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小白鼠附屬性腺所分泌蛋白 α 238; (P12) 和精子作用之 機制(2/3)

計畫類別：個別型計畫

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執行單位：國立臺灣大學生化科學研究所

計畫主持人：陳義雄

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行政院國家科學委員會補助專題研究計畫 ☐ 成果報告
☒ 期中進度報告

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執行單位：台灣大學生化科學研究所

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中文摘要:

執行本專題時，從儲精囊液中發現了一個 34 kDa 的醣蛋白，進一步鑑定該蛋白是 *Ceacam 10* 基因的衍生物。此蛋白主要表現於儲精囊，其他生殖腺的表現則十分少。該蛋白於儲精囊內腔表皮細胞的表現與發育有關，受到雄性素的調控。進一步也證實該蛋白可以促進精子的游動。

ABSTRACT

CEACAM10 was purified from mouse seminal vesicle secretion by a series of purification steps including ion exchange chromatography on a DEAE-Sephacel column and ion exchange HPLC on an SP column. It was shown to be a 36-kDa glycoprotein with an N-linked carbohydrate moiety. The CD spectrum of CEACAM10 in 50 mM phosphate buffer at pH 7.4 appeared as one negative band arising from the β -form at 217 nm. In the reproductive glands of adult mice, CEACAM10 was expressed predominantly in seminal vesicles. Both CEACAM10 and its mRNA were demonstrated on the luminal epithelium of the mucosal folds in the seminal vesicle. The amount of *Ceacam10* mRNA in the seminal vesicle was correlated with the stage of animal maturation. Castration of adult mice resulted in cessation of *Ceacam10* expression, while treatment of the castrated mice with testosterone propionate in corn oil restored the *Ceacam10* expression in the seminal vesicle. During the entire course of pregnancy, *Ceacam10* might be silent in the embryo. A cytochemical study illustrated the presence of the CEACAM10-binding region on the entire surface of mouse sperm. CEACAM10-sperm binding greatly enhanced sperm motility in vitro.

INTRODUCTION

Mammalian sperm display an intriguing sense of timing in undergoing some modification during their transit in the reproductive tract before encountering an egg. Studying how the lumen of the reproductive tract affects sperm function is a prerequisite to unraveling the molecular mechanisms underlying the complex modification of the sperm. Factors that affect sperm motility have been reported in the seminal plasma of several mammals including the boar [1-3], bull [4], mouse [5], and human [6].

The seminal vesicle is a male accessory sexual gland found in many species of more than 4,000 mammalian species alive on the earth today. After puberty, the gland secretes a fluid called seminal vesicle secretion (SVS), which accumulates in its lumen. SVS contains both protein and non-protein components. On ejaculation, SVS squirts into the urethra, contributing the major part of the liquid portion of seminal plasma that is the complex biological fluid formed from mixing of various fluid in the male reproductive tract. It has been found that extirpation of the seminal vesicle from mice and rats greatly reduces fertility [7, 8], demonstrating the importance of SVS to sperm modification under natural circumstances. SVS differs extensively in terms of volume and composition in various species of mammals. However, rodents have proven to be good experimental animals for the molecular study of mammalian reproduction, so attempts have been made to isolate the proteins involved in sperm modification by mouse SVS, which contains several minor proteins and seven well-defined major proteins designated SVS I-VII, named in decreasing order of molecular mass according to their mobilities in SDS/PAGE [9]. Previously, we demonstrated that SVS VII enhances sperm motility [10], and two of the minor proteins modulate sperm activity. One is a caltrin-like trypsin inhibitor/P12, which suppresses Ca^{2+} uptake by sperm [11], and the other is a seminal vesicle autoantigen, which serves as a decapacitation factor [12,13].

Here we report the purification and identification of an androgen-stimulated 36-kDa glycoprotein, a minor protein component of mouse SVS that is able to enhance sperm motility in vitro. We have demonstrated that its core protein is derived from the *Ceacam10* gene [14], which is a member of the cell adhesion molecule (CAM) subgroup belonging to the carcinoembryonic antigen (CEA) family.

