BIOLOGY OF REPRODUCTION **62,** 606–615 (2000)

Expression of Human Proacrosin in *Escherichia coli* and Binding to Zona Pellucida[1]

Laura I. Furlong,[3] Ulf Hellman,[4] Alejandro Krimer,[5] Jorge G. Tezón,[3] Eduardo H. Charreau,[3] and Mónica H. Vazquez-Levin[2,3]

Instituto de Biología y Medicina Experimental-Consejo Nacional de Investigaciones Científicas y Técnicas,[3] (1428) Buenos Aires, Argentina
Ludwig Institute for Cancer Research,[4] Uppsala Branch Biomedical Center, S-751 24 Uppsala, Sweden
Biosidus,[5] Buenos Aires, Argentina

ABSTRACT

Proacrosin is a multifunctional protein present in the sperm acrosome. This study characterizes the expression of human proacrosin in bacteria and assesses zona pellucida binding activity. The cDNA encoding human proacrosin was subcloned in pGEX-3X and pET-22b vectors. In the pGEX system, expression of the full-length fusion protein was not detected. In the pET system, an expression product with an apparent molecular size similar to that expected for the proenzyme (Rec-40, 42–44 kDa) was recognized by a monoclonal antibody to human acrosin, AcrC5F10. A 32–34-kDa protein (Rec-30), not recognized by AcrC5F10 on Western blots, was the major expression product. Proteins of 21 (Rec-20) and 18 (Rec-10) kDa were recovered as insoluble expression products as were Rec-40 and Rec-30, and truncated products from the C terminus were detected in the soluble fraction. Rec-40 and Rec-30 coexisted at any culture time tested. Immune serum raised against Rec-30 (AntiRec-30) stained the acrosomal region of permeabilized human spermatozoa and recognized the recombinant proteins and proacrosin from human sperm extracts. Amino acid sequence analysis indicated that Rec-30, Rec-20, and Rec-10 are N-terminal fragments of proacrosin. The recombinant proteins Rec-40, -30, -20, and -10 were found to interact with homologous ^{125}I-zona pellucida glycoproteins.

INTRODUCTION

Acrosin (EC 3.4.21.10), a sperm-specific serine protease, is synthesized as a single-chain zymogen, proacrosin. In humans, proacrosin is a protein of 402 residues, with an apparent molecular mass of 55 kDa due in part to glycosylation [1, 2]. During the acrosome reaction, proacrosin is converted to the mature enzyme β-acrosin and is released from the acrosome [3]. Proacrosin residues 24–322 have sequence homology with the serine protease superfamily [4]. The remaining C-terminal region is not present in other members of the superfamily and is unique because of its high proline content [2, 4], and its processing is a prerequisite for activation to the mature β-acrosin form [5, 6].

Acrosin is thought to aid to sperm penetration by limited and specific proteolysis of zona pellucida (ZP) glycoproteins, facilitating the passage of motile spermatozoa through the egg's extracellular matrix [3]. In addition to the proteolysis of oocyte ZP, acrosin protease activity is probably involved in the dissolution of the acrosomal matrix and in the regulated release of the acrosomal contents at the time of the acrosome reaction [7]. Independent of its proteolytic activity, boar and rabbit proacrosin possess high affinity (K_d: 1.2×10^{-8} to 5×10^{-8} M) for sulphated carbohydrates of the ZP [8–10].

Recently, gene knockout experiments have brought into question the proposed roles for proacrosin in the fertilization process. Mice lacking the proacrosin gene ($Acr^{-/-}$) were still able to have offspring [11, 12]. However, further analysis of this model showed that in mixed insemination experiments, no $Acr^{-/-}$ offspring was obtained, and sperm from $Acr^{-/-}$ mice were at a disadvantage during fertilization when competing with sperm from wild-type animals ($Acr^{+/+}$) [12]. Moreover, the sperm from $Acr^{-/-}$ animals showed a delayed release of the acrosomal contents after the acrosome reaction [13]. This evidence supports the participation of proacrosin in the penetration process, probably in concert with other acrosomal proteins.

The cDNA and genomic sequences of mammalian proacrosin have been elucidated in a number of species [1, 2, 5, 14–25]. Since the molecular cloning of proacrosin coding sequences, the production of recombinant acrosin in the boar and rabbit has been employed to characterize acrosin ZP binding sites. Four groups have reported the expression of proacrosin fragments as recombinant proteins in a prokaryotic system and their binding to homologous ZP glycoproteins [10, 26–28]. All these studies used fusion expression strategies for the production of the recombinant proteins. However, the expression of the full-length proacrosin was not obtained. In addition, a study in the human species has not yet been reported.

Hence, the expression of human proacrosin cDNA in bacteria was initiated with the aim of finding experimental conditions that allow the synthesis of the full-length protein. For this purpose, both fusion and direct expression strategies were employed. Additionally, the ZP binding ability of the recombinant protein was evaluated.

MATERIALS AND METHODS

Materials

The acrosin cDNA clone carried in the PHA plasmid [1] was kindly provided by Prof. Engel (Institut für Humangenetik, Göttingen, Germany). DNA amplification assays by polymerase chain reaction (PCR) were performed using the PCR SuperMix from Life Technologies (Gaithersburg, MD). DNA sequence analysis was carried out using the Sequenase Version 2.0 DNA Sequencing kit from U.S. Biochemical Corporation (Cleveland, OH). The pET-22b and

[1]This work was supported by the following grants to M.H.V.-L.: PLACIRH (207/95), Consejo Nacional de Investigaciones Científicas y Técnicas from Argentina (PIP 4404).
[2]Correspondence: Mónica H. Vazquez-Levin, Instituto de Biología y Medicina Experimental-Consejo Nacional de Investigaciones Científicas y Técnicas, Vuelta de Obligado 2490, (1428) Buenos Aires, Argentina. FAX: 54 11 4786 2564; e-mail: mhvaz@dna.uba.ar

Received: 4 January 1999.
First decision: 8 February 1999.
Accepted: 8 October 1999.

ISSN: 0006-3363. http://www.biolreprod.org

pGEX-3X expression systems were obtained from Novagen (Madison, WI) and Pharmacia (Amersham Pharmacia Biotech, Uppsala, Sweden), respectively. Restriction enzymes and T4 DNA Ligase were from New England Biolabs (Beverly, MA). The monoclonal antibody AcrC5F10 was purchased from Biosonda (Santiago, Chile). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Construction of Expression Vectors Containing the Proacrosin cDNA

For all procedures, standard molecular biology protocols were used [29]. Subcloning of cDNA encoding human proacrosin in the expression vectors pET-22b and pGEX-3X was carried out in two steps. First, a PCR fragment from PHA encoding the N-terminal region of the protein was generated with the primers 5′COMBO (5′TTG**GGATC<u>CATATG</u>**AAAGATAACGCCACGTGTGAT3′) and 3′ECO (5′TCGT**GAATTC**AGCAAGCTGCCT3′).

The 5′COMBO primer was designed to introduce the *Nde*I and *Bam*HI (bold-underlined and bold letters above, respectively) sites in the 5′ region of the proacrosin sequence for subcloning in the pET-22b and pGEX-3X vectors, respectively. Complementarity with the cDNA sequence started at nucleotide 15 of the primer. The 3′ECO flanking sequence primes with the cDNA's internal *Eco*RI site (bold letters) located on position 234 of the cDNA, being complementary to nucleotides 222 to 249.

