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# Evidence for the Involvement of Zinc in the Association of CRISP1 with Rat Sperm During Epididymal Maturation[1]

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## ABSTRACT

**Rat epididymal protein CRISP1 (cysteine-rich secretory protein 1) associates with sperm during maturation and participates in fertilization. Evidence indicates the existence of two populations of CRISP1 in sperm: one loosely bound and released during capacitation, and one strongly bound that remains after this process. However, the mechanisms underlying CRISP1 binding to sperm remain mostly unknown. Considering the high concentrations of $Zn^{2+}$ present in the epididymis, we investigated the potential involvement of this cation in the association of CRISP1 with sperm. Caput sperm were coincubated with epididymal fluid in the presence or absence of $Zn^{2+}$, and binding of CRISP1 to sperm was examined by Western blot analysis. An increase in CRISP1 was detected in sperm exposed to $Zn^{2+}$, but not if the cation was added with ethylenediaminetetra-acetic acid (EDTA). The same results were obtained using purified CRISP1. Association of CRISP1 with sperm was dependent on epididymal fluid and $Zn^{2+}$ concentrations and incubation time. Treatment with NaCl (0.6 M) removed the in vitro-bound CRISP1, indicating that it corresponds to the loosely bound population. Flow cytometry of caput sperm exposed to biotinylated CRISP1/avidin-fluorescein isothiocyanate revealed that only the cells incubated with $Zn^{2+}$ exhibited an increase in fluorescence. When these sperm were examined by epifluorescence microscopy, a clear staining in the tail, accompanied by a weaker labeling in the head, was observed. Detection of changes in the tryptophan fluorescence emission spectra of CRISP1 when exposed to $Zn^{2+}$ supported a direct interaction between CRISP1 and $Zn^{2+}$. Incubation of either cauda epididymal fluid or purified CRISP1 with $Zn^{2+}$, followed by native-PAGE and Western blot analysis, revealed the presence of high-molecular-weight CRISP1 complexes not detected in fluids treated with EDTA. Altogether, these results support the involvement of CRISP1-$Zn^{2+}$ complexes in the association of the loosely bound population of CRISP1 with sperm during epididymal maturation.**

*CRISP1, epididymis, fertilization, gamete biology, sperm, sperm maturation, zinc*

## INTRODUCTION

In most invertebrates and nonmammalian vertebrates, the sperm that leave the testes already have the ability to fertilize the egg. In mammals, however, testicular sperm are not yet able to fertilize an oocyte, and require additional posttesticular changes during their transit through the epididymis to achieve fertilization competence. Numerous reports have demonstrated that during this maturation process, many epididymal secretory proteins associate with the sperm surface and play an important role in the acquisition of fertilizing ability [1, 2]. One such protein is rat epididymal DE, described by Cameo and Blaquier [3] more than 30 yr ago and also known as CRISP1 for being the first identified member of the cysteine-rich secretory protein family. In addition to CRISP1, three other CRISP proteins have been described in mammals: CRISP2, expressed in the testis [4–6]; CRISP3, with a wider distribution, including nonreproductive organs [7–9]; and CRISP4, exclusively expressed in the epididymis [10, 11]. Besides these CRISP proteins that are highly enriched in the male reproductive tract, other nonmammalian members of the family have been described in venoms from several snakes and lizards [12, 13]. All CRISP proteins are characterized by the presence of the plant pathogenesis-related 1 (PR-1) domain in the N-terminal region, which is connected by a short hinge to the cysteine-rich domain in the C-terminal region of the molecule [14, 15].

Rat CRISP1 is produced and secreted by the epididymal epithelium in an androgen-dependent manner and associates with the sperm surface during epididymal maturation [16–19]. Different observations indicated that one population of CRISP1 is loosely associated with the surface and easily removable by ionic strength, whereas another population of the protein is strongly bound to the cells and behaves as an integral membrane protein [20, 21]. The weakly attached protein significantly increases in sperm from caput to cauda epididymis, but the amount corresponding to the tightly bound CRISP1 remains constant along epididymal transit [20]. Our results also revealed that the loosely associated CRISP1 is released during capacitation, whereas the strongly attached protein remains on the sperm surface after the acrosome reaction [20, 22] and participates in sperm-zona pellucida interaction [23] and gamete fusion [24]. Furthermore, although CRISP1-deficient mice were fertile, sperm lacking CRISP1 exhibited an impaired ability to fertilize both zona-intact and zona-free eggs in vitro [25], confirming the proposed roles for the protein in fertilization. Evidence of an additional role for CRISP1 during the capacitation process comes from experiments showing a regulatory activity of the protein on sperm protein tyrosine phosphorylation [25, 26] and on the progesterone-induced acrosome reaction [26]. Based on the finding that CRISP proteins from snake venoms as well as testicular CRISP2 possess an ion-channel regulatory activity [13, 27], it

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was suggested that CRISP1 might interfere with the uptake of ions required for the capacitation process [21].

Although considerable research has gone into determining the role of CRISP1 during the fertilization process, the mechanisms involved in the association of this protein with sperm remain mostly unknown. Despite the fact that a population of CRISP1 is easily released from the cell by saline treatments and, thus, ionically associated with sperm, initial attempts to load caput epididymal sperm with CRISP1 under different in vitro incubation conditions were unsuccessful (unpublished data), suggesting that CRISP1-sperm interaction might require the participation of other epididymal fluid components. In this regard, evidence indicates the presence of high concentrations of $Zn^{2+}$ in the rat epididymis [28] that might be involved in sperm maturation [29], and crystallographic studies revealed the existence of $Zn^{2+}$-binding sites in several snake venom CRISPs [30–32]. However, whereas it has been reported that $Zn^{2+}$ in seminal plasma can interact with the sperm surface either as a free ion or associated with proteins [33, 34], little is known about the participation of $Zn^{2+}$ in the association of proteins with sperm within the epididymis.

