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Involvement of a Rab8-like protein of *Dictyostelium discoideum*, Sas1, in the formation of membrane extensions, secretion and adhesion during development

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Establishment of cell–cell adhesions, regulation of actin, and secretion are critical during development. Rab8-like GTPases have been shown to modulate these cellular events, suggesting an involvement in developmental processes. To further elucidate the function of Rab8-like GTPases in a developmental context, a Rab8-related protein (Sas1) of *Dictyostelium discoideum* was examined, the expression of which increases at the onset of development. *Dictyostelium* cell lines expressing inactive (N128I mutant) and constitutively active (Q74L mutant) Sas1 as green fluorescent protein (GFP)-Sas1 chimeras were generated. Cells expressing Sas1Q74L displayed numerous actin-rich membrane protrusions, increased secretion, and were unable to complete development. In particular, these cells demonstrated a reduction in adhesion as well as in the levels of a cell adhesion molecule, gp24 (DdCAD-1). In contrast, cells expressing Sas1N128I exhibited increased cell–cell adhesion and increased levels of gp24. Counting factor is a multisubunit signalling complex that is secreted in early development and controls aggregate size by negatively regulating the levels of cell adhesion molecules, including gp24. Interestingly, the Sas1Q74L mutant demonstrated increased levels of extracellular countin, a subunit of counting factor, suggesting that Sas1 may regulate trafficking of counting factor components. Together, the data suggest that Sas1 may be a key regulator of actin, adhesion and secretion during development.

INTRODUCTION

The formation of tissues during development relies on the ability of cells to interact with each other, carry out developmentally regulated secretion, and reorganize cytoskeletal components. Various plasma membrane-associated proteins, collectively termed cell adhesion molecules (CAMs), enable the assembly of tissues by interacting with CAMs on neighbouring cells, with molecules in the extracellular matrix (ECM), and with the actin cytoskeleton (reviewed by Buckley *et al.*, 1998). In addition to providing structural integrity, the ECM proteins also participate in signal transduction that regulates tissue morphogenesis.

Dictyostelium discoideum offers numerous advantages as an experimental model system in which to study the role of cell–cell adhesion, developmentally regulated secretion, and actin in developmental processes. It possesses a simple and well-defined life cycle consisting of a vegetative phase and a developmental phase (reviewed by Loomis, 1975). During the vegetative phase, the organism survives as a free-living amoeba in the presence of sufficient nutrients. Starvation results in a switch to development during which individual cells emit pulses of cAMP to which other cells respond chemotactically. Streams of cells interact through cell–cell adhesions, and form a multicellular aggregate of about 10^5 cells. The aggregate then undergoes a programme of cell-type-specific gene expression and cytodifferentiation to produce an intermediate 'slug', and finally, a fruiting body that contains a stalk supporting environmentally stable spores.

Several of the CAMs that participate in *Dictyostelium* development have been described (reviewed by Coates & Harwood, 2001). *Dictyostelium* cells form their initial contacts through interactions of a plasma membrane-associated Ca^{2+}-dependent cadherin-like protein known as gp24 (DdCAD-1) (Yang *et al.*, 1997). In early development this CAM is transported from the cytosol to the external surface of the plasma membrane and a small fraction of this CAM is released into the extracellular

Abbreviations: CAM, cell adhesion molecule; CF, cell-counting factor; CMF, cell-density sensing factor; DB, developmental buffer; ECM, extracellular matrix; F-actin, filamentous actin; FACS, fluorescence-associated cell sorting; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein.

milieu. The second and third adhesion molecules to participate in cell–cell interactions during development are gp80 and gp150, respectively (Faix *et al.*, 1992; Wang *et al.*, 2000). Following the incorporation of gp80 and gp150 at cell–cell contact sites, filamentous actin (F-actin) is recruited to the contact site, whereas gp24 is removed from the contact site. The loss of gp24 is postulated to be necessary to weaken the adhesive forces between cells to facilitate streaming; however, the manner by which it is removed is unknown.

