long-time exposure.

Reactivation of latent brain cysts

Latently infected mice were prepared as described above. Dexamethasone (dexamethasone 21-phosphate disodium salt; Sigma) was dissolved at a concentration of 10 mg/liter in the drinking water, and the latently infected mice were allowed free access to the dexamethasone-containing water. After 28 days of dexamethasone treatment, the brains of treated mice were excised and chopped into small pieces. The pieces of brain were crushed onto glass slides and observed under fluorescence microscopy.

Anti-BAG1 antiserum

The truncated BAG1 gene (aa 94-229,) with introduced *Bam* HI and *Sma* I sites, was synthesized and ligated with the pGEX-4T-3 vector (Amersham Pharmacia Biotech, PA), which was digested with *Bam* HI and *Sma* I. *E. coli* (BL21) was transformed with the resulting plasmid and cultured in LB medium containing 100 μg/ml ampicillin at 37°C until OD_{600} reached 0.3. Isopropyl-β-D(-)-thiogalactopyranoside was then added to the medium (final concentration, 1 mM). Following 3 hr of incubation, the transgenic *E. coli* were pelleted by centrifugation at 3,000 rpm for 10 minutes at room temperature to remove the supernatant. Soluble, bacterial proteins were eluted using B-PER™ Reagent (PIERCE, USA) according to manufacturer's instructions. From the soluble proteins, glutathione *S*-transferase (GST) fusion protein (GST-BAG1)-was purified using Glutathione Sepharose™ 4B (Amersham Bioscience, Sweden) according to manufacture's instructions. A total of 50 μl of purified GST-BAG1 solution (containing 16.3 μg/μl GST-BAG 1) was mixed with 100 μg of adjuvant, TiterMax Gold (TiterMax USA. INC., GA). A total of 100 μl of the mixture was subcutaneously injected into each of three 6-week-old female BALB/c mice. Thirty-two days after the first immunization, a second immunization was performed by injecting 200 μl of the mixture. Serum (anti-BAG1 antiserum) was harvested from immunized mice 20 days after the second immunization, and was stored at -20°C until further use.

Immunostaining

Free PLK/DUAL tachyzoites, prepared as previously described (Unno *et al.* 2008), were incubated for 45 min at 37°C in an air incubator with anti-SAG1 mAb (3T × 19 MAb TP3, HyTest Ltd, Turku, Finland) or anti-BAG1 serum 50× diluted in PBS

containing 3% FCS. After washing them three times with PBS containing 3% FCS, the tachyzoites were incubated for 45 min at 37°C in an air incubator with Alexa Fluor® 350-labeled goat anti-mouse IgG (H+L) (Invitrogen). After washing them three times with PBS containing 3% FCS, the reacted tachyzoites were mounted with ProLong® Gold antifade reagent (Invitrogen) onto glass slides and covered with a coverslip. The glass slides were incubated for 30 min at room temperature in the dark and observed by fluorescence microscopy.

Results

Construction of transgenic *T. gondii* expressing dual fluorescent proteins

To produce transgenic parasites expressing stage-specific dual fluorescent proteins, we constructed a transfer vector, pminiHXGPRT/DUAL. The transfer vector contained genes coding for DsRed Express and GFP under the control of SAG1 and BAG1 promoters, respectively (Fig.1A). *T. gondii* P(LK) HXGPRT- strain tachyzoites were transformed with the transfer vector, pminiHXGPRT, and the stably transformed clone was designated as PLK/DUAL. The PLK/DUAL tachyzoites showed red fluorescence under pH 7.0 culture conditions (Fig.1 B, upper panel and Fig.1 C, upper panel). Although 293 zoites were observed, the number of red fluorescence negative zoites was only a few (6/293). During *in vitro* culture in pH 7.0 media for one month, with repeated passages, the proportion of fluorescent negative zoite did not increase. When PLK/DUAL tachyzoites were cultured under a stage conversion-inducing condition (pH 8.1 medium), a number of green fluorescence-positive tachyzoites were observed (Fig.1 B, lower panel and Fig.1 C, lower panel). Green fluorescence-positive tachyzoites were not detected in the pH 7.0-culture condition (Fig.1 B upper panel and Fig.1 C, upper panel). To confirm that the green fluorescence expression under the pH 8.1 condition was to the result of autofluorescence observed only under high pH conditions, but was rather caused by specific GFP expression in PLK/DUAL zoites, a transgenic *T. gondii* strain, PLK/RED, which expressed DsRed Express but no GFP, was also cultured in the pH 8.1 media. As shown in Fig. 1 D, green fluorescence-positive zoites were detected in Vero cell cultures infected with PLK/DUAL, but not in those infected with PLK/RED. These data suggest that PLK/DUAL expressed GFP during the bradyzoite stage.