For subcloning in the pET system, the *Nde*I site was selected for the 5′ cloning site to provide the initiation signal for protein synthesis. The 200-base pair (bp) PCR fragment was digested with *Nde*I and *Eco*RI, and directionally subcloned in the pET-22b plasmid. The *Eco*RI fragment of 1150 bp from PHA (containing nucleotides 234–1266 of the cDNA and 118 additional nucleotides 3′ from the proacrosin stop codon) was subcloned in the *Eco*RI site of pET carrying the 5′ coding region of proacrosin. The vector pET-proacrosin directs the expression of proacrosin, with a methionine residue introduced as the first amino acid to provide the initiation signal for protein synthesis, followed by the residues of the light and heavy chains. This gives a polypeptide of 403 residues with a predicted molecular weight of 43 955. The clone selected was named 525.

For subcloning in the pGEX, the PCR fragment was digested with *Bam*HI and *Eco*RI, and subcloned in frame with the glutathione S-transferase (GST) coding sequence. The *Eco*RI fragment of 1150 bp from PHA was then subcloned into the pGEX containing the PCR fragment. The resulting plasmid pGEX-proacrosin codes for the fusion protein GST-proacrosin with an expected molecular mass of 73 kDa, containing the GST polypeptide (29 kDa), followed by the sequence IEGRGIHM introduced by the subcloning strategy and the 402 residues of human proacrosin. The clone selected for expression analysis was named 409.

In both expression systems, proacrosin synthesis starts at the amino acid 1 (Lys) of the light chain.

All clones were subjected to nucleotide sequence analysis to confirm the sequence of the fragment generated by PCR and the reading frame of the insert.

Escherichia coli *Cultures and Protein Expression*

For the pET expression system, overnight cultures of *E. coli* BL21 (DE3) cells transformed with pET-proacrosin (525 clone) or pET-22b (pET clone) plasmids were inoculated in Terrific broth medium (2.4% bactoyeast extract, 1.2% bactotryptone, 0.4% glycerol, 0.17 M KH_2PO_4, 0.072 M K_2HPO_4) containing 50 μg/ml carbenicillin and cultured at 37°C until A_{600} = 0.6–1, and expression was induced by the addition of 1 mM isopropyl-thio-β-D-galactoside (IPTG). Induction of protein expression was performed at 37°C, 30°C, or 25°C. Bacteria were harvested at zero, 0.5, 1, 2, and 5 h after induction, depending on the assay performed. Cell pellets were kept at −70°C until used. To obtain cellular lysates and subcellular fractions, a method adapted from that described by Castellanos-Serra et al. [30] was used: pellets were resuspended in a 1:10 culture volume of buffer B (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate [DOC], 50 mM benzamidine, 1 mM *p*-aminobenzamidine, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) supplemented with 1 M urea. The suspensions were sonicated 3 times for 30 sec at maximal power or until the solution was no longer viscous to obtain the total cellular fraction (Sonifier Cell Disruptor, model W 140; Heat Systems-Ultrasonics, Inc., Plainview, NY). Soluble and insoluble fractions (1 M urea S and P, respectively) were obtained after centrifugation for 15 min at 10 000 × *g*. The 1 M urea P fraction was resuspended in 1:10 culture volume of buffer B supplemented with 4 M urea and sonicated for 30 sec, and the supernatant and pellet fractions (4 M urea S and P) were obtained after centrifugation. The same procedure was followed using buffer B supplemented with 8 M urea and the corresponding supernatant, and pellet fractions (8 M urea S and P) were obtained. To study expression at different time points after induction, the cellular lysates were prepared as follows: bacterial cells were resuspended in 1:10 culture volume in buffer B with 4 M urea, the suspension was sonicated as described above, and the P and S fractions were obtained after centrifugation. All these procedures were performed on ice.

In the pGEX expression system, *E. coli* BL21 (DE3) cells carrying pGEX-proacrosin (409 clone) and pGEX-3X as a control (pGEX clone) were cultured in YT medium (0.5% bactoyeast extract, 0.8% bactotryptone, 0.25% NaCl, pH 7.0) containing 100 μg/ml ampicillin. Cells were grown at 37°C until A_{600} = 0.6–1, and expression was induced by the addition of 0.1 mM IPTG. Cells were harvested at zero time and 2 h after induction, and cell pellets were kept at −70°C until used. The cells were resuspended in a 1:10 culture volume in PBS, 1% Triton X-100, 1% DOC, 2 mM EDTA, and a cocktail of protease inhibitors, and sonicated 3 times for 30 sec. The total cellular fraction was centrifuged at 10 000 × *g* for 15–20 min at 4°C, and the soluble (S) and insoluble (P) fractions were obtained.

Electrophoresis and Immunoblotting

SDS-PAGE was performed in 10–12% polyacrylamide gels with 0.1% SDS [31]. Samples for analysis were prepared in Laemmli sample buffer and boiled for 10 min. In some assays, the sample was made 5% 2-β-mercaptoethanol. For comparison, all SDS-PAGE lanes in each gel contained proteins recovered from the same amount of cells. Protein apparent molecular weight (M_r) was estimated by comparison with reduced molecular weight standards (Bio-Rad, Hercules, CA) run in parallel.

Proteins were visualized by staining with Coomassie R-250 brilliant blue stain (CBB). For Western blot analysis, proteins were electroblotted as previously described [32] onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes and immunostained with different antibodies.

The monoclonal antiserum against purified human acrosin AcrC5F10 (IgG type 1) was employed in a 1:2000 dilution in blocking solution (5% skim milk in PBS containing 0.02% Tween 20). AcrC5F10 antiserum specifically recognizes human proacrosin in sperm extracts as a 53–55-kDa doublet [33]. Biotinylated goat anti-mouse IgG or peroxidase-conjugated goat anti-mouse IgG was used as second antibody. To evaluate expression of recombinant pGEX-proacrosin on pGEX lysates, blots were developed using a 1:500 dilution of a rabbit anti-GST antiserum in blocking solution and a 1:500 dilution of biotinylated goat anti-rabbit IgG in blocking solution. Membranes were incubated with avidin-peroxidase (when biotinylated second antibody was used) and developed with diaminobenzamidine (DAB) as chromogenic substrate, or with enhanced chemiluminescence (ECL kit; Amersham Life Science, Buckinghamshire, UK). Blot immunostaining was performed at room temperature with constant shaking.

Sperm Extract Preparation

Acid sperm extracts were prepared as previously described [6]. Semen samples were obtained from fertile donors. Aliquots of acid sperm extracts were subjected to SDS-PAGE under nonreducing conditions and electroblotted onto nitrocellulose membranes as described above. Using this procedure, acrosin is obtained in the proenzyme form (molecular mass: 53–55 kDa).

Development of a Polyclonal Antiserum Against the Expression Product Rec-30 (AntiRec-30)

A polyclonal antiserum against the expression product Rec-30 was prepared in New Zealand rabbits. The 4 M urea P fraction obtained as described above was subjected to preparative SDS-PAGE under reducing conditions. After protein staining with 0.05% CBB in water, the area containing Rec-30 was identified and excised from the gel. The protein was eluted by diffusion in 0.1 M Tris pH 8, 0.1% SDS at 4°C for 24 h. An initial s.c. injection of Rec-30 with complete Freund's adjuvant was followed by three boosters with incomplete adjuvant 4 wk later. Ten days after the last booster, the serum was collected and was tested against human spermatozoa, human sperm extracts, and bacterial lysates. Western blots of human sperm extracts and 4 M urea P fraction from pET and 525 clones were developed with the serum (1:1000 dilution in blocking solution), using biotinylated goat anti-rabbit IgG and avidin-peroxidase or peroxidase conjugated goat anti-rabbit IgG (1:1000 in blocking solution), and developed with DAB. Animal handling was conducted in accordance with the Guiding Principles for the Care and Use of Research Animals (Society for the Study of Reproduction).

