. At least in pig, AnxA2 is found devoid of S100A10 in the outer surface of the apical plasma membrane of oviductal epithelial cells, indicating that it binds to sperm as a monomer or in association with proteins different from S100A10. In the apical cytoplasm of pig oviductal epithelial cells, AnxA2 is associated with S100A10. In primary culture of porcine oviductal cells, the expression of *ANXA2* is increased by progesterone, while the expression of *S100A10* is increased by progesterone and estradiol. The widespread detection of both proteins in the oviduct of mammals indicates a probable conserved function in this organ. In summary, S100A10 and AnxA2 are widespread in the mammalian oviduct but AnxA2 binds sperm in vivo devoid of S100A10 and may be related to reservoir formation.

Keywords Fallopian tube · Sperm reservoir · Annexin · Oviduct · S100A10

Introduction

The oviduct is a dynamic organ in which sperm selection, fertilization and the first stages of embryo development take place. This organ is largely responsible for sperm selection. When spermatozoa enter the oviduct in pigs (Hunter 1984), cattle (Hunter and Wilmut 1984), sheep (Hunter et al. 1982), mice (Suarez 1987), hamsters (Smith and Yanagimachi 1991), horses (Thomas et al. 1994), dogs (Pacey et al. 2000), cats (Chatdarong et al. 2004), rabbits (Baranda-Avila et al. 2010) and humans (Pacey et al. 1995), a subpopulation of them binds to the epithelial cells forming a reservoir. This process is considered to select for high quality, help control polyspermy, lengthen lifespan, regulate capacitation and select normal sperm (for review, see Talevi and Gualtieri 2010). Sperm binding presents localization differences among diverse species, including sperm retention at the uterus and vagina in rabbit, cat and dog (Barberini et al. 1991; Chatdarong et al. 2004; England et al. 2013). At the oviduct, in vivo, sperm bind predominantly at the crypts of the uterotubal junction (UTJ) and caudal isthmus in sows (Tummaruk and Tienthai 2010), at the caudal isthmus in cattle (Hunter and Wilmut 1984) and at the ciliated cells in the distal UTJ in bitches (England et al. 2013). In cats, the UTJ acts as sperm reservoir before ovulation, whereas the isthmus is a sperm

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reservoir around the time of ovulation (Chatdarong et al. 2004). The mechanisms responsible for sperm binding and detachment from the oviductal reservoir are under constant study; however, the molecular mechanisms involved require further investigation. Sperm binding to oviductal epithelial cells has been attributed to spermadhesins in porcine (Liberda et al. 2006) and to adsorbed seminal plasma proteins (BSP) in cattle (Gwathmey et al. 2006).

Previous work has linked oviductal annexins to sperm reservoir formation in cattle (Ignotz et al. 2007) and pig (Teijeiro et al. 2009). In cow, Ignotz et al. (2007) purified AnxA1, 2, 4 and 5 from oviductal apical plasma membrane preparations based on affinity to BSP and concluded that annexins are candidates for sperm binding to the oviduct. In pig, AnxA1, 2 and 5 were isolated from oviductal epithelial cell extracts based on affinity to sperm periacrosomal membrane proteins (Teijeiro et al. 2009). The localization of the isolated annexins by immunohistochemistry showed that AnxA1 and AnxA5 are present mainly in the cytoplasm of pig oviductal epithelial cells, while AnxA2 is found mainly at the cilia of oviductal epithelial cells (Teijeiro et al. 2009). This led to the conclusion that, in pig, AnxA2 is the strongest candidate for sperm binding to the oviduct.

Annexins are a multifunctional family of membrane phospholipid-binding proteins that are able to bridge or attach biological structures (reviewed in Grieve et al. 2012). AnxA2 is a member that can be found in the nucleus, cytoplasm or extracellular matrix of a variety of cell types and as a monomer or in heterotetrameric form bound to S100A10 (AIIt), both forms associated with different biological functions. S100A10 is a member of a group of small Ca2+-binding proteins with a molecular weight of 10–12 kDa (the S100 proteins; Donato 1999), which is unique in that it is locked in a permanently open conformation, comparable to the Ca2+-bound configuration of the other S100 proteins (for review, see Liu et al. 2015). It is also the most frequent binding partner of AnxA2, described more than 30 years ago (Erikson et al. 1984). Extracellularly, AnxA2's largest studied function is as a cell surface protector for the plasminogen receptor S100A10 (reviewed in Madureira et al. 2012). In addition, AnxA2 also interacts with extracellular receptors such as Toll-Like receptor 4 (TLR4) (Swisher et al. 2010) and ligands such as gastrin (Singh et al. 2007). Also in the cell surface, AnxA2 has been shown to function in signal transduction (Drücker et al. 2013). At this location, the AIIt complex is associated not only to the plasminogen receptor function but also to cell recognition for phagocytosis, to adhesion of different cell types in the bone marrow (Jung et al. 2007) and of endothelial and cancer cells (Myrvang et al. 2013).

The presence of AnxA2 in the porcine and bovine oviduct, its localization mainly at the cilia of oviductal epithelial cells, structures that have been involved in sperm attachment in porcine (Suarez et al. 1991), AnxA2's ability to bind to pig sperm periacrosomal membranes in vitro and to BSP in cow and its frequent association to S100A10, led us to hypothesize that AnxA2 might perform its sperm binding function in association with S100A10. Thus, in this work, we search for the presence of S100A10 and analyze the subcellular location of both proteins in the oviduct. Also, as the oviductal epithelium is prone to constant renovation in response to ovarian hormones, we reasoned that *ANXA2*'s and *S100A10*'s expression may respond to female hormone variations. To examine this hypothesis, we performed hormone treatment experiments on primary culture of pig oviductal epithelial cells and computational analysis of putative promoter regions on pig *ANXA2* and *S100A10* genes, searching for estrogen and progesterone response elements (ERE and PRE). As the sperm reservoir is present in the oviduct of numerous mammals and AnxA2 is involved in reservoir formation in cow and pig, we considered it important to analyze whether AnxA2 and S100A10 are also present in the oviduct of other mammals and particularly in the human oviduct.

