Commitment to cyst formation in *Giardia*

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*Giardia* trophozoites differentiate into infectious cysts (encystment) in response to physiological stimuli; encystment is crucial for *Giardia*’s transmission, survival and pathogenesis. *In vitro*, *Giardia* encysts when bile sequesters lipids necessary for this lipid auxotroph, and *in vivo* they encyst to infect new hosts. In this study, we investigated, for the first time, commitment to encystment in *Giardia* using both molecular and cellular techniques. We show that after 3–6 h in inducing conditions, encysting trophozoites continue to encyst regardless of whether the inducing stimulus remains. We propose that a trophozoite’s inability to revert to a growing or dividing trophozoite represents a commitment to encystment. The onset of commitment correlated with the appearance of encystment specific vesicles (ESVs) and encystment specific protein synthesis. These observations suggest the involvement of regulatory pathways with the ability to ‘remember’ a transient signal long after its removal; a property that enables encysting trophozoites to complete the encystment process should the unfavourable triggering condition(s) change. The ability to form cysts in response to transient signals or, as we have highlighted in this paper, the ability of a small percentage of the population to form cysts without an inducer is vital for the maintenance of infection within populations.

INTRODUCTION

*Giardia intestinalis* (syn. *duodenalis*, *lamblia*), a ubiquitous human intestinal protozoan parasite causes giardiasis, an infection which may present asymptomatically or cause symptoms such as severe diarrhoeal disease and failure to thrive in infants. Giardiasis is one of the most frequent causes of non-viral waterborne diarrhoea worldwide and is associated with mild to severe malnutrition, growth retardation and impaired cognitive development in children (Adam, 2001; Cotton et al., 2011). Infection begins when cysts are ingested and triggered to excyst, releasing trophozoites that colonize the small intestine (Adam, 2001). As trophozoites travel through the host intestine, some differentiate into infective cysts in a process known as encystment. Encystment is induced *in vitro* by exposing trophozoites to either low cholesterol conditions or alkaline pH and high bile conditions (Lopez et al., 2003). Encystment involves the expression of encystment specific proteins that are responsible for synthesis of a protective cyst wall made up of a unique β-1,3-α-acetylgalactosaminoglycan homopolymer (termed giardin) (Macechko et al., 1995; Sun et al., 2003; Luján, 2011). The CWPs are transported to the cell membrane via novel regulated secretory granules, encystment specific vesicles (ESVs). Regulation of encystment appears to be at a transcriptional level; a number of transcription factors have been characterized and shown to be involved in encystment specific gene expression (Sun et al., 2002, 2006; Wang et al., 2007; Huang et al., 2008; Pan et al., 2009; Wang et al., 2010).

Encystment serves as an important transmission and virulence factor that allows *Giardia* to survive outside its host while being transmitted from one host to another (Adam, 2001, Svärd et al., 2003). The environment colonized by *Giardia* is characterized by fluctuating physiological conditions mediated by the metabolic and physiological state of the host. To survive the environmental changes it encounters while travelling through the host’s intestine, *Giardia* must be able to sense and respond rapidly to these changes. Whether *Giardia* trophozoites encyst or not as they colonize the host is a very important cellular decision as encystment takes place at the expense of proliferation; a trophozoite that enters the encystment pathway forfeits the opportunity to grow and proliferate. Additionally, the encystment process is an energy intensive process that requires synthesis of new macromolecules, but on the other hand, it is vital for the maintenance of infection in host populations.

