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Role of α-Actinin 2 in Cytoadherence and Cytotoxicity of *Trichomonas vaginalis*

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Trichomonas vaginalis is a pathogen that triggers severe immune responses in hosts. *T. vaginalis* α-actinin 2 (Tvα-actinin 2) has been used to diagnose trichomoniasis. Tvα-actinin 2 was dissected into three parts; the N-terminal, central, and C-terminal portions of the protein (#1, #2, and #3, respectively). Western blot of these Tvα-actinin 2 proteins with pooled patients' sera indicated that #2 and #3, but not #1, reacted with those sera. Immunofluorescence assays of two different forms of *T. vaginalis* (trophozoites and amoeboid forms), using anti-Tvα-actinin 2 antibodies, showed localization of Tvα-actinin 2 close to the plasma membranes of the amoeboid form. Fractionation experiments indicated the presence of Tvα-actinin 2 in cytoplasmic, membrane, and secreted proteins of *T. vaginalis*. Binding of fluorescence-labeled *Trichomonas* to vaginal epithelial cells and prostate cells was decreased in the antibody blocking experiment using anti-Tvα-actinin 2 antibodies. Pretreatment of *T. vaginalis* with anti-rTvα-actinin 2 antibodies also resulted in reduction in its cytotoxicity. Flow cytometry, ligand-binding immunoblotting assay, and observation by fluorescence microscopy were used to detect the binding of recombinant Tvα-actinin 2 to human epithelial cell lines. Specifically, the truncated N-terminal portion of Tvα-actinin 2, Tvα-actinin 2 #1, was shown to bind directly to vaginal epithelial cells. These data suggest that α-actinin 2 is one of the virulence factors responsible for the pathogenesis of *T. vaginalis* by serving as an adhesin to the host cells.

Keywords: *Trichomonas vaginalis*, α-actinin 2, cytoadherence, cytotoxicity

Introduction

Trichomonas vaginalis is known to be an important pathogen causing the most prevalent non-viral sexually transmitted disease worldwide and has been used for the study of host-parasite co-rrelationship [1]. Host immunogens and protozoan factors cause a distinct interaction during *T. vaginalis* infection in the vaginal tract. There are a few studies about the host-parasite co-rrelationship, especially trophozoites of *T. vaginalis* interacting with mucin [2], and then contact with vaginal epithelial cells (VECs) at iron-regulated surface proteins of *T. vaginalis* [3]. Adherence of the host epithelium by *T. vaginalis* also occurs via a specific interaction with the extracellular matrix basement membrane glycoproteins [4].

In the pathogenesis of *T. vaginalis*, iron is one of the virulence factors that modulates diverse aspects of *T. vaginalis*, including cytoadherence, metabolic activity, and resistance to complement lysis [5, 6]. *T. vaginalis* has ways of acquiring iron from lactoferrin [3]. *T. vaginalis* takes up lactoferrin via specific receptors, which trigger iron accumulation and activity of pyruvate ferridoxin oxidoreductase [7]. Iron availability from lactoferrin caused enhanced binding to the host epithelial cells by increased amount of adhesins [8]. *T. vaginalis* also interacts with erythrocytes, which provide both lipid and iron for the parasite [9], and the action of two adhesion proteins, AP51 and AP65, triggers iron acquisition from hemoglobin by *T. vaginalis* [10]. Cultivation of *T. vaginalis* in iron-depleted medium shows no pathology, but could cause subcutaneous abscesses in mice [11].

Adhesion of fluorescence-labeled *T. vaginalis* to various

host cells has been observed by fluorescence microscopy [12]. Studies using erythrocytes as target cells demonstrated that hemolysis by *T. vaginalis* is mediated by perforins and the cytoskeleton [13]. Iron-regulated surface proteins of *T. vaginalis* (AP65, AP51, AP33, and AP23) have been postulated to play essential roles in the adherence of *T. vaginalis* to VECs [3]. Interestingly, a mutant *T. vaginalis* lacking its lipoglycan, TvLG, indicated that this surface molecule also plays a role in attachment of the parasite to host epithelial cells via specific interactions with galectin-1 of the host cell [14]. An evaluation of the adherence and cytotoxicity of 26 *T. vaginalis* strains to prostate and ectocervical epithelia, as well as erythrocytes, demonstrated variable pathogenicity among different *T. vagnialis* strains, but all strains showed contact-dependent cytolysis [15].

Among the immunogenic proteins of *T. vaginalis* identified in the previous investigation [16], *T. vaginalis* α-actinin 2 (Tvα-actinin 2) had further been studied, showing its ability to modulate host immune responses, using mouse dendritic cells as well as human vaginal and prostate epithelial cells [17]. Higher serological responses against Tvα-actinin 2 in prostate cancer cases compared with the control cases indicated a relationship between anti-*T. vaginalis* seropositivity and risk for prostate cancer in men [18, 19]. Reactive epitopes of α-actinin 2 have been finely mapped using sera from both male and female trichomoniasis patients [20]. Using a custom spot membrane loaded with overlapped 11-mer peptides covering Tvα-actinin 2, 13 epitopes were found to be scattered in α-actinin 2 when tested against women's sera, whereas only 4 of the 15 epitopes in the C-terminal portion of α-actinin 2 were

reactive to men's sera.

In this study, the role of Tvα-actinin 2 in parasite adherence to the prostate cell line RWPE-1 and to VECs was examined by direct binding assays, and the domain of Tvα-actinin 2 responsible for cytoadherence was also defined.

Materials and Methods

Parasite and In Vitro Transformation

T. vaginalis KT4 [21] was grown in Diamond's Trypticase Yeast extract Maltose (TYM) medium [22] with 10% horse serum (Gibco BRL, Germany) at 37ºC in 5% CO_2, and transferred into fresh medium daily. *T. vaginalis* trophozoites were converted into the amoeboid form by incubating them in fibronectin-coated plates (3 μg/ml) for 30 min.

Bacterial and Human Cell Cultures

Plasmids used for molecular manipulations are listed in Table 1. *Escherichia coli* BL21 (DE3) cells were grown at 37ºC in Luria-Bertani medium (0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7.5) supplemented with 100 μg/ml ampicillin (Sigma-Aldrich, USA) in order to maintain the plasmids. Medium components were obtained from BD-Difco (USA).

Human VECs (ATCC CRL-2614; American Type Culture Collection, USA) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Human prostatic epithelial cells (RWPE-1, ATCC CRL-11609; American Type Culture Collection) were grown using Keratinocyte Serum Free Medium (K-SFM; Gibco BRL) containing epidermal growth factor (0.2 ng/ml), bovine pituitary extract (0.05 mg/ml), FBS (5%), penicillin (100 U/ml), and streptomycin (100 μg/ml). These human epithelial cells were

Table 1. Primers and plasmids used in this study.

