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Binding of fibronectin by *Trichomonas vaginalis* is influenced by iron and calcium

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We have reported that *Trichomonas vaginalis*, the causative agent of the most common, non-viral sexually transmitted disease, bound to cover slips coated with fibronectin (FN) (Crouch & Alderete, *Microbiol*., 1999; 145: 2835–43). In this study, we extend that observation by showing that FN binding is specific, and we present data on the requirements of FN binding by *T. vaginalis*. Immunofluorescence and immuno-gold labelling readily detected FN throughout the trichomonal surface. Parasites bound to ^{125}I-labelled FN in a time- and concentration-dependent fashion. In the absence of protease inhibitor, iodinated FN was released from the trichomonad surface. Unlabelled FN specifically competed for binding in a concentration-dependent fashion with the ^{125}I-labelled FN. Interestingly, the amount of FN bound by *T. vaginalis* organisms was dependent on iron. High-iron-grown trichomonads acquired lower numbers of molecules but with 10-fold higher affinity than low-iron-grown organisms. Further, we show that for iron-replete organisms, calcium (Ca^{2+}) at physiological levels increased amounts of FN bound. The increase in binding was rapid, occurring within 5 min of Ca^{2+} addition, and required *de novo* protein synthesis. Co-incubation of live parasites with Ca^{2+} in the presence of FN was necessary to increase the amount of FN bound. Treatment of trichomonads with okadaic acid, but not other phosphatase inhibitors, resulted in a 50% decrease in binding of FN, regardless of the presence of Ca^{2+}, suggesting a role for phosphatase in FN association. These results indicate that depending on the iron status of *T. vaginalis* organisms *in vivo*, Ca^{2+} may influence trichomonad recognition and binding to FN during host parasitism.

Key words: extracellular matrix (ECM), fibronectin (FN), host–parasite, *Trichomonas vaginalis*, virulence.

Introduction

The flagellated protozoan, *Trichomonas vaginalis*, causes one of the most prevalent sexually transmitted diseases worldwide [1]. The disease, trichomonosis, is non-self-limiting, and it is of interest to study aspects of the host–parasite interrelationship that lead to understanding of host infection, persistent colonization and pathogenesis. Symptoms in patients with trichomonosis range from mild to severe inflammation with a foul-smelling

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discharge and severe irritation [2–4]. Furthermore, trichomonosis has been associated with increased risk for adverse pregnancy outcome [2–8], cervical cancer [9] and HIV seroconversion [10, 11]. Parasitism of the vaginal tract by *T. vaginalis* is a multi-step process involving distinct mechanisms of interaction with macromolecules, cells and tissues. Trichomonads appear to interact in a specific manner with mucin, the predominant component of mucus, as an initial event leading to host colonization [12]. Then, organisms come in contact with the vaginal epithelium where four iron-regulated surface proteins of *T. vaginalis* mediate cytoadherence to the vaginal epithelial cells (VECs) [13–16].

In the constantly changing environment of the vagina, a number of host and parasite factors may contribute to limiting cytoadherence. For example, hormone-mediated exfoliation of the squamous VECs and the known cytotoxicity caused by trichomonad cysteine proteinases likely lead to desquamation of the vaginal and cervical epithelia [17–21]. This local erosion may permit the parasites to gain access to immature VECs and extracellular matrix-basement membrane components. As *T. vaginalis* does not interact with immature VECs but survives in the vagina to cause a non-self-limiting infection under conditions that may be nutrient limiting, it is hypothesized that trichomonads have developed other mechanisms of colonization. Indeed, our group and others have shown that trichomonads attached to fibronectin (FN) [22, 23] and laminin (LM) [24, 25].

For eukaryotic cell interactions with FN, it is known that divalent cations play a crucial role [26–30]. Integrins require magnesium (Mg^{2+}) and calcium (Ca^{2+}) for stability and optimal FN binding. Furthermore, integrin–FN interactions initiate intracellular Ca^{2+}-dependent signalling cascades that result in cellular responses to environmental changes. Signals are relayed to the transcriptional machinery through a series of kinases and phosphatases, which lead to *de novo* gene expression [31–38].

Therefore, we felt it important to further examine the interaction of *T. vaginalis* with FN. We now show that binding of FN is specific. We found that both iron and calcium influence the association of trichomonads with FN. To our knowledge this is the first report showing a role for Ca^{2+} in the modulation of a biological property of the amitochondriate *Trichomonas vaginalis*. The significance of our findings with respect to the pathogenesis of trichomonosis is discussed.

Results

Visualization of FN associated with *T. vaginalis* surface and of live organisms bound to FN

We showed earlier the association of live *T. vaginalis* organisms to FN immobilized on cover slips [23]. We hypothesized that the surface of trichomonads bound FN in a specific fashion and now wanted to characterize the binding of FN by *T. vaginalis* organisms. We, therefore, visualized by immunofluorescence the secondary rhodamine-conjugated antibody (Materials and Methods) detection of the anti-FN serum antibody binding to FN associated with non-permeabilized parasites after incubation with FN. The FN (orange colour) on trichomonads was distributed over the entire surface [Fig. 1(a)]. Furthermore and not unexpectedly based on the fluorescence, immunocytochemical evidence was obtained showing 10 nm gold particles distributed throughout the trichomonad surface [Fig. 1(b), arrows point to clustering of gold particles]. No similar immunoreactivity was seen under the same experimental conditions omitting the primary antibody nor using prebleed, normal rabbit serum as a control (not shown). Specific surface binding of FN and antibody recognition was further evident by the absence of gold particles within parasite structures.

Binding by *T. vaginalis* of ^{125}I-FN and release of bound FN by trichomonad cysteine proteinases

The time course of binding of iodinated FN to live vs glutaraldehyde-fixed *T. vaginalis* organisms is illustrated in Fig. 2. We previously used fixed trichomonads to more accurately quantitate binding of host proteins by trichomonads [39]. Binding was maximal by 30 min at 37°C, and steady state binding was evident for an additional 30 min. We showed previously diminished attachment of trichomonads to FN on cover slips after 30 min due to cysteine proteinases synthesized by *T. vaginalis* [23]. Accordingly, to ascertain whether bound FN was