Immunocytochemistry of Human Spermatozoa with AntiRec-30

Ejaculated spermatozoa devoid of seminal plasma were fixed in 2% formaldehyde for 10 min at room temperature. Fifty thousand spermatozoa were loaded per well of eight-spot microscope slides and allowed to dry at 37°C. Then cells were washed in PBS for 5 min, permeabilized in methanol for 10 min at 4°C, and incubated with AntiRec-30 serum in PBS, 0.02% Tween 20 for 1 h at room temperature in a humid chamber. After 3 washes of 5 min each in PBS, anti-rabbit IgG fluorescein isothiocyanate isomer I conjugate (FITC) was added and incubated for 1 h at room temperature in the darkness. After 3 washes of 5 min each in PBS, the smears were mounted and observed in a Zeiss fluorescence microscope equipped with epiillumination (Carl Zeiss, Inc., Thornwood, NJ).

Rec-30, Rec-20, and Rec-10 Amino Acid Sequencing

For N-terminal amino acid analysis, the 4 M urea P fraction from the 525 clone in Laemmli sample buffer with 5% β-mercaptoethanol was subjected to SDS-PAGE and transferred onto PVDF membrane. The blot was stained with CBB and the bands of interest (Rec-30, Rec-20, and Rec-10) excised for analysis. Quantitative amino acid analysis was carried out in an Applied Biosystems 420A Amino Acid Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA) using PTC chemistry, and automated sequencing was performed on an Applied Biosystems 477A Protein Sequencer, using Edman chemistry, both procedures according to the manufacturer's instructions. Amino acid sequence analysis of the N-terminal region of Rec-30, Rec-20, and Rec-10 was performed in the LANAIS-PRO, UBA-CONICET (National Protein Sequencing Facility).

For Rec-30 internal peptides analysis, the 4 M urea P fraction from the 525 clone in Laemmli sample buffer with 10 mM dithiotreitol was heated at 100°C for 5 min. Then 20 mM iodoacetamide was added, incubated for 20 min, and loaded in the gel. Gel bands containing Rec-30 were treated for in-gel digestion essentially as recently described [34]. The generated protein fragments were extracted from the gel and isolated by microbore reversed-phase liquid chromatography on a Kromasil C18 (1 × 150 mm) column from Column Engineering (Ontario, CA) operated in a SMART system (Amersham Pharmacia Biotech). Selected purified peptides were analyzed by automated Edman degradation in a Procise 494A from Perkin-Elmer Applied Biosystems, operated according to instructions from the manufacturer.

AcrC5F10 Epitope Mapping

Identification of the epitope recognized by AcrC5F10 was performed using the SPOTscan method. Overlapping hexapeptides with an offset of one amino acid covering proacrosin residues 264–363 were synthesized according to the SPOT synthesis method [35]. The filter containing the array of peptides was developed with the monoclonal antibody according to the standard protocol. Briefly, the filter was blocked overnight with membrane blocking solution (MBS): 20% casein-based blocking buffer concentrate (No. SU-07-250; Genosys Biotechnologies, Cambridge, UK), 5% (w:v) saccharose in Tris-buffered saline 0.05% Tween 20 (T-TBS), pH 7. After a 10-min wash in T-TBS, the filter was incubated with AcrC5F10 diluted 1:400 in MBS for 2 h. Then the filter was washed 3 times with T-TBS and incubated with the second antibody (alkaline phosphatase-conjugated affinity-purified goat anti-mouse IgG Fcγ fragment; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 2 h, then washed two times with T-TBS and two times with citrate-buffered saline. The membrane was incubated in color-developing solution, and the reaction was stopped with PBS.

Radiolabeling of ZP Glycoproteins and Neoglycoproteins

Oocytes were obtained from patients undergoing ovulation stimulation and in vitro fertilization in an assisted fertilization-embryo transfer program. Unfertilized metaphase

II oocytes were kept for 2 wk at 4°C in 0.1 M Tris, 1.5 M $(NH_4)_2SO_4$, 0.5% Dextran pH 7, and then transferred to PBS containing glycerol and stored at −20°C until used. For ZP isolation, the oocytes were placed under a stereomicroscope in PBS 0.1% Triton X-100, and the ZP were mechanically removed using glass micropipettes. After being washed two times in PBS 0.1% Triton X-100, the ZP were resuspended in 50 mM Na_2HPO_4 pH 7.4. The ZP were heat-solubilized by incubation at 70°C for 2 h. Then solubilized ZP glycoproteins were radiolabeled with ^{125}I-Na following the chloramine T method [36] in the presence of 0.1% SDS. The iodinated ZP glycoproteins were evaluated by SDS-PAGE and showed two major bands of 50–72 and 89–98 kDa as previously reported [37]. NacGlc-BSA, Man-BSA, and BSA (Sigma catalog numbers A1034, A4664, A4503, respectively) were labeled by the same procedure used for ZP glycoprotein iodination.

^{125}I-ZP and ^{125}I-Neoglycoprotein Binding Assays

Rec-40 was isolated by SDS-PAGE under reducing conditions as mentioned above for the isolation of Rec-30. Human proacrosin was isolated from acid extracts and treated thereafter using the same procedure, except that preparative electrophoresis was performed under nonreducing conditions. The eluted proteins were precipitated with methanol-chloroform [38] to remove the detergent. Proteins were solubilized in 8 M urea in PBS, and different amounts (from 5 to 20 pmol) were immobilized onto nitrocellulose membrane using a Dot Blot device (Bio-Rad). Dots containing immobilized protein were cut off from the membrane as individual squares and blocked in 5% BSA, 0.05% Tween 20 in PBS for 2 h. Then dots were incubated with the radiolabeled ZP glycoproteins in 1% BSA, 0.05% Tween 20 in PBS for 1 h at different concentrations depending on the assay performed (from 1×10^6 to 7.5×10^6 cpm/ml). The dots were washed in 0.5% Tween 20, dried, and counted in a Gamma 4000 Beckman counter (Beckman Instruments, Palo Alto, CA). All procedures were performed at room temperature with constant shaking. Nonspecific binding was defined as the amount of radioactivity bound to the nitrocellulose in the absence of immobilized protein. Specific binding was obtained by subtracting nonspecific binding from the total radioactivity bound to the nitrocellulose in the presence of immobilized protein. All determinations were done in duplicate, and assays were repeated at least twice. Protein determinations were performed according to Lowry [39] using BSA as standard. Alternatively, Western blots containing 4 M urea P fractions from the 525 and pET control clones were incubated with the solubilized ^{125}I-ZP glycoproteins or ^{125}I-neoglycoproteins according to the same procedure as described for the dot blot assays.

RESULTS

Expression of Recombinant Proacrosin in the pET System

The cDNA encoding human proacrosin was subcloned in the pET-22b expression vector. On the basis of the subcloning strategy, we expected to obtain a 403-residue protein corresponding to the 402 amino acids of the proacrosin sequence with an additional N-terminal methionine residue (M_r 43 955 protein, as deduced from the amino acid sequence).