Materials and methods

Chemicals

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich (Buenos Aires, Argentina).

Ethics statements

This study was approved by the Institutional Bioethical Board for the use of human samples and by the Bioethical Board for use of Laboratory Animals for the use of animal samples, both from Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina.

Oviducts collection

Domestic mixed breed cat and dog oviducts (10 of each) were obtained by surgery from Instituto Municipal de Salud Animal (Rosario, Argentina). Rabbit (New Zealand) oviducts (10) were obtained by surgery from Bioterio de Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina. Cow (10, beef cattle) and pig (Large White) oviducts were obtained from a local slaughterhouse and transported to the laboratory on ice. Human oviducts (five women) were obtained from women subjected to endoscopic salpingectomy for non-malignant cause with the corresponding acceptance of the patient and written consent was obtained from all donors, at Hospital Provincial del Centenario, Argentina.

Preparation of oviductal cell protein extracts

For western blot detection of AnxA2 and S100A10 among whole cell protein content from pig, human, cow, cat, dog and rabbit, oviducts were opened longitudinally and epithelia from the isthmus sections were scraped with the blunt side of a scalpel blade. The cells were collected in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 5 mM EDTA and 50 mM DTT). Protein concentration was determined by the Qubit® device (Invitrogen, Buenos Aires, Argentina) according to the manufacturer's instructions.

Western blot

Oviductal cell protein extracts were used for 15 % SDS-PAGE according to Laemmli (1970), using the Miniprotean 3 System (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to nitrocellulose membranes (GE Healthcare, Argentina). Membranes were blocked with 5 % dry milk in TBS (25 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 1 h, incubated with HH7 anti-AnxA2 monoclonal antibody, raised against a synthetic peptide corresponding to amino acids 1–18 of human AnnexinAII, (1:1000 v/v) (kindly gifted by Dr. Gerke from the Institute of Medical Biochemistry Center for Molecular Biology of Inflammation, University of Münster, Germany) and anti-S100A10 (cow) monoclonal antibodies (1:1000 v/v, BD transduction Laboratories #610070), a kind gift from Dr. Melle (Core Unit Chip Application, Institute of Human Genetics and Anthropology, Universitätsklinikum Jena, Germany). Then, membranes were washed twice for 15 min with TBS and incubated with anti-mouse IgG-horseradish peroxidase (Amersham Biosciences, UK) 1:5000 v/v in TBS, during 1 h at room temperature. After washing twice with TBS and once with TTBS (TBS plus 0.05 % Tween-20), peroxidase activity was revealed using enhanced chemiluminescence detection with ECL (Amersham Bioscience) according to the manufacturer's instructions. Monoclonal antibodies anti-α tubulin (sc-8035) (1:1000 v/v dilution) and anti-actin (sc-1616) (1:1000 v/v dilution), used as procedure and cellular compartment controls, were provided by Santa Cruz Biotechnology (CA, USA). When these controls were not appropriated, membranes were stained with Ponceau Red to check quantitative transference of proteins. For relative quantification, films were analyzed with Gel-Pro Analyzer v.4.0 (Media Cybernetics).

Immunohistochemistry

The isthmic portions of porcine and human oviducts were separated by dissection into 1-cm segments and fixed in 4 % formaldehyde. Tissue was dehydrated, embedded in paraffin, cut into 5-μm sections and mounted on slides optimized for immunohistochemistry (Frosted HiFixNH; TNT, Argentina). Tissue sections were deparaffinised in xylene and then rehydrated through graded dilutions of ethanol (100, 95, 70 and 35 %), followed by two washes in TBS. Antigen retrieval was performed by microwaving slides for 20 min in 10 mM trisodium citrate (pH 6). Endogenous peroxidases were inactivated by incubating slides for 30 min in 0.3 % H_2O_2 in methanol, followed by three 5-min washes in TBS. Sections were blocked with 5 % bovine serum albumin, 0.2 % Triton X-100 in TBS for 60 min and then treated with HH7 anti-AnxA2 antibodies (1:100) or anti-S100A10 antibodies (1:50) 1 h at 37 °C followed by anti-mouse IgG-horseradish peroxidase (diluted 1:100) for 1 h. Horseradish peroxidase activity was visualized by development with 3,3′-diaminobenzidine tetrahydrochloride and stopping the reaction by washing in water. Counterstaining was performed by Haematoxylin staining. Controls were run by omission of the primary antibody (data not shown).

Preparation of oviductal cell suspensions and protein extracts from different cell fractions

Porcine oviductal epithelial cells were prepared based on previous reports (Marini and Cabada 2003). Briefly, isthmuses were opened longitudinally and the epithelial cells were scraped using the blunt side of a scalpel blade. The cell-containing suspension was centrifuged at 100*g* for 3 min, suspended in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and incubated for 1 h at 4 °C. After incubation, cells were centrifuged at 100*g* for 3 min and supernatants were recovered, centrifuged at 16,000*g* for 15 min and stored at −20 °C for further protein analysis. Then, epithelial cells were suspended in distilled water and disrupted in Potter homogenizer. Fractions enriched in membranes were prepared by differential centrifugation as described elsewhere (Marini and Cabada 2003). Detergent protein extracts were prepared by incubation of membrane fractions with 0.2 % Triton X-100, 10 mM Tris-HCl, pH 7.5 for 1 h at 4 °C. After incubation, membrane fractions were centrifuged at 105,000*g* at 4 °C for 1 h, the supernatant was recovered and membrane fractions were suspended in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5 and incubated for 1 h at 4 °C for high ionic strength protein extraction. After centrifugation at 105,000*g*, the supernatant was recovered and stored at −20 °C until use.