RESULTS

Protein characterization of CEACAM10 purified from mouse SVS

The fresh preparation of soluble SVS was divided into Fr. I to IV by ion exchange chromatography on a DEAE-Sephacel column [Fig. 1(A)]. The Fr. III sample was further resolved into peaks a to e [Fig. 1(B)] by ion exchange HPLC on an SP column. Peak e was a FAD-dependent sulfhydryl oxidase (unpublished observation). On reducing SDS/PAGE gel, each of the a-to-d peak samples gave one rather broad 36-kDa band that could be stained with either Coomassie Blue or periodic acid-Schiff reagent, demonstrating their glycoprotein nature [Fig. 1(C), lanes 1, 3, 5, and 7]. Each protein sample could be deglycosylated either by trifluoromethane sulfonic acid or exhaustive digestion with N-glycosidase F to a core protein which was identified as a sharp band between 26 and 28 kDa by SDS/PAGE [Fig 1(C), lanes 2, 4, 6 and 8], indicating a similar molecular mass of the protein cores. Apparently, peaks a to d were glycoproteins with an N-linked carbohydrate moiety. They were purified to homogeneity.

Automated Edman degradation for each of peak a-to-d samples for 14 cycles gave reliable data, which were assembled to the N-terminal sequences. AVPPXVTADNNVLL was determined from the peak a sample. Two amino acids were detected in each cycle during protein analysis for each of the peaks b to d. The actual yield of the two sequences in an individual cycle was such that the ratio of the major sequence to the minor one was estimated to be 2.5 to 3.0:1. Assembly of the major and the minor sequences gave a peptide sequence of AQVTVEAVPPXVTA and QVTVEAVPPXVTA, respectively. The three N-terminal peptides were completely confirmed in the *Ceacam10*-deduced protein consisting of 265 amino acid residues in all positions except that X (asparagines), one potential site for an N-linked carbohydrate in CEACAM10, was not identified in the protein sequencing [Fig. 2(B)]. The post-translational cleavage at the peptide bond between Glu and Ala, Thr and Ala, or Ala and Gln in the signal peptide of the putative CEACAM10 sequence gives rise to peak a or peaks b to d proteins. As a result, peaks a to d share a very similar protein core with a slight difference in their N-terminal sequences. Thereafter, we combined them for further study. Among the SVS protein components on SDS/PAGE gel, antibody against CEACAM10 immunoreacted only to a 36-kDa protein band corresponding to the antigen, showing high specificity of the antibody. Taken together, these data indicate that peaks a to d are translational products of the *Ceacam10* gene.

Each of peaks a to d was digested with trypsin, and the digests were subjected to HPLC on a C₁₈ column. The chromatographic patterns of the four trypsin-digested samples were very similar. One representative chromatogram is shown in Fig. 2(A). Three amino acids were identified in each cycle of automated Edman degradation of peak 9 on Fig. 2(A). These data could be assembled to three peptide sequences of VFYWK, ETIYSN, and AIYWYR in CEACAM10. The peptide sequence of NDEGAYALDMLFQNF in CEACAM10 was completely confirmed by automated Edman degradation of peak 18 [see Fig. 2(B)].

CEACAM10 was stable in 10 mM Tris-HCl at pH 8.0, but it was degraded to an 18-kDa protein component in 5% acetic acid (not shown). The CD spectrum of this protein at pH 7.4 shows a negative band with a minimum mean residue ellipticity of 12,000 deg cm² dmole⁻¹ at

217 nm [Fig. 2(C)]. In addition, a positive band appeared as the CD profile extended below 200 nm. The spectral profile in the UV region shows some resemblance to that of the β form of protein conformation [23-26], suggesting the presence of a considerable amount of β -form and/or a β -turn in the protein molecule.