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Abbreviations: ESV, encystment specific vesicle; CWP, cyst wall protein; NET, non-encysting trophozoite; ET, encysting trophozoite.
Other microbes, such as bacteria and amoeba, that deal with frequent environmental fluctuations possess adaptive strategies, such as developmental commitment, to facilitate responses to the changing growth conditions. In response to conditions of nutrient limitation, vegetative cells of Bacillus subtilis stop growing and enter a developmental pathway that results in the formation of spores. Sporulating B. subtilis exhibit developmental commitment that ensures differentiation irrespective of the changes in the extracellular environment (Dworkin & Losick, 2005). Katoh et al. (2007) showed that there is a point of commitment during Dictyostelium differentiation, which provides the cells within a starving population with a physiological cue to continue development, and prevents responses to nutritional cues that might inappropriately block development. There have been no previous studies, of which we are aware, that examine if parasitic flagellates such as Giardia exhibit commitment to encystment even if that stimulus is removed.

In this paper, we have addressed the question of whether initiation of Giardia encystment is an irreversible process or if it can be reversed should conditions become favourable. Evidence is presented in the current study that Giardia trophozoites induced to encyst become committed to cyst formation after as little as 3–6 h of induction even though the initial stimulus is removed. This developmental commitment increases Giardia’s chances of survival and contributes to its success as a parasite.

**METHODS**

*Giardia* cultivation and encystment. *Giardia intestinalis* (strain WB clone 6 from ATCC) with an 8-10 h cell cycle (Reiner et al., 2001) was grown anaerobically and axenically at 37°C in TYI-S-33 medium (Keister, 1983) with or without 1 mg ml⁻¹ bovine bile (Sigma-Aldrich) (growth medium). Encystment medium was made as previously described (Lopez et al., 2003). However, before encystment was induced, trophozoites were washed in growth medium without bile and grown in growth medium without bile for at least 24 h. With the exception of the assay for heterogeneity (Fig. 4), dividing trophozoites – which are motile and non-adherent – were removed by pouring off the supernatant of growing cultures. The remaining non-dividing trophozoites, which adhere to the plastic culture flask in confluent cultures, were used to initiate encystment. These cells were collected by chilling the culture flasks with growth medium in an ice water bath for approximately 10 min. Cells were then washed and transferred into encystment medium and incubated at 37°C. Cells grown in 96-well plates were covered with Parafilm and grown in an anaerobic incubator (Coy laboratories, type B).

Protein gel separation and Western blot analysis of cyst wall protein 1 and 2 protein during encystment. SDS-PAGE was performed under reducing conditions using the Novex Tris-Glycine SDS-PAGE system from Invitrogen. Cells, harvested by chilling, were centrifuged at 2000 g for 5 min. Each cell pellet was washed twice in 1× PBS and incubated in 200 μl of lysis buffer (10 mM magnesium sulfate, 50 mM HEPES, 0.1% (v/v) Triton X-100, pH 7.7). Protein concentration was determined using the Bradford method (Bradford, 1976) and measurements were performed with a microplate reader (BioTek Synergy HT). For SDS-PAGE, 20 μg of protein was diluted in 2× Novex Tris-Glycine SDS sample buffer (Invitrogen), 2× NuPAGE sample reducing agent (Invitrogen) and distilled water according to manufacturer’s direction to a total volume of 25 μl. This mixture was heated at 85°C for 2 min, allowed to cool and loaded on a 4–20% (w/v) Tris-Glycine SDS-polyacrylamide gel (Invitrogen) in a XCell SureLock Electrophoresis Cell (Invitrogen) containing 1× Tris-Glycine SDS running buffer (Invitrogen). Electrophoresis was performed for 1 h at 160 V.

For Western blots, proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane in transfer buffer (20 mM Tris, 150 mM glycine, and 20%, v/v, methanol) for 2 h at 20 V in a semi dry transfer unit (Fisher Scientific). The nitrocellulose membrane was blocked with 5% (w/v) non-fat milk in Tris-buffered saline (25 mM Tris/HCl, pH 7.4, 140 mM NaCl and 3 mM KCl) containing 0.1% (v/v) Tween 20 and then incubated overnight with appropriate antibodies. For CWP1 and 2, a mAb (generous gifts from Professor Hugo Lujan) was used at a dilution of 1:1000 in Tris-buffered saline. For anti-α-tubulin (housekeeping protein) a mAb (Sigma-Aldrich) was used according to manufacturer’s instructions. Nitrocellulose membranes were washed for approximately 1 h in Tris-buffered saline containing 0.1% (v/v) Tween 20 before incubation in the