Stage-specific expression of fluorescent proteins by PLK/DUAL

To investigate whether DsRed Express and GFP were expressed stage specifically,

PLK/DUAL zoites were stained with anti-SAG1 antibody or anti-BAG1 antiserum. Under the pH 7.0-condition, before the antibody staining procedure, almost all parasites expressed Ds Red Express but no zoite expressed GFP (Fig.1 B, C). When the parasites cultured in pH 7.0-condition, all zoites were stained by anti-SAG1 antibody but specific staining was not revealed by anti-BAG1 antiserum (Fig.2 A). SAG1-/DsRed Express + zoite was not observed. Although some parasites looked like SAG1+/DsRed Express – (Fig. 2 A, arrows), it must be a result of artificial reduction of fluorescent intensity by the staining procedure because almost all zoites showed strong red fluorescence before the staining procedure (Fig. 1 B). These results indicate that the DsRed Express-positive zoites, cultured under the pH 7.0-condition, were in the tachyzoite stage.

When PLK/DUAL zoites were cultured under stage conversion-inducing conditions, either high pH or the presence of a p38 MAPK inhibitor, a number of zoites expressed not only DsRed Express, but also GFP (Fig.1 B, C, D, and Fig.2 B). The intensity of GFP expression varied between individual zoites (Fig. 2 B). Zoites cultured in medium containing a p38 MAPK inhibitor were stained with anti-BAG1 antiserum (Fig.2 B). Fig. 2 B demonstrates a zoite that was strongly expressing GFP and was BAG1 positive, as well as a zoite showing weak GFP expression that was weakly BAG1 positive. Although only a few zoites are shown in Fig. 2 B, all observed zoites in this culture demonstrated a similar trend (data not shown). These results indicate that BAG1 and GFP expression patterns were synchronized; in other words, GFP expression indicated bradyzoite stage-specific expression.

DsRed Express and GFP *in vivo* expression in the PLK/DUAL strain

To confirm *in vivo*, stage-specific expression of DsRed Express and GFP by the PLK/DUAL strain, C57BL/6J mice were infected with PLK/DUAL tachyzoites. The brain samples were observed at acute, latent, and reactivating phases. In the acute phase, disseminated red fluorescence-positive zoites were observed in the brain samples (Fig.3 A and Fig.3 D, left panel). However, GFP expression was not detected (Fig. 3 A). During the latent phase, green fluorescence-positive cyst and green fluorescence-positive bradyzoites within the cyst were observed in the brain samples (Fig.3 B and Fig.3 D, center panel). As shown in Fig. 3 B, red fluorescence was not detected in these cysts by short-time exposure (1/3.0 sec). Whole brain tissues from one latently infected mouse were examined by short-time exposure. Analysis resulted in neither red fluorescence-positive cysts nor tachyzoites. The expression of fluorescent proteins was detected in the parasites but secretion into the

parasitophorous vacuole was not observed (Fig.3 D). To investigate whether reactivated tachyzoites from latent cysts also expressed DsRed Express, latent cysts were reactivated by administration of dexamethasone to the latently infected mice. After 28 days, red fluorescence-positive zoites were observed in the brain; however, green fluorescence-positive zoites were not detected (Fig.3 C). These results indicate that stage specificity could be identified in the PLK/DUAL living zoites by fluorescence.