Name	Relevant characteristics[a]	Source
Primers		
α-actinin2-1F	CG<u>GAATTC</u>GATGTCTGTTCGTCGTGAAGG (EcoRI)	
α-actinin2-1R	CGG<u>CTCGAG</u>TGCGAAAGCCTTCTCGCAGGCG (XhoI)	
α-actinin2-2F	ACGC<u>GTCGAC</u>GCTTGCAAGGAACTCGGCAT (SalI)	
α-actinin2-2R	TTTTTT<u>GCGGCCGC</u>CTTGTAGTTGAGCTTGAATT (NotI)	
α-actinin2-3F	ACGC<u>GTCGAC</u>GTCACATACACATACTCTGA (SalI)	
α-actinin2-3R	TTTTTT<u>GCGGCCGC</u>TTGGTTGTAAAGTGAGT (NotI)	
Plasmids		
pET21b	Expression vector for a histidine-tagged protein	Novagen
pETα-actinin2	pET21b, 2,786-bp α-actinin 2 gene	[30]
pETact1	pET21b, 591-bp N-terminal portion of α-actinin 2 gene	This study
pETact2	pET21b, 1,358-bp central portion of -actinin 2 gene	This study
pETact3	pET21b, 843-bp C-terminal portion of α-actinin 2 gene	This study

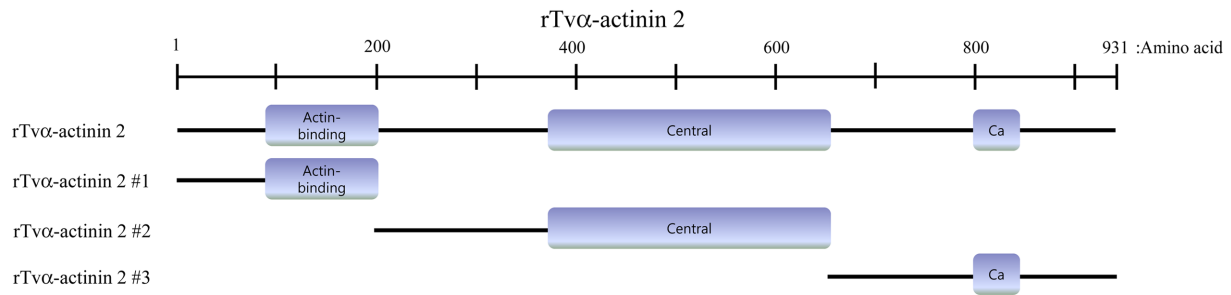[a]Underlined sequences indicate restriction sites.

Fig. 1. rTvα-actinin 2 protein of *T. vaginalis* and its truncated derivatives used in this study.
Four different rTvα-actinin 2 polypeptides were prepared containing the complete Tvα-actinin 2 open reading frame (rTvα-actinin 2), and the N-terminal (rTvα-actinin 2 #1), central (rTvα-actinin 2 #2), and C-terminal (rTvα-actinin 2 #3) regions. The three boxes above these numbers represent the actin-binding domain, central antigenic region, and Ca^{2+}-binding domain of Tvα-actinin 2 (Addis *et al.* [25]).

cultured to 80−90% confluence for use in the adhesion and cytotoxicity assays at 37ºC/5% CO_2.

Expression and Purification of Recombinant Tvα-Actinin 2 Polypeptides

The DNA fragment encoding Tvα-actinin 2 was divided into three portions (Fig. 1). The 5′-region of the Tvα-actinin 2 gene (591 bp) was made from the genome of *T. vaginalis* KT4 by PCR using the primers α-actinin2-1F and α-actinin2-1R (Table 1), and the central region of the Tvα-actinin 2 gene (1,358 bp) was amplified with another set of primers, α-actinin2-2F and α-actinin2-2R. The 3′-region of the Tvα-actinin 2 gene was amplified as an 843-bp DNA fragment, using the primers α-actinin2-3F and α-actinin2-3R. Each of the resulting Tvα-actinin 2 DNA fragments was cloned into pET21b (Novagen, Germany), yielding overexpression plasmids for the truncated α-actinin 2 proteins (pETact1, pETact2, and pETact3).

These truncated Tvα-actinin 2 proteins were expressed as histidine-tagged form in *E. coli* BL21 (DE3) with 1 mM isopropyl β-D-thiogalactoside, and then purified using nickel-nitrilotriacetic acid affinity chromatography as described by the manufacturer (Qiagen, Germany). Full-length recombinant Tvα-actinin 2 (rTvα-actinin 2) was prepared as previously described [17].

Western Blot Analysis

These rTvα-actinin 2 polypeptides (100 ng) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a nitrocellulose filter (Millipore, USA). The membrane was incubated with pooled sera (1:200 dilution) derived from trichomoniasis patients in a blocking solution (Tris-buffered saline (TBS; 50 mM Tris-Cl and 150 mM NaCl, pH 7.5), 5% skim milk, and 0.05% Tween 20), and incubated with alkaline phosphatase (AP)-conjugated anti-human IgG (Sigma-Aldrich). As a control, these proteins were also reacted with normal sera (1:200 dilution) derived from uninfected individuals. The immunoreactive protein was visualized using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate system (Promega, USA).

Immunofluorescence Assays (IFA)

Trophozoites and amoeboid forms of *T. vaginalis* were adhered to glass slides, and then incubated with 100% methanol at −20ºC for 10 min. After 1 h incubation in a blocking buffer (phosphate-buffered saline (PBS: 1.7 mM KCl, 137 mM NaCl, 2 mM KH_2PO_4, and 10 mM Na_2HPO_4, pH 7.3), 5% goat serum, and 3% bovine serum albumin (BSA)), the cells were reacted with anti-Tvα-actinin 2 polyclonal antibodies (1:800 dilution) [17] at 4°C. Following three washes with PBS, the cells were reacted with Alexa-Fluora 555-conjugated anti-rat IgG (1:400 dilution; Life Technologies, USA) at 37°C for 2 h. The slides were washed with PBS and mounted with 1.5 µg/ml 4′6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) anti-fade mounting medium (VECTASHIELD; Vector Laboratories, USA). The cells were then observed under an Axiovert 200 fluorescent microscope or LSM710 confocal microscopy (Carl Zeiss, Germany).

Protein Fractionation from Amoeboid Form of *T. vaginalis*

Cytoplasmic and membrane fractions of the *T. vaginalis* were prepared using the subcellular Proteome Extraction Kit (Merck, Germany). One microgram of protein samples was separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes and blocked with the blocking solution for 1 h at room temperature. The membranes were treated with anti-Tvα-actinin 2 antibodies at 4°C overnight, and reacted with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling, USA). An Enhanced Chemiluminescence System (ECL; GE Healthcare Life Science, UK) was used for signal detection.