Predominant *Ceacam10* expression in the luminal epithelium of the seminal vesicle

We examined the distribution of CEACAM10 and its RNA message in the tissue homogenates of reproductive glands, including the seminal vesicle, epididymis, testis, coagulating gland, vas deferens, prostate, uterus, and ovary. The RNA message was predominantly detected in the seminal vesicles [Fig. 3(A)]. This was confirmed by the results of Western blot analysis showing that CEACAM10 was abundant in the seminal vesicle; a trace appeared in the epididymis and prostate only after over autoradiography [Fig. 3(B)]. When equal amount of the total RNA from the homogenate of a non-reproductive organ was compared with that of the seminal vesicle, very little to none *Ceacam10* mRNA was found in brain, heart, lung, liver, spleen, kidney, stomach, small intestine, muscle, skin, and thymus (not shown).

CEACAM10 was mainly immunolocalized to the luminal epithelium of the mucosal folds in the seminal vesicle slides of adult mice [Fig. 4(A)]. The smooth muscle layer contained almost none. The strong immunochemical staining in the lumen supports the view that CEACAM10 accumulates in the lumen as a result of its secretion from the luminal epithelium. Further, we separated mucosal epithelial cells and smooth muscle cells from the tissue slices by LCM. *Ceacam10* transcripts were relatively abundant in the mucosal cells, but only trace amounts of the RNA message appeared in the smooth muscle cells [Fig. 4(B)].

The developmental profiles of *Ceacam10* mRNA in seminal vesicles and embryos

The amounts of *Ceacam10* mRNA in the seminal vesicles of mice at different ages were compared. The RNA message first appeared at a considerable level in 3-week-old mice. Thereafter, the amount of transcript began increasing rapidly at 4 weeks and reached a maximum in 7-week-old mice [Fig. 5(A)].

We analyzed mouse embryos from 4.5 to 18.5 days postcoitus (d.p.c.). The 4.5-to-6.5 d.p.c. samples included early stage embryos, extra-embryonic tissue, and maternal uterus; the 7.5-to-9.5 d.p.c. samples included embryos and extra-embryonic tissues, and the 10.5-to-18.5 d.p.c. samples were solely embryos. The RNA message in the embryo samples was present in trace amounts on 5.5 d.p.c, increased remarkably from 6.5 d.p.c. to a maximum on 9.5 d.p.c., and rapidly declined thereafter to an almost undetectable level until delivery [Fig. 5(B)].

Since seminal vesicle growth is known to be androgen-dependent, we examined how androgen influenced on *Ceacam10* expression in the seminal vesicles of adult mice that had been castrated 3 weeks earlier (Fig. 6). *Ceacam10* mRNA was undetectable in the total RNA prepared from the control castrates that had received daily injection of corn oil only when compared with normal adults. Induction of *Ceacam10* mRNA was observed in the castrates treated with testosterone (5 mg/kg per day) for 8 consecutive days.

Enhancement of sperm motility by CEACAM10 in vitro

Fig. 7(A) shows micrographs of sperm with indirect fluorescence staining. No fluorescence was seen on the epididymal spermatozoa after they were treated successively with anti-CEACAM10 antibody and rhodamine-conjugated anti-rabbit IgG, demonstrating a lack of CEACAM10 on the cell surface. When spermatozoa were preincubated with 1 μ M CEACAM10

in a blocking solution at room temperature for 45 min, rhodamine fluorescence was prominent on the middle piece, relatively weak on the tail and faint on the head [Fig. 7(A), d]. No fluorescence was seen when the antiserum was replaced with normal serum [Fig. 7(A), b]. We also observed CEACAM10 on the surface of the ejaculated sperm, in spite of high background on the micrograph [Fig. 7(B)]. Apparently, sperm have CEACAM10-binding sites that cover the entire cell surface.

Most spermatozoa freshly retrieved from the caudal epididymis of mice in modified Tyrode's buffer were mobile with tail beating visible. The result of CASA for the cell incubation at specified conditions revealed that 90.0 μ M CEACAM10 in cell culture greatly enhanced sperm motility relative to the motility of control cells at any incubation time [Fig. 7(B)].