Isolation of apical plasma membranes (APM) by peeling was based on the method described by Teijeiro and Marini (2012). Batches of ten porcine oviducts were trimmed from reproductive tissues and the isthmus section of each oviduct was separated and opened longitudinally. Then, each section was stretched and subjected with pins. Whatman filter paper (0.18 mm thick; Whatman International, Maidstone, UK) strips were pre-wetted with deionized water, put over the

exposed epithelium and incubated for 5 min. After peeling, the APM retained at the surface of the paper were harvested by rehydration of strips in 15-ml polystyrene conical tubes with 5 ml of deionized water. Then, proteins from the harvested APM were obtained by incubation in RIPA buffer.

Porcine oviductal cell membranes and cytoplasmic proteins were obtained as previously reported by Teijeiro and Marini (2012). Briefly, epithelial cells obtained by scrapping epithelia were disaggregated by passing through a 21-gauge needle to separate the cells. After this, the suspensions were washed twice in TALP by 5 min centrifugation at 650*g* at 4 °C. The cells were disrupted in deionized water in Potter homogenizer and centrifuged at 1000*g* 10 min at 4 °C. The supernatant was ultracentrifuged for 1 h at 105,000*g* at 4 °C. Fractions enriched in membranes were recovered from the pellet and the supernatant of these fractions was considered as cytoplasmic fraction.

In situ biotinylation of oviductal luminal proteins

The biotinylation protocol was based on the method described by Holt et al. (2005) and Teijeiro and Marini (2012). Batches of ten oviducts were trimmed from reproductive tissues and washed in ice-cold PBS. The isthmus section of each oviduct was separated, flushed with 2 ml of ice-cold PBS/CM (PBS containing 1.3 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.2) and then with 2 ml of the biotinylating reagent EZ-Link® Sulfo-NHS-SS-Biotinylation kit (Pierce, Thermo Scientific, USA) at 0.5 mg/ml in PBS/CM. The isthmic sections were clamped at each end, filled with the same solution and incubated 30 min at room temperature. After incubation, isthmus sections were emptied and the excess of biotin was inactivated by flushing with quenching solution (Pierce). The oviduct sections were opened longitudinally and epithelia were scraped and transferred to a 50-ml conical tube. Scraped cells were rinsed three times with 5 ml of TBS by centrifugation at 500*g* for 3 min at 4 °C. After rinsing, cells were suspended in lysis buffer (Pierce) and sonicated at 1.5 %, five 1-s bursts. Cells were incubated 30 min on ice, vortexing every 5 min for 5 s and then centrifuged at 10,000*g* for 2 min at 4 °C. Biotinylated proteins were purified by affinity to NeutrAvidin™ Agarose (Pierce) according to the manufacturer's instructions and then subjected to SDS-PAGE and detection by incubation with 1 μg/ml peroxidase-conjugated streptavidin (Pierce). Labeled proteins were revealed using enhanced chemiluminescence detection as described for western blot. As a control, tissue was processed without biotinylating reagent.

Immunoprecipitation

For immunoprecipitation assays, 200 μl of protein extracts containing 600 μg of protein and obtained by incubation with 0.2 % Triton X-100, 10 mM Tris-HCl pH 7.5 from membrane-enriched fractions as described previously, were incubated overnight at 4 °C with 2 μl of HH7 anti-AnxA2 antibodies. One hundred microliters of protein A/G Sepharose (Pierce) were washed three times with PBS and non-specific binding sites blocked by 2 h incubation with 2 % BSA-PBS. After washing with PBS, the resin was incubated with the solution containing HH7 anti-AnxA2 antibodies and protein extracts for 2 h. Following incubation, the resin was washed 5 times with PBS and antibody-protein complexes were recovered by incubation in 20 μl of SDS-PAGE loading buffer. Then, purified protein complexes were subjected to western blot for AnxA2 and S100A10 detection. As a control, irrelevant mouse IgG was used and only the expected bands corresponding to mouse IgG were detected.

Primary oviductal cell culture

Freshly collected porcine oviducts were dissected free from surrounding tissues and washed twice in sterile PBS at 4 °C. Then, they were cut, flushed with sterile PBS and squeezed by pressure with tweezers. Batches of cells from 10 oviducts corresponding to diverse ovarian status were used. Laminae of porcine oviduct epithelial cells were selected on the basis of ciliary beating, washed by centrifugation at 1500*g* for 5 min and incubated in M199 medium supplemented with 10 % (v/v) fetal bovine serum (Internegocios, Argentina), 50 μg/ml gentamycin (Gibco Life Technologies, Argentina) and antibiotic antimicotic solution (100 UI penicillin, 10 μg streptomycin and 0.25 μg amphotericin B per mL; Sigma-Aldrich A5955). Cultures were performed at 39 °C, 5 % CO2 in air, in 100-mm Petridishes (Greiner bio-one). Fresh media changes were performed every 48 h until 80 % of confluence. Then, cells were incubated for 24 h in DMEM:F12 medium without phenol red and fetal bovine serum and with the corresponding antibiotics. After that, cells were incubated for 24 h with different concentrations of β-estradiol (Sigma E-8875) or progesterone (Sigma P-0130) using ethanol as a solvent. Primary culture controls were performed using the same volume of ethanol. Cell viability was assessed by the 0.2 % Trypan blue exclusion method. Cells were harvested by incubation for 10 min with trypsin at 37 °C, centrifugation, PBS wash (twice) and were suspended in RIPA buffer for protein extraction. Three independent experiments were performed and three repetitions from each primary culture pool were processed.

Bioinformatics

The sequence of the pig AnxA2 gene (ID: 406192) located in chromosome 1, contig NC_010443.4 and the sequence of pig S100A10 gene (ID: 100515138) located in chromosome 4, contig NC_010446.4, were subjected to analysis against a genome database to find corresponding promoter sequences. The relevant promoter sequences found were evaluated for

regions homologous to known hormone response elements using MatInspector Professional software (Genomatix suite v.3.2, free trial) on the vertebrate's transcription factor library.

