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Epitopes within recombinant α-actinin protein is serodiagnostic target for *Trichomonas vaginalis* sexually transmitted infections

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Abstract

To date there is no commercially-available serodiagnostic for women and men infected with *Trichomonas vaginalis*. Thirteen epitopes of the immunogenic *T. vaginalis* α-actinin (106.2-kDa) are detected by sera of women patients, and 5 epitopes, a subset of the 13, are detected by sera of men. A truncated recombinant protein called ACT-P2 (63.5-kDa) encoding the 5 epitopes is used for screening by ELISA for antibody in sera of women and men. Because ACT-P2 is poorly expressed in *E. coli*, we wanted alternative recombinant α-actinin proteins as serodiagnostic targets. We, therefore, constructed plasmids encoding two novel, small recombinant proteins of ~25-kDa comprised of 15-mer peptides, each peptide of which contains one of the 13 epitopes. We refer to these novel proteins as α-actinin::string-of-epitopes1 (ACT::SOE1) and ACT::SOE2. We found the proteins to be unrecoverable from insoluble inclusion bodies without denaturing conditions, which rendered the proteins unsuitable for antibody detection. Thus, we synthesized a third ACT::SOE3 protein (72.4-kDa) with 7 epitopes that was synthesized in high amounts and was readily purified. Monoclonal antibodies to α-actinin detected ACT::SOE3 and ACT-P2 by ELISA. Further, we show that ACT::SOE3 is equal to ACT-P2 as a target protein for detection of serum IgG in

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positive sera of women and men. Data indicate that ACT::SOE3 is a target for screening of populations at-risk for this STI. Finally, the paper discusses the findings with regard to Point-of-Care diagnostic targets and vaccine candidates.

Keyword: Microbiology

1. Introduction

Trichomonas vaginalis causes a non-reportable sexually transmitted infection (STI) with undesirable outcomes to the health of women [1, 2]. Men having intercourse with a partner with trichomonosis become infected, but reports, without clinical and experimental evidence, have indicated clearance of the parasite by men. One notable feature of this STI, despite its high prevalence [3, 4, 5], is persistence among infected individuals, perhaps owing to the asymptomatic nature following host colonization. In men, the parasite has been observed in the prostate, and *T. vaginalis* DNA has been detected in hyperplastic prostate tissue [6, 7]. It has been hypothesized that seropositivity and, therefore, exposure to *T. vaginalis* is preparatory to prostate cancer (PCa) [8, 9, 10]. Indeed, a model for *T. vaginalis*-mediated development and progression of PCa has been proposed [11].

Epidemiological studies indicate that there is a need for a rapid, inexpensive and accurate serum antibody-based diagnostic with targets of high specificity for the screening of large cohorts of at-risk women and men. This laboratory invented the commercially-available lateral flow, immuno-chromatograhic Point-of-Care (POC) test (OSOM^{TM} Trichomonas Rapid Test, Sekisui Diagnostics, Lexington, MA, USA) for trichomonosis [12]. However, shortcomings of this strip test are that it does not detect trichomonad protein from men secretions, such as urine. Other nucleic acid amplification tests (NAATs) [13, 14] are available for women and men with this STI, but these NAATs are not POC tests, require trained personnel and expensive equipment, and are unsuitable for large scale screening in non-sterile settings and at under-developed parts of the world.

Both women and men make serum antibody to the immunogenic α-actinin protein of *T. vaginalis* [8, 9, 10, 15, 16]. Thirteen epitopes of α-actinin to which women and men make antibody were recently identified, and, importantly, the epitopes have no amino acid sequence identity to other proteins [17], reinforcing the notion that antibody to this protein is indicative of exposure to *T. vaginalis*. Equally noteworthy is the fact that the α-actinin protein and epitopes are invariant, as indicated recently [17]. Presently, a truncated recombinant version of α-actinin called ACT-P2 is the target for screening sera for antibody. A major drawback, however, is the low amounts of ACT-P2 synthesized by *E. coli*. Thus, an alternative α-actinin target is needed that is expressed in large amounts and is readily purified. In this report an approach is utilized as recently described [18] to produce two proteins of ~25-kDa that incorporate all 13 epitopes of the

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trichomonad α-actinin protein identified earlier [17]. These two proteins are referred to as α-actinin::string-of-epitopes1 (ACT::SOE1) and ACT::SOE2. Unfortunately, these proteins were found in inclusion bodies, and denaturing conditions for purification rendered the proteins unsuitable for detection by antibody. A version of α-actinin called ACT::SOE3 was synthesized that had 7-epitopes including the 5 epitopes detected by both women and men. This protein is synthesized in large amounts and is readily purified. Immunoassay data indicates that this protein is an ideal target for detection of serum antibody present in the sera of individuals with this STI [17, 18]. Finally, the paper discusses the approaches that may lead to development of novel highly-specific diagnostic immunotargets with potential for use as vaccines.