In view of all of this, the aim of the present study was to investigate whether $Zn^{2+}$ in epididymal fluid is involved in the association of CRISP1 with sperm during epididymal maturation. Our observations support the idea that the cation participates in the loose binding of CRISP1 to sperm via the formation of high-molecular-weight complexes with the epididymal protein.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats (age, 3–6 mo) were used for the present study. The animals were housed under a 12L:12D photoperiod with ad libitum access to food and water. Experiments were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* published by the National Institutes of Health.

### Collection of Epididymal Sperm

Sperm were collected from the caput or cauda epididymal regions by cutting the corresponding tissues in a buffered solution containing 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 0.15 M NaCl (pH 6.5; MES buffer) at 37°C and allowing sperm to disperse for 10 min. After removal of the tissue fragments, the medium containing sperm was placed in a 1.5-ml microcentrifuge tube, and the cells were washed by centrifugation at $500 \times g$ for 5 min. Sperm were counted using a Neubauer hemocytometer. For viability assessment, sperm suspensions were incubated with one volume of prewarmed 0.5% eosin (yellowish; Sigma-Aldrich) in saline solution, and the incorporation of the dye was determined by light microscopy. The percentage of viability was calculated as the number of sperm that did not incorporate the dye over the total number of sperm counted.

### Collection of Epididymal Fluids

Cauda epididymal fluid was obtained by retrograde flushing of the cauda region with 1–1.5 ml of 0.15 M NaCl solution by introducing a syringe in the vas deferens. Caput epididymal fluid was obtained by placing the caput region in 1–1.5 ml of 0.15 M NaCl and gently mincing the tissue with fine scissors. In both cases, fluids were centrifuged at $700 \times g$ for 10 min to remove sperm and twice at $10\,000 \times g$ for 15 min to eliminate remaining debris. The supernatants were recovered and ultracentrifuged at $70\,000 \times g$ for 45 min to remove epididymal membrane vesicles. The resulting supernatants are referred to as diluted fluids. Nondiluted cauda epididymal fluid was obtained by applying air pressure through the tubules with a syringe introduced in the vas deferens. Protein concentrations were determined by the Bradford method (Bio-Rad).

### Protein CRISP1 and Anti-CRISP1 Antibodies

CRISP1 was purified from rat epididymal homogenates according to the protocol described by Garberi et al. [35, 36], with slight modifications. Briefly, the biochemical procedures included 1) $(NH_4)_2SO_4$ precipitation, 2) affinity chromatography in Sepharose-Concanavalin A (Amersham Biosciences), and 3) ion-exchange chromatography in DEAE-Sephadex (Sigma-Aldrich). Using these procedures, a protein with a purity of 95% (according to silver staining) was obtained. Subsequent analysis of purified CRISP1 by mass spectrometry using an ESI-ion trap LCQ-Duo mass spectrometer (Thermo Fisher Scientific) revealed that all tryptic peptides within the sample corresponded to CRISP1. Polyclonal antibody anti-CRISP1 (8 mg/ml) was produced by immunization of rabbits with purified CRISP1 as previously described [37].

### Biotinylation of CRISP1

Purified CRISP1 was incubated with freshly prepared Biotin 3-sulfo-N-hydroxysuccinimide ester (Sigma-Aldrich) in PBS at a biotin:protein ratio of 50:1 at room temperature for 30 min. The reaction was quenched by the addition of 0.1 volume of 0.5 M glycine in PBS. Biotinylated CRISP1 was then dialyzed against PBS and stored at −20°C until used. As a control, a CRISP1-free preparation was subjected to the same biotinylation protocol (mock preparation).

### In Vitro CRISP1-Binding Assay

Caput sperm were suspended at a concentration of $1 \times 10^6$ sperm/100 μl in MES buffer and, unless otherwise specified, coincubated with either epididymal fluids (0.05 μg protein/μl) or purified CRISP1 (0.15 μM) in the presence of $ZnCl_2$ (1 mM) for 3 h at 37°C with gentle shaking. In some cases, binding of CRISP1 to sperm was performed in the presence of 3.3 mM ethylenediaminetetra-acetic acid (EDTA). For evaluation of the effect of other divalent cations, $MgCl_2$, $CaCl_2$, or $CoCl_2$ (all 1 mM) was used. At the end of the incubation period, sperm were washed twice with 500 μl of 0.15 M NaCl by centrifugation at $500 \times g$ for 8 min, and sperm extracts (see below) were used for Western blot analysis. To investigate whether exposure to $Zn^{2+}$ promotes CRISP1 precipitation, cauda epididymal fluid (0.05 μg protein/μl) was incubated for 3 h in the absence or presence of $ZnCl_2$ (1 mM) and then subjected to centrifugation as described above. In those cases in which biotinylated CRISP1 (0.75 μM) was used, sperm were incubated with the protein or the mock preparation for 2.5 h under the conditions described above and then exposed to ExtrAvidin-fluorescein isothiocyanate (FITC) (1:50; Sigma-Aldrich) for 30 min. Samples were then analyzed using a BD FACSAria flow cytometer (BD Biosciences) or examined with a Nikon Optiphot microscope equipped with epifluorescent optics.