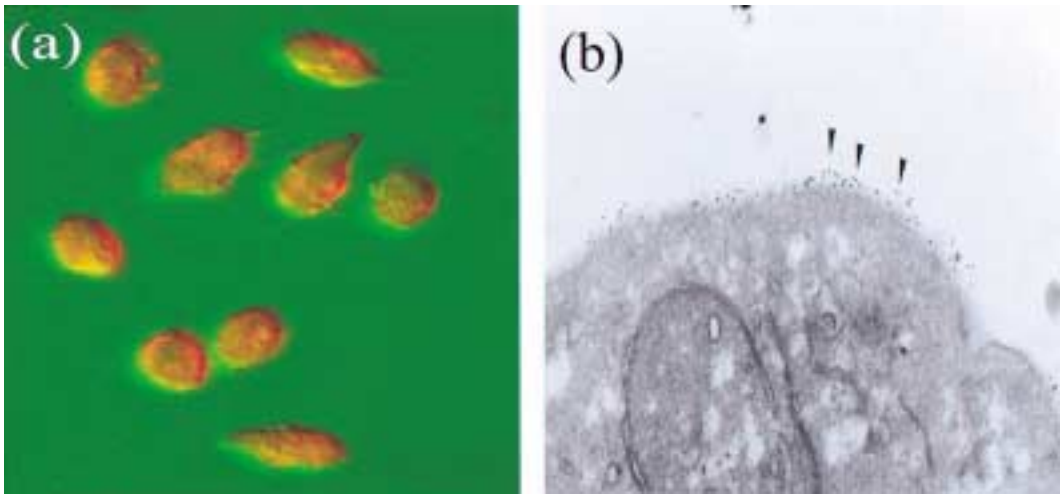

Figure 1. Immunofluorescence and immuno-cytochemical evidence showing association of FN with the surface of *T. vaginalis* organisms. (a) Fixed, non-permeabilized trichomonads were incubated with FN in MB buffer followed by treatment with rabbit anti-FN serum (Materials and Methods). No immunoreactivity was ever detected with prebleed normal rabbit serum as a control. Immunofluorescence was examined with an Axiophot II Zeiss epifluorescent microscope. (b) Trichomonads prepared as described in Materials and Methods were washed in MB buffer prior to parasite samples incubated with rabbit anti-FN antibody followed by goat anti-rabbit antibody conjugated to 10 nm gold particles. Samples were examined using a Jeol 1210 electron microscope.

being affected by the proteinases, we placed live trichomonads with bound iodinated FN at 37°C, and as seen for a representative experiment in Table 1, approximately 55% of the bound FN was released from the parasite surface by 30 min of incubation. By comparison, in the presence of the cysteine proteinase inhibitor, N-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), little or no bound FN was released from the surface of trichomonads. Analysis of the released ^{125}I-FN by SDS-PAGE and autoradiography showed that the FN was degraded and the extent of degradation was time dependent (data not shown).

We then performed saturation-binding kinetics, and although live organisms achieved saturation binding at a lower concentration of FN [Fig. 3(a)], higher amounts of FN bound to fixed [Fig. 3(b)] trichomonads. Finally, Scatchard analysis (data not shown) was performed on the representative data from these experiments. Fixed organisms acquired 10-fold more FN with 2 000 000 molecules bound/trichomonad. In contrast, live organisms achieved saturation at 10-fold lower concentrations of FN. It is noteworthy that the number of FN molecules acquired by live trichomonads passaged daily in normal TYM-serum medium was variable, and such variations have been recorded for other properties of *T. vaginalis* [13, 16, 46]. Binding studies performed weekly over several weeks indicted a range between 100 000 to 500 000 molecules of FN bound by live organisms.

Concentration-dependent competition of FN binding

While competition by unlabelled FN of iodinated FN was seen using live trichomonds, reproducible data was obtained with fixed organisms, further reinforcing the idea that proteinases (Table 1) [23], and possibly membrane dynamics affect binding of FN by live parasites. Unlabelled FN competed for iodinated FN binding in a concentration-dependent manner (Fig. 4). The amount of 0.2 nM FN decreased ^{125}I-FN binding by 50%. Unlabelled transferrin, as a negative control [39], did not compete for labelled FN association. This result and those presented above suggest that the binding of FN at the trichomonad surface is specific. We also performed competition assays for trichomonad binding to ^{125}I-FN with the cell-binding domain and the integrin-binding tripeptide, RGD, contained within the cell-binding domain [41–43]. The peptide had no specific competition for binding with iodinated FN; however, the cell-binding domain inhibited binding of ^{125}I-FN by

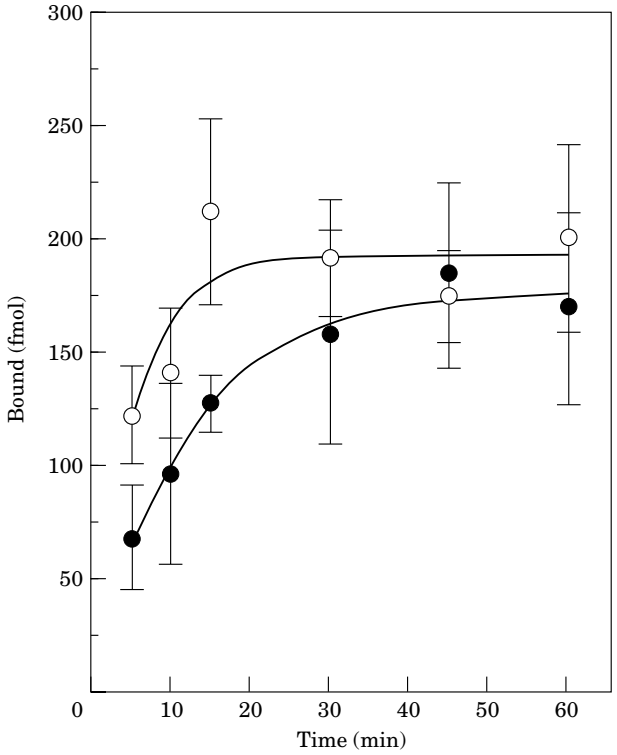

Figure 2. Representative time course binding of ^{125}I-labelled FN by *T. vaginalis* grown in normal medium. Live (●) and glutaraldehyde-fixed (○) trichomonads were suspended at a density of 5×10^5 in 0.1 ml volume of MB buffer and incubated with 20 μg of ^{125}I-FN at 37°C. The amount of cell-associated radioactivity was determined as described in Methods. Each point represents the mean of triplicate samples from an individual experiment. The specific activities of the radiolabelled FN used with fixed vs live trichomonads were 49 and 148 cpm/fmol, respectively.

50%, suggesting that trichomonads recognize non-RGD sequences within this domain and possibly other FN domains.