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**Fig. 1.** Effect of interrupting encystment induction on *Giardia* cyst formation. Commitment to encystment was analysed by interrupting induction with growth medium. (a) Interruption of encystment does not prevent cyst formation in induced trophozoites; some trophozoites go on to form cysts in the absence of the inducing medium. Cyst concentration was determined by haemocytometer counts after water resistance treatment. (b) A single cell assay of commitment was performed as described in Methods. Error bars represent SEM for n of 3. ‘Induction interrupted’ samples were analysed after 72 h.
secondary rabbit anti-mouse IgG Ab with horseradish peroxidase conjugated (Fisher Scientific; 1:20,000 dilution). Membranes were incubated with the secondary Ab for 1 h and washed twice in Tris-buffered saline containing 0.1% (v/v) Tween 20. The membrane was developed with ECL (Invitrogen) and the chemiluminescent signals were exposed to X-ray film (Kodak) which was developed with an Automatic X-Ray Film Processor (AFP) imaging system from ImageWorks (model Mini-Medical 90). Novex sharp pre-stained protein standards from Invitrogen were used as molecular mass markers. Populations induced for the times indicated served as controls to which the populations, in which encystment was interrupted, were compared. Flow cytometric analysis was also performed to determine the expression of these proteins in individual cells.

**Immunofluorescence and flow cytometric analyses.** For either immunofluorescence or flow cytometric analyses, trophozoites were fixed in 3% (v/v) formalin diluted in 10 mM PBS at pH 7.4 with 0.02% (v/v) Triton X-100 for 15 min. Fixed cells were then washed in 1 × PBS, incubated overnight with either the CWp1 or CWp2 mAb. The mAb-labelled cells were then washed twice in 1 × PBS and exposed to the rabbit anti-mouse IgG FITC conjugated secondary antibody for 1 h. After washing the cells twice in 1 × PBS, they were observed microscopically, or for flow cytometry, a BD flow cytometer (model: FACScan) was used and data analysed with Flowjo v.7.2.4 (Tree Star). Controls in both methods were treated with only the FITC conjugated 2 Ab. Fluorescence microscopy was performed with a Zeiss differential interference contrast microscope (DIC) with fluorescence (Axioplan 2). The microscope was equipped with a Hamamatsu C4742-95 camera used for taking all fluorescence micrographs. For single cell assays, an Olympus IX71 inverted microscope with a Hamamatsu C4742-95 camera was used.

**Effect of interrupting encystment induction on induced *Giardia* populations.** Trophozoites (approximately 10⁶) in exponential growth were induced to encyst in encystment medium for 0, 3, 6, 12, 18, 24 and 72 h in 15 ml centrifuge tubes. At each of these times, cells in encystment medium were centrifuged, washed once in growth medium without bile, transferred into growth medium and cultured for a total of 72 h. Populations only induced for the times indicated served as controls for the populations in which encystment was interrupted (by replacing the encystment medium with growth medium) and these two populations were compared to assess the effect of encystment interruption on cyst formation. To obtain the total cyst number, harvested cells were suspended in distilled water and maintained at 4 °C for about 48 h (Gillin et al., 1989). After this period, water resistant structures were harvested by centrifugation for 5 min at 4 °C to remove non-encysting and dead trophozoites. This pellet was then resuspended in 1 ml of 1× PBS and cells and cysts were counted with a haemocytometer. Immunofluorescence and flow cytometric analyses of these populations were performed as described above.