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FIGURE LEGENDS

Figure 1. Purification of 36-kDa glycoproteins from mouse SVS proteins.

(A). Fractionation of soluble mouse SVS proteins by ion exchange chromatography on a DEAE-Sephacel column.

(B). Resolution of Fr. III fraction from (A) by ion-exchange HPLC on an SP column.

(C). Demonstration of the glycoprotein nature. Each of the peaks a to d from (B) was digested with N-glycosidase F. The parent proteins (lane 1, peak a; lane 3, peak b; lane 5, peak c; lane 7, peak d) and their deglycosylated forms (lanes 2, 4, 6 and 8) were identified by SDS/PAGE on a 12% polyacrylamide gel slab. The proteins in the gel were stained with Coomassie blue.

Figure 2. Identification of the 36-kDa glycoprotein derived from *Ceacam10* and its circular dichroism.

(A). The trypsin-digested sample of peak c from Fig. 1(B) was resolved by reverse-phase HPLC on a C₁₈ column (see Materials and Methods).

(B). The protein sequence was deduced from the reading frame of *Ceacam10* cDNA (GenBank accession no. NM_007675). The initial and stop codons are underlined. The potential N-linked glycosylation sites are denoted by open boxes. The deduced protein sequence and the amino acid sequences determined directly from protein analysis for peaks a to d in Fig. 1(B) and peaks 9 and 18 in Fig. 2(A) agree in all positions except that Asn¹¹ from the cDNA-deduced protein was not identified in protein sequencing. The cleavage points for the generation of mature protein are indicated by an arrow.

(C). Circular dichroism of CEACAM10 in 50 mM phosphate buffer at pH 7.4 at room temperature.

Figure 3. Distribution of *Ceacam10* and its protein among the reproductive glands

Total RNA (20 µg) or protein extract (50 µg) prepared from the homogenates of each sexual gland was analyzed by Northern blot procedure (A) or Western blot procedure (B) (see Experimental).

Figure 4. *Ceacam10* expression in the luminal epithelium of the seminal vesicle.

(A). Immunolocalization of CEACAM10 to the luminal epithelium of the seminal vesicle. Tissue slices were histochemically stained for CEACAM10 with antibody against the protein, biotin-conjugate goat-anti-rabbit IgG, and alkaline phosphatase-conjugated streptavidin (a). The specimens were stained as in (a) except that the antibody was replaced by normal serum (b). For contrast, the specimens were further stained with Nuclear Fast Red. Photographs were taken with bright-field illumination: MF, mucosal fold; SM, smooth muscle; LF, luminal fluid. The staining of Nuclear Fast Red is in pink and the signals of CEACAM10 protein, demonstrated by staining of alkaline phosphatase activity, are in dark blue. Bar = 20 µm.

(B) Demonstration of *Ceacam10* mRNA in the luminal epithelial cells of the seminal vesicle. The epithelial cells of mucosal folds (MF) or smooth muscle cells (SM) in a tissue slice (8 µm) of mouse seminal vesicle were selectively captured and transferred to films by LCM. The tissue slides before and after LCM were stained and observed (see text for details). A *Ceacam10* cDNA fragment (237 bp) or a *Gapd* cDNA fragment (557 bp) was amplified from the total RNA

of MF or SM by RT-PCR. The level of *Gapd* mRNA was used as an internal control. Bar = 100 μ m.

Figure 5. Developmental profile of *Ceacam10* mRNA in embryos and seminal vesicles.

Ceacam10 mRNA and *Gapd* mRNA in the total RNA prepared from mouse seminal vesicles at different ages (A) or in mouse embryos collected on various days post-coitus (d.p.c) (B). The RNA messages were measured by the Northern blot procedure as described in the text.

Figure 6. Androgen dependence of *Ceacam10* mRNA expression in seminal vesicles of adult mice.