Statistical analysis

Statistical analysis was performed for AnxA2 and S100A10 relative detection on western blots. When F test results were significant in ANOVA, individual means were further tested by Tukey's multiple range test (Motulsky 1995). Differences with respect to the control without hormones and between treatments were indicated as #, ##, ### and *. Statistical significance, $p<0.05$.

Results

Detection and immunohistochemical localization of S100A10 in human and pig oviducts

To search for the presence of AnxA2's most frequent partner S100A10 in human and porcine oviductal epithelial cells, western blot experiments of protein extracts obtained in RIPA buffer were performed with anti-S100A10 antibodies. A band was noted at the expected apparent molecular mass (10 kDa) (Fig. 1a). When the same blots were used for AnxA2 detection, a band corresponding to the expected apparent molecular mass (38 kDa) was observed (Fig. 1a).

The localization of S100A10 was analyzed by immunohistochemistry and compared with that of AnxA2 (Fig. 1b). S100A10 appears to localize to the apical cytoplasm of human and porcine oviductal ciliated epithelial cells and shares this location with AnxA2 (Fig. 1b). AnxA2 localizes mainly at the cilia of ciliated cells, as far as the method allows these structure's discrimination (Fig. 1b), as reported previously for pig (Teijeiro et al. 2009).

Subcellular fractionation of pig oviductal epithelial cells and AnxA2 and S100A10 detection

AnxA2 is considered to perform its cytoplasmic and cell bridging activities in association with S100A10 (Luo and Hajjar 2013). To connect sperm with the oviduct, the proteins should be present at the outer side of the apical membranes of oviductal epithelial cells. To analyze this hypothesis, extracellular peripheral membrane proteins of the oviductal epithelial cells were extracted by PBS treatment, based on the ionic strength of its NaCl component. This moderate ionic treatment allowed detecting AnxA2 very clearly by western blot among the extracted proteins; however, S100A10 was not detected (Fig. 2a, line 1). When membrane fractions were prepared and proteins extracted with Triton X-100, AnxA2 was detected, as was a slight S100A10 band (Fig. 2a, line 2). The membranes were subsequently treated with 0.5 M NaCl and both AnxA2 and S100A10 were clearly detected by western blot in the supernatant (Fig. 2a, line 3). The extraction of both proteins with detergent and successive salt treatment indicates that AIIt is bound to membranes and probably in different configurations with distinct binding affinities. However, AnxA2 is also present at the extracellular membrane surface of the cells, as it could be detached from the cells by PBS treatment.

To further analyze the localization of these proteins in the oviductal cells, protein extracts from apical plasma membranes were prepared by peeling (Teijeiro and Marini 2012) and compared with cytoplasmic preparations and membrane-enriched extracts obtained by cellular fractionation, by western blot with anti-AnxA2 and anti-S100A10 antibodies (Fig. 2b). Detection of actin and tubulin were used as compartment control, considering that beta-actin is a membrane-cytoskeletal adaptor protein and is therefore associated with cell membranes, while alpha-tubulin should only be found in the cytoplasmic fraction. AnxA2 was detected in both membrane preparations; however, S100A10 was absent from APM (peeling) preparations (which are also devoid of tubulin) and present in the cytoplasmic and membranes extracts (Fig. 2b). This result is in accordance with the previous, indicating absence of S100A10 from the extracellular membrane surface of the cell's apical domain (Fig. 2a). It also indicates that S100A10 is not associated to the inner leaflet of the apical plasmatic membrane, as it is not present in APM. S100A10 is probably bound to membranes that belong to the endomembrane system and probably through AnxA2.

To clearly establish the presence or absence of AnxA2 and S100A10 at the extracellular membrane surface of apical plasma membranes, in situ biotinylation of proteins exposed at the lumen of porcine oviducts was performed, followed by cell disaggregation and protein extracts preparation. The specificity of this technique for only extracellular surface proteins, despite the fragility of the oviduct, has been already demonstrated (Holt et al. 2005; Teijeiro and Marini 2012). In the context of this work, if biotinylation would proceed beyond the surface of the tissue or cell rupture would occur, cytoplasmic proteins biotinylation would include S100A10. However, purified biotinylated proteins contained AnxA2 but not S100A10, as detected by western blot (Fig. 2c). This confirms the absence of S100A10 from the extracellular membrane surface of the cells and also extracellular location for AnxA2, in association to the plasma membrane. The control performed without biotinylating reagent shows minor bands of in vivo biotinylated proteins.

Interaction between AnxA2 and S100A10 in pig oviductal epithelial cells

The association of AnxA2 and S100A10 to form AIIt is related to the proteins function. To search for a functional link between