Labeling of *T. vaginalis* and Blocking Experiments

T. vaginalis cells were stained with the fluorescent Cell Tracker Orange CMTMR dye (Invitrogen, USA). Briefly, *T. vaginalis* cells (1×10^6 cells/ml) were incubated in pre-warmed cell tracker dye solution (0.5 µM in TYM medium without serum) for 30 min at 37ºC. The cells were harvested by centrifugation, and then incubated in TYM medium supplemented with 10% FBS at 37ºC for 30 min. The parasites were then washed twice: once with TYM medium and once with complete K-SFM.

Prior to cytoadherence assays, *T. vaginalis* trophozoites (1×10^8) were pre-incubated with anti-Tvα-actinin 2 antibodies for 1 h at a concentration of 30 μg/ml. As a control, pre-immune rat serum was used to monitor the nonspecific effect of serum on the cytoadherence of *T. vaginalis* to the host cells.

Cytotoxicity Assays and Blocking Experiments

VECs (3×10^4 cells/well) inoculated into 96-well plates were cultured to confluence at 37ºC/5% CO_2. *T. vaginalis* cells (3×10^5 cells/well) at log phase were added to the confluent VECs, and then incubated for 4 h at 37ºC/5% CO_2. Lysis of VECs was measured by determining the level of released lactate dehydrogenase (LDH) using the Cytotox[96] Non-Radioactive Cytotoxicity Kit (Promega). The level of released LDH was determined as follows: [(OD$_{490}$ of experimental LDH – OD$_{490}$ of spontaneous LDH)/(OD$_{490}$ of maximum LDH – OD$_{490}$ of spontaneous LDH)] × 100. The spontaneous LDH was the activity of LDH released from untreated VECs, whereas the maximum LDH was the LDH activity caused from complete lysis of VECs due to Triton X-100 treatment.

To examine the role of Tvα-actinin 2 in cytotoxicity of *T. vaginalis* to VECs, *T. vaginalis* trophozoites (1×10^8) were pre-incubated with anti-Tvα-actinin 2 antibodies for 1 h at a concentration of 30 μg/ml. In order to monitor the nonspecific effect of serum on the cytotoxicity of *T. vaginalis* to VECs, pre-immune rat serum was used as a control.

Labeling of rTvα-Actinin 2

To estimate the binding of rTvα-actinin 2 to the host cells, Tvα-actinin 2 proteins were labeled with a fluorescent probe using an Alexa-Fluor 555 Microscale Protein Labeling Kit (A30007; Molecular Probes, USA). One hundred microgram of recombinant polypeptides was reacted with Alexa-Fluor 555 succinimidyl ester, and then passed through the spin-filters supplied with the kit to remove unincorporated probe. Fluorescence of the proteins was measured at 280 and 555 nm in order to check labeling of the protein as directed by the manufacturer. Alexa-Fluor 555-labeled α-actinin 2 (100 μg/ml) was stored at –20ºC before being used for the binding assays.

Fluorescence-Activated Cell Sorter (FACS) Analysis

FACS analysis was carried out by using a BD FACS Calibur flow cytometer (BD Biosciences, USA). For the adhesion assay, Cell Tracker Orange CMTMR-stained *T. vaginalis* can be detected in excitation and emission spectrum peak wavelengths of approximately 488 and 565 nm. Additionally, Alexa-fluor 555-labeled proteins have fluorescence excitation and emission maxima of 555 and 565 nm. To detect CMTMR-stained parasites or Alexa-fluor 555 dye-labeled proteins in the presence of VECs or RWPE-1, voltage sets were made based on the auto-fluorescent signal of the VECs and RWPE-1. Finally, FACS data analyses were performed using FlowJo software (FlowJo LLC, USA).

Fluorescence Microscopy

rTvα-actinin 2 proteins were conjugated with a fluorescent probe using an Alexa-Fluor 555 Microscale Protein Labeling Kit as described above. VEC cells (2×10^4 cells per well) were grown on glass coverslips in 24-well plates for 24 h. The cells were incubated with 3% BSA for 30 min in DMEM without serum followed by a 1 h treatment with 10 μg/ml Alexa-Fluor 555-labeled polypeptides at 37ºC, and then fixed in 3.7% paraformaldehyde/PBS at room temperature for 30 min. After being washed with PBS three times, the cells were observed under an Axiovert 200 fluorescence microscope.

Ligand-Binding Immunoblotting Assay

Each well of a 24-well culture plate was seeded with VECs (2×10^5), and incubated overnight at 37ºC in the presence of 5% CO_2. After removing the medium and washing twice with DMEM, the cells were incubated in 1 ml of serum-free DMEM containing 10 μg of the rTvα-actinin 2 proteins as previously described [23]. After five washes with DMEM, the cells were disrupted with 150 μl of lysis buffer (10 mM Tris-HCl, pH 8.4, and 0.8 % SDS) and boiled for 10 min. To examine whether rTvα-actinin 2 was stably bound to VEC monolayers, a portion of the lysate (15 μg of protein) was subjected to SDS-PAGE and then transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat dry milk in TBS with 0.05% Tween 20 for 1 h at room temperature, treated with anti-Tvα-actinin 2 antibodies at 4ºC overnight, and subsequently reacted with AP-conjugated anti-rat IgG (Sigma-Aldrich).

In the case of truncated rTvα-actinin 2 proteins, #1, #2, and #3, the membranes were treated with anti-histidine Abs (IG Therapy, Korea) instead of anti-rTvα-actinin 2 antibodies. They were then incubated with HRP-conjugated secondary antibodies (Cell Signaling), and the immunoreactive bands were visualized using an ECL System (GE Healthcare Life Science). To monitor the protein level of VECs in each treatment, the VEC lysates were monitored for their β-actin levels using anti-human β-actin antibodies (Millipore).

Statistical Analyses

Results are indicated as the means ± standard deviations obtained from three independent experiments. Data were analyzed by pair-wise comparison using the Student's *t*-test (SYSTAT program, SigmaPlot ver. 10; Systat Software Inc., USA). Differences were considered statistically meaningful if the *p*-values were <0.05.