Total bacterial lysates obtained after induction at 37°C and prepared in Laemmli sample buffer were subjected to electrophoresis under reducing and nonreducing conditions,

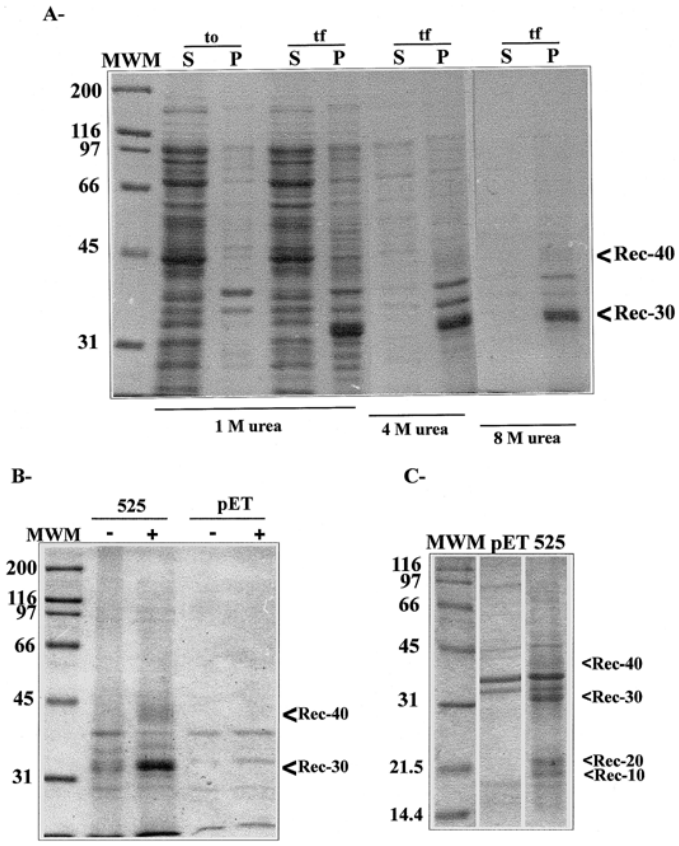

FIG. 1. Expression of recombinant proacrosin in the pET system. Induction of protein expression in the pET-22b control clone (pET) and pET-proacrosin clone (525) was carried out for 5 h at 37°C. Soluble (S) and insoluble (P) fractions from the pET and 525 clones at zero (t0) and 5 h after induction (tf) were subjected to SDS-PAGE and stained with CBB. Unless otherwise noted, samples were reduced by adding 5% 2-β-mercaptoethanol before electrophoresis. **A**) Partial solubilization of bacterial proteins from the 525 clone with increasing concentration of urea. Rec-40 (42–44 kDa) and Rec-30 (32–34 kDa, arrows) were recovered in the 1 M, 4 M, and 8 M urea P fraction from tf cultures but were not detected in the P fraction obtained from t0 cultures in 10% polyacrylamide gels. **B**) Samples from 4 M urea P from pET and 525 tf cultures were reduced (+) or not reduced (−) with 5% 2-β-mercaptoethanol before electrophoresis in 10% polyacrylamide gels. **C**) The 4 M urea P fractions from pET and 525 clones were subjected to 12% SDS-PAGE and stained with CBB. The expression products Rec-40, Rec-30, Rec-20, and Rec-10 are indicated. MWM: Molecular weight markers ($M_r \times 10^{-3}$).

Western-blotted, and immunostained with AcrC5F10. Proteins from bacterial lysates obtained from the pET-proacrosin clone (525) gave a specific positive signal with AcrC5F10. Nevertheless, the immunostaining pattern did not allow the identification of the expression product(s) in the lysate (data not shown).

To improve the bacterial lysate preparation, disruption of cells was carried out in the presence of Tris, EDTA, detergents, urea, and a cocktail of protease inhibitors; in addition, bacterial DNA was sheared by sonication. Most bacterial proteins were solubilized with increasing concentrations of urea (Fig. 1A). Two expression products were identified in the insoluble fraction on 10% polyacrylamide CBB-stained gels: an abundant product of 32–34 kDa, called Rec-30, and a minor 42–44-kDa product, Rec-40. Both products were absent in bacterial lysates obtained from a 525 clone before induction (Fig. 1A) as well as from a pET control clone (Fig. 1B). Detection of Rec-30 and Rec-40 on CBB-stained gels was found to depend upon the

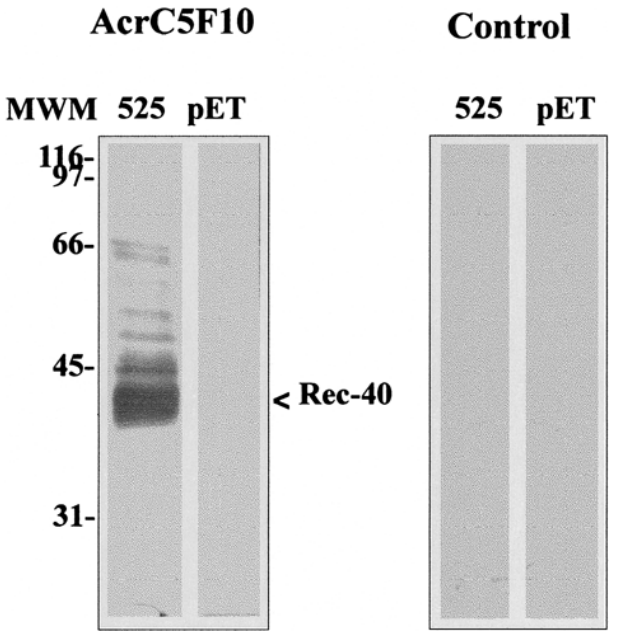

FIG. 2. Expression of recombinant proacrosin in the pET system. AcrC5F10 immunostained blot of 4 M urea P fraction from pET and 525 clones after induction of protein expression. SDS-PAGE was performed in 10% gels. Control: Blot developed in the absence of AcrC5F10 antibody. The blot was developed with the chromogenic substrate DAB. The arrow shows the Rec-40 expression product, which cross-reacted with the monoclonal antibody AcrC5F10. MWM: Molecular weight markers ($M_r \times 10^{-3}$).

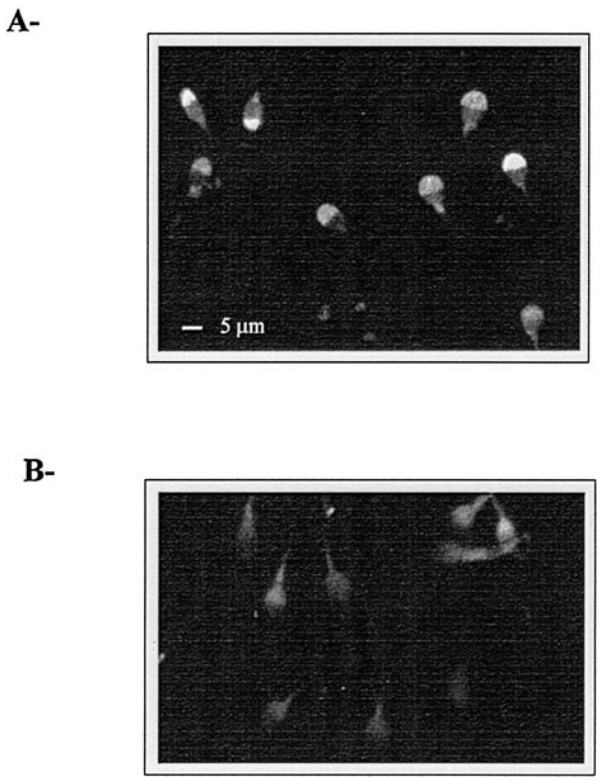

FIG. 3. Immune serum developed against Rec-30 reacts with the acrosomal region of fixed and permeabilized human spermatozoa. **A**) Immunocytochemistry of permeabilized human spermatozoa incubated with AntiRec-30 serum. **B**) The same as in **A**, except that the spermatozoa were incubated with preimmune serum.

use of disulfide bond reducing agents before electrophoresis (Fig. 1B). Two additional expression products of 18 kDa (Rec-10) and 21 kDa (Rec-20) were found associated to the 4 M urea P fraction on CBB-stained 12% polyacrylamide gels (Fig. 1C).