**Effect of interrupting encystment induction on induced single *Giardia* cells.** Single cells (visible trophozoites only) from the serial dilution of cultures in growth medium were transferred into each well of a 96-well microtitre plate (BD Biosciences). These trophozoites were induced to encyst for 0, 3, 6, 12, 18, 24 and 48 h then centrifuged at 2000 g for 5 min, the pellet was then washed in growth medium and finally resuspended in fresh growth medium and incubated at 37 °C for a total of 72 h. Plates were monitored by light microscopy after the induction period and after the 72 h time point to determine whether or not a single trophozoite had formed a cyst (accessed by roundness of the cells, lack of motility and refractivity). Populations only induced for the time points indicated served as controls to which the populations in which encystment was interrupted were compared to assess the effect of interruption.

**RESULTS**

**Trophozoites become committed once induced to form cysts**

Commitment to encystment was measured by determining the point at which transfer of encysting trophozoites into non-inducing conditions fails to prevent cyst formation. This in turn was elucidated based on increased cyst formation after transfer into non-inducing conditions. The total number of cysts formed increased when encysting trophozoites were transferred into non-inducing conditions, i.e. growth medium (Fig. 1) at the times examined. Fig. 1(a) shows that trophozoites induced to encyst become committed to encystment after as short as 3 to 6 h; by 12 h after induction of encystment, most, if not all, of the cells that were going to encyst were committed. Although these data show that trophozoites become committed to encystment, it does not show if trophozoites that were encysting were encysting at the time of interruption of induction were the same ones that eventually went on to form the cysts counted after the interruption time point. To verify that trophozoites encysting at the time of interruption of encystment induction really go on to form cysts, a single cell assay was conducted in which encystment was interrupted in individual trophozoites induced to encyst for different time points. Trophozoites were then assessed for encystment at the end of the experiment. In Fig. 1(b), this single cell assay showed that trophozoites induced to encyst for various times also go on to form cysts even if encystment induction is interrupted. The time dependence of encystment in the single cell assay (Fig. 1b) is similar to that seen in the population analyses (Fig. 1a); as in the population assay, the number of trophozoites committed to encystment increased with

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**Co-culture of trophozoites with either encysting or non-encysting trophozoites.** To determine if non-encysting trophozoites (NET) in an induced population formed cysts, or encysting trophozoites (ET) promote or prevent encystment of induced trophozoites, we co-cultured cells from 12 h, 18 h, 24 h and 48 h induced populations with trophozoites from a confluent non-induced population using a 12-well plate and corresponding 0.4 µm pore size cell culture inserts (Corning). NET and ET were collected as described previously. Co-culture was carried out at a 2:1 ratio of induced to growing trophozoites; growing trophozoites were kept in the wells of the culture plates while the ET and NET were kept in the cell culture inserts using the volume specified by the manufacturer. The ability of ET and NET to promote encystment was tested in growth medium while their ability to inhibit cyst formation was tested in encystment medium. Plates were covered and sealed with Parafilm and incubated anaerobically in an anaerobic chamber (Coy laboratories, type B) at 37 °C for 48 h. The cell suspensions in these plates were then analysed for cyst formation. Controls were included to verify that there was no exchange of cells between chambers and inserts; for this, insert with trophozoites but without trophozoites in the well, and wells with trophozoites in the well but not in the inserts were used. The controls to which the co-cultured samples were compared included cyst formation without co-culture under inducing conditions and non-inducing conditions; the inserts for these controls contained neither NET nor ET cells while the culture wells contained fresh trophozoites incubated in encystment or growth medium, respectively.
time. The single cell assay showed that trophozoites became committed to encyst by 6 h after induction (Fig. 1b), while Fig. 1(a) suggests commitment occurs after 3 h. Interestingly, commitment to encystment correlated with the appearance of ESVs; trophozoites with visible ESVs at the time of induction interruption (replacing the encystment medium with non-inducing medium) encysted in the absence of the inducing conditions.