2. Materials and methods

2.1. ACT-P2, ACT::SOE1, ACT::SOE2 and ACT::SOE3

The *T. vaginalis* α-actinin protein of 931-amino acids (106.2-kDa) and the subclone ACT-P2 protein of 558-amino acids (64.1-kDa) have been described [17]. Both the α-actinin and ACT-P2 have been used to examine the relation of serum antibody in men and prostate cancer [8, 9, 10]. The thirteen epitopes of α-actinin detected by seropositive women and the five epitopes that are a subset of the thirteen detected by seropositive men permitted construction of a plasmid encoding thirteen 15-mer peptides each with an epitope. Fig. 1 shows the 232-amino acids of the first two recombinant proteins called ACT::SOE1 (with −GG- linkers) (part A1) and ACT::SOE2 (with −KK- linkers) (part A2). The coding region of ACT::SOE3 corresponded to amino acids 250 and 879 (Fig. 1B). The DNA coding sequence for each was synthesized by GenWay Biotech, Inc (San Diego, CA) and cloned in pET-23a(+) expression vector with the ampicillin (Amp) resistance gene for transformation of *E. coli* BL21DE3 cells. The correct reading frames were confirmed by sequencing of the plasmid DNA prior to and after transformation of *E. coli*. ACT-P2 was originally prepared in *E. coli* XL1-Blue (Strategene, La Jolla, CA) containing a plasmid with the truncated α-actinin DNA and with the kanamycin (Kan) resistance gene [8, 9, 17]. The fusion protein with hexa-histidine at the carboxy-terminal end was able to be purified, as before [17, 18].

2.2. Growth of *E. coli* and purification of recombinant proteins

The bacteria were grown on Luria Broth (LB) agar plates containing either 25 μg/ml Kan or 100 μg/ml Amp, and a starter culture of LB medium inoculated with recombinant *E. coli* was grown as before [17, 18]. Aliquots of *E. coli* were obtained of uninduced and induced *E. coli* for SDS-PAGE and staining of acrylamide gels for visualization of synthesis of recombinant protein.

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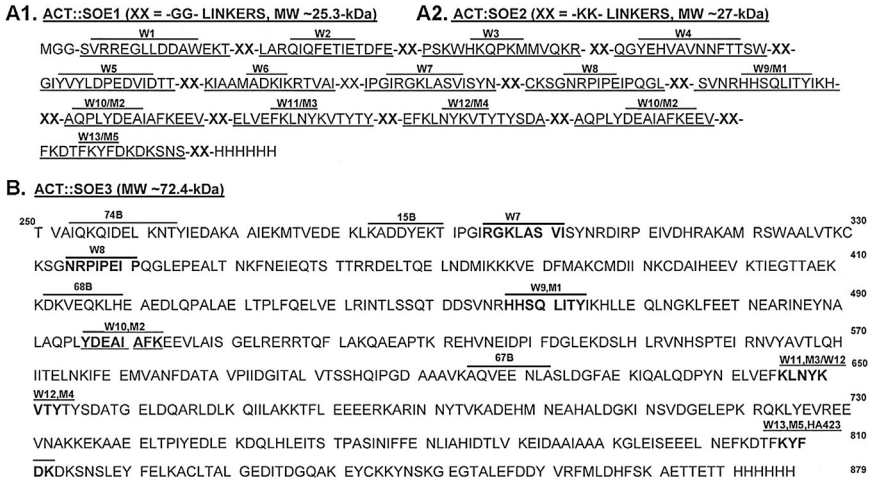

Fig. 1. Sequences of recombinant ACT::SOE1 and ACT::SOE2 proteins encoding for 13 epitopes (A1 and A2) and ACT::SOE3 encoding 7 epitopes (B) of α-actinin of *T. vaginalis*. Parts A1 and A2 show the amino acid sequences of proteins called ACT::SOE1 (A1) and ACT::SOE2 (A2) that illustrates 15-mer peptides within which are epitopes detected by the highly positive sera of women (W1 through W13) and men (M1 through M5). The 15-mer sequences were linked with either two glycine (-GG-) (A1) or two lysine (-KK-) (A2) residues. The six histidine (H) residues at the carboxy terminus are hexa-histidine added for purification as described in Materials and Methods. Part B presents the amino acid sequence of ACT::SOE3 showing the positions of 7 epitopes in bold detected by positive sera of women (W7-W13) and men (M1-M5) and the MAb HA423. The new monoclonal antibodies (MAbs) 15B, 67B, 68B, and 74B that were generated in this study to α-actinin are as labeled and underlined above the amino acid sequences. Abbreviations: W1 through W13, epitopes detected by positive women sera; M1 through M5, epitopes detected by positive men sera; 74B, 15B, 68B, and 67B, epitopes detected by new MAbs; HA423, MAb generated to α-actinin (9, 17); HHHHHH, hexa-histidine; kDa, kilodaltons; MW, molecular weight.