Low-level DsRed Express expression in PLK/DUAL cysts

As described above, red fluorescence-positive PLK/DUAL latent cysts were not detected by short-time exposure (Fig.3 B). To determine whether PLK/DUAL bradyzoites in the latent cysts express a trace amount of DsRed Express or none at all, PLK/DUAL latent cysts were observed using a long time exposure. The results demonstrated that a portion of the bradyzoites in latent cysts weakly showed red fluorescence (Fig. 4 left panel). Bradyzoites in latent PLK/GFP cysts did not show red fluorescence (Fig. 4 right panel). These results indicate that the weak red fluorescence in the PLK/DUAL bradyzoites was not because of GFP bleed-through into the red fluorescent channel, but rather was caused by the presence of DsRed Express molecules in the bradyzoites.

Discussion

The present study demonstrated the use of a transgenic *T. gondii* strain, PLK/DUAL, as a tool to investigate stage conversion in *T. gondii*. The transgenic parasites express DsRed Express during the tachyzoite stage and GFP during the bradyzoite stage. Several transgenic parasite-expressing fluorescent proteins have been reported as tools for investigation for stage conversion in *T. gondii* and/or visualization of parasites in host tissue (Vanchinathan *et al.* 2005; Takashima *et al.* 2008; Nishikawa *et al.* 2008). This method is a powerful tool with which to observe one-way stage conversion, from tachyzoite to bradyzoite, or from bradyzoite to tachyzoite. However, none of these methods have allowed for the observation of stage conversion in both directions. Vanchinathan P. *et al.* (2005) constructed a transgenic parasite that fluoresced only during the bradyzoite stage. Using this transgenic parasite, stage conversion from tachyzoites into bradyzoites could easily be detected as an emission of fluorescence. However, it was impossible to detect reactivation of the parasite (stage conversion from bradyzoite to tachyzoite) using fluorescence. We have also

reported a transgenic parasite that fluoresced only during the tachyzoite stage (Takashima *et al.* 2008), and have visualized *T. gondii* reactivation in brain tissue. However, it is difficult to observe entrance into a latent state (stage conversion from tachyzoite to bradyzoite), because bradyzoites in brain cysts do not show bright fluorescence. To better understand the life cycle of *T. gondii* in the intermediate host, it is important to study the mechanisms of stage conversion between tachyzoites and bradyzoites in both directions. Although much knowledge regarding stage conversion has been independently provided by several research groups (Takashima *et al.* 2008, Vanchinathan *et al.* 2005), various strains of *T. gondii* were used for these studies. Because of the diverse properties among parasite strains (Grigg *et al.* 2001; Gross *et al.* 1997), an assortment of independent reports does not directly result in an overall understanding of the *T. gondii* life cycle. The PLK/DUAL strain, in which stage conversion is visualized in both directions by dual fluorescence, could provide a greater understanding of the *T. gondii* life cycle in the intermediate host. *T. gondii* cysts are primarily formed in brain and muscle tissue of the host (Turner 1978). Latent cysts have also been reported in the placenta (Dubey 1987). This indicates that *T. gondii* tachyzoites can convert to bradyzoites in the placenta. In addition, it was reported that there is lagged-time between *T. gonii* infection in the placenta and the fetus in some pregnant mouse models (Shiono *et al.*, 2007). There is a possibility that the bradyzoites existing in the cysts in the placenta could reactivate and reach the fetus later. Thus to understand the behavior of *T. gondii* in the placenta, observation of stage conversion in both directions is necessary. Therefore, the PLK/DUAL strain could contribute to further understanding of the mechanisms of *T. gondii* vertical infections.

Stage conversion between tachyzoite and bradyzoite is associated with morphological and molecular biological changes, including stage-specific antigen expression, many of which have been identified (Lyon *et al.* 2002). To construct transgenic *T. gondii* stage-specifically expressing fluorescent proteins, the promoters of the SAG1 and BAG1 genes were utilized, thereby specifically targeting expression in tachyzoites and bradyzoites, respectively (Ferguson. 2004). In addition, it has been reported that stage-specific expression of SAG1 and BAG1 is controlled at the transcriptional level (Cleary *et al.* 2002; Bohne *et al.* 1997). SAG1 gene transcription occurs during the tachyzoite stage, and decreases drastically during stage conversion from tachyzoite to bradyzoite (Cleary *et al.* 2002). By contrast, the upregulation of BAG1 mRNA occurs early during differentiation from tachyzoite to bradyzoite (Bohne *et al.* 1997). To confirm stage-specific expression of fluorescent proteins by