### Preparation of Protein Samples

Sperm protein extracts were prepared by incubating caput or cauda epididymal sperm in Laemmli sample buffer [38] (without 2-mercaptoethanol) for 5 min. Samples were then boiled, centrifuged at $5000 \times g$, and the supernatants subjected to Western blot analysis. To analyze the removal of CRISP1 from sperm by ionic strength, caput and cauda epididymal cells as well as caput sperm previously exposed to cauda epididymal fluid and $ZnCl_2$ were incubated for 30 min with 0.6 M NaCl. Following this incubation, suspensions were centrifuged at $500 \times g$ for 8 min, and the resulting supernatants were transferred to a new tube and proteins precipitated by addition of one volume of 10% ice-cold trichloroacetic acid. Finally, protein extracts were suspended and boiled in Laemmli sample buffer and subjected to Western blot analysis. Epididymal fluid and purified CRISP1 were either incubated with Laemmli sample buffer, boiled and subjected to SDS-PAGE, or mixed with 5% glycerol and 0.5% bromophenol blue for native-PAGE analysis.

### PAGE and Western Blot Analysis

Protein samples were separated in nonreducing and either native (7.5%) or SDS (12%) polyacrylamide gels according to the method described by Hames [39] or Laemmli [38], respectively, and electrotransferred onto nitrocellulose membranes [40]. After blocking with 2% skim milk in PBS-0.1% Tween 20, the membranes were probed with anti-CRISP1 (1:50 000), followed by peroxidase-conjugated secondary antibody (Vector). Monoclonal antibodies against β-tubulin (1:50 000; clone D66; Sigma-Aldrich) were used to assess protein loading. In all cases, the immunoreactive proteins were detected by an ECL Western Blotting kit (Amersham Life Science).

### Measurement of Fluorescence Spectra

The intrinsic fluorescence of tryptophan residues from CRISP1 (3 μM in MES buffer) was analyzed as a function of $ZnCl_2$ concentration (0–4 mM) and recorded on a JASCO FP-6500 fluorescence spectrophotometer. Emission spectra were scanned at 25°C from 300 to 430 nm, with the excitation

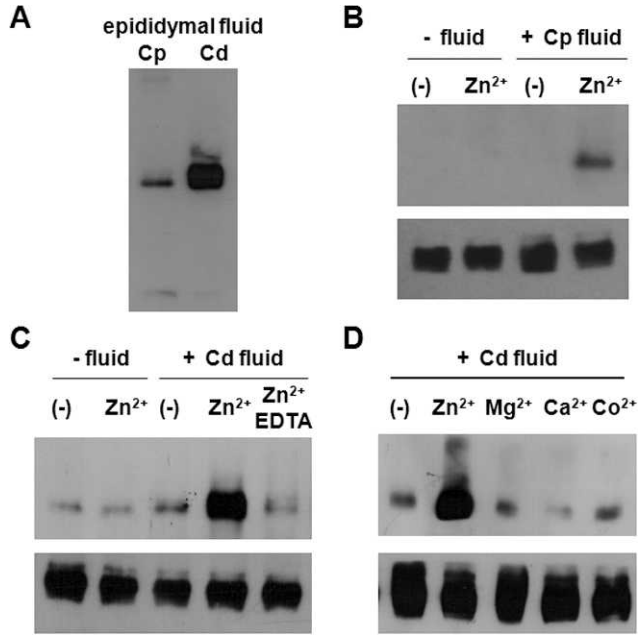


FIG. 1. Effect of zinc on the association of epididymal fluid CRISP1 with sperm. **A**) Caput (Cp) and cauda (Cd) epididymal fluid (diluted in saline solution and free of both sperm and membrane vesicles) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-CRISP1. **B** and **C**) Immature caput epididymal sperm were incubated in vitro for 3 h with caput or cauda epididymal fluid (0.05 μg protein/μl) in the absence (–) or presence of 1 mM $Zn^{2+}$ added either with or without 3.3 mM EDTA. **D**) Caput sperm were exposed to cauda epididymal fluid in the presence of 1 mM $Mg^{2+}$, $Ca^{2+}$, or $Co^{2+}$. In **B**–**D**, the association of CRISP1 with the cells was examined by SDS-PAGE followed by Western blot analysis using anti-CRISP1 (**top**) and anti-β-tubulin (**bottom**).

wavelength set at 295 nm. Data collected at 344 nm and corresponding to the maximum fluorescence intensity were used for the analysis.

### *Statistical Analysis*

Flow cytometry results were expressed as the relative mean fluorescence intensity ± SEM. Statistical significance of the data was analyzed using Student *t*-test. Results were considered to be significant at $P < 0.05$.