Purification of the recombinant proteins was performed as detailed recently [17, 18]. Briefly, for ACT-P2 and ACT::SOE3 *E. coli* pellets were suspended in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol (β-ME), and 0.1% Triton-X100) and sonicated 10 times each at RT for 30 s. Tubes were placed on ice for 30 s after each 30 s sonication pulse. After centrifugation, supernatant was applied to a Ni^{2+}-NTA superflow affinity column (Qiagen Inc., Valencia, CA, USA). The ACT::SOE1 and ACT::SOE2 proteins required solubilizing in lysis buffer containing urea, and the protocol used was that recommended by Qiagen. Multiple attempts made using various procedures to refold ACT::SOE1 and ACT::SOE2 in the absence of denaturing conditions were unsuccessful.

2.3. Sera from women and men for ELISA and assignment of positive to negative (P/N) scores to serum reactions

The source of sera from women and men has been described [17, 18]. Ethical approval was obtained to collect the serum samples used in this study by IRBs at

http://dx.doi.org/10.1016/j.heliyon.2017.e00237

the collaborating institution (Washington University-Saint Louis, MO) as well as by IRB at Washington State University. The sera were obtained from the Health Professionals Follow-up Study of cancer and heart disease in men [8, 9, 10], and informed consent was obtained from all patients to use their samples in research.

Sera were used for detection of antibody to ACT-P2 in relation with PCa [8, 9, 10]. Another target protein is called AEG::SOE described in an earlier report [18]. Where indicated, highly seropositive sera of both women and men were pooled. ELISA was used to screen large numbers of sera of women and men for detection of antibody to ACT-P2. The ELISA protocol was done as has been detailed before [8, 9, 10] and recently [17, 18]. Briefly, individual wells of microtiter plates were coated with 1 μg of purified ACT-P2, ACT::SOE3, and AEG::SOE as before [17, 18] and stored at 4 °C until use. Plates were washed three times (3X) with PBS-Tween 20 (0.05% Tween 20 in PBS (pH 7.0) followed by blocking with 200 μl of ELISA-grade BSA (Sigma Chemical Co., St. Louis, MO) in PBS (eBSA-PBS), pH 7.4, for 2 h at 37 °C. After washing the Plates 3X with PBS-Tween 20, 50 μl of a 1:25 (vol/vol) dilution of serum was added to wells. Where necessary, a 50 μl volume of undiluted hybridoma supernatant of the various monoclonal antibodies (MAbs) was also added. Plates were incubated at 37 °C for various time points as indicated. After washing with PBS-Tween 20, wells were incubated with 50 μl of freshly-prepared secondary horseradish peroxidase-conjugated goat anti-human IgG Fc antibody or goat anti-mouse IgG antibody (both diluted 1:1,500 in PBS) (Sigma) and incubated at 37 °C for 60 min. Color development was done by adding 50 μl of substrate solution (ABTS; phosphate-citrate buffer with 0.03% sodium perborate, Sigma) and incubating the plates at room temperature for 15 min. Absorbance values were obtained spectrophotometrically at 405-nm wavelength using an ELISA reader (Bio-TEK Instruments, Inc., Winooski, VT).

We previously assigned scores from 0 to 4+ to serum based on the calculation of P/N values obtained using ACT-P2 as the target [8, 9, 10]. For this study, cutoff points for seropositivity were obtained by dividing the average OD_{405nm} of the seropositive control serum by the corresponding OD_{405nm} of seronegative control serum. The lowest seronegative control was assigned a score of 0 (zero). Values from 0 to the next lowest was given a score of 1+. Values from 1+ to the next lowest was assigned a score of 2+, etcetera. For ELISA data presented in this study, therefore, P/N for 0 = 1 to <1.75; P/N for 1+ = 1.76 to < 2.40; P/N for 2+ = 2.41 to 3.15; P/N for 3+ = 3.16 to <4.25; and P/N for 4+ = >4.25. The P/N scores ≤2 were negative as evidenced by lack of detection of any *T. vaginalis* proteins by immunoblot [17]. Scores ≥3 were positive and have antibody to α-actinin and other trichomonad proteins [17].

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2.4. Synthesis of 11-mer overlapping peptides spanning the α-actinin amino acid sequence for MAb epitope identification

To identify the epitopes detected by the MAbs generated to α-actinin, 11-mer peptides overlapping by 8 amino acids were synthesized from the amino acid sequence spanning the entire α-actinin protein, as before [17] (Sigma-Genosys, The Woodlands, TX). One to 10 mg of each lyophilized peptide provided in individual tubes was dissolved in 100 μl of a 1:1 (vol/vol) mixture of distilled water:acetic acid. If the peptide was insoluble, 100 μl acetonitrile was then added and vortexed for 5-min. The mixture was then diluted into carbonate buffer [17, 18] to give a concentration of 1 μg/50 μl added to each well of microtiter plates. Wells were dried o/n at 37 °C. Finally, ELISA was performed using the protocol recently detailed for epitope identification [17].

2.5. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Standard procedures for SDS-PAGE using 8% acrylamide have been described [19, 20, 21, 22, 23, 24]. Total proteins of *E. coli* expressing ACT-P2 or ACT::SOE3 were obtained by microfuge pelleting of 1.5 ml bacteria induced by IPTG. Pelleted bacteria were suspended in electrophoresis dissolving buffer [17, 18] and boiled for 10-min prior to SDS-PAGE and staining of acrylamide gels with Coomassie Brilliant blue.