Results

Reactivity of Recombinant Tvα-Actinin 2 Proteins against Patients' Sera

T. vaginalis α-actinin 2 was previously identified as one of the immunogens recognized by sera of *T. vaginalis*-infected women [24]. Full-length rTvα-actinin 2 protein was expressed in *E. coli* and purified via Ni^{2+}-NTA affinity chromatography. ELISA was performed with 10 female patients' sera (data not shown). Nine of 10 sera were reactive

A   Silver staining
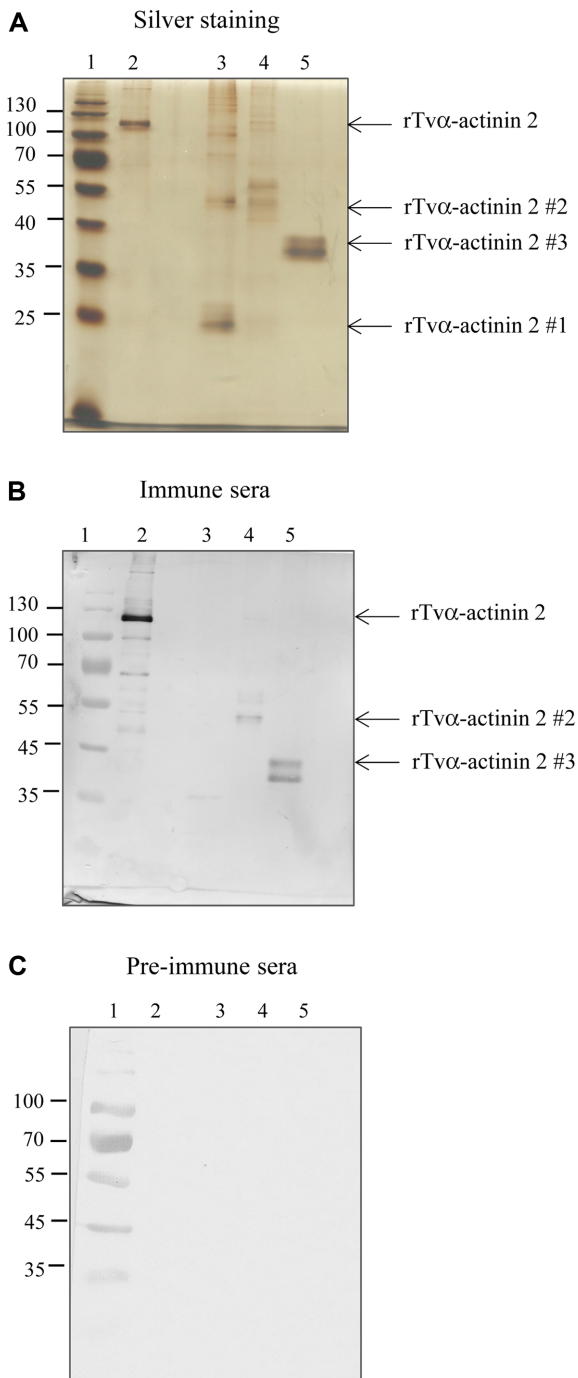

B   Immune sera

C   Pre-immune sera

Fig. 2. Reactivity of rTvα-actinin 2 polypeptides to a pool of immunopositive sera.

(**A**) A silver-stained SDS-PAGE gel of rTvα-actinin 2 proteins. (**B**) Western blot analysis of these Tvα-actinin 2 proteins with sera derived from patients with trichomoniasis. (**C**) Western blot analysis of these Tvα-actinin 2 proteins with a negative control serum showing no reactivity to rTvα-actinin 2 by ELISA. Lane1: protein size marker; lane 2: full-length rTvα-actinin 2; lane 3: rTvα-actinin 2 #1; lane 4: rTvα-actinin 2 #2; lane 5: rTvα-actinin 2 #3.

to this recombinant protein. In the subsequent experiment, a pool of nine sera was used for immunopositive sera, and the human serum showing no reactivity to rTvα-actinin 2 served as a negative control (Fig. 2).

Analysis of the deduced amino acid sequence of Tvα-actinin 2 revealed that this protein is composed of an N-terminal actin-binding domain, a central domain rich in α-helices, and a C-terminal region with Ca^{2+}-binding activity [25]. Three truncated recombinant Tvα-actinin 2 proteins were prepared (Fig. 2A) and used to define the protein regions reactive to patients' sera. Western blot analysis of these partial rTvα–actinin 2 proteins with the pooled patients' sera indicated that the central (#2) and C-terminal portions (#3), but not the N-terminal part (#1) of Tvα-actinin 2, reacted with patients' sera (Fig. 2B). In contrast, these proteins did not show any reactivity with the pre-immune serum used as a negative control (Fig. 2C).

Localization of Tvα-Actinin 2 in *T. vaginalis*

Using antibodies specific to rTvα-actinin 2, IFAs were performed to examine the intracellular location of Tvα-actinin 2 in two different forms of *T. vaginalis*, trophozoites and amoebic form (Figs. 3A and 3B/3C, respectively). Tvα-actinin 2 was mainly detected in the cytoplasm of *T. vaginalis* trophozoites. A conformational change into the amoeboid form occurred upon contact of *T. vaginalis* trophozoites with fibronectin. Interestingly, the amoebic form of *T. vaginalis* showed localization of Tvα-actinin 2 near the plasma membranes. Observation of the amoebic *T. vaginalis* at higher magnification indicated that Tvα-actinin 2 also locates near the membranes.

In the subsequent experiment, fibronectin-induced amoebic *T. vaginalis* cells were fractionated into cytoplasmic, membrane, and secreted proteins, and then analyzed by western blot analysis using anti-Tvα-actinin 2 antibodies (Fig. 3D). Interestingly, this protein was found in all the three fractions of *T. vaginalis*, indicating that Tvα-actinin 2 is secreted or present at the surface of *T. vaginalis*.

Additional IFAs were performed to observe the location of Tvα-actinin 2 when *T. vaginalis* was incubated with VECs or RWPE-1 (Figs. 3E and 3F, respectively). Most *T. vaginalis* cells were in the amoeboid form upon incubation with both the vaginal and prostate epithelial cell lines and, under these conditions, IFAs of *T. vaginalis* with anti-rTvα-actinin 2 antibodies showed localization of Tvα-actinin 2 near its plasma membrane.