**Expression of cyst wall protein 1 and 2 (CWP1, 2) during encystment**

In order for encysting trophozoites to become committed to the encystment pathway, the expression of the proteins required for the formation of cysts should be stably maintained once induced. To determine if expression of encystment specific proteins becomes committed during encystment, the expression of cwp1 and cwp2 in encysting trophozoites whose encystment has been interrupted was assessed by Western blot analysis. Fig. 2 shows CWP1 and 2 are both detectable by Western blot after 12 h in inducing conditions (not interrupted). However, the 6 h interrupted induction populations show that expression of both proteins occurs earlier than the 12 h time point; although there was no protein detected at 6 h after induction (not interrupted), CWP expression is markedly increased in the 6 h interrupted population, suggesting that CWP expression occurs by 6 h after induction of encystment and is stably maintained after interruption of induction. These proteins increased markedly in the 12 h interrupted and again in the 18 h interrupted induction. At 18 h, the induced but not interrupted groups show similar levels of CWP1 to the interrupted group. Levels of CWP2 were lower in the normal, non-interrupted induced population than seen in the interrupted group; however, unlike CWP1, this was seen even up to 18 h. The reason for this presumably relates to the fact that in the interrupted population, cells have had 24 h to form cysts and agrees with the data shown in Fig. 1. In the non-interrupted induced population not all cells are at the same stage of encystment; however, by 18 h of induction expression of CWP1 and 2 are similar in both groups. It is clear from these data that the encystment pathway remains active after removal of inducing conditions, and this supports the observation that trophozoites become committed to encystment after a short period of time (Fig. 1).

**Flow cytometric analysis of encystment**

The effect of induction interruption on encystment specific protein expression was also assessed by flow cytometry.
where a FITC-conjugated secondary antibody was used to determine the presence of CWP2 in individual cells. Fig. 3 supports the data shown in Fig. 2 in that those populations where induction is interrupted show CWP2 levels that are greater than those seen in the induced non-interrupted population. It should also be noted that two clear populations could be seen in the 12 h interrupted group; this is not seen in the 12 h normal induction group. It is important to note that after 18 h both cell treatments produce profiles that exhibit two clear populations, however the fluorescence intensity is different; increased encystment is observed after interruption of encystment induction. After 24 h the distribution of staining in both populations is like that seen in the 72 h encystment control.

**Intercellular communication during encystment does not appear to occur**

The involvement of extracellular signalling molecules in *Giardia* encystment induction was assessed with spent medium from *Giardia* populations that had been induced to encyst for 6, 12, 18, 24 and 72 h. The encystment levels obtained from the use of spent encystment medium suggests that encystment in non-encysting cells was not induced by signals from encysting cells. Encystment obtained using the spent medium was not due to cholesterol depletion, as cholesterol was replenished in the spent medium by adding 10% (v/v) calf serum. The encystment level obtained when serum was added to the spent encystment medium was comparable to that obtained with just spent encystment medium alone again.

![Flow cytometric analysis of CWP2 levels in induced and induction interrupted cell populations. The data include a 72 h encystment control and the number of cells analysed for each profile was no less than 10⁵. NET, Non-encysting trophozoites; ET, formed cysts or encysting trophozoites.](image-url)
indicating that there was no induction of encystment in non-encysting cells by encysting cells.

**Giardia populations exhibit heterogeneity**

*Giardia* populations were accessed for phenotypic heterogeneity during encystment induction and vegetative growth. Trophozoites were induced to encyst for different time periods and the level of encystment determined. Fig. 4(a) shows that cysts (approx. 5–8%) are present even at the start of the induction process (T0), which suggests that cysts are formed even without an inducer present. To verify that the encystment observed was not a result of nutrient depletion, the growth medium in a vegetative culture was replaced with fresh medium every 24 h; Fig. 4(a) shows that cyst formation persisted under favourable growth conditions with abundant nutrients (72GM). During encystment, there was a typical increase in the number of *Giardia* trophozoites that became cysts; however, by 72 h (approximately nine cell cycles) in encystment medium, approximately 20% of the population remained undifferentiated (Fig. 4a, c), resulting in a heterogeneous population. This heterogeneity persisted when the non-encysting trophozoites from encysting populations were cultured to confluence and subjected to another round of encystment (Fig. 4b).