2.6. Generation of new MAbs to α-actinin

Established protocols were used to immunize mice with purified α-actinin to yield hybridomas for generation of MAbs [25]. The MAbs were generated by the MAb Core Facility of the College of Veterinary Medicine at Washington State University. After confirming that mouse anti-α-actinin serum had high-titered IgG reactive with ACT-P2 and ACT::SOE3, hybridomas were prepared and screened by ELISA with immobilized α-actinin as before [17, 18] followed by single-cell cloning. Four MAbs of IgG_1 isotype (15B, 67B, 68B, and 74B) were identified for further analysis in this study.

2.7 Reproducibility

Data in this report are from representative experiments. All experiments were performed three or more times under identical conditions. ELISAs on microtiter plates coated with proteins were done in quadruplicate, and averages and standard deviations were calculated.

http://dx.doi.org/10.1016/j.heliyon.2017.e00237

3. Results

3.1. Expression, synthesis and purification of recombinant SOE proteins

First a novel SOE protein was synthesized of ~25-kDa comprised of thirteen 15-mer peptides within which were the identified α-actinin epitopes [17]. Fig. 1 (parts A1 and A2) shows the 232-amino acid sequence of the recombinant proteins called ACT::SOE1 and ACT::SOE2 containing the epitopes detected by sera of women patients (underlined W1 through W13) and men (M1 through M5) [17]. The difference between the proteins is the di-amino acid linkers. Both proteins were synthesized in large amounts, but, unfortunately, the proteins aggregated into inclusion bodies. ACT::SOE1 and ACT::SOE2 were only solubilized in denaturing conditions that neither permitted detection by serum antibodies nor with the original monoclonal antibody (MAb) HA423 generated to α-actinin [17].

As ACT-P2 is synthesized in low amounts, selection of a portion of the α-actinin protein different from ACT-P2 [17] might permit synthesis of greater amounts of protein for analysis. Fig. 1 (part B) shows the sequence of the 604 amino acid protein called ACT::SOE3, which has seven epitopes, including the five detected by sera of both women and men (underlined W7 through W13 and M1 through M5). The synthesis by *E. coli* of recombinant ACT::SOE3 and SDS-PAGE of the protein purified by NTA-affinity chromatography is shown in Fig. 2.

3.2. ACT::SOE3 reactions with positive control sera of women and men and MAb HA423

Next an ELISA was performed with microtiter wells individually coated with 1 μg each of ACT-P2 [17] and ACT::SOE3. Further, the recombinant protein of 111

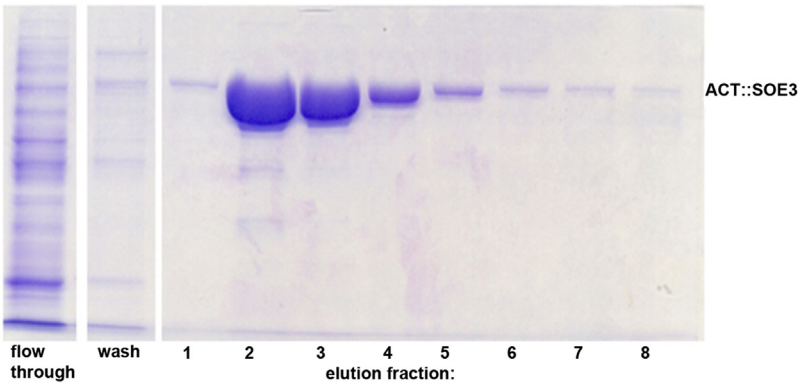

Fig. 2. Expression and purification of recombinant ACT::SOE3 by Ni-NTA affinity chromatography. SDS-PAGE and Coomassie-brilliant blue stained gels of the flow through after chromatography of lysate followed by wash buffer, as before (18), and elution fractions of purified ACT::SOE3. Preparation of lysate is as described in Materials and Methods.

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amino acids (M_r of 13.35-kDa) called AEG::SOE from an earlier study [18] was included. This AEG::SOE protein encoded six 15-mer amino acids, two each incorporating immunogenic epitopes of fructose-1,6-bisphosphate aldolase, α-enolase, and glyceraldehyde-3-phosphate dehydrogenase. It is noteworthy that these enzyme epitopes have no sequence identity with amino acid sequences of similar enzymes in databanks [18].