Binding of *T. vaginalis* to VEC and RWPE-1 Cells

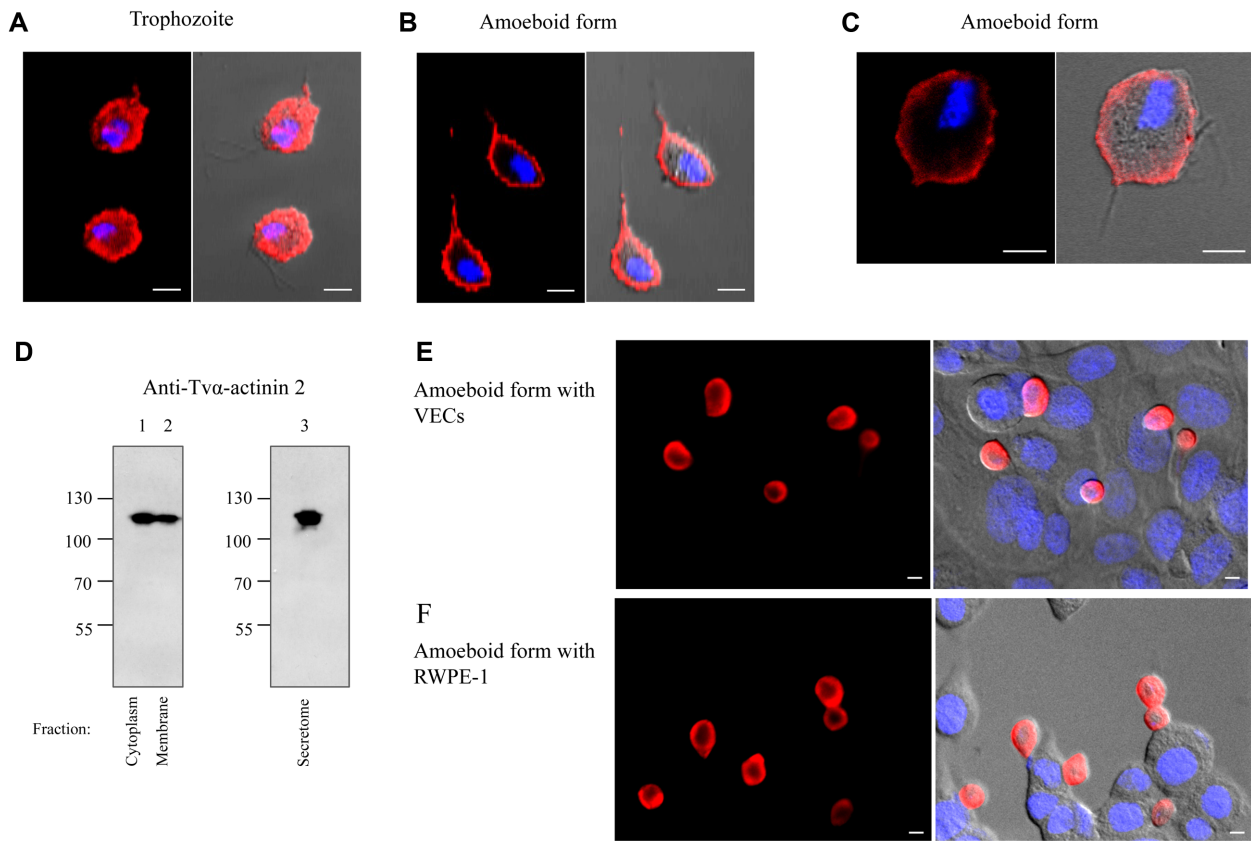The ability of the pathogenic trichomonads to adhere to

Fig. 3. Localization of Tvα-actinin 2 in *T. vaginalis*.

Using rTvα-actinin 2 antibodies, the localization of Tvα-actinin 2 was examined in *T. vaginalis* trophozoites (**A**), amoeboid forms (**B**, **C**), *T. vaginalis* incubated with vaginal epithelial cells (VECs) (**E**), and *T. vaginalis* incubated with prostate cells (RWPE-1) (**F**). The scale bars represent 5 μm. (**D**) *T. vaginalis* exposed to fibronectin was used for the fractionation experiments into cytoplasmic, membrane, and secreted proteins. The protein fractions were analyzed by western blot assay using anti-Tvα-actinin 2 antibodies.

host cells is required for its survival and a prerequisite to host cell killing [26]. Here, adherence of *T. vaginalis* to VEC or RWPE-1 was quantified by flow cytometry analysis using fluorescence-labeled *T. vaginalis* (Figs. 4A and 4B for VEC; Fig. 4C for RWPE-1).

An experiment was then performed to examine whether masking of Tvα-actinin 2 on the *T. vaginalis* surface inhibited the parasites' adherence to these cells (Fig. 4B for VEC; Fig. 4C for RWPE-1). For both cell lines, a significant decrease in cytoadherence of *T. vaginalis* was observed upon pretreatment with anti-Tvα-actinin 2 antibodies. As a control, *T. vaginalis* incubated with pre-immune serum was used for the adherence tests, and no effect on VEC or RWPE-1 binding ability was observed.

Role of Tvα-Actinin 2 in Cytotoxicity of *T. vaginalis* to VECs

We examined whether incubation of *T. vaginalis* with anti-Tvα-actinin 2 antibodies affected the pathogen's ability

to kill VECs. After VECs were incubated with *T. vaginalis* or *T. vaginalis* pretreated with anti-Tvα-actinin 2 antibodies, the viability of the VECs was monitored by measuring the released LDH (Fig. 4D). VECs treated with *T. vaginalis* at a multiplicity of infection of 10 for 4 h demonstrated 82% LDH release relative to the LDH of the total cells used in the assays. The cytotoxicity of *T. vaginalis* pretreated with anti-Tvα-actinin 2 antibodies was decreased to 59% in contrast with 80% cytotoxicity of pre-immune serum-treated *T. vaginalis*.