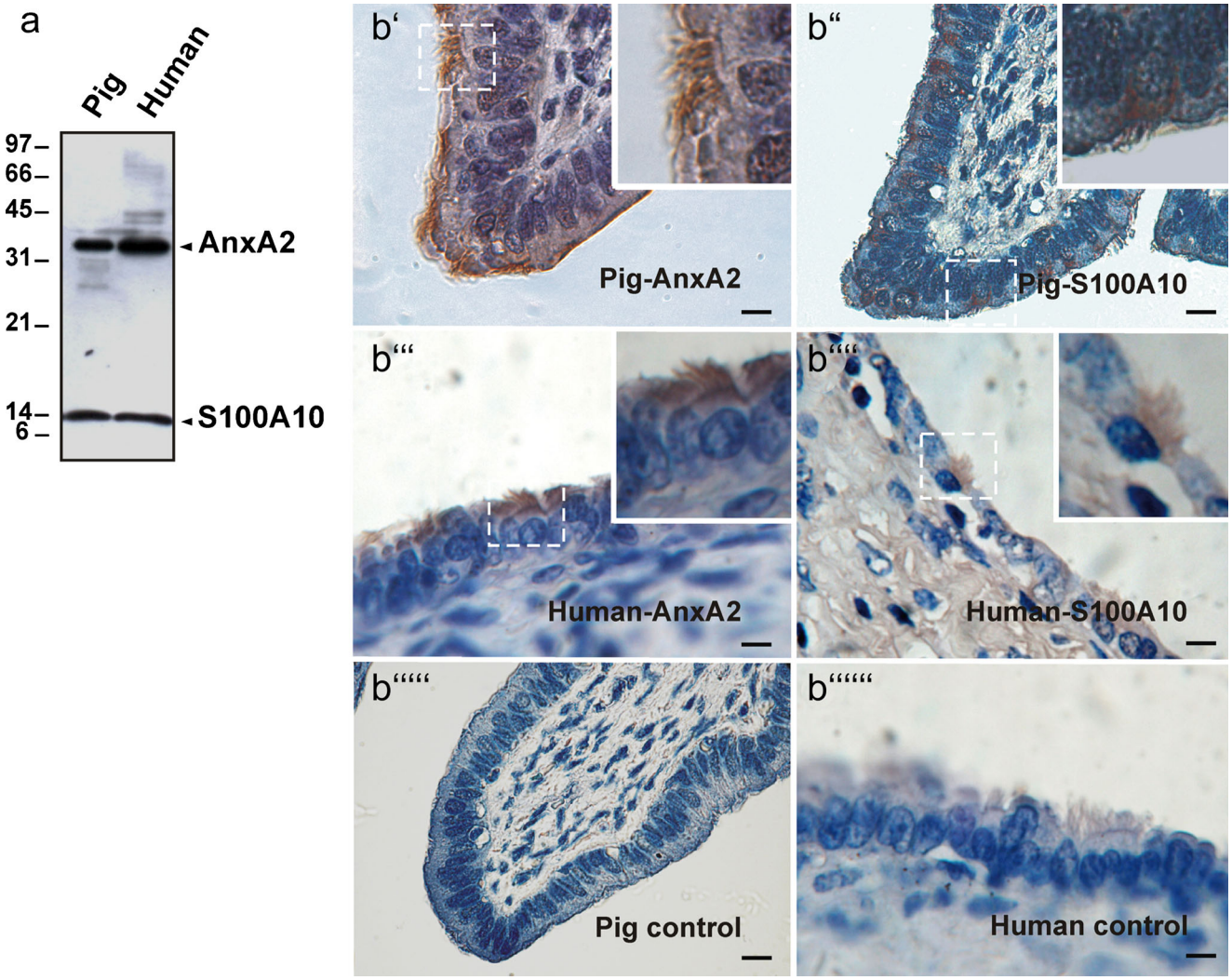

Fig. 1 Annexin A2 and S100A10 are present at the apical cytoplasm of human and pig oviductal epithelial cells. **a** Western blot of human and pig oviductal cell extracts (30 μg of protein) with HH7 anti-AnxA2 and anti-S100A10 antibodies. **b** Immunohistochemical localization of AnxA2 at the cilia of pig (**b'**) and human (**b"'**) and S100A10 at the apical cytoplasm of pig (**b"**) and human (**b""**) oviductal epithelial cells. **b""'** (pig) and **b"""** (human) controls were performed without primary antibody and counterstained with hematoxilin. *Bars* 20 μm. *Inset* ×2.5 digital magnification. Representative results of five porcine and five human oviducts are shown

AnxA2 and S100A10 at the cytoplasm of the oviductal epithelial cells, immunoprecipitation experiments were performed. Protein extracts from membrane fractions prepared as described previously were used for immunoprecipitation with anti-AnxA2 antibodies. As shown in Fig. 2d, S100A10 co-immunoprecipitates, showing interaction between AnxA2 and S100A10 on the intracellular membranes. Control with irrelevant mouse IgG allowed the detection only of IgG (Fig. 2d).

Ovarian hormones influence the amounts of AnxA2 and S100A10 proteins in pig oviductal cells

Cyclic changes on the oviductal epithelia are dependent on ovarian-derived steroid hormones, estradiol and progesterone (Hess et al. 1994). To analyze possible variations on the amount of AnxA2 and S100A10 proteins in oviductal cells in response to progesterone and estradiol, primary oviductal cell cultures were used. The culture medium was supplemented with varying amounts of ovarian hormones, chosen based on reported blood concentrations (Mariscal et al. 1998). After culture, western blot analysis of protein extracts prepared with RIPA was performed (Fig. 3a) and followed by optical densitometry analysis relative to tubulin content (Fig. 3b). An increase of AnxA2 protein was noted in cells cultured in the presence of progesterone at all the concentrations tested with respect to controls (no hormone treatment) and to estrogen treatment (Fig. 3a, b). S100A10 protein increased on cells cultured with estradiol with respect to no hormone treatment, showing the highest level at 25 pg/ml estradiol. When cells were cultured with progesterone, S100A10 also showed higher levels than without hormones but lower than in the presence of estradiol (Fig. 3a, b).