Table 1 presents results from a representative experiment. Pooled negative sera of women and men were compared to pooled highly seropositive sera of women (positive 1) and of men (positive 2) as well as pooled positive sera of women and men combined (positive 3) [8, 9, 10, 17, 18]. The sera were diluted 1/25, a dilution

Table 1. Representative ELISA comparing negative and positive sera* for detection of IgG antibody to ACT-P2‡, ACT::SOE3‡, and AEG::SOE‡ as targets.

	targets‡		
sera* and antibodies†	**ACT-P2**	**ACT::SOE3**	**AEG::SOE**
goat anti-human IgG†	0.055, 0.056‡‡	0.051, 0.050	0.052, 0.051
negative*	0.105, 0.110	0.109, 0.137	0.104, 0.180
positive 1*	0.510, 0.502	0.344, 0.357	0.488, 0.570
positive 2	0.364, 0.375	0.341, 0.320	0.365, 0.375
positive 3	0.490, 0.485	0.475, 0.480	0.500, 0.528
MAb HA423†	0.388, 0.386	0.060, 0.053	0.053, 0.050
MAb ALD30A†	0.056, 0.059	0.065, 0.058	0.560, 0.550
MAb L64†	0.055, 0.055	0.051, 0.052	0.051, 0.050

*Negative and positive serum are as defined recently [16, 17]. Positive 1 and 2 are pooled sera of ten women and ten men, respectively. Positive 3 is 1:1 (vol:vol) combined positive 1 and positive 2 pooled sera. Negative is pooled sera of ten each women and men sera without antibody to trichomonad proteins as determined by immunoblot assays (Materials and Methods) [16].

‡ACTP2 is a recombinant, truncated version of the parental α-actinin protein of *T. vaginalis* [7, 8, 9, 10, 13, 17]. The use of the recombinant AEG::SOE protein as a target for detection of serum antibodies to *T. vaginalis* is as recently detailed [18]. One microgram of each purified target protein was immobilized on individual wells of microtiter plates as described previously for ELISA [8, 13, 17]. ACT::SOE3 is as shown in Fig. 1B.

†The peroxidase-conjugated goat anti-human IgG (Fc fraction) is the secondary antibody used for ELISA for detecting human antibody and gave values equal to the use of 2% eBSA-PBS alone. The MAb HA423 is directed to the W12/M4 epitope of ACT-P2 and full-length α-actinin as shown in Fig. 1B [16]. MAb ALD30A is directed to the epitope of glyceraldehyde-3-phosphate that is within the 15-mer peptide of GAP3 of the recombinant AEG::SOE recently described [17]. MAb L64 is an irrelevant control antibody that reacts with a cytoplasmic protein of *T. vaginalis* [34] and is of the same IgG_1 isotype as MAb HA423 and ALD30A.

‡‡Absorbance values were obtained at 405 nm. The standard deviation was calculated for the average of all ELISA performed in quadruplicate samples. All experiments were performed using quadruplicate wells and were performed no less than on three different occasions.

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previously found to be optimal for screening sera for antibody to α-actinin. The positive sera readily reacted with each protein target, as evidenced by high absorbance values greater than the negative sera or secondary antibody alone. As expected, MAb HA423 detected ACT-P2 and was unreactive with AEG::SOE, which lacks the α-actinin epitope (Fig. 1B). Interestingly, MAb HA423 did not detect ACT:SOE3. As a control, the MAb ALD30A reactive to an epitope of AEG::SOE [18] was negative for both ACT-P2 and ACT::SOE3 and positive only with AEG::SOE. MAb L64 detects a low M_r cytoplasmic protein of *T. vaginalis* and was an additional negative control.

3.3. ACT::SOE3 is a target for detection of IgG antibody in the sera of women and men

ACT-P2 has been used as the target for screening for IgG antibody in the sera of men [8, 9, 10, 17], and since women patients with trichomonosis have serum antibody to α-actinin [15, 16], it was necessary to compare by ELISA the reactivity of sera at different dilutions between ACT::SOE3 and ACT-P2. Table 2 presents results of representative ELISA absorbance values for pooled positive sera of ten

Table 2. Antibody in pooled sera[*] of women and men detected by ELISA using ACT::SOE3[‡] and ACT-P2[‡] as targets.

sera dilutions[**]	women sera	men sera
ACT::SOE3		
negative[*] 1:25	0.110 ± 0.001[†]	0.109 ± 0.002
1:25[**]	0.512 ± 0.005	0.472 ± 0.006
1:50	0.464 ± 0.008	0.403 ± 0.005
1:100	0.373 ± 0.006	0.296 ± 0.003
1:250	0.230 ± 0.003	0.206 ± 0.004
1:500	0.185 ± 0.003	0.171 ± 0.003
ACT-P2		
negative 1:25	0.101 ± 0.001	0.105 ± 0.001
1:25[**]	0.483 ± 0.003	0.366 ± 0.005
1:50	0.423 ± 0.005	0.333 ± 0.005
1:100	0.366 ± 0.004	0.235 ± 0.004
1:250	0.242 ± 0.004	0.162 ± 0.003
1:500	0.195 ± 0.003	0.130 ± 0.001

[‡,*,†]as for Table 1.

[**]dilutions (v/v) were prepared in eBSA-PBS as per Table 1 and Materials and Methods.

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women and ten men compared with negative sera. ELISA was also performed with the 10 positive sera individually of women and of men, and ELISA values were similar to those using pooled sera (data not shown). That the 1/250 and 1/500 dilutions of the sera gave higher absorbance readings than negative controls suggests that these highly positive sera have high-titered IgG toward the immunogenic α-actinin.