## RESULTS

As a first approach to study the possible participation of $Zn^{2+}$ in the association of CRISP1 with sperm, immature caput epididymal sperm were incubated in vitro with either caput or cauda epididymal fluid (diluted and free of sperm and membrane vesicles) as the source of CRISP1 (Fig. 1A), in the presence or absence of $Zn^{2+}$, and the binding of CRISP1 to the cells was then examined by Western blot analysis. Results revealed an increase in CRISP1 only in extracts from sperm that had been exposed to the fluids in the presence of $Zn^{2+}$ (Fig. 1, B and C) and not in those for which the cation had been added together with EDTA (Fig. 1C). A higher amount of CRISP1 bound to sperm was observed when cauda instead of caput epididymal fluid was used, consistent with CRISP1 content in each fluid. To exclude the possibility that exposure of the fluids to $Zn^{2+}$ induces protein precipitation, cauda epididymal fluid, either alone or incubated with $Zn^{2+}$, was also examined by Western blot analysis. Under these conditions, no CRISP1 was detected in the samples (data not shown). When other divalent cations, such as $Mg^{2+}$, $Ca^{2+}$, or $Co^{2+}$, were used, no enhanced CRISP1 binding to caput sperm was observed (Fig. 1D), supporting the specific participation of $Zn^{2+}$ in the association of CRISP1 with sperm. It should be noted that sperm exposed to $Zn^{2+}$ exhibited viability rates similar to those of sperm incubated under control conditions (with $Zn^{2+}$, 61.8% ± 5.0%; without $Zn^{2+}$, 66.2% ± 4.9%; n = 5, not significant), indicating that the cation did not exert any deleterious effect on sperm.

The potential role of $Zn^{2+}$ in the association of CRISP1 with sperm was further characterized by incubating sperm with different concentrations of either cauda epididymal fluid or $Zn^{2+}$ and for different periods of time. Results shown in Figure 2 indicate that the binding of CRISP1 to sperm was dependent on both protein (Fig. 2A) and cation (Fig. 2B) concentrations as well as on the coincubation time (Fig. 2C). Considering that the weakly bound population of CRISP1 in sperm increases during epididymal transit, the above observations showing an enhanced binding of CRISP1 to the cells at increasing concentrations of the protein suggested that $Zn^{2+}$ might be involved in the loose association of CRISP1 with sperm. To assess this hypothesis, caput sperm were first exposed to cauda epididymal fluid in the presence of $Zn^{2+}$ and then subjected to high ionic strength (0.6 M NaCl) to remove weakly bound peripheral proteins. Caput and cauda sperm that had not been incubated with CRISP1 in vitro were used as controls. Results revealed that whereas the saline treatment did not remove CRISP1 from control caput sperm, it was capable of releasing the protein from caput sperm previously exposed to epididymal fluid and $Zn^{2+}$ and from control cauda cells (Fig. 2D).

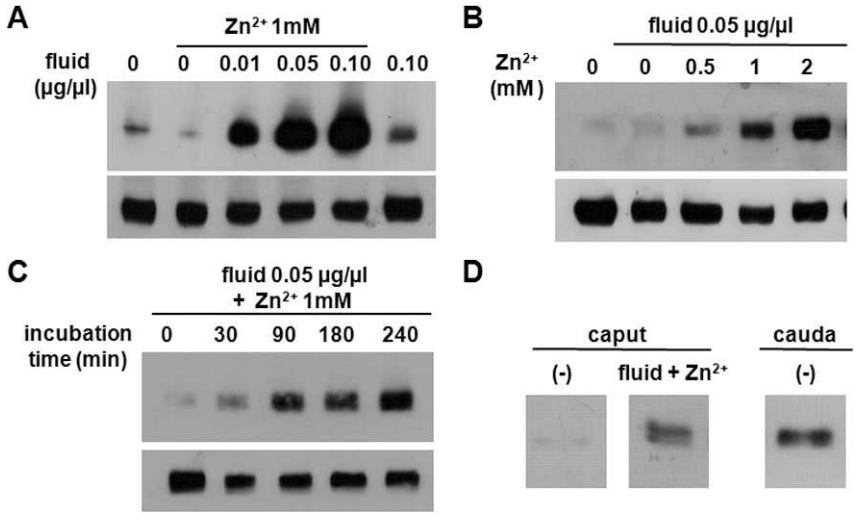


FIG. 2. Characterization of the zinc-mediated association of CRISP1 with sperm. Caput sperm were incubated with different concentrations of either cauda epididymal fluid (0–0.10 μg protein/μl; **A**) or $Zn^{2+}$ (0–2 mM; **B**) during different times (0–240 min; **C**). In all cases, the presence of CRISP1 was examined by Western blot analysis using anti-CRISP1 (**top**) and anti-β-tubulin (**bottom**). **D**) Caput sperm were coincubated with cauda epididymal fluid (0.05 μg protein/μl) and 1 mM $Zn^{2+}$ for 3 h, washed twice with 0.15 M NaCl, and then exposed to 0.6 M NaCl. After 30 min, sperm suspensions were centrifuged and the resulting supernatants examined by Western blot analysis using anti-CRISP1. Caput and cauda epididymal sperm exposed neither to epididymal fluid nor to $Zn^{2+}$ (–) and subjected to the saline treatment were used as controls.