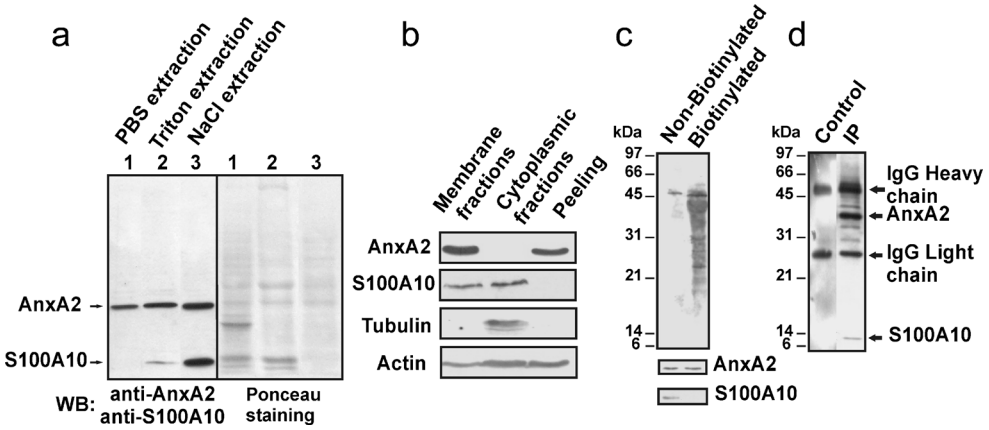

Fig. 2 AnxA2 is present devoid of S100A10 at the outer surface of porcine oviductal cells apical membranes and in combination with S100A10 at their apical cytoplasm Western blots with anti-AnxA2 and anti-S100A10 antibodies of protein extracts (30 μg) from **a** the surface of oviductal epithelial cells with PBS extraction (*1*) and from membrane enriched fractions successively extracted with 0.2 % TritonX-100 (*2*) and 0.5 M NaCl (*3*), Ponceau staining of the membrane is shown; and **b** membrane-enriched, cytoplasmic and APM (*peeling*) fractions, prepared as described in "Materials and Methods"; actin and tubulin detection were used as cellular compartment control. (**c**) Proteins exposed at the oviductal lumen were biotinylated and purified based on biotin content, after electrophoresis and blotting of the purified fraction (*Biotinylated*), biotinylated proteins detection (*upper*) and western blot with anti-AnxA2 and anti-S100A10 antibodies (*lower*) were performed; extract from tissue without biotinylating reagent treatment (*Non-Biotinylated*) was used as control. (**d**) Immunoprecipitation of proteins from membrane-enriched oviductal cell extracts with anti-AnxA2 was followed by western blot with AnxA2 and anti-S100A10 antibodies (*IP*) and a control with irrelevant mouse IgG (*Control*) and only the expected bands corresponding to mouse IgG were detected. Representative results of three repetitions are shown

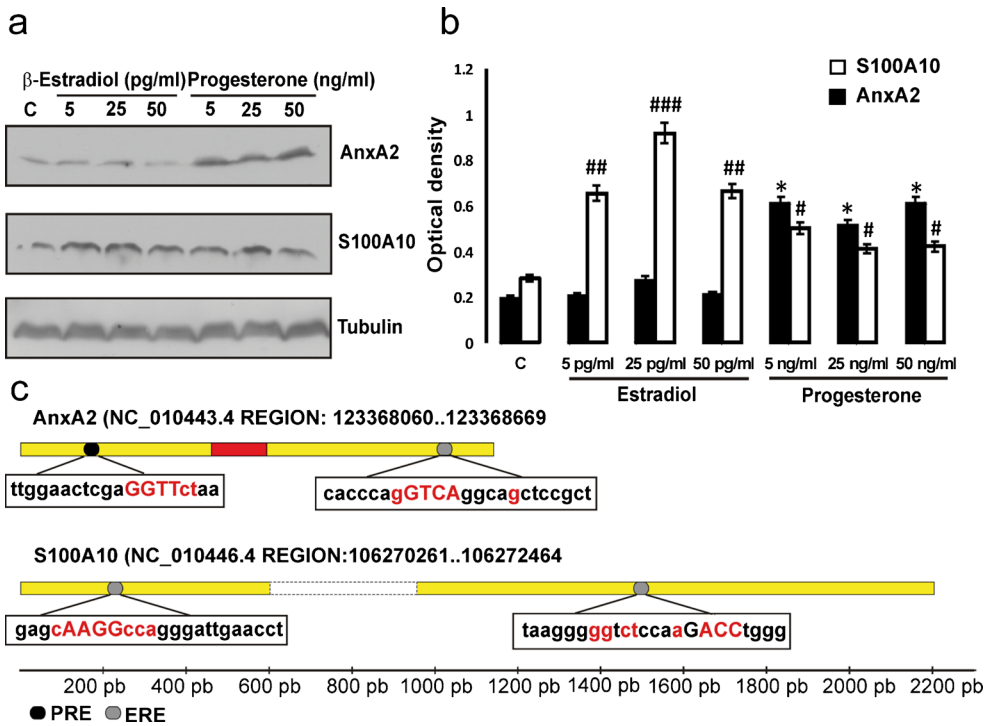

Fig. 3 *ANXA2* and *S100A10* expression in porcine oviductal epithelial cells primary culture with ovarian hormones. **a** Detection of AnxA2 and S100A10 by western blot in primary cell culture extracts (30 μg of protein) from porcine oviductal cells treated with the noted concentrations of ovarian hormones, three independent experiments using batches of 10 oviducts were performed. **b** Relative densitometric analysis of AnxA2 and S100A10 detected in (**a**). #, ##, ### and * indicate significant differences with respect to the control without hormones and between treatments, $p<0.05$, $n=3$, *whiskers* standard errors. **c** Graphic representation of the bioinformatics analysis of: *ANXA2*, region 123368060 to 123368669 from GeneBank NC_010443.4, composed by two predicted promoters, is shown. The two putative promoters show overlapping (in *red*). A PRE (●) is predicted for the upstream promoter and an ERE (○) for the more downstream one; *S100A10*, region 106270261 to 106272464 from GeneBank NC_010446.4, composed of two predicted promoters, is shown. An ERE (○) is associated to each predicted promoter region. ERE and PRE sequences are shown in *boxes*. *Discontinuous lines* indicate DNA sequences unrelated to the promoters

Bioinformatic analysis of *ANXA2* and *S100A10* promoters

To give insight into the possible mechanisms related to the influence of ovarian hormones on *ANXA2* and *S100A10* expression in the porcine oviduct, bioinformatics tools were used. For AnxA2, contig NC_010443.4 located in chromosome 1 was analysed in silico, showing two overlapping promoter regions with relevant associated RNAs (locus GXP_2003513, strand +; localization 133367987–123368668 and locus GXP_4922954, strand +, localization 123367516–123368116; Fig. 3c). Bioinformatics evaluation for regions homologous to known hormone response elements showed that one of the predicted promoters presents a PRE (Fig. 3c) in accordance with the experimental results (Fig. 3a, b). Thus, it is possible that *ANXA2* might be regulated by progesterone at the transcription level, using this promoter.