As the 1/25 dilution of sera and the 240-min incubation period of serum with ACT-P2 have been used in earlier studies to examine for seropositivity [8, 9, 10, 17, 18], the extent of reactivity at 60 min and 240 min was compared. As shown in Table 3, the pooled positive sera of women and men as well as the mouse antiserum to α-actinin were highly reactive at both time points when compared to negative sera and secondary antibody alone. As above, ELISA was performed on the ten individual positive sera of either women or men, and each positive serum gave high absorbance values at the dilutions as shown here.

3.4. Testing individual sera with P/N values of 0 to 4+ at different dilutions

Finally, to test whether ACT::SOE3 was equal to ACT-P2 as a target for serum antibody, ELISA was done using numerous sera of women and men that have been used in earlier reports (8–10, 17) and that have assigned P/N scores of 0, 1+, 2+, 3 + and 4+ (Materials and Methods). Only 3+ and 4+ sera have antibody to α-actinin and other trichomonad proteins by immunoblot assays [17]. Sera with P/N scores of 0, 1+, and 2+ scores represent background reactivities and have no antibody to trichomonad proteins [17]. In addition serum dilutions of 1/25, 1/50, 1/100, and 1/250 were tested.

Table 3. ELISA performed at different incubation times with ACT-P2[‡] and ACT::SOE3[‡] proteins with pooled negative and positive combined sera[*] of women and men.

	ACT-P2		ACT::SOE3	
antibodies[‡]	**60**[†]	**240**[†]	**60**	**240**
goat anti-human IgG	0.050 ± 0.001[‡]	0.051 ± 0.001	0.049 ± 0.001	0.051 ± 0.001
negative[*]	0.090 ± 0.001	0.106 ± 0.001	0.076 ± 0.001	0.088 ± 0.00
positive[*]	0.425 ± 0.004	0.430 ± 0.005	0.462 ± 0.007	0.445 ± 0.00
mouse anti-SOE3 serum	0.528 ± 0.006	0.480 ± 0.005	0.592 ± 0.008	0.558 ± 0.008

[‡]as for Table 1.

[*]pooled combined negative and positive sera of ten women and ten men were derived from the sera used for Table 3. Sera were diluted 1:25 in eBSA-PBS.

[†]60 and 240 refer to minutes of incubation with the various antibodies.

http://dx.doi.org/10.1016/j.heliyon.2017.e00237

Table 4 presents ELISA results for sera of women and men. Importantly, a total of 14 sera of women (W) and men (M) were tested for each score of 0, 1+, 2+, 3+, and 4+, and the findings were equivalent to those summarized in Table 4. As expected, the 0, 1+ and 2+ sera remained negative regardless of serum dilution. Sera with values of 3+ were positive at the 1/25 dilution but became negative a higher dilutions. On the other hand, for both ACT-P2 and ACT::SOE3, the 4+ highly positive sera diluted 1:50 remained positive at 3+ and 4+. At the 1/100 dilution, four sera remained positive for ACT-P2, and two were positive for ACT::SOE3. These data show that the 1/25 dilution is optimal for detection of serum antibody to these target proteins. Equally noteworthy is that the data indicate that 4 + positive sera of both women and men have increased titers of IgG compared to 3 + positive sera to α-actinin.

Table 4. Testing by ELISA of representative women and men sera of different positive to negative (P/N)[†] scores at different dilutions.

sera dilution	**A. P/N serum designations against ACT-P2[‡]:**													
	0[†]	**0**	**1+**	**1+**	**2+**	**2+**	**3+**	**3+**	**4+**	**4+**	**4+**	**4+**	**4+**	**4+**
	W[*]	**M[*]**	**W**	**M**	**W**	**M**	**W**	**M**	**W**	**W**	**W**	**M**	**M**	**M**
1:25	0	0	1+	1+	2+	2+	3+	3+	4+	4+	4+	4+	4+	4+
1:50	0	0	0	0	1+	1+	2+	2+	4+	3+	4+	2+	4+	3
1:100	0	0	0	0	0	1+	1+	1+	4+	3+	3+	2+	3+	0
1:200	0	0	0	0	0	0	0	1+	2+	1+	1+	1+	2+	0
	B. P/N serum designations against ACT::SOE[‡]:													
	0[†]	0	1+	1+	2+	2+	3+	3+	4+	4+	4+	4+	4+	4+
	W[*]	M[*]	W	M	W	M	W	M	W	W	W	M	M	M
1:25	0	0	1+	1+	2+	2+	3+	3+	4+	4+	4+	4+	4+	4+
1:50	0	0	0	0	1+	1+	2+	2+	4+	3+	3+	3+	3+	3+
1:100	0	0	0	0	0	0	1+	1+	4+	2+	3+	2+	2+	1+
1:200	0	0	0	0	0	0	0	1+	3+	1+	2+	1+	0	0

[†]Relative P/N values were calculated as described before [7, 8, 9, 10] and as presented in Materials and Methods. Serum designations refers to the 0, 1+, 2+, 3+, and 4+ scoring of the sera following ELISA using standards as controls [7, 8, 9, 10]. This scoring enable the examination of dilutions on the relative reactivities against the recombinant ACT-P2 and ACT::SOE3 proteins.