FN binding is influenced by iron and calcium

Fig. 5 shows that trichomonads grown in low-iron-medium bound higher amounts of FN compared to high-iron-medium. This effect was specific as other cations like Mg^{2+} and Mn^{2+} did not produce similar differences, as shown before for other iron-regulated properties [14–16]. However, in examining for the specificity of iron, we observed that calcium also influenced the extent of FN acquisition but only among high-iron parasites. As can be seen for the representative experiment in Fig. 6, ≥ 0.5 mM Ca^{2+} increased amounts of FN bound by high-iron organisms compared to either the absence of Ca^{2+} or the presence of Mn^{2+} as a control. Low-iron parasites had little or no increased amounts of associated FN in the presence of Ca^{2+}. Consistent with the data of Fig. 5, amounts of FN in the absence of Ca^{2+} showed that high-iron organisms [Fig. 6(a)] bound less FN than did low-iron trichomonads FN (part b). These data indicate a role for Ca^{2+} in modulating the extent of FN bound by high-iron *T. vaginalis* organisms.

Table 2 summarizes Scatchard plot analysis of data from the representative experiments of Figs 5 and 6 and shows, as expected, that low-iron-grown *T. vaginalis* bound twice the number of FN molecules with 10-fold lower affinity than high-iron-grown trichomonads. In the presence

Table 1. Representative experiment showing release of ^{125}I-FN from parasite surface[a]

Treatment with TLCK	Incubation (min)	FN bound[b] (fmol)	Cell-associated FN (%)
No[c]	0	1765	100
	15	1142	65
	30	812	46
	45	726	41
Yes[c]	0	1897	100
	15	2211	117
	30	1662	88
	45	2009	106

[a] Live trichomonads were incubated with ^{125}I-FN as described in Materials and Methods.
[b] The amount of cell-associated ^{125}I-FN remaining on parasites was determined by comparing with the zero time point (100%). Subsequent to the interaction with iodinated FN, the organisms were washed and suspended in MB buffer for the various time points. Trichomonads were then washed, and cell-associated ^{125}I-FN was determined (Materials and Methods). The specific activity of iodinated FN was 117 cpm/fmol.
[c] TLCK inhibits the activity of the trichomonad cysteine proteinases.

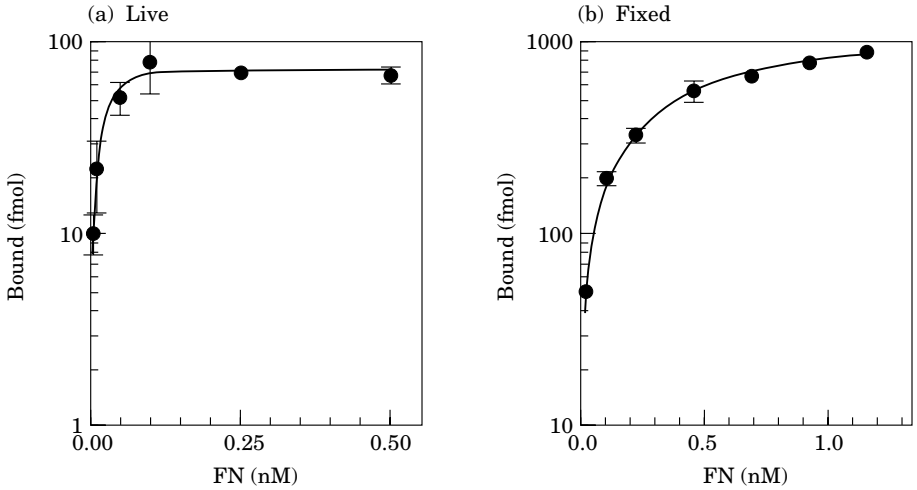

Figure 3. Representative experiment demonstrating concentration-dependent binding of ^{125}I-FN by live (a) and glutaraldehyde-treated (b) *T. vaginalis* grown in normal medium. Trichomonads were suspended at a density of 500 000 in 0.1 ml MB buffer containing 400 ?M TLCK and incubated with increasing amounts of ^{125}I-FN for 20 min at 37°C. After washing, cell-associated radioactivity was measured. As indicated in the text, Scatchard plot analysis of saturation kinetics of ^{125}I-FN binding data presented in (a) and (b) was performed. The data plots representing relative binding of ^{125}I-FN by live vs fixed parasites were also derived. Data was used to determine the relative K_d as the concentration of FN required to obtain half saturation. Each data point represents the mean value of triplicate sample for one representative experiment. The specific activities of the iodinated FN used with fixed vs live trichomonads were 38 and 37 cpm/fmol, respectively.

of Ca^{2+}, the number of molecules bound was similar regardless of iron status but overall increase was seen in the absence of Ca^{2+}. In this case, the FN-binding affinity with Ca^{2+} was 20-fold higher for the high-iron cells. Finally, Ca^{2+}-mediated increased binding of FN as seen for high-iron parasites (part a) was also obtained for organisms grown in normal TYM-serum medium. This was noteworthy because it permitted us to use trichomonads grown in normal medium without manipulating iron content for subsequent experiments.

Ca^{2+}-induced increase in FN binding requires protein synthesis

Fig. 7 presents data of a representative experiment in which Ca^{2+} was added to trichomonads at 5 min intervals during the typical 20 min incubation of a FN binding assay. Addition of Ca^{2+} 5 min before the end of the binding assay was sufficient to increase binding of iodinated FN by 100% (bar d compared to bar e), while a 10 min incubation with Ca^{2+} (bar c) resulted in a greater than 200% increase in the amount of FN bound. It seemed reasonable to test whether the Ca^{2+} effect on FN acquisition required *de novo* protein synthesis. In data not shown, trichomonads pretreated with cycloheximide decreased by 60% the amounts of FN bound in the presence of Ca^{2+} for a 20 min period when compared to untreated organisms with Ca^{2+} alone. Not unexpectedly, cycloheximide-treated trichomonads without Ca^{2+} bound low amounts of ^{125}I-FN as that seen for untreated samples, similar to the levels seen in bar e. Importantly, treatment with this protein synthesis inhibitor did not affect trichomonal viability, as evidenced by continued motility of and exclusion of trypan blue by parasites [17]. These data indicate that parasites require *de novo* protein synthesis to reach maximal binding of FN in the presence of Ca^{2+}.