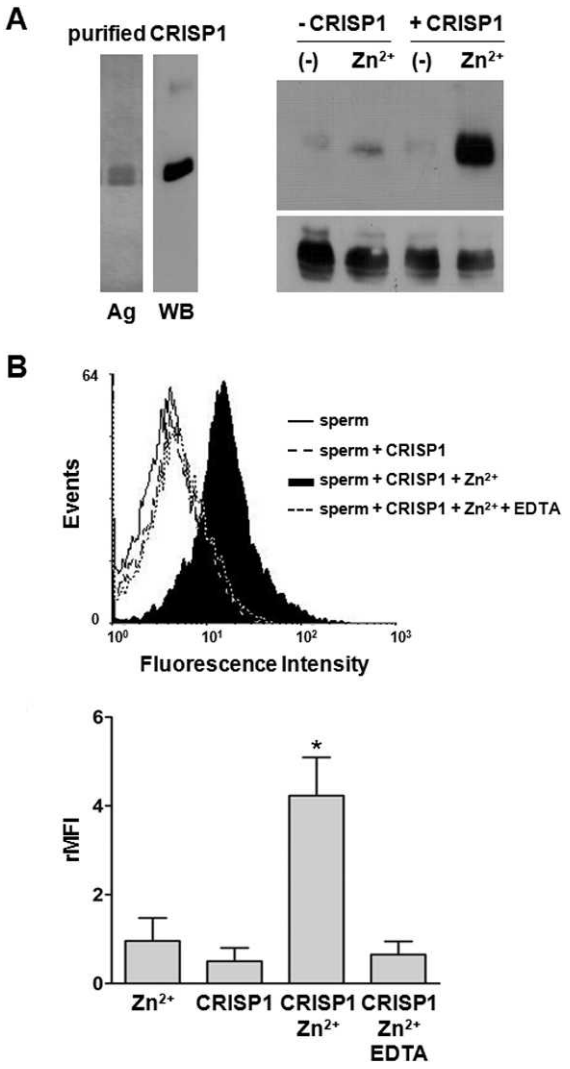


FIG. 3. Effect of zinc on the binding of purified CRISP1 to sperm. **A**) Purified CRISP1 was separated by SDS-PAGE and the protein either detected by silver staining (Ag) or transferred to nitrocellulose and immunoblotted using anti-CRISP1 (WB; **left**). Caput epididymal sperm were incubated in vitro for 3 h with purified CRISP1 in the absence (–) or presence of 1 mM $Zn^{2+}$ and examined by Western blot analysis using anti-CRISP1 or anti-β tubulin (**right**). **B**) Caput sperm were coincubated for 2.5 h with 0.75 μM biotinylated CRISP1 in the absence or presence of 1 mM $Zn^{2+}$ added with or without 3.3 mM EDTA. At the end of the incubation, cells were exposed to ExtrAvidin-FITC for 30 min and analyzed by flow cytometry. Data are representative of three independent experiments (**top**), and relative mean fluorescence intensity (rMFI; **bottom**) was calculated as (MFI experimental − MFI control)/MFI control, where control corresponds to cells exposed only to ExtrAvidin-FITC. Data are presented as the mean ± SEM of three independent experiments. *$P < 0.05$.

To investigate the possibility that other cauda epididymal fluid components could be participating in the in vitro association of CRISP1 with sperm, binding assays in the absence or presence of $Zn^{2+}$ were carried out using purified CRISP1 (Fig. 3A). Detection of CRISP1 in caput sperm incubated under these conditions suggests that the presence of $Zn^{2+}$ would be sufficient for the association of CRISP1 with sperm. As another approach to explore the participation of $Zn^{2+}$ in the binding of CRISP1 to sperm, caput cells were incubated with biotinylated CRISP1 in the presence of the cation and analyzed by flow cytometry. In agreement with Western blot experiments, results showed that exposure of sperm to both CRISP1 and $Zn^{2+}$ produced an increase in fluorescence intensity that was not observed when the cation was added together with EDTA (Fig. 3B).

To examine the localization of the in vitro-bound CRISP1 in the cells, caput sperm exposed to biotinylated CRISP1 in the presence or absence of $Zn^{2+}$ were analyzed by epifluorescence microscopy. No labeling was observed in control cells (Fig. 4A), but most (60%–70%) of the sperm exposed to both the protein and $Zn^{2+}$ exhibited a clear staining in the tail accompanied by a weaker labeling in the head (Fig. 4B). Addition of the cation together with EDTA resulted in a complete absence of labeling in all the cells.

To gain insights regarding the molecular mechanisms involved in the $Zn^{2+}$-mediated association of CRISP1 with sperm, a possible interaction between the protein and the cation was explored by evaluating the effects of $Zn^{2+}$ on the intrinsic tryptophan fluorescence emission of CRISP1. Because tryptophan fluorescence can be affected by its molecular environment, variations in the intrinsic fluorescence of this amino acid are used to monitor protein structural changes. As shown in Figure 5A, fluorescence intensity decreases as a function of $Zn^{2+}$ concentrations supporting the binding of the cation to CRISP1. In agreement with these observations, the three-dimensional structure of CRISP1 based on the crystallographic data of $Zn^{2+}$-bound snake venom pseudecin (48% identity with CRISP1) shows that the protein could adopt the proper conformation to form two potential $Zn^{2+}$-binding sites (see illustrative model in Fig. 5A).

In view of these observations, and considering reports showing that $Zn^{2+}$-protein interactions might lead to the formation of oligomeric structures [41, 42], the next step was to explore the possible effect of $Zn^{2+}$ on CRISP1 protein oligomerization. For this purpose, either purified CRISP1 or diluted cauda epididymal fluid were incubated with $Zn^{2+}$ and examined by native-PAGE and Western blot analysis using anti-CRISP1 as first antibody. In both cases, high-molecular-weight bands, not detected in the absence of $Zn^{2+}$, were observed (Fig. 5B). To examine the formation of such molecular complexes in vivo, nondiluted cauda epididymal fluid, not exposed to $Zn^{2+}$, was analyzed as described above. In this case, results also revealed the presence of a high-molecular-weight band, which was not detected in the fluid pretreated with EDTA (Fig. 5C). When the protein corresponding to this band was extracted from the nitrocellulose membrane and reexamined by SDS-PAGE and Western blot analysis, a band of 32 kDa was detected, confirming the presence of CRISP1 in the fluid complexes (Fig. 5D).