For *S100A10*, the corresponding sequence located in chromosome 4, contig NC_010446.4, was analyzed. Again, two relevant promoter sequences were predicted, with relevant associated RNAs (locus GXP_2006651, strand+, localization 106271232–106272464 and locus GXP_4943704, strand+, localization 106270261–106270861). Further bioinformatics analysis showed that both promoters were associated to EREs (Fig. 3c).

AnxA2/S100A10's detection in the oviducts of cow, cat, dog and rabbit

The previous results show a probable biological function for AnxA2 in sperm binding at the extracellular domain of porcine oviductal epithelial cells and another for the AIIt complex at the membranes present in the cytoplasm. To find out if the proteins are widespread in the mammalian oviduct, we searched for the presence of both proteins in whole protein cell extracts obtained with RIPA from oviductal epithelial cells of the domestic animals cow, cat, dog, and rabbit. As shown in Fig. 4, AnxA2 and S100A10 were detected by western blot in all the species tested.

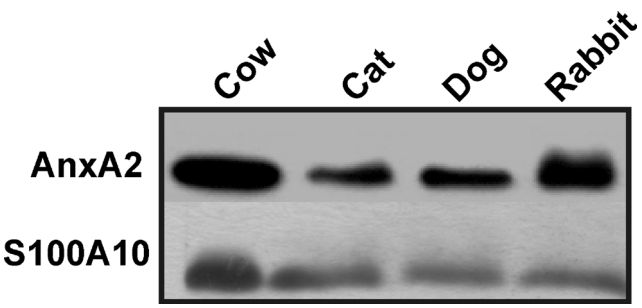

Fig. 4 AnxA2 and S100A10 are detected in the oviducts of cow, cat, dog and rabbit. Western blot of membrane-enriched protein extracts (30 μg of protein) from oviducts of the named animals with anti-AnxA2 and anti-S100A10 antibodies

Discussion

This study focuses on the presence and localization of AnxA2 and its partner S100A10 in the oviduct. Previous work conducted by Teijeiro et al. (2009) demonstrated that AnxA2 localizes at the cilia of sow oviductal epithelial cells and interacts, in vitro, with boar sperm periacrosomal membranes. AnxA2 has been also reported in cilia of cow oviductal cells, linked to sperm binding to form a reservoir, through proteins absorbed to the sperm head (Ignotz et al. 2007). Since AnxA2 has been described to interact with S100A10 to accomplish several structure-linking functions, we searched for the possible presence of S100A10 in the porcine oviduct. In this regard, we first described the presence of S100A10 in the oviduct (Fig. 1) and its localization mainly at the apical cytoplasm of the porcine oviductal epithelial cells (Figs. 1b, 2b, c). The protein is not only detected in the porcine model but also in the human oviduct, sharing subcellular localization (Fig. 1b) despite the different origin of the oviducts (postmorten and surgical, respectively). In view of the multiple publications reporting varied cellular locations for both proteins and a relationship between location and biological functions (reviewed in Gerke and Moss 2002; Grieve et al. 2012 and Luo and Hajjar 2013), we reasoned that it would be important to further analyze the cellular location of both proteins.

We find that AnxA2 but not S100A10, can be easily freed from pig oviductal epithelial cell's surface (Fig. 2a, lane 1). This surprising result differs from the reported in other cell types, where at least 90 % of extracellular AnxA2 is present as the AIIt heterotetramer at the extracellular surface (Vedeler et al. 2012); however, it is in agreement with that reported for reproductive tissues in sheep (Zhang and Wu 2007). In order to further analyze the previous results indicating the extracellular location of AnxA2 and intracellular location of S100A10 and AnxA2, we proceeded to prepare protein extracts from membrane-enriched fractions of porcine oviductal epithelial cells by successive treatment with detergent and high ionic strength. The extraction of S100A10 upon both successive treatments and the increased amount extracted after the second procedure, ionic strength-based processing (Fig. 2a), suggest that S100A10 is probably associated to the membranes in two distinct forms, with different affinity for the membranes. Extensive studies of the AIIt complex support a model in which AnxA2 binds to anionic phospholipids, calcium, heparin, DNA or F-actin through its carboxyl-terminal core region, while the amino-terminal tail binds S100A10 by hydrophobic contact (for review, see Bharadwaj et al. 2013). Two models are supported for AIIt, in one of them AnxA2 molecules bind to the membrane and the S100A10 dimer resides on the outer side of the complex, facilitating interaction with other proteins and membranes (Gerke and Moss 2002; Illien et al. 2010). The other model proposes that the two monomeric AnxA2 molecules are bridged by a central dimer

made up of S100A10 (Ayala-Sanmartin et al. 2008). As AIIt was dissociated from oviductal epithelial cell membranes by successive treatments with detergent and high concentration of NaCl, the complex might be bound to the membranes in both reported conformations, showing differential binding properties. Thus, AIIt may be present in the endomembrane system structures at the apical cytoplasm in the two previously reported conformations.