PLK/DUAL zoites, immunostaining was performed. Although zoites showing weak and no red fluorescence were observed after SAG-1 staining (Fig. 2 A), this loss of red fluorescence of zoite may be artifact by staining procedure because almost all PLK/DUAL showed red fluorescence strongly before staining (Fig. 1 B). DsRed Express positive and GFP negative zoites exhibited SAG1-expression, but no BAG1-expression (Fig. 2 A). When PLK/DUAL zoites were cultured medium containing SB202190, a number of zoites expressed BAG1, as well as GFP (Fig. 2 B). Strongly BAG1-positive zoites exhibited brighter GFP expression compared with BAG1-negative or BAG1-slightly positive zoites (Fig.2 B). These results indicate that DsRed Express and GFP expression patterns coincided with SAG1 and BAG1, respectively; in other words, tachyzoites were DsRed Express and SAG1 positive, and bradyzoites were GFP and BAG1 positive. *In vivo* analysis demonstrated that zoites in the acute and reactivating phases, most likely tachyzoites, exhibited DsRed Express expression; however, GFP expression was undetectable (Fig.3 A and C) By contrast, zoites within latent cysts, most likely bradyzoites, exhibited GFP expression, and DsRed Express expression was undetectable (Fig.3 B). Therefore, *in vivo* PLK/DUAL zoite stages could also be identified by fluorescence. Although it is necessary to consider the effect of expressed fluorescent proteins to immune reactions and parasites' behaviors, the PLK/DUAL strain could be a tool for investigation of in vivo stage-conversion of *T. gondii*. The intensity of the DsRed Express expressed by PLK tachyzoites was strong enough to detect zoites in the brain tissue from the outside (Takashima *et al.*, 2008). However, despite of the usage of strong bradyzoite-specific BAG1 promoter (Bohne *et al.* 1997), the intensity of green fluorescence of GFP expressed by bradyzoites was not enough to detect latent cysts in that manner. Trying out other fluorescent proteins and bradyzoite specific promoters, it might be possible to construct more useful recombinant parasites, of which latent cysts can be identified by strong fluorescence without either squashing tissue or making thin sections.

Bohne W. et al. (1993) observed a parasitophorous vacuole containing both tachyzoites and bradyzoites during the *in vitro* stage-converting phase. It was also reported that there is a point when parasites are expressing both SAG1 and BAG1 (Ferguson, 2004). We also observed a PLK/DUAL cyst containing both of red fluorescence +/green fluorescence - and red fluorescence +/green fluorescence + zoites in the brain on the 28 days after PLK/DUAL infection (data not shown). It suggested that PLK/DUAL also has SAG1/BAG1 double positive phase during stage-conversion. Although SAG1 is regarded as a tachyzoite-specific antigen, latent

cysts of the *T. gondii* ME-49 strain have been shown to contain some SAG1-slightly positive zoites (Silva *et al.* 1998). The PLK strain is a clonal derivative of ME-49 (Bohne *et al.* 1998). Therefore, we attempted to detect low levels of red fluorescence in the PLK/DUAL latent cysts using a long time exposure. As shown in Fig. 4, weak red fluorescence was detected in PLK/DUAL latent cysts. Considering that weak expression was detected not only in the cyst shown in Fig. 4, but also in all observed PLK/DUAL cysts (data not shown), it is unlikely that the weak red fluorescence indicates reactivation. We have previously reported that latent cysts of the PLK/RED strain exhibit much brighter red fluorescence at the point of inception of reactivation (Takashima *et al.* 2008). Our present results suggest low-level SAG1 promoter activity during the latent phase in the PLK strain.

It has been reported that *T. gondii* modifies many signaling cascades within the host cell (Seaij et al., 2007, Fouts and Boothroyd 2007). It has also been demonstrated that tachyzoites and bradyzoites can induce host transcriptional changes (Fouts and Boothroyd 2007). However, it is not yet known what biological activity exists between tachyzoite- and bradyzoite-infected cells to induce various host transcriptional changes in different manners. To elucidate the difference in the mechanisms of biological activity, that is, their reactions to exogenous stimuli, it is necessary to identify the stages of the infected parasites without killing them. As well, it is necessary to observe the parasite and/or host cells over a period of time. The PLK/DUAL strain could be an effective tool for investigating the biological activities of tachyzoite- and/or bradyzoite-infected host cells.