## DISCUSSION

Previous studies examining the interaction between CRISP1 and the sperm surface revealed the existence of two populations of CRISP1 in epididymal sperm: one loosely associated and removable by exposure to ionic strength, and one strongly bound that behaves as an integral membrane protein [20]. In the present work, we have further characterized the interaction of CRISP1 with sperm by showing the involvement of $Zn^{2+}$ in the loose association of this protein with sperm during epididymal maturation.

In vitro-binding assays carried out in the presence or absence of $Zn^{2+}$ revealed that CRISP1 present in both caput and cauda epididymal fluid was able to associate with caput sperm only when exposed to $Zn^{2+}$. Considering that the epididymal fluids also contain $Zn^{2+}$, these observations indicate that the cation concentration in the diluted fluid used in our studies would be insufficient for promoting the

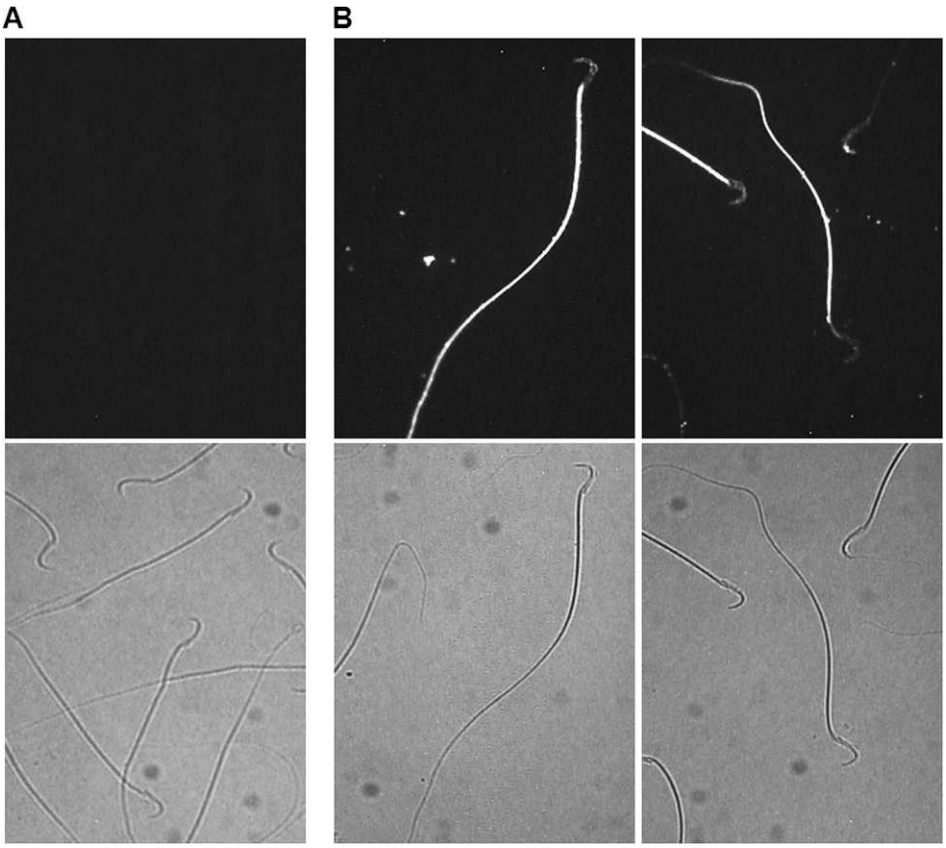


FIG. 4. Localization of in vitro-associated CRISP1 in sperm. Caput sperm were incubated with 0.75 μM biotinylated CRISP1 (**A**) or 0.75 μM biotinylated CRISP1 together with 1 mM $Zn^{2+}$ (**B**), then exposed to ExtrAvidin-FITC, and finally observed by fluorescence microscopy (**top**) or light microscopy (**bottom**). Cells either incubated with CRISP1 in the presence of both 1 mM $Zn^{2+}$ and 3.3 mM EDTA or exposed to a mock preparation and 1 mM $Zn^{2+}$ were completely unlabeled as controls (shown in **A**). Original magnification ×400.

association of CRISP1 with sperm. The need of $Zn^{2+}$ for CRISP1 binding to sperm was further indicated by the fact that no association of the protein with sperm was observed when the cation was added together with EDTA. Although EDTA is not a $Zn^{2+}$-specific chelator, the finding that other divalent cations (i.e., $Mg^{2+}$, $Ca^{2+}$, or $Co^{2+}$) did not promote the binding of CRISP1 to sperm supports the specific involvement of $Zn^{2+}$ in this interaction.

An autometallographic analysis of different sections of the epididymis revealed that $Zn^{2+}$ levels within the lumen increased from caput to cauda [29]. Because CRISP1 concentrations in epididymal fluid also increase from the proximal to the distal regions of the organ [43] (present study), it could be hypothesized that sperm acquire CRISP1 during epididymal transit as a consequence of their exposure to increasing concentrations of both $Zn^{2+}$ and CRISP1. This is further supported by our results showing higher amounts of CRISP1 in sperm exposed to cauda than caput epididymal fluid and by the finding that CRISP1 association with sperm was dependent on both epididymal fluid and $Zn^{2+}$ concentrations. In this regard, it is important to note that the concentrations of $Zn^{2+}$ at which binding of CRISP1 to sperm was observed are considered to be physiological [44, 45] and do not differ from those reported in the male reproductive tract (i.e., 1 mM in the epididymis of prepubertal rats [46] and 1.0 and 2.0 mM in dog [47] and human [48] seminal plasma, respectively).