Immunostaining of 4 M urea P fraction Western blots from 525 and pET clones with AcrC5F10 showed a strong and specific signal on Rec-40. Under the conditions assayed, neither Rec-30, Rec-20, nor Rec-10 were reactive to AcrC5F10 antibody (Fig. 2).

Development of a Polyclonal Antiserum Against Rec-30

If Rec-30 were a protein related to human proacrosin, an antiserum against this product should cross-react with the sperm protein. An immune serum against Rec-30 (AntiRec-30) was developed and tested against human spermatozoa and protein extracts. Immunocytochemical analysis performed on permeabilized human spermatozoa with AntiRec-30 serum showed specific staining over the acrosomal region of the sperm head (Fig. 3A). Spermatozoa stained with preimmune serum showed no fluorescence in the acrosomal region (Fig. 3B). On immunoblot assays, the immune serum specifically recognized a 53–55-kDa protein doublet with the same electrophoretic mobility of that detected with AcrC5F10 (Fig. 4A). The antiserum raised against Rec-30 cross-reacted with Rec-40, Rec-20, and Rec-10, indicating that these are related products (Fig. 4B). No protein was immunostained by AntiRec-30 on Western blots of the 4 M urea P fraction from the pET lysate (Fig. 4, A and B).

Amino Acid Sequence Analysis of Rec-30, -20, and -10

To confirm the identity of the Rec-30, Rec-20, and Rec-10 expression products, amino acid sequence analysis was performed. Analysis of two internal peptides of Rec-30 showed the following sequences: KIIIHEK and KAPLQERYVEK, sequences identical to the residues 98–108 and 108–114, respectively, of human proacrosin [1, 2]. N-Terminal amino acid analysis showed the sequence MKDNAT for Rec-30, MKDNATXD for Rec-20, and XXDNATXDG for Rec-10 (X: no amino acid residue assigned to this position). The 9 N-terminal residues of human proacrosin are KDNATCDG [1, 2]. These results indicated that Rec-30, Rec-20, and Rec-10 have sequences corresponding to the N-terminal region of the proenzyme.

Identification of the AcrC5F10 Epitope and Detection of C-Terminal Expression Products

Since N-terminal fragments of human proacrosin were not detected by AcrC5F10 on Western blots (Rec-30, -20, and -10), and the epitope recognized by the antibody had not been identified, this sequence was searched within the C-terminal region of proacrosin. For this purpose, a peptide library consisting of overlapping peptides covering proacrosin residues 264–363 was synthesized. This region belongs to the C-terminal region of the protein. By this approach, the sequence recognized by the antibody was delimited to the residues 305–309 (LPWYF) of human proacrosin (Fig. 5A).

The results in the pET system clearly showed expression of recombinant proteins comprising the full-length protein and N-terminal fragments. However, there was no certainty whether C-terminal polypeptides were produced under the culture conditions used. Since AcrC5F10 epitope is localized in the C-terminal portion of proacrosin, an ECL system

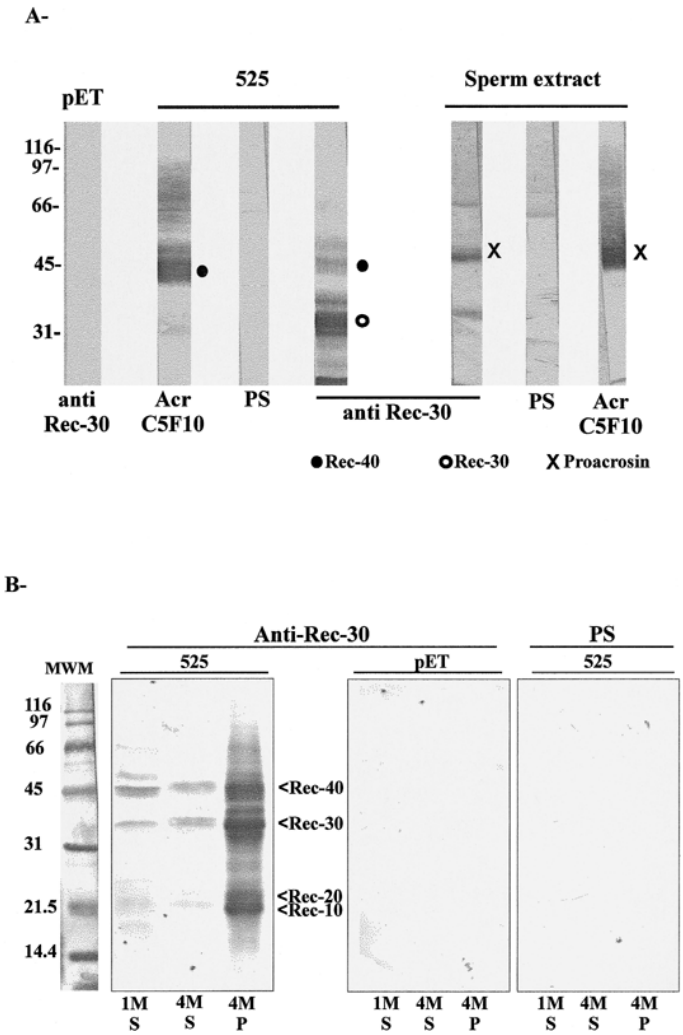

FIG. 4. **A**) AntiRec-30 immunostained blots of human sperm extracts containing proacrosin (sperm extract), and 4 M urea P fractions from pET and 525 clones. The protein extracts were subjected to 10% SDS-PAGE before electroblotting. Blots were also incubated with preimmune serum (PS) and AcrC5F10 antibody, and developed with DAB as chromogenic substrate. Rec-40, Rec-30, and proacrosin are indicated. **B**) Twelve percent SDS-PAGE of pET and 525 S and P fractions immunostained with AntiRec-30 serum and developed with DAB. The expression products Rec-40, Rec-30, Rec-20, and Rec-10 are indicated. MWM: Molecular weight markers ($M_r \times 10^{-3}$).