Binding of Tvα-Actinin 2 to VECs and RWPE-1 Cell Lines

We then investigated whether Tvα-actinin 2 binds directly to host cells, by (i) flow cytometry analysis, (ii) observation of the cell lines incubated with fluorescence-labeled rTvα-actinin 2 proteins, and (iii) ligand immunoblotting binding assays.
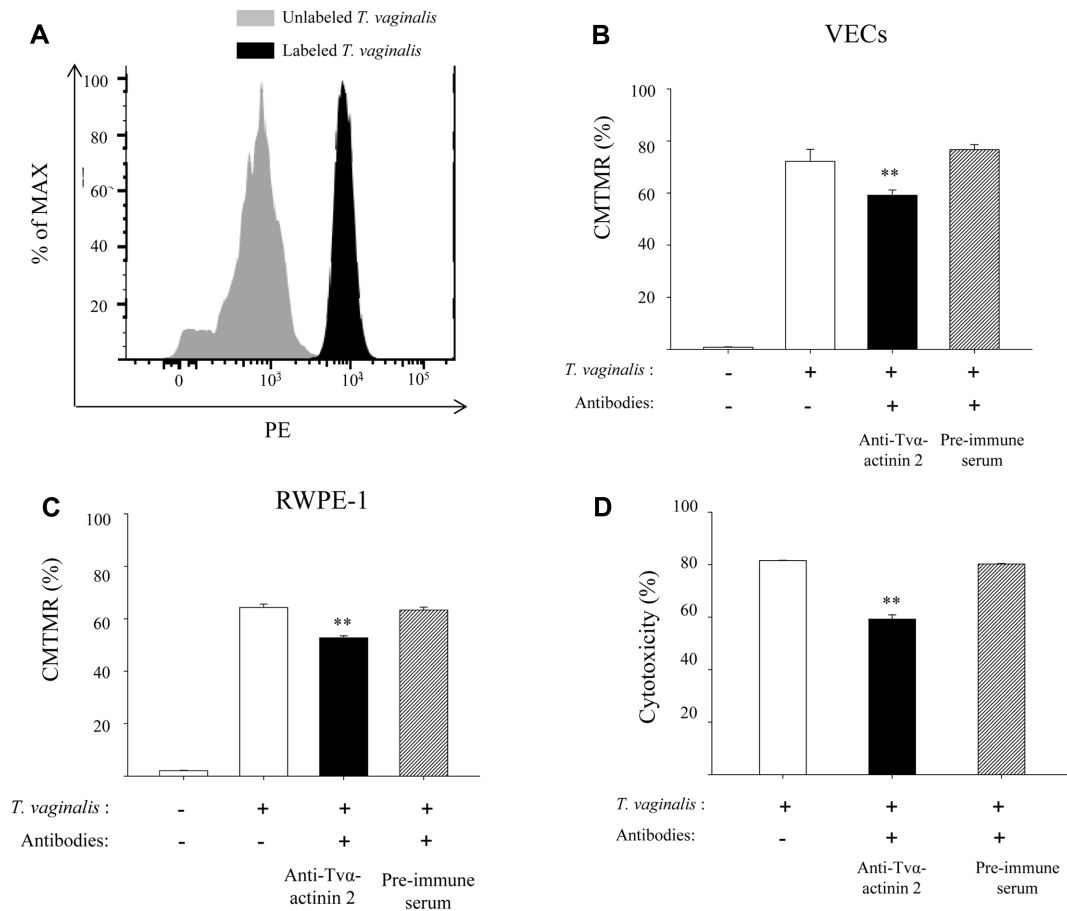
After VEC and RWPE-1 cells were incubated with

Fig. 4. Role of Tvα-actinin 2 in *T. vaginalis* cytoadherence and cytotoxicity to vaginal epithelial cells (VECs) and prostate epithelial (RWPE1) cells.

(**A**) A histogram showing the binding of fluorescence-labeled *T. vaginalis* to VECs determined by flow cytometric analysis. *T. vaginalis* was stained with 0.5 μM CMTMR to determine staining intensity compared with unstained *T. vaginalis*. Stained trichomonads were incubated with VECs at an MOI of 10 for 6 h and washed in free growth medium five times to discriminate unbound cells. (**B, C**) Bar graphs that show average adherence of CMTMR-stained *T. vaginalis* or CMTMR-stained *T. vaginalis* with VECs (**B**) and RWPE-1 (**C**) pretreated with anti-Tvα-actinin 2 antibodies for 1 h at a concentration of 30 μg/ml. Pre-immune rat serum was used as a control to monitor the nonspecific effect of serum on the binding of *T. vaginalis* to VECs. (**D**) Role of Tvα-actinin 2 in cytotoxicity of *T. vaginalis* to VECs. *T. vaginalis* trophozoites were pre-incubated with anti-Tvα-actinin 2 antibodies for 1 h at a concentration of 30 μg/ml. Pre-immune rat serum was used as a control to monitor the nonspecific effect of serum on the cytotoxicity of *T. vaginalis* to VECs. Statistical analyses for pair-wise comparisons were performed using Student's *t*-tests to evaluate the statistical significance of these results. Differences with $p < 0.05$ were considered significant. $**p < 0.001$.

fluorescence-labeled rTvα-actinin 2, they were analyzed by FACS (Fig. 5A). Significant portions of the cell lines (65% and 51%) were labeled with fluorescent proteins in the case of VEC and RWPE-1, respectively. Binding of labeled rTvα-actinin 2 was also observed with a fluorescence microscope, as shown in Fig. 5B.

In addition, VECs were incubated with rTvα-actinin 2, and cell lysates were prepared for use in the western blot analysis with anti-rTvα-actinin 2 antibodies (Fig. 5C(a)). The lysates prepared from the VECs incubated with rTvα-actinin 2 indicated that rTvα-actinin 2 binds to VECs

(lane 3). As a loading control, the amount of human β-actin in the VEC lysates was also monitored (Fig. 5C(b)). A similar level of human β-actin was observed in each lysate sample, indicating that the absence of rTvα-actinin 2 in lane 2 of Fig. 5C(a) was not caused from a lower level of protein of human cell lysates loaded into the same lane.

Determination of the Binding Domain of Tvα-Actinin 2 to Cell Lines

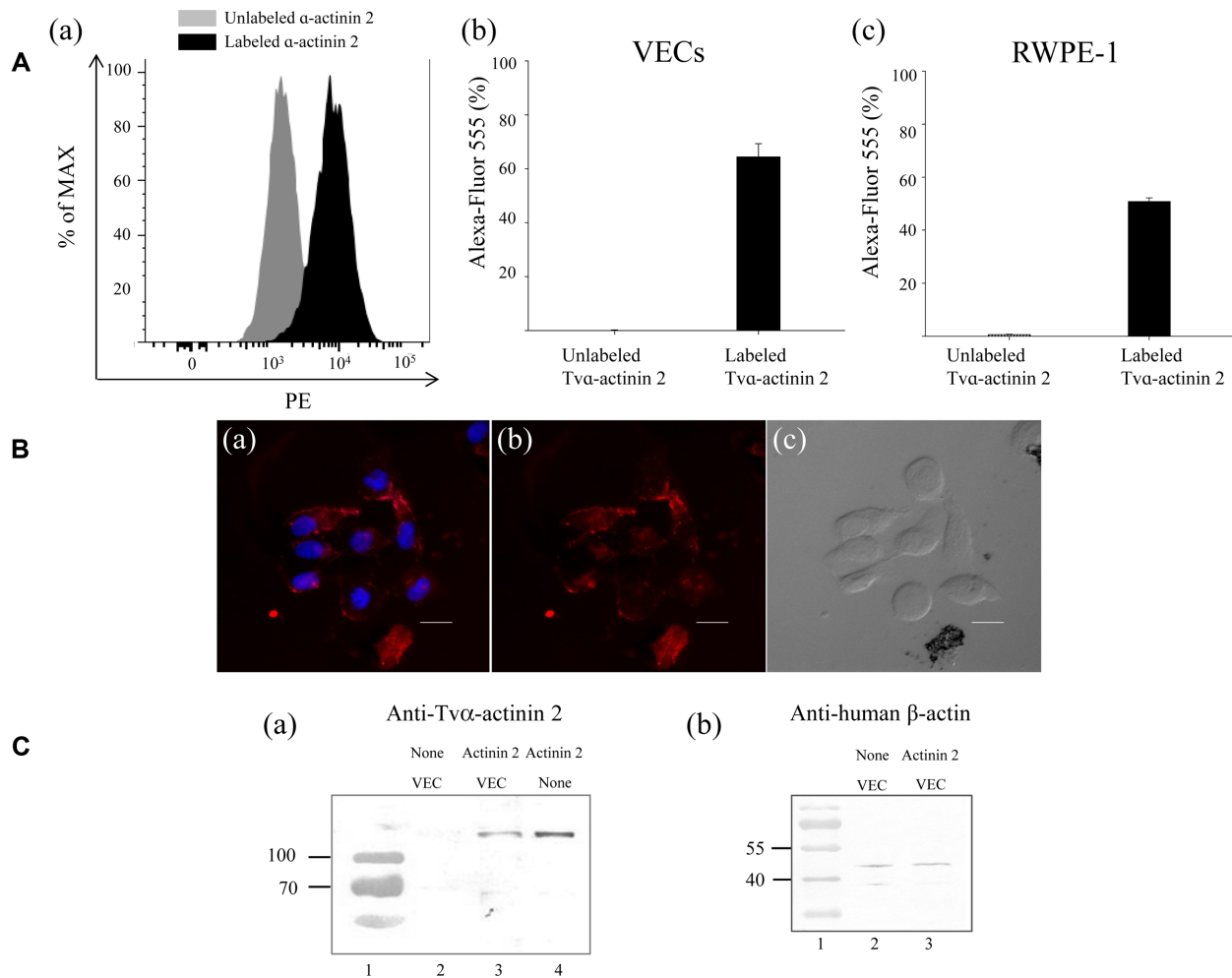Additional experiments were performed to define the domain of rTvα-actinin 2 involved in the adherence of