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Figure Legends

Fig.1 Construction of transgenic *T. gondii* expressing DsRed Express and GFP. (A) Schema of the transfer vector, pminiHXGPRT/DUAL. Genes coding DsRed Express and GFP are under the control of the SAG1 and BAG1 promoter, respectively. (B) Vero cells, infected with PLK/DUAL tachyzoites, were cultured in pH 7.0 medium (upper panels) and pH 8.1 medium (lower panels). Left and center

panels show red and green fluorescence detected by fluorescence microscope, as detected by 1/3.0 sec and 1.5 sec exposure, was shown respectively. Right panels show overlay images of red and green fluorescence. Scale bar = 50.0 μm. Arrows in the expanded Fig. indicate a green fluorescent zoites. (C) Fluorescent PLK/DUAL zoites in a vacuole. PLK/DUAL tachyzoites cultured in pH 7.0 medium (upper panels) and pH 8.1 medium (lower panels) were observed by large magnification. Scale bar = 10.0 μm. (D) PLK/DUAL or PLK/RED zoites released from infected Vero cells cultured in pH 8.1 medium (left and light panels, respectively). GFP and DsRed Express were detected by Fl-1 and Fl-2, respectively.

Fig.2 Stage-specific expression of DsRed Express and GFP by PLK/DUAL. (A) Purified free zoites from PLK/DUAL-infected Vero cells cultured in pH 7.0-medium. The purified free zoites were stained with anti-SAG1 antibody (upper panels) or anti-BAG1 antiserum (lower panels). Goat anti-mouse IgG (H+L)-Alexa 350, secondary antibodies were used for both experiments. Transmitted light images, red fluorescence images, green fluorescence images, overlay images of red and green fluorescence, and blue fluorescence images (□-SAG1 or □-BAG1) are shown. The blue fluorescence images were converted into gray scale. Arrow in the upper panels indicates zoites of wich red fluorescence had reduced by the staining procedure. (B) Purified free zoites from PLK/DUAL-infected Vero cells cultured in medium containing SB202190. The purified free zoites were stained with anti-BAG1 antiserum. Goat anti-mouse IgG (H+L)-Alexa 350 secondary antibody was used. Transmitted light images, red fluorescence images, green fluorescence images, overlay images of red and green fluorescence, and blue fluorescence images (□-BAG1) are shown. Arrowhead and arrow indicate red and green fluorescent zoites, respectively. The blue fluorescence images were converted into gray scale. Bar = 20.0 μm.

Fig.3 Red and green *in vivo* fluorescence in the PLK/DUAL strain. (A-C) PLK/DUAL fluorescence in the brains of infected C57BL/6J mice during the acute phase (A), latent phase (B) and reactivating phase (C). Red fluorescence was detected by short exposure (1/3.0 sec.). Green fluorescence was detected by long exposure (1.5 sec). Transmitted light images, red fluorescent images, green fluorescent images, and overlay images of transmitted, red fluorescent, and green fluorescent images are shown. (D) Area shown by the dotted frames in Fig.3 A (left pannel), B (center pannel) and C (right pannel) were observed by confocal laser microscopy. Bar = 5.0

μm. Note that images in Fig. 3 D is shown as mirrored images of Fig. 3 A, B and C, because of the difference in structure of fluorescent microscope and confocal laser microscope.

Fig.4 Expression of DsRed Express in latent cysts of the PLK/DUAL strain. Latent cysts of the PLK/DUAL (left panels) and PLK/GFP (right panels) strains in the brains of infected C57BL/6J mice are shown. Red fluorescence was detected by long exposure (1.5 sec). Green fluorescence was detected under the conditions described in Fig. 3. Bar = 20.0 μm

Fig. 1

A
BAG1 promoter
SAG1 promoter
Hind III
Bam HI
pminHXGPRT
HXGPRT Expressing Unit
Promoter of BAG1 or SAG1gene
GFP gene
DsRed-Express DR gene
3' GRA1 poly A signal
B
Red
Green
Overlay
pH 7.0
pH 8.1
50 μm
C
Transmitted light
Red
Green
Overlay
pH 7.0
pH 8.1
10μm
D
PLK/DUAL
PLK/RED
Red
pH 8.1
129
2839
0
2711
FL2-Height
FL1-Height
Green