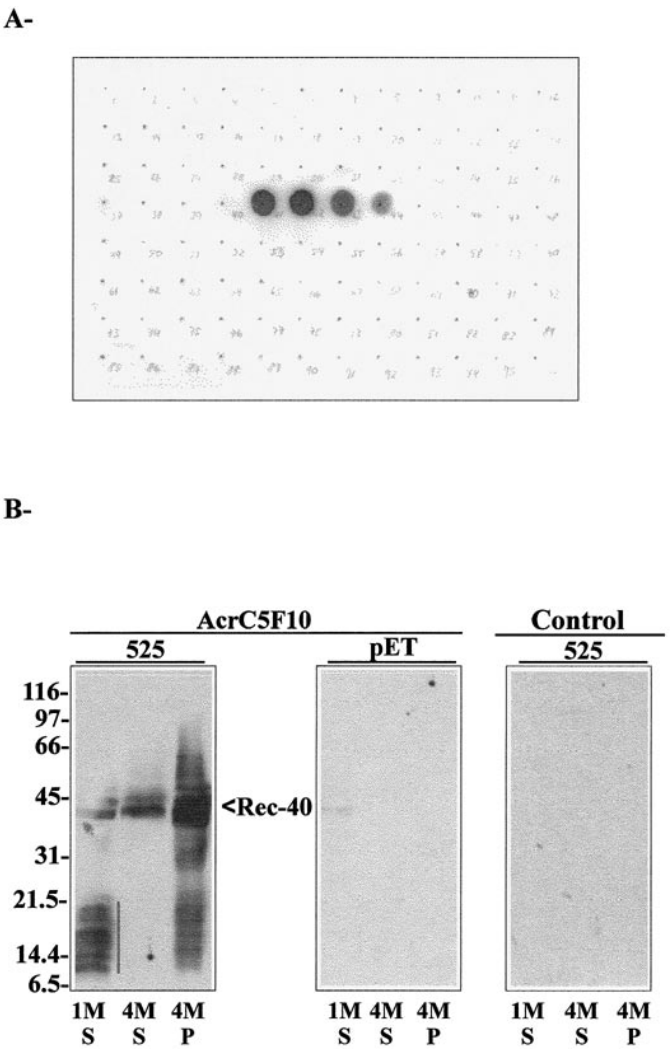

FIG. 5. Identification of the AcrC5F10 epitope and detection of C-terminal expression products in the pET system. **A**) AcrC5F10 epitope analysis. The array of 95 overlapping hexapeptides covering the C-terminal 264–363 residues of human proacrosin is shown. The stained spots represent the individual peptides recognized by AcrC5F10 antibody, comprising amino acid residues 305–309. **B**) AcrC5F10 immunostained blot of S and P fractions from pET and 525 clones. The protein extracts were subjected to 12% SDS-PAGE before electroblotting. The blot was developed with ECL. Control: blot developed in the absence of AcrC5F10 antibody. The 10- to 17-kDa expression products associated with the 1 M urea S fraction are indicated with a vertical bar. MWM: Molecular weight markers ($M_r \times 10^{-3}$).

was used to develop AcrC5F10 immunoblots of 525 and pET soluble and insoluble fractions, with the purpose of identifying those products. Six bands corresponding to recombinant polypeptides of 10, 11, 12, 14, 15, and 17 kDa were detected in the 1 M urea S fraction (Fig. 5B). In the 4 M urea P fraction from the 525 clone, in addition to Rec-40, several bands of low molecular mass were found. However, the molecular size of these protein bands did not correspond either to Rec-30, Rec-20, or Rec-10 (Fig. 5B).

Time Course of Expression of Recombinant Proacrosin

It could be hypothesized that Rec-30 arose as a proteolytic product of Rec-40 during the prolonged time of cell culture. The protein expression pattern at 0.5, 1, 2, and 5 h after induction was analyzed. The AcrC5F10 and AntiRec-30 immunostained blots showed that both expression products could be detected as soon as 30 min after induction, with Rec-30 the major expression product at any time analyzed (Fig. 6, A and B).

Bacterial clones carrying pET-proacrosin plasmid cultured at lower temperatures (25–30°C) did not prevent the expression of truncated products or the accumulation of recombinant proteins as inclusion bodies (data not shown).

Expression of Recombinant Proacrosin in the pGEX System

Fusion protein strategies are usually employed to increase the yield of recombinant protein expression. This is achieved by preventing protein proteolysis or accumulation as inclusion bodies. Moreover, the presence of mRNA secondary structures that prevent translation can be destabilized using this approach [40]. In addition, these systems often provide advantages for the purification of the expres-

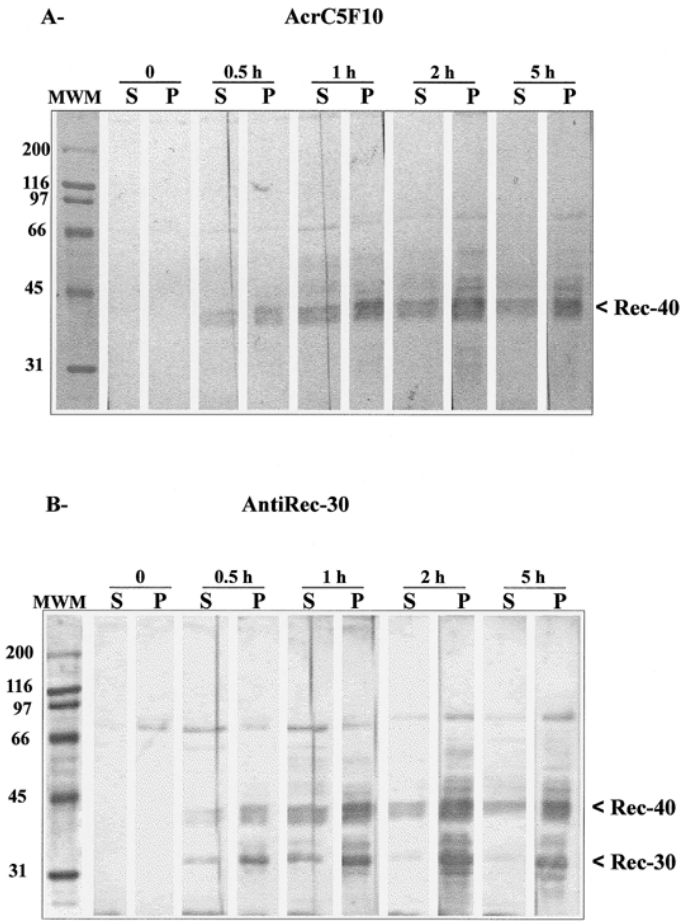

FIG. 6. Expression analysis throughout the induction time. Samples at 0, 0.5, 1, 2, and 5 h after induction at 37°C were obtained from a 525 clone culture. The bacterial P and S fractions were obtained as described in *Materials and Methods* and were subjected to 10% SDS-PAGE. **A**) AcrC5F10 immunostained blot. Arrow, signal given by Rec-40. **B**) AntiRec-30 immunostained blot. The same blot tested with AcrC5F10 was stripped and immunostained with AntiRec-30 serum. Rec-30 was detected at every time it was analyzed after induction. MWM: Molecular weight markers ($M_r \times 10^{-3}$).

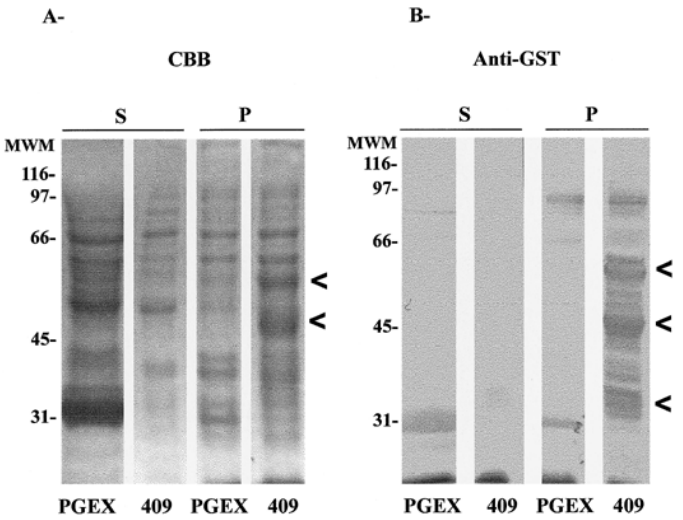

FIG. 7. Expression of recombinant proacrosin in the pGEX system. Ten percent SDS-PAGE of S and P fractions of the pGEX-3X control clone (pGEX) and the 409 clone obtained after 2 h of induction at 37°C. Samples were reduced before electrophoresis. **A**) CBB-stained gel. Arrows: 55- and 44-kDa expression products present in 409 P fraction. **B**) Anti-GST immunostained Western blot. The 55-, 44-, and 33-kDa fusion products are shown. MWM: Molecular weight markers ($M_r \times 10^{-3}$).

sion products. In order to improve expression of recombinant human proacrosin, and to prevent its proteolysis to minor products, a fusion protein strategy was tested. Analysis of the 409 clone S and P fractions revealed the presence of two major expression products of 55 kDa and 44 kDa after SDS-PAGE and protein staining with CBB (Fig. 7A). These products were associated with the insoluble fraction of the bacterial lysates. Immunostaining with anti-GST immune serum confirmed that these expression products were fusion proteins, and revealed an additional band of an apparent molecular mass of 33 kDa (Fig. 7B). A similar pattern was obtained with AntiRec-30 serum (data not shown). Under the conditions assayed, a fusion protein of 73 kDa expected for the expression of the full-length proacrosin sequence was not detected.