Fig. 5. Binding of rTvα-actinin 2 protein to the cell lines.
(**A**) Flow cytometric analysis of rTvα-actinin 2 binding to vaginal epithelial cells (VECs) and prostate epithelial (RWPE1) cells. A histogram showing the binding of fluorescence-labeled α-actinin 2 of *T. vaginalis* to cell lines determined by FACs. As a control, VECs were incubated with unlabeled rTvα-actinin 2 and analyzed. (**B**) Labeled rTvα-actinin 2 protein binding to VECs. (a) VECs incubated with labeled rTvα-actinin 2 (red) merged with DAPI (blue); (b) VECs incubated with labeled rTvα-actinin 2 (red); (c) VECs incubated with labeled rTvα-actinin 2 under differential interference contrast. The bars represent 20 μm. (**C**) Ligand-binding immunoblotting assays. VECs (2×10^5) were incubated with 10 μg of the rTvα-actinin 2 proteins. After five washes, the cells were lysed, and analyzed by western blot assay using anti-Tvα-actinin 2 antibodies. The same VEC lysates were analyzed for their β-actin content as a loading control by western blot assay. Lane 1: protein size marker; lane 2: VECs alone; lane 3: VECs with rTvα-actinin 2; lane 4: rTvα-actinin 2 (5 ng).

rTvα-actinin 2 to VECs (Fig. 6). The deduced amino acid sequence of Tvα-actinin 2 revealed that this protein is composed of an N-terminal actin-binding domain, a central domain rich in α-helices, and a C-terminal region with Ca^{2+}-binding activity [24]. Tvα-actinin 2 protein was separated into three parts as histidine-tagged forms (Fig. 1). Truncated rTvα-actinin 2 proteins were determined for their cytoadherence to VECs by flow cytometry analysis (Fig. 6A). Compared with VECs without labeled protein (5.8%), all three truncated and labeled Tvα-actinin 2 proteins

demonstrated significant binding to VECs (23~87%). Among them, Tvα-actinin 2 #1, containing the N-terminal domain of the protein, showed the highest binding ability. Binding of these three truncated rTvα-actinin 2 proteins to VECs was observed using a fluorescence microscope (Fig. 6B). rTvα-actinin 2 proteins were tagged with red fluorescent Alexa-Fluor 555, and then added to VECs. Only the VECs treated with rTvα-actinin 2 #1 demonstrated red fluorescence, whereas VECs incubated with rTvα-actinin 2 #2 or #3 showed no fluorescence signal.
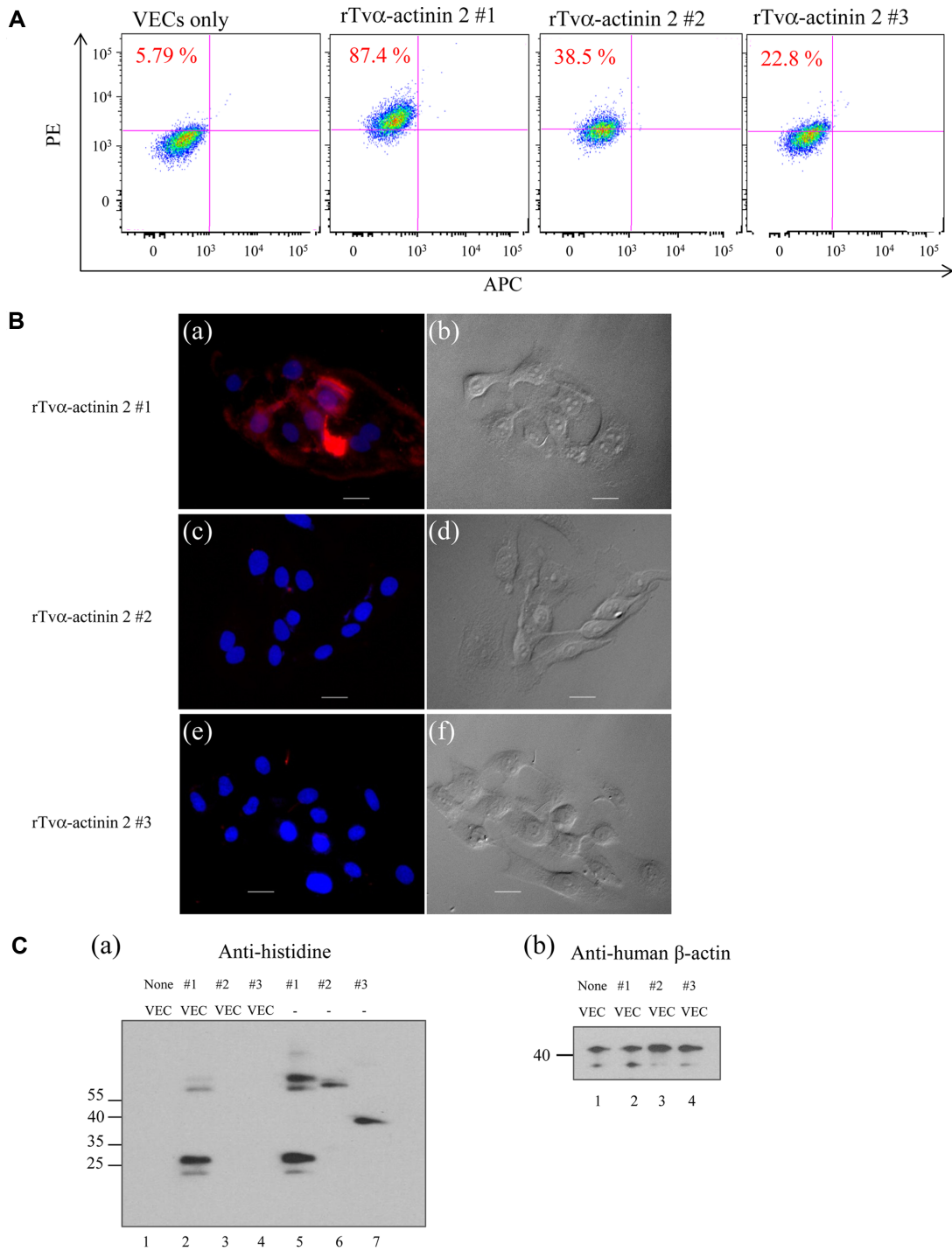
Fig. 6. Determination of the Tvα-actinin 2 domain that binds to vaginal epithelial cells (VECs).
(**A**) Flow cytometric analysis of the truncated rTvα-actinin 2 protein binding to VECs. (**B**) Binding of the truncated rTvα-actinin 2 proteins to VECs. VECs with labeled, truncated rTvα-actinin 2 (red) merged with DAPI (blue) are presented on the left side and VECs under differential interference contrast are presented on the right side. The bars represent 20 μm. (**C**) Ligand-binding immunoblotting assays. VECs (2×10^5) were incubated with 10 μg of one of the three truncated rTvα-actinin 2 proteins, #1, #2, and #3. After five washes, the cells were lysed, and analyzed by western blot assay using anti-histidine antibodies. The same VEC lysates were analyzed for their β-actin content as a loading control by western blot assay. Lane 1: VECs alone; lane 2: VECs with rTvα-actinin 2 #1; lane 3: VECs with rTvα-actinin 2 #2; lane 4: VECs with rTvα-actinin 2 #3; lanes 5−7: rTvα-actinin 2 #1−#3 reacted with anti-histidine antibodies (5 ng each).