Previous results from our group indicated that whereas the amount of the strongly bound population of CRISP1 in sperm remains almost constant during maturation, the weakly bound population increases as sperm pass through the successive regions of the epididymis [20]. In view of this, our results showing an enhanced association of CRISP1 with sperm at increasing concentrations of the protein suggest that the in vitro-bound protein corresponds to the weakly associated CRISP1 population in sperm. This was confirmed by the finding that a hypertonic salt solution was able to remove the in vitro-associated CRISP1 from the immature cells. Considering evidence suggesting that bovine and porcine sperm lose $Zn^{2+}$ when washed with saline solution [49], the release of the in vitro- and the in vivo-associated CRISP1 from sperm exposed to high ionic strength could be explained by the loss of $Zn^{2+}$ from the cells caused by the saline treatment. Interestingly, both the loosely bound population of CRISP1 [20] and $Zn^{2+}$ [50, 51] are released from sperm during capacitation, opening the possibility that removal of CRISP1 during capacitation could also be a result of the loss of $Zn^{2+}$ described as one of the initial steps in the capacitation process [50]. This parallel behavior of CRISP1 and $Zn^{2+}$ during salt treatment and capacitation supports a possible interaction between the weakly bound protein and the cation, being in agreement with reports indicating that $Zn^{2+}$ in the epididymis is present not only as free ions but also in association with macromolecules [29].

Although we cannot exclude that other factors in epididymal fluid could be contributing to CRISP1-sperm interaction, our observations showing that purified CRISP1 also associates with sperm when exposed to $Zn^{2+}$ suggest that the presence of the cation would be sufficient to allow binding of the protein to the cells. This result differs from previous observations by Roberts et al. [43] showing that CRISP1 could bind to rat epididymal sperm in the absence of $Zn^{2+}$. The different incubation protocols and antibodies used in each case might explain the discrepancy between the two studies.

In agreement with the Western blot observations, flow cytometry showed that biotinylated CRISP1 associates with sperm when exposed to $Zn^{2+}$ but not when the cation was added with EDTA, confirming that CRISP1 was, indeed, binding to the cells. This was further supported by immunolocalization studies showing that sperm coincubated with the

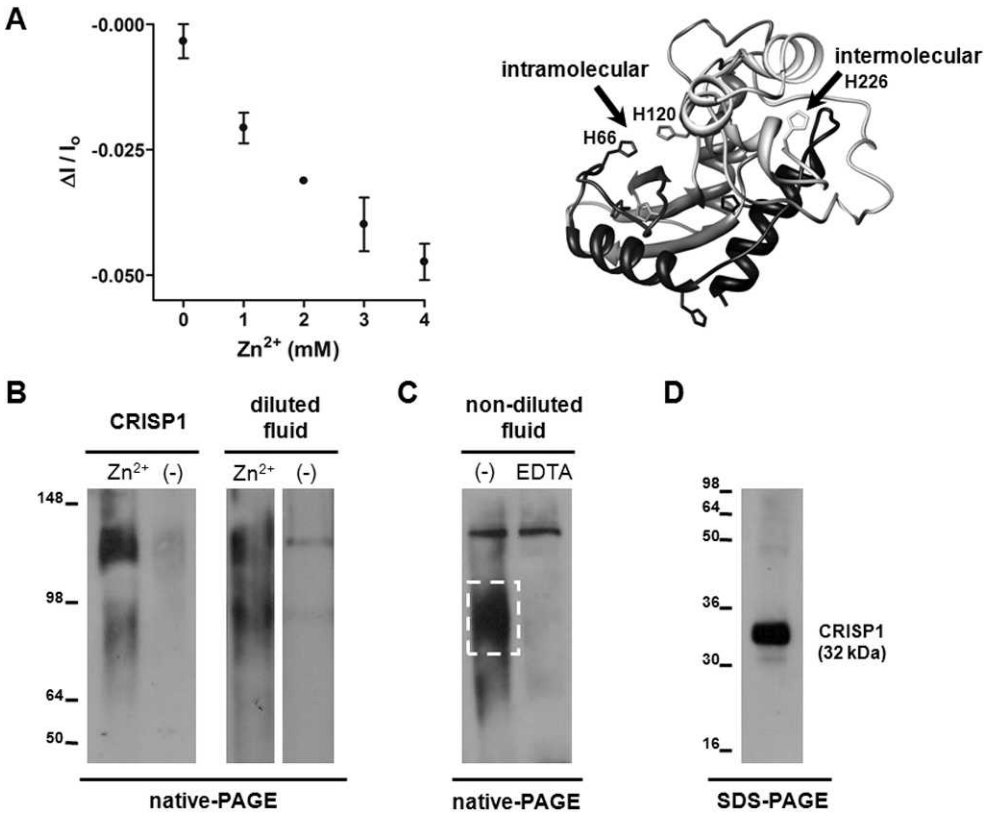


FIG. 5. Interaction between CRISP1 and zinc. **A**) CRISP1 was incubated with increasing concentrations of $Zn^{2+}$, and the intrinsic fluorescence intensity (I) of tryptophan from CRISP1 was measured by setting the excitation and emission wavelengths at 295 and 344 nm, respectively (**left**). Data are representative of three independent experiments. Homology-based CRISP1 three-dimensional structure showing the two potential (intra- and intermolecular) $Zn^{2+}$-binding sites is also shown (**right**). **B** and **C**) Purified CRISP1 and diluted cauda epididymal fluid incubated for 3 h in the absence (–) or presence of 1 mM $Zn^{2+}$ (**B**) as well as nondiluted cauda fluid incubated for 30 min in the absence (–) or presence of 3.3 mM EDTA (**C**) were subjected to native-PAGE (7.5%) and immunoblotted using anti-CRISP1. **D**) The high-molecular-weight band detected in nondiluted epididymal fluid (dotted square; **C**) was extracted from the nitrocellulose membrane by SDS and reexamined by SDS-PAGE and Western blot analysis with anti-CRISP1.