Recombinant Protein ^{125}I-ZP Glycoprotein Binding Activity

The expression products from the pET-system were tested for their ability to recognize human ZP glycoproteins. For this purpose, Western blots containing the inclusion body proteins were incubated with solubilized ^{125}I-ZP glycoproteins. The 4 M urea P fraction from the pET clone was used as a control. As can be seen in Figure 8, protein bands with the molecular sizes of Rec-30, Rec-20, and Rec-10 were recognized by the iodinated ZP glycoproteins, in addition to some bands corresponding to bacterial polypeptides present both in the 525 and pET insoluble fraction. The intensity of these bands decreased when the incubation of the iodinated probe was performed in the presence of 10 μM dextran sulphate (5000 Da), suggesting that part of the interaction was due to sulphated residues of the ZP glycoproteins.

The same protein pattern was obtained when the blots were incubated with the iodinated neoglycoproteins mannose (Man)-BSA and *N*-acetyl-D-glucosamine (NAcGlc)-BSA. Iodinated BSA did not recognize any polypeptide on the Western blot, indicating that the interaction between the recombinant proteins and neoglycoproteins was mediated by the sugar portion of the probe (Fig. 8).

Since no signal was detected for Rec-40 on the Western blot, its ability to interact with ZP glycoproteins was assessed by the dot blot assay. Rec-40 was isolated from the inclusion bodies by preparative electrophoresis and then immobilized on nitrocellulose membrane, as described in the *Materials and Methods* section. Rec-40 binding to ZP glycoproteins was augmented with increasing concentrations of radiolabeled ZP and reached saturation (Fig. 9A). Binding was linear between 5 and 20 pmol of immobilized Rec-40 (Fig. 9B). Under the experimental conditions tested, 15 pmol of Rec-40 retained 73% of denatured human proacrosin binding to ZP glycoproteins (data not shown).

DISCUSSION

Four research groups have reported the expression of boar and rabbit proacrosin fragments as recombinant proteins in bacteria and have evaluated their ability to recognize homologous ZP glycoproteins [10, 26–28]. In the boar, acrosin fragments comprising the 275 N-terminal residues of proacrosin were obtained as inclusion bodies [26] or as soluble fusion proteins [27]. In the rabbit, several fragments of proacrosin were synthesized, excluding the proline-rich

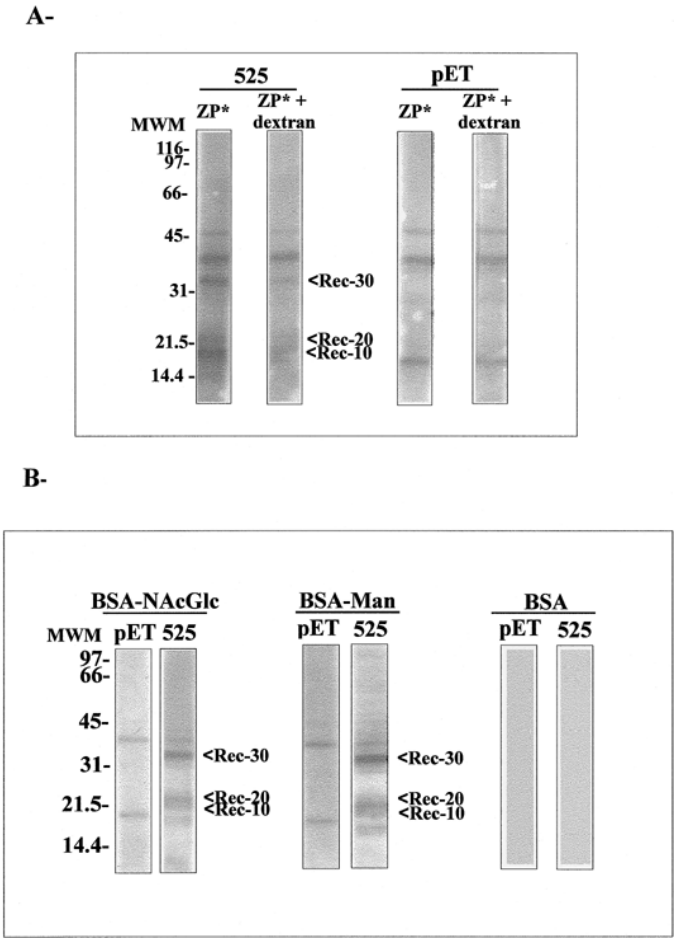

FIG. 8. Binding of ^{125}I-ZP glycoproteins and ^{125}I-neoglycoproteins to recombinant proteins on Western blots. The 4 M urea P fractions from 525 and pET clones were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane. **A**) The blots were incubated with ^{125}I-ZP glycoproteins with or without dextran sulphate. **B**) The blots were incubated with ^{125}I-neoglycoproteins and ^{125}I-BSA. MWM: Molecular weight markers ($M_r \times 10^{-3}$).

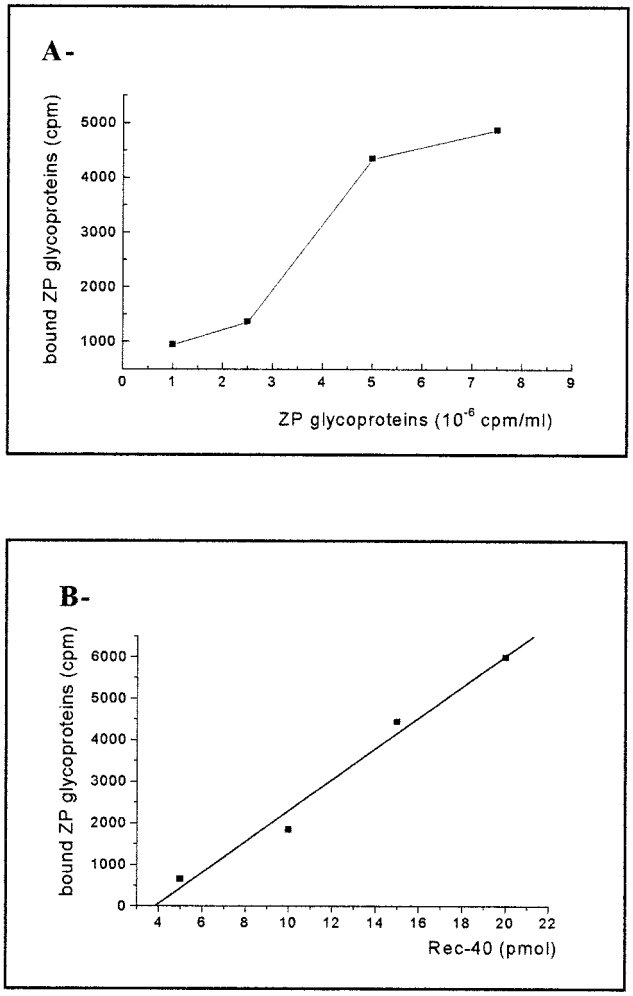

FIG. 9. Binding of ^{125}I-ZP glycoproteins to Rec-40. **A**) Binding of ^{125}I-ZP glycoproteins to Rec-40 was saturable. Rec-40 (20 pmol) was immobilized on nitrocellulose and incubated with increasing concentrations of the iodinated probe. **B**) Binding of ^{125}I-ZP glycoproteins to increasing quantities of Rec-40. Increasing amounts of Rec-40 were immobilized on nitrocellulose and incubated with 2.5 10^6 cpm/ml iodinated probe.