Fig. 2

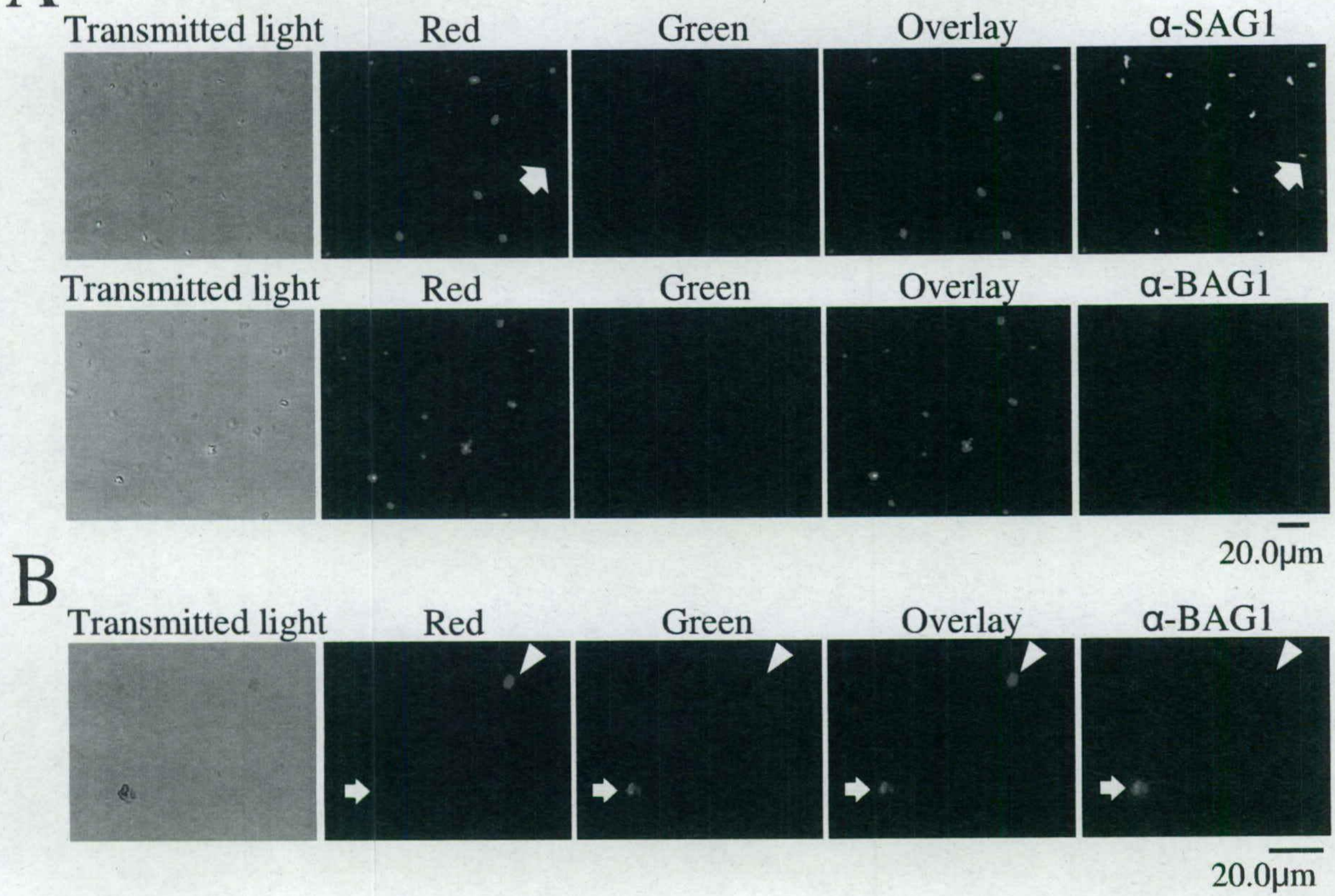
A
Transmitted light
Red
Green
Overlay
α-SAG1
Transmitted light
Red
Green
Overlay
α-BAG1
20.0μm
B
Transmitted light
Red
Green
Overlay
α-BAG1
20.0μm