Co-incubation with FN and Ca^{2+} is necessary for maximum FN acquisition

Last, we wanted to examine whether association of Ca^{2+} with the trichomonal surface was required for FN binding. After interaction of trichomonads with FN in a typical binding assay in the presence or absence of Ca^{2+}, the organisms were washed in buffer with or without EDTA. Fig. 8(a) shows that regardless of Ca^{2+} induction,

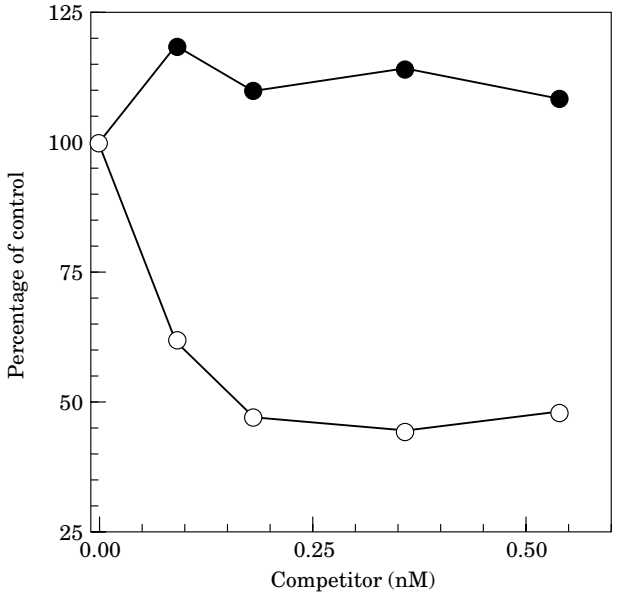

Figure 4. Representative experiment showing competition for ^{125}I-FN binding by fixed *T. vaginalis* with unlabelled FN (○) and transferrin (●) as a negative control. A 0.1 ml suspension of 200 000 parasites was incubated with increasing concentrations of unlabelled FN or transferrin and 4 μg ^{125}I-FN for 20 min at 37°C. The amounts of associated ^{125}I-FN were measured as described in Materials and Methods. Individual data points represent the mean of triplicate samples from a representative experiment. Parasites for this experiment were grown in normal medium. The specific activities of iodinated FN used for unlabelled FN and transferrin were 173 and 53 cpm/fmol, respectively.

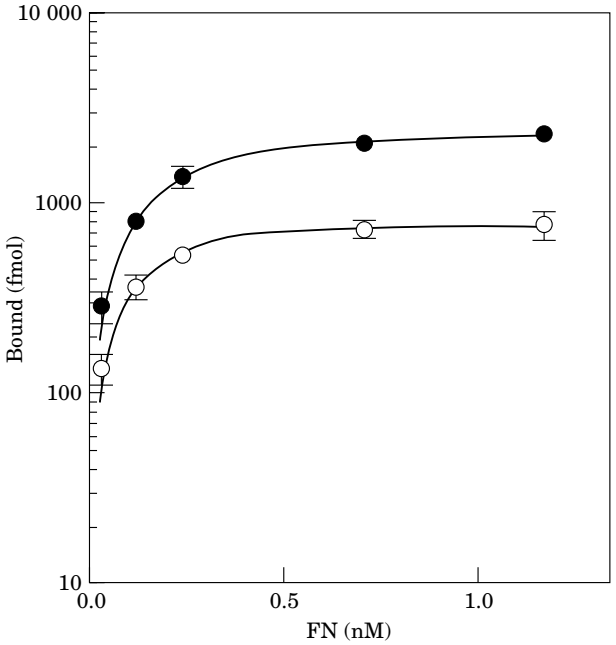

Figure 5. Saturation binding to FN using fixed *T. vaginalis* organisms grown in high- and low-iron-medium. High- (○) and low-iron (●) grown trichomonads were generated as described in Materials and Methods. Glutaraldehyde-treated parasites at a density of 500 000 in 0.1 ml MB buffer were incubated with increasing concentrations of iodinated FN for 20 min at 37°C. Trichomonad-associated radioactivity was measured as described in Methods. Individual data points represent the means of triplicate samples of a representative experiment. The specific activities of the FN used in this representative experiment was 79 cpm/fmol.

washing of parasites with EDTA had no effect on the amount of FN bound when compared to the absence of EDTA.

Next, we tested whether pretreatment and, therefore, induction of organisms with Ca^{2+} alone led to increased FN binding [Fig. 8(b)]. A binding assay was performed with organisms pretreated with Ca^{2+}, and Ca^{2+}-pretreated parasites were washed in the presence or absence of EDTA. Trichomonads were then incubated with iodinated FN in the absence of the divalent cation. Pretreatment of trichomonads with Ca^{2+} did not enhance FN acquisition above that of control untreated organisms, indicating that Ca^{2+} was required during the incubation with FN.

Finally, we performed an experiment similar to that seen in Fig. 8(b) to test whether Ca^{2+} was needed during incubation to achieve maximum acquisition. As presented in Fig. 8(c), organisms were pretreated with or without Ca^{2+}, followed by washing in buffer with or without EDTA to remove any associated cation. The parasites were then interacted with FN again with or without Ca^{2+} in the binding assay. Not unexpectedly, the presence of Ca^{2+} during the interaction resulted in increased FN bound regardless of EDTA washing of cells prior to the binding assay. However, in a third set of samples handled identically, EDTA was added during the incubation with FN plus or minus Ca^{2+}. In this case, almost complete abrogation of FN binding was observed. Identical experiments performed with low-iron-grown parasites showed that while these organisms were unresponsive to the addition of Ca^{2+} in the interaction buffer, as expected (Fig. 6), the presence of EDTA during the interaction with FN also resulted in a dramatic ($\geq$50%) decrease in levels of FN bound (data not shown). These data reinforce the idea

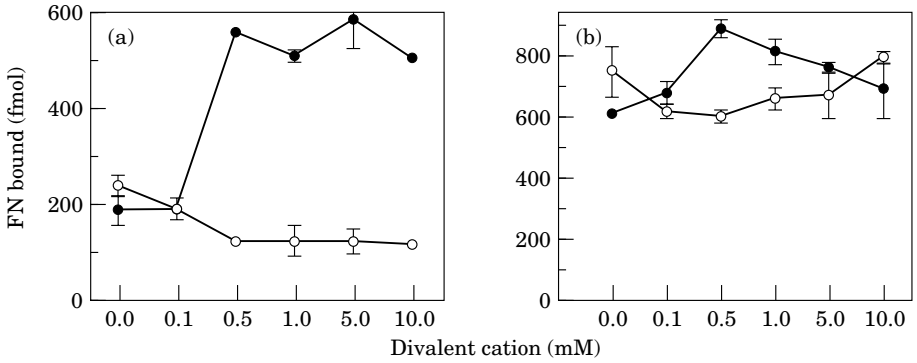

Figure 6. A representative experiment showing that calcium increases ^{125}I-FN binding by high-iron-grown *T. vaginalis*. Trichomonads were grown in medium supplemented with (a) or depleted of (b) iron as described in Materials and Methods. Organisms were washed in MB buffer prior to incubation for 20 min at 37°C in MB buffer containing 10 μg iodinated FN and increasing concentration of $CaCl_2$ (●) or $MnCl_2$ (○). Results similar to $MnCl_2$ were obtained with $MgCl_2$. Each data point represents the mean of triplicate samples. As experiments in (a) and (b) were performed separately, the specific activities of the iodinated FN were 176 and 59 cpm/fmol, respectively.