[‡]as described in Table 3.

[*]abbreviations: W, pooled sera of women; M, pooled sera of men, as described in Table 1.

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3.5. ELISA reactions by new MAbs to ACT::SOE3

Because of the lack of binding by MAb HA423 to ACT::SOE3, new MAbs were generated using mice immunized with α-actinin. Table 5 shows the ELISA results on ACT::SOE3 using the new MAbs as probe. The MAbs 15B and 74B gave high absorbance values with both ACT-P2 and ACT::SOE3. MAb 67B reacted strongly with ACT::SOE3 and gave absorbance readings for ACT-P2 above the negative control MAb L64. Further, MAb 68B detected ACT::SOE3 but gave values lower than that seen for ACT-P2. The highly immunogenic nature of α-actinin is shown by high absorbance values obtained using mouse anti-α-actinin serum.

3.6. Identification of ACT::SOE3 epitopes detected by MAbs to ACT::SOE3

The epitope amino acid sequences detected by the new MAbs to ACT::SOE3 are illustrated in Fig. 1B (labeled and underlined above the amino acid sequence): MAb 74B, 253-**I**QKQ**I**DE**L**KNT-263; MAb 15B, 282-KADDYEKT-289; MAb 68B, 412-DK**V**EQK**L**HEAE-422; and MAb 67B, 616-AQ**V**EEN**L**A-622. The peptides with these epitope amino acid sequences were soluble in aqueous solution. Some epitopes had hydrophobic residues (bold, underlined) that did not affect their solubility. Further, most amino acids within ten amino acids adjacent to the epitopes were hydrophilic and consistent with eliciting antibody following

Table 5. ELISA reactions of MAbs* with recombinant ACT-P2† and ACT::SOE3† proteins as targets.

	targets	
antibodies	**ACTP2**	**ACT::SOE3**
L64 control*	0.055 ± 0.001‡	0.051 ± 0.001
HA423*	0.455 ± 0.004	0.050 ± 0.001
15B*	0.431 ± 0.003	0.427 ± 0.003
67B	0.111 ± 0.003	0.352 ± 0.004
68B	0.428 ± 0.005	0.188 ± 0.001
74B	0.483 ± 0.004	0.488 ± 0.005
mouse anti-α-actinin serum‡	0.527 ± 0.006	0.595 ± 0.007

*The MAbs labeled 15B, 67B, 68B, and 74B were generated as described in Materials and Methods and are directed to different epitopes as shown in Fig. 1B. The MAbs L64 and HA423 are as for Table 1. As expected, the MAbs were unreactive with the purified recombinant AEG::SOE protein (Table 1) [17]. All MAbs are IgG_1 isotype.

†ACT-P2 and ACT::SOE3 and absorbance values are as described in Table 1.

‡The mouse anti-α-actinin serum was from mice immunized with purified α-actinin protein prior to generation of hybridomas producing the new MAbs (Materials and Methods).

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immunization of mice with purified protein. The MAbs recognized neither the epitope detected by the MAb HA423 nor the other epitopes found previously to react with highly seropositive sera of women and men (Fig. 1B). The absence of detection of other peptides during epitope-mapping experiments provided internal controls to show specificity in reactions of MAbs with epitopes.

4. Discussion

Importantly, the 13 epitopes of the α-actinin protein of *T. vaginalis* have no amino acid sequence identity to the four human α-actinin 1 homologs, none of which possess the epitopes detected by positive sera of women and men [17]. Equally noteworthy is the fact that the gene and protein are not found among other microbial pathogens, including organisms that cause other STIs, as discussed recently [17]. Further, the trichomonad α-actinin has little sequence identity to proteins of other eukaryotes, including *Tritrichomonas suis*, and the yeasts *Candida albicans* and *Saccharomyces cerevisiae* [17]. Equally noteworthy, this laboratory has in the past examined sera and vaginal washes of both patients with trichomonosis and controls with other infections, such as candidiasis, urinary tract infections, syphilis, chlamydia, and group B streptococci [26]. Importantly, only patients with trichomonosis had antibody to *T. vaginalis* as evidenced by ELISA using either whole cells or aqueous extracts [27]. Thus, it is not surprising that this protein may be a highly specific target for serodiagnosis of at-risk individuals. Because the presence of antibody to these epitopes is indicative of exposure to this STI, such antibody to this protein is useful for identifying patients with trichomonosis. Although ACT-P2 is used for screening sera for seropositivity to *T. vaginalis* [8, 9, 10, 17], there is a need for an alternative version of α-actinin given the low amounts of ACT-P2 synthesized in *E. coli*, which is a significant limiting factor for large scale production and for screening large populations of individuals at-risk for trichomonosis.