Fig. 3

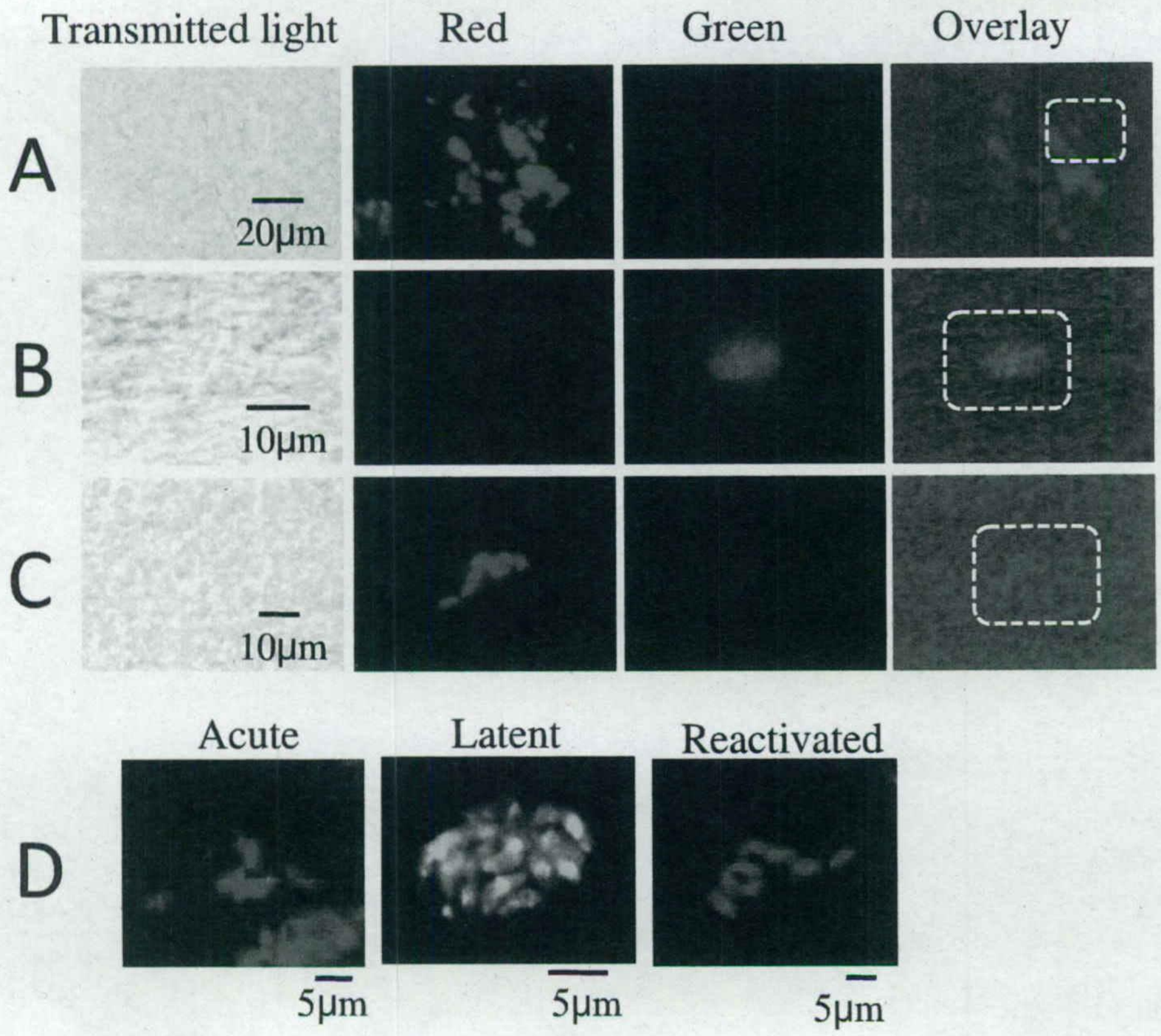
Transmitted light
Red
Green
Overlay
A
20μm
B
10μm
C
10μm
D
Acute
Latent
Reactivated
5μm
5μm
5μm

Fig. 4