Table 2. Influence of iron and calcium on FN-binding kinetics of high- and low-iron-grown trichomonads

Iron status	No addition		With 1 mM calcium	
	K_d[b]	Number of molecules bound[c]	K_d	Number of molecules bound (fold increase)
Low[a]	0.40	500 000	0.40	1 100 000 (2)
High	0.04	230 000	0.02	1 400 000 (6)

[a] Low- and high-iron-grown trichomonads were obtained as described in Materials and Methods.
[b] The relative binding affinity (Kd), with or without addition of 1 mM $CaCl_2$, is defined as the amount of iodinated FN domains (nM) required to reach half saturation of binding. The relative binding affinity determination and Scatchard plot analysis were performed on the data presented in Figs 5 and 6.
[c] The number of molecules bound was determined by Scatchard analysis.

that regardless of the iron status of trichomonads, Ca^{2+} and/or possibly another divalent cation, is required for FN binding.

Trichomonad binding to FN involves a phosphatase independent of extracellular Ca^{2+}

As signaling may be a feature of Ca^{2+} induction of FN binding, we treated trichomonads with the kinase inhibitors staurosporine, H7, and wortmannin (Materials and Methods) prior to interaction with FN in the presence and absence of Ca^{2+}. Trichomonads treated in this manner retained the same level of binding as untreated organisms (data not shown). Only pretreatment of *T. vaginalis* organisms with the phosphatase inhibitor okadaic acid decreased FN binding by 50% (data not shown). The phosphatase inhibitors __-naphthyl acid phosphate, cypermethrin, dephosphatin and tautomycin had no effect. This extent of decreased FN acquisition was seen both in the absence and presence of Ca^{2+}, suggesting that a specific phosphatase is involved in the regulation of FN binding, independent of Ca^{2+}.

Discussion

The menstrual cycle plays a key role in modulating the vaginal environment. Fluctuations in

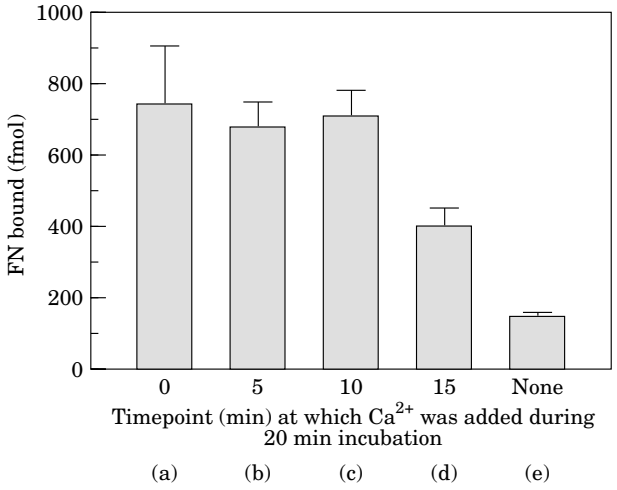

Figure 7. Experiment showing the rapid increase in FN binding by $CaCl_2$. Trichomonads grown in normal medium were prepared for a binding assay with ^{125}I-FN (sp. act., 48 cpm/fmol). The interaction buffer was supplemented with 0.5 mM Ca^{2+} at 5 min intervals within the standard 20 min assay. All samples were then collected and washed, and bound radioactivity was measured for calculation of amounts of FN bound.

iron sources and other nutrients and desquamation of vaginal epithelial cells (VECs) are regulated in part by the hormones estrogen and progesterone [44]. Therefore, it would not be surprising if *T. vaginalis* has evolved multiple mechanisms for colonization and persistence within this constantly changing host environment. Trichomonads must first penetrate the mucus layer [12] prior to adherence to superficial VECs, a process mediated by four iron-regulated adhesin proteins [13–16]. However, in this complex environment, a combination of host and parasite factors may contribute to limit cytoadherence. Changes in iron levels at the site of infection, the normal desquamation of the vaginal epithelium, trichomonal cytotoxicity of epithelial cells and the vaginal discharge following infection may be critical obstacles for successful long-term host parasitism. Since trichomonads overcome such obstacles to establish a non-self-limiting infection, it seems plausible that *T. vaginalis* may recognize and bind to extracellular matrix sites below the superficial epithelium. Not surprisingly, *T. vaginalis* binds to at least two extracellular matrix (ECM) glycoproteins, FN and LM [22–25], and this report provides evidence for the specific and complex binding to FN.

It is noteworthy that trichomonads have at least 10-fold greater affinity for FN than other microbial pathogens, including *Treponema pallidum*, *Trypanosoma cruzi*, *Staphylococcus aureus* and *Streptococcus pyogenes* [45]. Trichomonads also bound greater numbers of FN molecules, as evidenced by both calculations of number of molecules associated with organisms and by the intensity of fluorescence and immuno-gold labelling (Fig. 1). This may suggest that attachment to this glycoprotein may be important for more than just colonization and persistence within the host. Indeed, we have reported the ability of *T. vaginalis* to coat itself with host proteins, one of which was FN [24, 39]. This property may be an important mechanism for immune evasion and possibly other unknown functions, such as protection of the parasite surface from its own secreted cysteine proteinases [24], and the proteinases known to reside in ECM and basement membrane (BM) sites [46–49].

The number of molecules of FN bound was highest under low-iron (Figs 5 and 6), albeit with a 10-fold decrease in K_d (Table 2). That iron does not modulate the level of parasite association with FN on glass cover slips [23] is not in conflict with these findings as this is likely due to the greater sensitivity of measuring ^{125}I-FN acquisition. Our data supports the hypothesis that ECM binding would be especially important during conditions unfavourable to cytoadherence, such as a low-iron environment [40]. In this case, a lower K_d due to low iron might permit an increased motility for trichomonads to more favourable host sites. Other organisms modulate their ability to bind FN in response to iron. *Candida albicans* grown in complex medium associates with high affinity to the gelatin-binding domain [50], whereas organisms cultured in defined medium supplemented with haemoglobin showed high affinity for the FN cell-binding domain and decreased binding capacity for gelatin-binding domain. Future work will clarify whether *T. vaginalis* organisms have preferred associations with specific FN domains. Nonetheless, at this point, we know that the non-RGD region within the cell-attachment domain of FN is an effective competitor of FN acquisition.