Cytoplasmic, membrane enriched, and peeling-obtained APM extracts from pig oviductal epithelial cells were compared for AnxA2 and S100A10 content. AnxA2 was present in the membrane and APM protein extracts and S100A10 in the cytoplasmic and membrane fractions but not APM extracts (Fig. 2b). This supports the previous results indicating the presence of AnxA2 at the extracellular surface of the apical plasma membrane and of S100A10 only at the apical cytoplasm (Fig. 2a). The absence of AIIt from the APM extracts indicates that it is not associated to the inner leaflet of the cytoplasmic membrane; thus, it must be bound to membranous structures present at the apical cytoplasm. Also, as APM are capable of binding motile sperm (Teijeiro and Marini 2012), the lack of detectable S100A10 in this extract allows the inferring that S100A10 is not necessary for sperm binding. As conclusive evidence of the lack of detectable S100A10 in the extracellular domain of pig oviductal epithelial cells, in situ biotinylation of oviductal luminal proteins was performed and AnxA2 but not S100A10 was detected at the oviductal surface (Fig. 2c). Altogether, the results support that, in pigs, AnxA2 is present at the oviductal epithelial cells surface devoid of S100A10, probably associated to other proteins and that AIIt is associated to endomembrane structures in two conformations with different binding characteristics, which may correspond to that described by Gerke and Moss (2002) and by Ayala-Sanmartin et al. (2008). It is of note that monomeric AnxA2 and the heterotetrameric AIIt complex present different biochemical properties, which are related to their biological functions (Waisman 1995; Gerke and Moss 2002).

At the endomembrane system, a functional interaction between AnxA2 and S100A10 was inferred in pig by immunoprecipitation with anti-AnxA2 antibodies (Fig. 2d). The complex, associated to intracellular membranes in oviductal epithelial cells, may function at several of the previously described biological activities for AIIt in membrane association: membrane organization and trafficking, cell polarization, endocytosis, exocytosis and modulation of membrane aggregation (Rescher and Gerke 2004; Gerke et al. 2005; Illien et al. 2010; Drücker et al. 2013). However, the AIIt complex may also promote AnxA2 translocation to the cell surface, as has been shown in other cellular models (Valapala et al. 2014).

At the extracellular membrane surface, AnxA2 might assemble with other proteins to form a multi-protein sperm receptor complex as it does in other cell types where it exhibits receptor functions (Allen et al. 2012). In epithelia, AIIt associates with epithelial E-cadherin and endothelial VE-cadherin (Yamada et al. 2005; Su et al. 2010) and, recently, E-cadherin has been shown to participate in bovine sperm interaction with the oviduct (Caballero et al. 2014). It is possible that an interaction between AnxA2 and E-cadherin in the oviductal epithelium mediates sperm–oviduct binding.

ANXA2 expression is regulated in varied tissues at the transcriptional and translational levels and the protein is also prone to post-translational modification (reviewed in Bharadwaj et al. 2013). In this work, we show that the amount of AnxA2 and S100A10 proteins in pig oviductal cells is higher upon progesterone treatment (Fig. 3a, b). We report the presence of a PRE related to the *ANXA2* promoter region (Fig. 3c), which might be indicative of possible regulation at the transcriptional level. The association of the PRE to the most downstream promoter predicted on *ANXA2* indicates that this promoter might be used for progesterone-regulated transcription in oviductal cells. PRE sequences were not found associated to the *S100A10* promoter. The regulation of *ANXA2* and *S100A10* expression by progesterone may alternatively proceed by a mechanism different than transcriptional regulation. In endothelial cells, AnxA2 plays an obligatory role in the regulation of S100A10 by protecting it from rapid ubiquitin-mediated degradation (He et al. 2008). The absence of a predicted PRE element associated to the *S100A10* promoter in the porcine genome, together with an increase of the S100A10 protein and of AnxA2 upon progesterone treatment, may be indicative of a mechanism similar to that of endothelial cells in the oviductal epithelium. The increase of S100A10 might be related to lower degradation instead of increased production. The increase of AnxA2 and S100A10 after treatment with progesterone has also been reported in sheep (Zhang and Wu 2007). Upon estradiol treatment, S100A10 showed an increase with respect to the control without hormones, with the highest levels found at 25 pg/ml (Fig. 3a, b), in agreement with the presence of an ERE associated to the predicted *S100A10* promoter region (Fig. 3c). However, regulation at any level other than transcription may not be ruled out. According to Mariscal et al. (1998), this concentration of estradiol is found in sow's blood at days 0–1 of the estrous cycle. Further studies are needed to understand the relationship between the estrus cycle and the regulation mechanisms of AnxA2 and S100A10 in the oviduct. The distribution of sperm in the oviduct is dependent on the estrus cycle. In pig, ovulation is accompanied by sperm ultrastructural changes and relocation of sperm from the mucosal crypts/interfolds towards the more central part of the mucosal surface (Mburu et al. 1997), where AnxA2 is predominantly localized. Further analysis of the possible link between the amount of AnxA2 present at the extracellular surface of the cells, where it can bind sperm and the ovarian hormones are required. Studies are being conducted in our laboratory to this end.

The experiments performed with pig oviducts in this study include fixed oviducts, primary cell culture, oviduct treatment ex situ, cell fractionation and protein extracts preparation by various methods; however, previously reported results indicate consistency of all the methods as to sperm attachment (Töpfer-Petersen et al. 2002; Holt et al. 2005; Ignotz et al. 2007; Teijeiro et al. 2009 and 2012) and there is a considerable coincidence in the results reported here using different techniques.

We extended this work to the detection of S100A10 and AnxA2 in the oviduct of four other mammals, selected for their importance as farm animals or pets (Fig. 4). The proteins were detected in all the oviduct extracts by western blot (Fig. 4), regardless of the location of the sperm reservoir of the particular species. This is in agreement with a conserved function other than sperm reservoir formation for AIIt in the oviduct of the studied mammals.

In summary, we report the presence of AnxA2 and S100A10 in the oviducts of human, pig, cow, cat, dog and rabbit. In the pig and human oviducts, we show that S100A10 localizes at the apical cytoplasm. In pig, we also show an association of S100A10 to the endomembrane system and to AnxA2. In the extracellular domain of the pig oviductal epithelial cells, AnxA2 devoid of S100A10 may be involved in sperm binding, either as a monomer or in association with other proteins. The ovarian hormones influence the amounts of AnxA2 and S100A10 found in pig oviductal epithelial cells. The most striking outcome of this work is that, at least in pigs, AnxA2 may exert the oviductal cell–sperm contact function without forming a complex with S100A10.

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