Fig. 5 shows flow cytometric analysis of encystment using anti-CWP2. Encysting trophozoites demonstrated heterogeneity in the expression of CWP2 during encystment (Fig. 5a). By 12 h after induction of encystment, the distribution of the population with regard to fluorescence intensity becomes broader (shifted to the right) and by 18 h, the population has divided into two distinct groups — one showing fluorescence intensity similar to the T0 control group (trophozoites) and the other showing fluorescence intensity consistent with encysting cells (cysts). Clearly, some trophozoites enter the encystment pathway while others do not. When NET from induced populations were separated from encysting cells, collected and subjected to a second round of growth and encystment induction, the same heterogeneity pattern persisted (data not shown) indicating that at least some trophozoites that were not encysting during the first cycle of encystment induction were capable of encysting in subsequent cycles.

**DISCUSSION**

Success of *Giardia* as a parasite depends on its ability to differentiate into cysts, and when to encyst or remain a trophozoite is central to this. For example, if trophozoites entering the duodenum during an infection encyst before they reach the jejunum, they forfeit the opportunity to divide and increase the probability of propagating the infectious population. On the other hand, trophozoites that are carried downstream must rapidly differentiate into cysts to be able to survive outside a host and reach another host. Clearly, it is beneficial to survival of *Giardia* that trophozoites encyst when faced with harsh environmental conditions, but it is equally important that trophozoites are able to resume vegetative growth in situations where the harsh environmental condition is only transient. Likewise, it is important that some *Giardia* trophozoites encyst spontaneously without the presence of an exogenous inducer so that there will always be small numbers of cysts (these may not always be detectable) entering the faecal stream to guarantee dissemination of *Giardia* even if an extreme event (for example, severe diarrhoea) were to evacuate trophozoites. That morphological heterogeneity persisted (Figs 4 and 5) after several cycles of encystment induction or vegetative cultivation as we have shown indicates that maintaining a heterogeneous population (both trophozoites and cysts) is an inherent property of the *Giardia* life cycle and is likely to be a mechanism for giving the parasite population a chance to recolonize a host and reach other hosts under both favourable and unfavourable conditions.

*Giardia* trophozoites, like most unicellular organisms, must respond to a constantly changing environment. Therefore, it is important that they respond rapidly to appropriate signals in order to survive. Furthermore, encystment is energy expensive (Sener et al., 2009); it takes place at the expense of continued growth and requires significant turnover of biological molecules. The process must therefore be tightly regulated to ensure that it only takes place when absolutely necessary. The use of regulatory pathways that can remember a signal even after it has been removed will allow for tight regulation of these processes (Bagowski & Ferrell, 2001; Xiong & Ferrell, 2003; Smits et al., 2005).