To our knowledge, this is the first time that Ca^{2+} has been shown to play a role in an important property of *T. vaginalis*. It is especially noteworthy that the effect by Ca^{2+} on FN binding

occurs within the range of physiological concentrations of Ca^{2+} at the site of infection (Fig. 1). Physiological Ca^{2+} concentrations in serum and other tissues are normally between 1.1 mM and 1.3 mM while vaginal fluids contain six-fold higher concentrations [51, 52]. Therefore, maximal acquisition of FN was seen for high-iron organisms during co-incubations with physiological amounts of Ca^{2+} (Figs 1 and 2). This increase in FN binding was not accompanied by a change in relative binding affinity (Table 2). Surprisingly, but not inconsistent with what is known of integrins [26–30, 53], data presented in Fig. 8(c) also suggest that Ca^{2+} or some other divalent cation is required for FN binding. Of interest will be the future identification of the trichomonad surface protein or proteins that binds FN. Finally, there are two high affinity and at least 12 weak affinity Ca^{2+} binding sites on the FN molecule itself, a number of which are located in the N-terminal region of FN [54]. As the effect of Ca^{2+} (or other divalent cation) requires the presence of FN [Fig. 8(b) and (c)], we cannot rule out that Ca^{2+} binding to the FN molecule may influence receptor recognition. There is precedence for our observations. It is noteworthy that studies on rabbit hepatocyte interaction with FN showed that binding was increased in the presence of Ca^{2+}

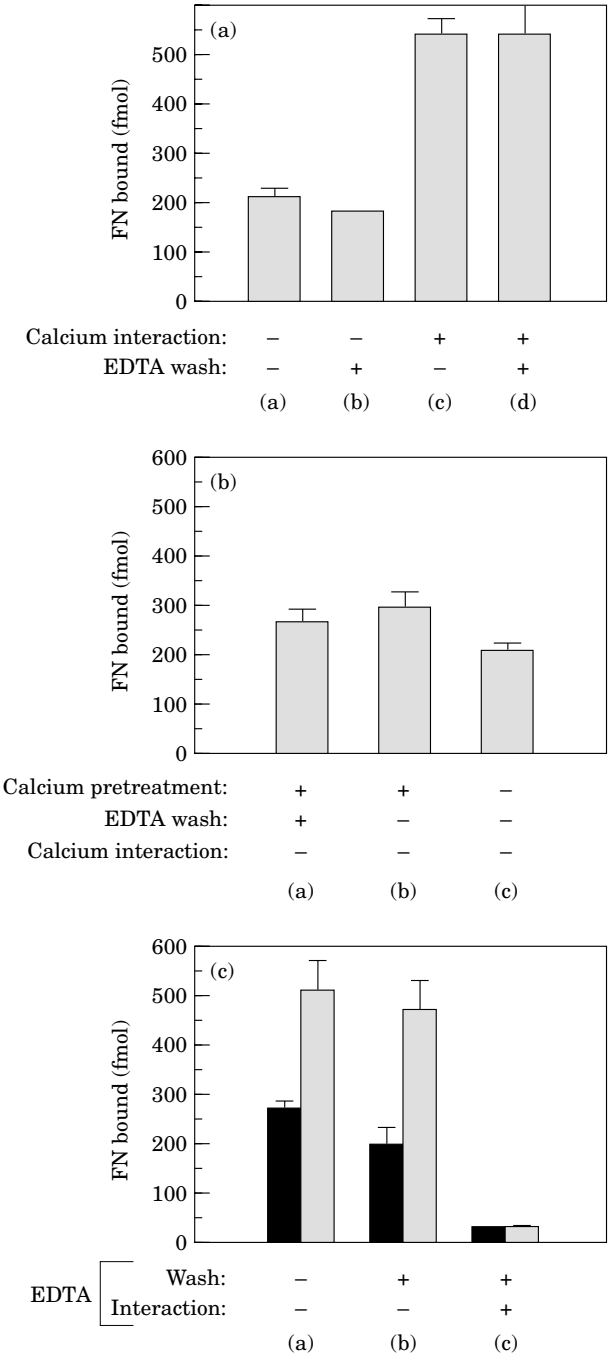

Figure 8. Ca^{2+} and FN together are required for increased FN binding by trichomonads grown in normal medium. (a) Organisms were harvested and a binding assay performed in MB buffer with 10 μg ^{125}I-FN (sp act., 228 cpm/fmol) and in the absence (bars a and b) or presence (bars c and d) of 1 mM $CaCl_2$. Following the 20 min incubation at 37°C, organisms were washed in MB buffer with (bars b and d) or without (bars a and c) 5 mM EDTA. Bound iodinated FN was measured as described. Organisms retained higher amounts of FN bound despite washing with EDTA after the incubation period. (b) Trichomonads were pretreated for 10 min with (bars a and b) or without (bar c) 1 mM Ca^{2+} in MB buffer followed by washing in buffer with (bar a) or without (bars b and c) 5 mM EDTA. The binding assay was performed without Ca^{2+}. Note the lack of any increased amounts of FN bound, indicating a requirement for Ca^{2+} during the incubation with FN in the binding assay. The binding assay was performed with 10 μg ^{125}I-FN (sp. act., 28 cpm/fmol) in MB buffer. (c) Trichomonads were pretreated with (■) or without (■) Ca^{2+} and washed in the presence (bars b and c) or absence (bar a) of 5 mM EDTA as described for part b. The parasites were interacted with FN again in the presence (■) or absence (■) of Ca^{2+} in the binding assay. For the third set of samples (bars in c), the interaction with FN with and without Ca^{2+} included 5 mM EDTA. Note the almost complete abolishment of FN acquisition when EDTA is included in the binding assay. The binding assay was also performed with 10 μg ^{125}I-FN (sp. act., 28 cpm/fmol) in MB buffer.

while amounts acquired were decreased in the presence of EDTA [27].

It is of interest whether there is any phosphorylation of proteins that leads, for example, to activation of transcription factors, something known to occur in mammalian cells and that permits the cells to respond to changes in the environment [31, 36–38]. Through electrophoretic analysis we were unable to detect newly synthesized or phosphorylated proteins upon incubation in the presence of FN with and without Ca^{2+} (data not shown). Along these lines and of interest here was the fact that a role for protein kinases was not found under the experimental conditions employed. That the protein kinase A and C inhibitors were ineffective may be explained on the basis of distinct structure-function properties for the trichomonad kinases and/or lack of participation in the regulation of FN binding by Ca^{2+} [55]. Treatment with one phosphatase inhibitor did decrease levels of surface-associated FN, and this inhibition was observed even in the absence of Ca^{2+} (data not presented). Furthermore, this phosphatase activity does not seem to depend on protein synthesis as determined by lack of inhibition of FN binding with cycloheximide. It is possible that dephosphorylation by a phosphatase is required for receptor activity, as exemplified by the β_1 integrin where dephosphorylation of the cytoplasmic tail seems to be required for enhanced interaction with FN [56].