These truncated rTvα-actinin 2 proteins were also used for ligand immunoblotting binding assays using anti-histidine antibodies (Fig. 6C). Only the rTvα-actinin 2 #1 protein, containing the actin-binding domain, was detected in VEC lysates (lane 2). On the other hand, the other two rTvα-actinin 2 proteins, #2 and #3, were not observed in VEC lysates (lanes 3 and 4, respectively). Western blot analysis of human β-actin in the VEC lysates indicated that similar amounts of proteins were used in the assay. These results demonstrated that rTvα-actinin 2 binds directly to VECs via its N-terminal region.

Discussion

After being identified as an immunogenic protein reacting to anti-*T. vaginalis* IgGs of female patients with trichomoniasis [24], Tvα-actinin 2 has even been used to monitor men's serological samples for trichomoniasis exposure and risk of prostate cancer [19]. To be functional in the host interaction, this protein in the pathogen should be secreted or be present on its outermost surface. In this study, *T. vaginalis* was transformed in vitro into the amoeboid form, and Tvα-actinin 2 was also observed near the membranes of the amoeboid form (Figs. 3B and 3C). Conversion of *T. vaginalis* into the amoebic form and the concurrent membrane localization of Tvα-actinin 2 were observed upon incubation with VECs and RWPE-1 (Figs. 3E and 3F, respectively). Fractionation experiments indicated that Tvα-actinin 2 was located in the plasma membranes and secreted from *T. vaginalis* (Fig. 3D).

Incubation of *T. vaginalis* with VECs induced the expression of certain *T. vaginalis* genes, including AP-65, AP-33, and Tvα-actinin 2 [27]. This is in agreement with the study of Addis *et al.* [25], in which the expression of Tvα-actinin 2 increased in the amoebic form of *T. vaginalis*. The membrane localization and induced expression of Tvα-actinin 2 in the amoebic form of *T. vaginalis* suggest its role in cytoadherence of *T. vaginalis* to the host cells, which may trigger an immune response or lead to death of the host cells. The role of Tvα-actinin 2 as an immunomodulator has already been demonstrated using human epithelial cell lines and mouse dendritic cells challenged with rTvα-actinin 2 [17]. In this study, an additional role of Tvα-actinin 2 as an adhesin was shown by the decreased number of *T. vaginalis* that adhered to VECs and RWPE-1 in the antibody-blocking experiment (Figs. 4B and 4C, respectively). Treatment with Tvα-actinin 2-specific antibodies resulted in the attenuated cytotoxicity to VECs (Fig. 4D),

caused from the decreased binding of antibody-treated *T. vaginalis*. Among the identified adhesins, Tvα-actinin 2 is the trichomonad's molecule showing immunomodulating activity.

Owing to the actin-binding domain at its N-terminus, this protein was identified as an actinin [28]. Direct interaction of Tvα-actinin 2 with actin was observed by actin-binding assays using polymerized actin (Lee and Park, unpublished results). Thus, it is likely that Tvα-actinin 2 becomes important under specific stages of *T. vaginalis* (*i.e.*, upon exposure to the host cells via direct interaction with actin). A search of the *T. vaginalis* genome demonstrated that five Tvα-actinin genes encode an N-terminal actin-binding domain, a C-terminal Ca^{2+}-binding domain, and a central variable domain [29]. Among these genes, Tvα-actinin 3 was identified to bind the iron-responsible element of *Tvcp4* RNA encoding *T. vaginalis* cysteine proteinase 4, thus participating in post-transcriptional iron regulation. The various roles and expression of these genes should be examined.

It is possible that Tvα-actinin 2 in the amoeboid form of *T. vaginalis* binds to the host epithelial cells via direct association with surface component(s) of the host cells. This study showed a direct binding of rTvα-actinin 2 protein to the epithelial cells via flow cytometry measurement (Fig. 5A), fluorescence microscopy using the labeled Tvα-actinin 2 proteins (Fig. 5B), and ligand immunoblotting assays (Fig. 5C). Experiments using the truncated Tvα-actinin 2 clearly demonstrated that the N-terminal actin-binding domain of Tvα-actinin 2 plays a role in host adherence (Fig. 6).

These data indicate that Tvα-actinin 2 plays a role in the pathogenesis of *T. vaginalis* by serving as one of the adhesins to the host cells. The N-terminal domain containing a putative actin-binding sequence was important for its adhesion to the host epithelial cells.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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