An approach that was published recently [18] was used in this study. In this earlier report, a recombinant protein called AEG::SOE was synthesized, and the protein was comprised of six epitopes, two of which were derived from three different proteins. Also important was the fact that these six epitopes had no sequence identity with other proteins in databanks. Here ACT::SOE3 recombinant protein was produced that had 7 of the 13 epitopes within the carboxy terminus of α-actinin and possesses the 5 epitopes of the ACT-P2 protein that are detected by positive sera of both women and men [8, 9, 10, 17]. ACT::SOE3 was synthesized and purified in large amounts and, importantly, possessed the same high specificity and sensitivity as ACT-P2 in being recognized by antibody of positive sera of women and men. Therefore, these data suggest that ACT::SOE3 may be a target for a rapid, accurate, and cost-effective POC test. Such a test would allow for identification of individuals either previously exposed to or with active infection

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caused by *T. vaginalis*. This seems warranted given the highly prevalent nature of trichomonosis and the adverse consequences to the health of patients [1, 2, 3, 4, 5, 6]. Another reason for moving toward a serum-based diagnostic is the possible relation of IgG seroconversion to *T. vaginalis* and PCa development and progression [8, 9, 10, 11].

A barrier to development of a serodiagnostic to *T. vaginalis* is the idea that serum antibody may be long-lasting and, therefore, would not be indicative of active infection and/or re-infection. Of interest would be the time after infection a host immunoglobulin response is seen and, following diagnosis and cure, the time that levels of antibody ablate. There are earlier reports from this laboratory that showed a short-lived nature of both serum and vaginal IgG to *T. vaginalis* cysteine proteinases after drug treatment of patients [26, 28], and these were the first reports to suggest the feasibility of a serodiagnostic test. On the other hand serum IgG to a prominent high M_r protein of 230-kDa was still detectable in vaginal washes of patients with trichomonosis at 4 weeks post-cure [29]. All this notwithstanding, for both women and men, there is a lack of data on the kinetics and temporal nature and extent of serum antibody responses to specific *T. vaginalis* proteins, such as α-actinin, following infection. It is likely that answers to these and other immunological questions may be obtained in clinical studies on patients with trichomonosis if a serum-antibody POC test for this STI is available.

In this report it is shown that highly seropositive 4+ sera of women and men have higher titers of IgG to α-actinin, as indicated by others [15, 16]. The presence of serum IgG antibody to epitopes unique to only trichomonad proteins, such as α-actinin and immunogenic enzymes (fructose-1,6-diphosphate aldolase, α-enolase, and glyceraldehyde-3-phosphate) [17, 18], reinforces the legitimacy of the approach taken here for identifying specific and novel targets that have no sequence identities to proteins of microbial pathogens, including those that cause other STIs [8, 9, 10, 17, 18].

It was surprising that the MAb HA423 reactive to α-actinin and ACT-P2 [17] did not detect ACT::SOE3, especially since ACT::SOE3 has the same epitope, as confirmed by nucleic acid sequencing of the DNA encoding the protein (Fig. 1B). This concern prompted us to generate new MAbs to α-actinin, which recognized ACT::SOE3. Equally surprising was the differential nature of immunoreactivities by these MAbs depending on ELISA. One explanation for this may be that ACT-P2 and ACT::SOE3 have altered conformations for epitope accessibility, perhaps owing to their different M_r. Although post-translational modification of the proteins by recombinant *E. coli* is possible, there is no evidence of this occurring.

It is noteworthy that, since these novel SOE proteins are comprised of immunogenic epitopes, the proteins may represent vaccine candidates. Reports on the availability of whole cell and lysate vaccines for the fetal-wasting bovine

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trichomonad *Tt. foetus* have suggested the possibility of a vaccine for *T. vaginalis* [30, 31]. As mentioned above, both serum and vaginal antibody responses among patients with trichomonosis have been observed [26, 27, 28], although there is no evidence of protection. For *T. vaginalis* whole cell vaccines may not be a viable option as numerous host serum proteins that play a role in pathogenesis associate with the parasite surface [22, 23, 32, 33, 34]. Further, trichomonads place metabolic enzymes on the surface that act as ligands for binding host pathogenicity proteins, such as plasminogen [22, 34], and it has been shown that antibody is directed to epitopes of these enzymes that have sequence identities with the host enzymes [18]. Thus, vaccinating with organisms coated with host proteins and metabolic enzymes may result in the induction of anti-host protein responses. Indeed, human serum antibody responses to α–enolase have been shown to cross-react with host tissues [35]. Therefore, if a future vaccine for this STI is to be considered, then it is plausible that the approach described here and recently [18] may lead to effective subunit vaccine candidates.

In conclusion, it has been established here and recently [17, 18] that the method to identify epitopes of trichomonad surface and non-surface protein immunogens can lead to novel recombinant SOE proteins. It can be envisioned that SOE proteins in the future may be produced that combine, for example, AEG::SOE [18] and ACT::SOE3 constructs. Such hybrid proteins may also include unique epitopes identified in the future to other trichomonad protein immunogens. Such a novel multi-protein hybrid SOE with exceptional specificity may function as an ideal POC diagnostic immunotarget and may also be vaccine candidates.

Declarations

Author contribution statement

John F. Alderete: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

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