In summary, our data continue to reinforce the idea of a highly evolved nature of this urogenital parasite capable of surviving in one of the most dynamic and constantly changing host environments. What is clear is the ability of *T. vaginalis* to effectively colonize the urogenital region through the existence on trichomonads of distinct functional proteins, such as adhesins for host epithelial cytoadherence and receptors for basement membrane associations. The existence on the parasite surface of such adhesins and FN receptors would be highly advantageous to survival at sites inside the host with different chemical-nutritional compositions. For example, amounts of lactoferrin, an *in vivo* source of iron for trichomonads [16, 39], fluctuate during the menstrual cycle. Nonetheless, analysis of parasites from fresh clinical isolates for properties modulated by iron, such as cytoadherence [13] and phenotypic variation [57], revealed that a large percentage of trichomonads from vaginal secretions were high-iron-organisms. These high-iron-trichomonads in the presence of physiological concentrations of Ca^{2+}, if now residing at sites of infection other than the surface epithelium for cytoadherence, would optimally associate with FN with high affinity. This mechanism would permit organisms to be highly parasitic and capable of associating with multiple host sites. Moreover, as the Ca^{2+} levels in vaginal secretions do not fluctuate, the low-iron parasites with decreased ability to cytoadhere [40] would possess high numbers of FN-binding sites and still be capable of extracellular matrix-basement membrane associations thereby enhancing survival and contributing to the non-self-limiting nature of infection. In this scenario, it can be hypothesized that Ca^{2+} might be activating a signalling pathway for up-regulating *de novo* expression of FN receptors, and possibly other virulence factors. Collectively, the data support the view that FN binding by *T. vaginalis* is a process requiring activation of the FN receptors by a Ca^{2+}-independent phosphatase and FN receptor ligation itself. The data further strengthen the hypothesis that both high- and low-affinity FN receptors whose expression is regulated by iron exist.

Materials and Methods

Culture and growth of *T. vaginalis*

The fresh clinical *T. vaginalis* isolate T016 [23] was grown no longer than four weeks by daily passage in Trypticase-yeast extract-maltose medium supplemented with 5% heat inactivated horse serum (TYM-serum). Parasites were grown to mid-logarithmic phase (20 h), after which washed trichomonads were suspended to the same final density in fresh TYM-serum for an additional 2 h incubation at 37°C. Parasites were harvested and washed three times in cold minimal binding (MB) buffer (120 mM NaCl, 1.3 mM KCl, 0.9 mM NaH_2PO_4, 5.5 mM glucose, and 26 mM $NaHCO^3$, pH 5.0) [23] prior to use in a binding assay (described below). For chemical fixation, organisms were washed three times in PBS and incubated 16 h at 4°C in PBS-3% glutaraldehyde (v/v). Treated parasites were then washed five times in cold PBS and incubated for 16 h at 4°C in PBS-500 mM glycine to neutralize fixative, after which the chemically-stabilized organisms were washed and stored

in PBS-0.1% sodium azide until use. Low-iron-grown trichomonads were obtained as previously detailed [23, 40]. Briefly, parasites at a starting density of 10^5 organisms/ml were cultured overnight in TYM-serum containing 50 μM of the iron chelator 2,2-dipyridil (2,2-DP; Sigma Chemical Co., St. Louis, MO, U.S.A.) for 24 h at 37°C [23, 40]. These organisms were then suspended in TYM-serum supplemented with 75 μM 2,2-DP for an additional 24 h incubation. To obtain high-iron organisms, low-iron parasites were washed once and suspended in TYM-serum medium containing 250 (μM ferrous ammonium sulfate (Sigma) and incubated for 20 h at 37°C. Low- and high-iron cultures were processed as above for the binding assay.

Radiolabelling of *T. vaginalis*

To examine for changes in surface protein patterns by Ca^{2+}, trichomonads were harvested and washed three times in PBS after a binding assay with unlabelled FN. Parasites were labelled for surface proteins by radioiodination with 0.1 mCi Na[^{125}I] [43]. To monitor possible changes in protein phosphorylation patterns upon addition of Ca^{2+} and FN, trichomonads were prelabelled for 2 h at 37°C in MB buffer (120 mM NaCl, 1.3 mM KCl, 0.9 mM NaH_2PO_4, 5.5 mM glucose and 26 mM $NaHCO_3$, pH 5.0) [23] containing 400 μM TLCK (Sigma) and 0.1 mCi ^{32}P-*ortho*-phosphate (185 MBq, NEN). A binding assay was then performed with unlabelled FN and Ca^{2+}, and samples were obtained at different time points. After washing in MB buffer containing TLCK, ^{125}I and ^{32}P-labelled organisms were lysed in electrophoresis dissolving buffer, and the samples were analysed by SDS-PAGE gels, and autoradiography using established protocols [14, 17, 24].

Purification and radioiodination of FN

FN was purified from human plasma from the University Hospital blood bank by gelatin-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) affinity chromatography as detailed previously [41–43]. Briefly, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) and 50 mM ε-amino-*n*-caproic acid were added together as protease inhibitors to prewarmed (37°C) plasma. Plasma was centrifuged for 15 min at 10 000 × *g* followed by rapid passage over of a Sepharose CL-4B precolumn (Amersham) prior to application to a gelatin-Sepharose column. The gelatin-Sepharose beads were first washed extensively in buffer consisting of 1 M NaCl, 50 mM Tris–HCl, 50 mM 6-aminohexanoic acid and 20 mM sodium citrate, pH 7.6, followed by a washing in this same buffer without 1 M NaCl. Bound FN was eluted with 100 mM NaCl containing 50 mM sodium citrate, pH 5.5, followed by a second elution with 4 M urea in TBS (150 mM NaCl and 10 mM Tris–HCl, pH 7.0). The pooled fractions containing FN were precipitated with 50% NH_4SO_2 (w/v) while stirring at 4°C for over 2 h. Precipitated FN was centrifuged for 15 min at 10 000 × *g*, and pelleted FN was suspended in PBS for dialysis at 4°C with two buffer changes each day for 3 days [41]. The protein concentration was determined using BCA. Iodination of FN was performed using the chloramine-T method [39, 58]. Briefly, FN at 1 mg/ml were incubated with 0.1 ml chloramine-T (2 mg/ml in PBS; Sigma) and 1 mCi Na[^{125}I] (185 MBq) for 5 min at RT. After addition of 0.1 ml sodium metabisulfite (4 mg/ml in PBS), iodinated FN was purified by G25 Sephadex chromatography. The concentration of ^{125}I-labelled protein, and the specific activity (cpm/fmol) was calculated for each experiment. The quality and purity of FN was always monitored by SDS-PAGE and autoradiography [17, 58].