Northern blot analysis for 1.1 kb *Ceacam10* mRNA in total RNA from seminal vesicles from normal adult mice (lane 1), adults castrated 3 wk previously and treated only with corn oil (lane 2), and adults castrated 3 wk previously and treated testosterone propionate in corn oil for 8 consecutive days (lane 3). Total RNA (20 μ g) was used for each experiment. *Gapd* mRNA was used as an internal control.

Figure 7. Analysis of sperm motility under the influence of CEACAM10

(A) Demonstration of the CEACAM10-binding zone on the epididymal spermatozoa. Fresh sperm cells were incubated with or without CEACAM10 as described in Materials and Methods. The cells on slides were incubated with normal serum (a and b) or affinity-purified anti-CEACAM10 antibody (c and d). The slides were incubated with rhodamine-conjugated anti-rabbit IgG and observed by a light microscope (a and c) or a fluorescence microscope (b and d). Bar = 10 μ m.

(B) Illustration of CEACAM10 on the ejaculated sperm. Freshly prepared cells, see Materials and Methods, on slides were incubated with normal serum (a and b) or affinity-purified anti-CEACAM10 antibody (c and d) and followed incubation with rhodamine-conjugated anti-rabbit IgG. The slides were observed by a light microscope (a and c) or a fluorescence microscope (b and d). Bar = 10 μ m.

(C) Freshly prepared mouse spermatozoa in modified Tyrode's solution (10^5 cells/ml) containing 1.8 mM CaCl_2 were incubated alone ($\circ$) or in the presence of 90 μ M CEACAM10 ($\bullet$) at 37°C for 0 to 60 min. Cell motility determined at each specified incubation time was expressed as a percentage of control cell motility at time zero. Points are mean $\pm$ S.D for three determinations. *, $P < 0.01$ in the paired statistical comparison with the corresponding control. Values were evaluated using one-way analysis of variance.

Fig. 1

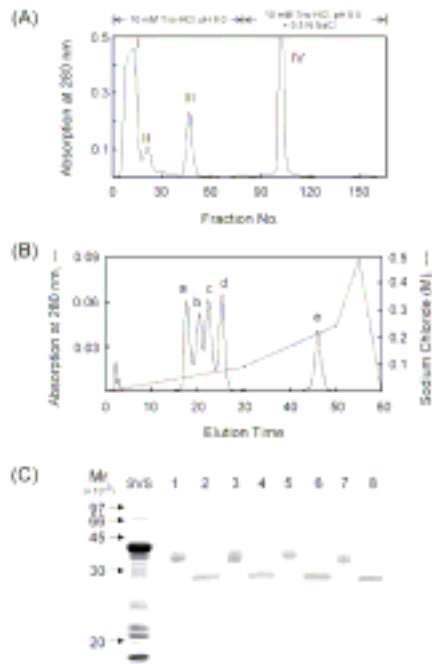


Fig. 2

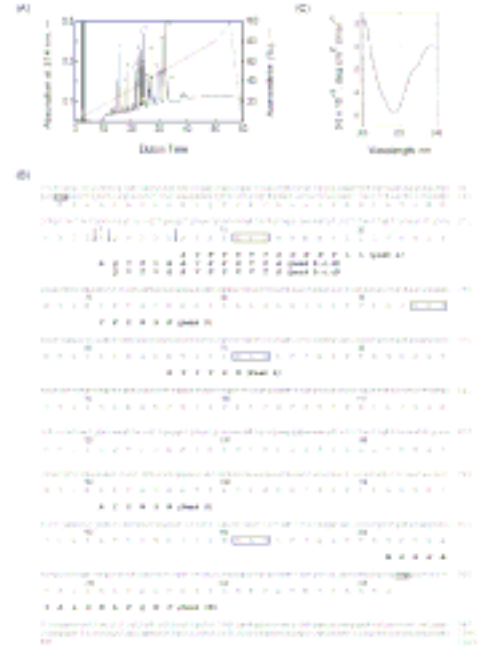


Fig. 3