Commitment to differentiation in unicellular organisms has been extensively studied in both prokaryotic and eukaryotic systems where the phenomenon has been attributed to the presence of feedback loops in the regulatory pathways involved (Ferrell, 2002; Ferrell & Xiong, 2001; Smits et al., 2005; Veening et al., 2008); when the concentration of a regulatory protein in a feedback loop reaches the critical concentration for activating the feedback loop, the feedback loop is turned on and its effect sustained. Once this is achieved, the downstream effect of the regulatory pathway is expressed even in the absence of the inducing signal — a mechanism described as hysteresis (Bagowski & Ferrell, 2001; Sha et al., 2003; Wang et al., 2006). There is evidence for the involvement of feedback loops in regulation of encystment; the *Giardia* transcription factor, Myb2, promotes upregulation of its own expression and that of four other important encystment specific genes — *cwp1, 2 and 3 and Gnp* — during encystment (Sun et al., 2002). The MAPK-ERK signalling pathway that has been proposed for regulation of encystment (Ellis et al., 2003) also has numerous feedback loops embedded in it (Ferrell, 2002; Xiong & Ferrell, 2003). The data presented here show that there is a point of commitment during encystment, after which encysting trophozoites complete the encystment process even in the absence of the inducer.
(Fig. 1). Even though commitment was observed at many of the induction times investigated, the earliest commitment detectable was after as little as 3–6 h in encystment conditions (Figs 1 and 2) and the majority of the population became committed to the encystment pathway after 12 h in encysting conditions (Fig. 1). Interestingly, the commitment
times correlate fairly closely with the decreases observed in the ability of exogenous glucose to stimulate oxygen uptake or metronidazole to inhibit cyst metabolism (Paget et al., 1998), and with the detectable appearance of mRNA for glucosamine-6-phosphate isomerase (deaminase) (Lopez et al., 2003), the first enzyme induced in the pathway required for the synthesis of giardan (Sener et al., 2009). That maximum commitment occurs by 12 h after induction fits in nicely with a cell cycle time of 9–12 h (Reiner et al., 2008). Consistent with cyst formation, encystment specific gene expression is stably maintained after interruption of induction (Fig. 2). The involvement of feedback regulatory loops that exhibit hysteresis will explain the stability in the expression of encystment specific proteins. These observations show that commitment to encystment with respect to cwp expression occurs at a translational level and is activated during the late phase of the induction process (Erlandsen et al., 1996), which explains the observation that most members in the population were committed to encystment after 12 h in encystment medium. It also suggests that during the very early stages of encystment, trophozoites may be capable of aborting the encystment process when the stressor is removed and conditions once again become favourable for growth.

Considering that the mRNA for the enzymes in the cyst wall carbohydrate pathway become apparent about 6 h after induction of encystment (Van Keulen et al., 1998), it appears that the expression of CWPs and their synthesis precedes that of the cyst wall polysaccharide into which they become incorporated to form a functional cyst wall. The data suggest that cyst wall protein assembly and transport are requirements for commitment to encystment since commitment to encystment occurred after the appearance of ESVs. ESV formation is one of the cellular hallmarks of encystment induction; several groups have showed that visible ESVs appear as early as 5 h after induction of encystment (Faubert et al., 1991; Hehl et al., 2000; Lujan & Touz, 2003). As reported earlier (Davids et al., 2004; Reiner et al., 2001; Touz et al., 2002), substances that inhibit formation of ESVs prevent differentiation of trophozoites into cysts, indicating that transport and assembly of CWPs is a key regulatory step during the encystment pathway.

With the use of techniques that allow for the study of individual members of Giardia populations, we have shown that Giardia populations bifurcate into subpopulations of the two Giardia cell types (cysts and trophozoites).
under both vegetative growth and encysting conditions. The heterogeneity observed is employed by Giardia most likely to maximize its chances of survival; the presence of both cell forms in a population allows the population to survive under both favourable and unfavourable conditions and at a lower energy expense. We propose that as in most bacterial systems that exhibit heterogeneity (Dubnau & Losick, 2006; Ferrell, 2002), regulatory pathways for Giardia differentiation employ a threshold mechanism that ensures that cyst formation is initiated only in cells with the necessary concentration of the regulatory proteins, and due to differences in the rate of gene expression, some cells are able to reach the concentrations required while others are not. With such a mechanism in place, and if conditions become favourable after a transient period of nutrient deprivation, members of the population with suboptimal concentrations of encystment regulatory proteins will be able to resume growth without the energy expense of both encystment and excystation. The use of a threshold mechanism in the regulation of encystment will also ensure that, when unfavourable conditions become prolonged, some trophozoites will be able to form cysts in order for the population to survive.

While the mechanisms governing the regulation of the decision-making processes in Giardia have yet to be determined, our data show clearly that Giardia employs commitment and phenotypic heterogeneity to increase its survival.

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