Immunofluorescence and immuno-electron microscopy of parasites with bound FN

Both immunofluorescence and immuno-electron microscopy were performed using standard protocols [22]. Following overnight growth, washed organisms were fixed with 4% paraformaldehyde in 100 nM cacodylate buffer, pH 7.2, for 2 h, washed in MB buffer, pH 5.0, and incubated in MB buffer containing 10 mg/ml FN for 3 h with occasional agitation. After three washes in MB buffer, the cells were allowed to adhere onto poly-L-lysine-coated cover slips followed by quenching with 50 mM NH_4Cl and 3% (w/v) bovine serum albumin (BSA). Trichomonads fixed onto cover slips were washed in PBS-2% BSA, pH 8.0, for 30 min followed by incubation with rabbit anti-FN antiserum or control, prebleed normal rabbit serum diluted 1:300 in PBS for 3 h. After washing in PBS, cover slips were incubated for 50 min with goat anti-rabbit

antibody conjugated with tetramethylrhodamine B isothiocyanate (1:100), followed by additional washes in PBS. No reactivity has ever been observed using prebleed normal rabbit serum as control. Each cover slip was mounted onto slides using N-propyl-gallate as an anti-fading agent, and examined with an Axiophot II Zeiss microscope equipped with UV epifluorescence. Images were acquired with a Hamamatsu chilled CCD camera C5985 and overlaid with DIC images.

Organisms prepared for immuno-gold labelling and transmission electron microscopy were washed three times in PHEM buffer (50 mM $MgCl_2$, 70 mM KCl, 10 mM EGTA, 20 mM HEPES, 60 mM PIPES, pH 6.8) at 37°C, and fixed overnight in 4% paraformaldehyde, 0.4% glutaraldehyde, and 1% picric acid in 100 mM cacodylate buffer, pH 7.0. Following three washes in MB buffer, pH 5.0, trichomonads were incubated in MB buffer containing 10 mg/ml FN for 3 h. Samples were subsequently washed in MB buffer, quenched with 50 mM NH_4Cl-3% BSA, and washed again in PBS-2% BSA, pH 8.0, for 30 min. For immunogold labelling, trichomonads were incubated with rabbit anti-FN antibody (1:300) for 3 h, and washed several times with PBS-1% BSA. Sections were incubated with 10 nM gold-labelled goat anti-rabbit IgG antibodies (1:100, BBInternational) for 1 h followed by three washes in PHEM buffer at 37°C. Samples were once again fixed overnight with 0.1 M cacodylate buffer-2.5% glutaraldehyde (v/v) at RT. Post-fixation was performed in 1% OsO_4 in cacodylate buffer containing 5 mM $CaCl_2$ and 0.8% $K_3Fe(CN)_6$. Cells were washed, dehydrated in acetone, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and observed in a Jeol 1210 electron microscope. Control samples were not incubated with primary antibody. The pre-embbeding technique was preferred as this permits immuno-labelling using whole cells and more accurate visualization of surface-associated FN. Further, as the whole cell labelling is performed before embedding the cells by the resin, the antigenic sites are better preserved for antibody recognition.

Trichomonad binding assays

All assays, unless specifically indicated, were performed at least three times using triplicate samples using the following procedure unless indicated otherwise. For a standard binding assay, 500 000 live trichomonads were suspended in MB buffer containing 400 μM TLCK and iodinated FN for a final volume of 0.1 ml/sample. Samples were incubated at 37°C for 20 min, or as indicated in the text, followed by three washes in MB buffer. The amount of labelled protein bound was measured by scintillation spectroscopy, and cpm was converted to fmol protein bound. As previous work had shown that glutaraldehyde-fixed trichomonads can generate more accurate quantitative results in amounts of protein acquired [39], organisms were fixed as described above and incubated with a mixture comprised of 4 μg ^{125}I-FN and increasing concentrations of unlabelled FN or of transferrin as a control serum glycoprotein.

Treatment of trichomonads with inhibitors

Organisms were suspended in MB buffer as above and incubated for 30 min at 37°C with or without 10 nM cycloheximide (Sigma). Parasites were then suspended in MB buffer containing 10 μg ^{125}I-FN in the presence or absence of 1 mM $CaCl_2$ and/or 10 nM cycloheximide. As a control for inhibition of protein synthesis after treatment with cycloheximide, trichomonads were incubated with ^{35}S-labelled methionine and cysteine (518 MBq) (EasyTag™ EXPRESS Protein Labelling Mix, NEN) for 20 min at 37°C prior to lysis in dissolving buffer for SDS-PAGE analysis. Autoradiography showed almost complete inhibition of protein synthesis, as before, in cycloheximide-treated protein samples.

Stock solutions of kinase inhibitors staurosporine (ST), H7 and wortmannin (WT) were also from Sigma. Bicinchoninic acid (BCA), all from Pierce Biochemicals, and phosphatase inhibitors α-naphthyl acid phosphate (NPA), cypermethrin (Cpn), dephosphatin (Dpn), okadaic acid (OA) and tautomycin (Taut), from Calbiochem (San Diego, CA, U.S.A.), were prepared as recommended by the manufacturers. ST was dissolved in ice cold 100% methanol to obtain a final stock concentration of 1 mM. H7 and NPA were diluted in sterile distilled water to a final concentration of 100 mM each. WT (20 mM), Cpn (100 mM), Dpn (10 mM), OA (100 μM), Taut (100 μM) were each dissolved individually in dimethylsulfoxide (DMSO; American Type Culture Collection, Rockville, MD, U.S.A.). All reagents were stored in the dark at either 4°C or −20°C as directed by the manufacturers. The

final concentration of DMSO and methanol during trichomonad treatment was 0.5%, neither of which was toxic, as evidenced by trypan blue exclusion and trichomonal motility. Parasites were suspended in MB buffer and treated with the various kinase inhibitors (1 μM ST, 100 μM H7 or 100 nM WT) and phosphatase inhibitors (10 mM NPA, 20 μM Cpn, 10 μM Dpn, 200 nM OA or 200 nM Taut) for 30 min at 37°C prior to interaction with iodinated FN. Trichomonads handled identically with 0.5% DMSO and MeOH were included as controls.

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