C-terminal portion of the protein [10]. None of these studies reported the expression of the full-length recombinant proacrosin. The aim of the present study was to obtain the full-length recombinant human proacrosin, characterize its expression in a bacterial system, and evaluate its ability to recognize homologous ZP-glycoproteins.

For the expression studies, two vectors were tested: one is a direct expression vector (pET-22b), and the other produces a fusion protein with GST at the N-terminal end of proacrosin (pGEX-3X). Using the pET expression system, a protein with a molecular size similar to that expected for the recombinant proenzyme was identified among other expression products (Rec-40, 42–44 kDa). In addition to Rec-40, a 32–34-kDa major expression product (Rec-30) and truncated products of 21 kDa (Rec-20) and 18 kDa (Rec-10) were obtained.

Detection of the recombinant proteins was achieved only after preparation of the bacterial lysates and subcellular fractions in the presence of protease inhibitors and shearing of the bacterial DNA. By treating the insoluble fraction of the lysate with detergents and increasing concentrations of urea, most of the bacterial proteins were removed from the aggregates, and expression products were clearly visualized. Results after disruption of the aggregates with urea and detergents suggested the presence of stabilizing hydrophobic interactions. Moreover, the addition of 2-β-mercaptoethanol was essential to identify the expression products, suggesting that the aggregates were also maintained by disulfide bridges. A similar behavior has been described for other eukaryotic proteins expressed in bacteria. The cytoplasm of *E. coli* cells is a reductive environment; in consequence, bacterial cytoplasmic proteins are found to have low cysteine content and few disulfide bonds [41]. However, it has been suggested that disulfide bridges are involved in aggregation of recombinant proteins in vivo [42] or after cell lysis [41]. This seems to be the case with Rec-40, Rec-30, Rec-20, and Rec-10. In fact, proacrosin contains 12 cysteine residues involved in disulfide bonds in the native protein, localized within the 250 N-terminal amino acids of the proenzyme [43].

Rec-30 was not recognized by AcrC5F10 on Western blots despite being the major expression product, as seen on CBB-stained gels. Rec-30 was found to be related to Rec-40, Rec-20, Rec-10, and proacrosin from human sperm extracts, since an antiserum raised against Rec-30 recognized these proteins immobilized on Western blots. Moreover, AntiRec-30 specifically immunostained the acrosomal

region of permeabilized human spermatozoa. Amino acid sequence analysis showed that Rec-30, Rec-20, and Rec-10 are N-terminal products of the proacrosin cDNA, and supported the results obtained with AntiRec-30 serum.

In order to determine whether Rec-30 arose after proteolysis of Rec-40 by host cell proteases during prolonged culture growth time (5 h), the presence of both expression proteins was analyzed at different time points after induction. Both products were found to coexist in the bacterial lysate at any time tested. Thus, Rec-30 may arise as a proteolytic product of Rec-40 as soon as it is synthesized, or from abnormalities during transcription or translation.

Expression of recombinant proteins as fusion products has been shown to increase stability of the recombinant protein, i.e., by circumventing degradation by host cell proteases and/or by destabilizing mRNA secondary structures that prevent translation [40, 41, 44]. However, this was not observed with proacrosin, since evidence presented in this and in other studies [10] shows the opposite. In the present study, no expression of the fusion protein containing the full-length sequence was detected. The report on expression of rabbit acrosin fragments as fusion proteins showed the difficulty of synthesizing constructs comprising the proline-rich C-terminal portion of the proenzyme, using various growth conditions and different bacterial strains [10]. These results suggest that, independently of the bacterial expression system employed, it is difficult to produce the whole proenzyme.

With a highly sensitive system used to develop immunoblots, several minor bands of apparent molecular size between 10 and 17 kDa were found associated to the soluble fraction with the monoclonal antibody AcrC5F10, but not with AntiRec-30 serum. Since the AcrC5F10 epitope is located in the C-terminal region of proacrosin, these results strongly suggest that the 10- to 17-kDa polypeptides are C-terminal fragments of proacrosin. This supports the hypothesis that the full-length proenzyme is posttranslationally processed by host cell proteases to give rise to the truncated products Rec-30, Rec-20, and Rec-10.

The results presented in this study show, for the first time, the expression of human recombinant proacrosin. This product has a molecular mass of 42–44 kDa, similar to that deduced from the amino acid sequence (43.8 kDa). Human sperm proacrosin has an apparent molecular mass of 53–55 kDa. The 10-kDa difference between this value and that deduced from the amino acid sequence might be explained by posttranslational modification of the native protein, i.e., glycosylation, as well as unusual electrophoretic behavior, as suggested for boar proacrosin [5].

In addition to the Rec-40 product, as reported in other species, N-terminal truncated forms are produced in the bacteria [10]. The major expression product synthesized in the study reported here has the same molecular size as the rabbit and boar acrosin proteins expressed in bacteria (corresponding to approximately 300 residues from the N terminus of proacrosin) [10, 26–28]. The evidence strongly suggests that a major difficulty would reside in the expression of the C-terminal portion of the protein. In the native protein, the C-terminal region is proteolytically processed during protease activation [5, 6]. In consequence, this protease-sensitive site could be the target of bacterial serine proteases during cell culture. However, the N-terminal amino acid sequence data indicates that no light chain processing of the recombinant proteins is observed during cell culture.

The ZP-binding activity of the recombinant proteins was evaluated by Western blot and dot blot analyses. Rec-30, -20, and -10 were found to bind ZP glycoproteins in the Western blot assay. The results obtained with ZP and neoglycoproteins suggest that the interaction may involve the sugar moieties NAcGlc and Man as well as sulphated residues of the ZP glycoproteins. Since Rec-10 still retained binding activity towards ZP glycoproteins, it could be proposed that in human proacrosin, at least part of the recognition site would be localized within the N-terminal 160 amino acids of the protein. Using site-directed mutagenesis, residues involved in ZP recognition have been identified in this region in boar and rabbit acrosin [10, 27, 28].

The absence of a signal for Rec-40 in the binding assay on Western blot was not surprising, since Rec-40 was detected as a faint band in CBB-stained gels. Therefore, Rec-40 isolated from the inclusion bodies was immobilized onto a nitrocellulose membrane, and its ability to interact with ZP glycoproteins was assessed. Binding of Rec-40 to ZP glycoproteins was found to be linear between 5 and 20 pmol of protein, and saturable. Using this approach, linear sequences of the protein were evaluated for ZP binding activity. However, it could not be ruled out that part of the interaction with the ZP glycoproteins could be associated with conformational sites of the protein. In order to evaluate these sites, solubilization and refolding is required. Taking this into consideration, studies were carried out for the solubilization and renaturation of the recombinant proteins. Several protocols were attempted; however, no successful refolding was achieved, as evaluated by testing the recombinant protein enzymatic activity.

This is the first report on the synthesis of recombinant human proacrosin in bacteria. The studies performed have allowed the characterization of the expression of human proacrosin and truncated products in a prokaryotic system. The recombinant proteins were found to have binding activity towards human ZP glycoproteins.

ACKNOWLEDGMENTS

The authors would like to thank Celia Melamed, Veterinarian, for helping in the immunization experiments with Rec-30; J.C. Biancotti, M.S., for his technical assistance in the spermatozoa immunocytochemical analysis; and Prof. Engel for providing the acrosin cDNA clone.

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