protein in the presence of the cation exhibited a clear staining in the flagellum, accompanied by a weaker labeling in the head. This localization differs from our previous indirect immunofluorescence (IIF) studies showing that CRISP1 localized only in the head region of either caput or cauda epididymal sperm [22]. Considering that CRISP1 has also been detected in the flagellum of cauda sperm using monoclonal antibodies against the protein [19, 43], we concluded that our polyclonal anti-CRISP1 antibody might have a poor ability to recognize the protein located in the tail. In this regard, the use of biotinylated CRISP1 and ExtrAvidin-FITC indicates that CRISP1 associates with both the tail and the head of sperm while passing through the increasing concentrations of CRISP1 present in epididymal fluid. The weaker labeling in the head observed during the present study compared to the strong head staining shown in caput sperm by IIF [22] might be attributed to the fact that the $Zn^{2+}$-mediated bound protein corresponds to the loosely associated population of CRISP1, whereas that detected by the antibody corresponds to the tightly bound population, as judged by the fact that no protein is released from caput cells by ionic strength.

Considering that tryptophan fluorescence can be affected by its molecular environment, our results showing the effect of $Zn^{2+}$ on the intrinsic tryptophan fluorescence emission of CRISP1 supports a direct interaction between the cation and the protein. In this regard, crystallographic studies revealed that highly conserved His residues present in some snake venom CRISPs (i.e., pseudecin, triflin, and natrin) form a putative divalent cation-binding site, which has been extensively accepted as a $Zn^{2+}$-binding site [30–32]. Moreover, the crystal structure of one of these venom proteins, pseudecin, suggests the existence of a second $Zn^{2+}$-binding site formed by two protein molecules [31]. The potential presence of these two $Zn^{2+}$-binding sites in the three-dimensional structure of CRISP1 based on the crystallographic data reported for pseudecin (48% identity with CRISP1) supports the possible formation of complexes between CRISP1 molecules and $Zn^{2+}$.

In agreement with the above observations, our results showed the presence of high-molecular-weight CRISP1 complexes in epididymal fluid exposed to $Zn^{2+}$. Although we cannot exclude the possibility that these complexes contain fluid components other than CRISP1 and $Zn^{2+}$, the detection of bands of the same molecular weight when purified CRISP1 was coincubated with the cation suggests that these high-molecular-weight complexes might be formed just by the association between CRISP1 molecules and $Zn^{2+}$. A possible explanation is that the interaction of the cation with its binding site in CRISP1 induces a conformational change that leads to protein oligomerization or, alternatively, that the metal forms bridges between CRISP1 molecules, leading to the formation of oligomers by intermolecular interactions [31, 41, 42]. The finding that nondiluted epididymal fluid also contained high-molecular-weight CRISP1 complexes not detected in the presence of EDTA indicates that such $Zn^{2+}$-CRISP1 oligomeric structures might also be formed in vivo. The observation that exposure to EDTA prevents both the formation of

CRISP1-$Zn^{2+}$ complexes and the binding of CRISP1 to sperm supports the involvement of these complexes in the association of CRISP1 with sperm during epididymal maturation. In this regard, it is interesting to note that CRISP1 has been detected by transmission-electron microscopy in the linking material between rat sperm heads present in the ''rosettes'' [52]. Although the biological significance of these sperm associations is still unknown, it has been proposed that rosette formation may allow an adequate coating of the sperm with CRISP1. Moreover, rosette formation begins at the distal corpus coincident with the association of the loosely bound population of CRISP1 with sperm. In view of this correlation, it is possible that CRISP1 within the linking material of the rosettes is part of $Zn^{2+}$-protein complexes such as those described in the present study.

As far as we know, the present study is the first to show the involvement of $Zn^{2+}$-protein complexes in the binding of an epididymal protein to the sperm surface during maturation. In this regard, the high concentration of $Zn^{2+}$ within the epididymis [28], together with the ability of other sperm proteins to bind $Zn^{2+}$ [53, 54], opens the possibility that this novel $Zn^{2+}$-protein complex mechanism is involved in the binding of not only CRISP1 but also other epididymal proteins to sperm. Moreover, the finding that $Zn^{2+}$ also contributes to the transfer of epididymal proteins from epididymosomes to sperm [45, 55] suggests that this cation might be involved in different mechanisms of association of epididymal secretory proteins with sperm during maturation.

In summary, these experiments provide evidence supporting the idea that the weakly bound population of CRISP1 associates with sperm in the epididymis by a $Zn^{2+}$-mediated mechanism that involves the formation of high-molecular-weight complexes between the protein and the cation. This information will contribute to a better understanding of both the role of $Zn^{2+}$ within the epididymis and the molecular mechanisms underlying the sperm maturation process.

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