Regulated secretion is also characteristic of *D. discoideum* development. When cells reach high density and during early development, *D. discoideum* amoebae secrete lysosomal hydrolases (Cardelli, 1993; Temesvari *et al.*, 1996), cell-density sensing factor (CMF) (Gomer *et al.*, 1991; Jain *et al.*, 1992; Yuen *et al.*, 1995) and cell-counting factor (CF) (Brock & Gomer, 1999; Roisin-Bouffay *et al.*, 2000; Brock *et al.*, 2002; Jang *et al.*, 2002; Tang *et al.*, 2002). CF is a 450 kDa protein complex that can regulate aggregate size during development. Countin, a subunit of CF, has been shown to negatively regulate adhesion by regulating the expression of CAMs (Roisin-Bouffay *et al.*, 2000). For example, in mutants (*smlA*) which oversecrete countin, there is decreased adhesion and delayed expression of gp24, whereas in mutants in which the gene for countin is disrupted (*countin*$^{-}$) there is increased adhesion and expression of gp24 (Roisin-Bouffay *et al.*, 2000). Like countin, CF45, another subunit of CF, also participates in group size regulation in *Dictyostelium*. Finally, at later stages in development ($\geqslant$20 h), secretion of the contents of prespore vesicles is necessary for the formation of the spore coat (reviewed by Srinivasan *et al.*, 2000).

While much is known about the types of proteins that become part of adhesions or that are secreted during *Dictyostelium* development, little is known about how these molecules are delivered to the cell surface and/or secreted. Presumably, the movement of these proteins relies on vesicle trafficking, suggesting that the Ras-related Rab GTPases, master controllers of this process (reviewed by Pfeffer, 2001; Segev, 2001), may be involved. Rabs share a number of homologous domains, including four GTP-interacting domains (G1–G4), five Rab-specific functional domains (F1–F5) and four subfamily-specific domains (SF1–SF4) (reviewed by Pereira-Leal & Seabra, 2000; Stenmark & Olkkonen, 2001). In general, phylogenetic analyses of the F1 and SF1–SF4 domains have revealed the existence of ten subfamilies of Rab GTPases. Rabs cycle between an active GTP-bound state and an inactive GDP-bound form and associate with transport vesicles through C-terminal lipid modifications (Schafer & Rine, 1992). After vesicle docking at target membranes, GTP hydrolysis, via the intrinsic GTPase activity of the Rab, converts the Rab to its GDP-bound form. Rab GTPase activity is regulated by GTPase-activating proteins (GAPs) and the activation of Rabs by nucleotide exchange relies on guanine nucleotide exchange factors (GEFs). Rabs are proposed to interact with GAPs and GEFs through their effector (F1) domain.

Rab8-related GTPases represent a subfamily of Rabs that include mammalian Rab8 (Peranen *et al.*, 1996; Imamura *et al.*, 1998; Hattula *et al.*, 2002; Lau & Mruk, 2003), Rab13 (Zahraoui *et al.*, 1994; Sheth *et al.*, 2000; Marzesco *et al.*, 2002), Rab10 (Chen *et al.*, 1993), Rab3A (Vadlamudi *et al.*, 2000) and yeast Sec4p (Guo *et al.*, 1999). These Rabs participate in cellular functions that may be vital to development as they regulate polarized secretion, actin cytoskeletal dynamics, and cell–cell adhesion. In support of this, mammalian Rab13 has been shown to participate in the maturation of epithelial tight junctions during embryogenesis (Sheth *et al.*, 2000). To further our understanding of the molecular factors that govern cell–cell adhesion, the actin cytoskeleton and regulated secretion in a developmental context, we have characterized a Rab8-like protein of *Dictyostelium*, Sas1. This protein, first identified by Saxe & Kimmel (1990), is present in vegetative cells; however, the level of transcript increases immediately at the onset of development, reaching a maximum level at 15 h into the developmental programme. This pattern of gene expression suggests that this protein plays an important role in early development and we demonstrate that, like other Rab8-related proteins, Sas1 may regulate the arrangement of actin, cell–cell adhesion and secretion. As a control, we also examined a second highly related Rab8-like protein of *Dictyostelium*, Sas2 (Saxe & Kimmel, 1990). The expression pattern of Sas2, characterized by low levels of transcript in vegetative cells and an increase in transcript beginning only after 15 h into development, suggests that it is a likely candidate for the regulation of cellular processes important at later stages in development.

METHODS

Analysis of the Sas cDNA clones. Searches of the National Center for Biotechnology Information (NCBI) databases were conducted on-line using BLAST (Altschul *et al.*, 1990). Alignments of amino acid sequences were generated with MacVector V 7.0 using the CLUSTAL W algorithm (Thompson *et al.*, 1994).

Strains and culture conditions. *D. discoideum* wild-type (Ax2) and mutant cell lines (see below) were grown axenically in HL5 medium as described previously (Temesvari *et al.*, 1996). Transformed cell lines expressing mutant Sas proteins (see below) were maintained by G418 selection (20 $\mu g\ ml^{-1}$) and folic acid (1×10^{-3} M) repression. Prior to all experimentation, mutant cells were cultured for 72 h in the absence of folic acid to induce expression of the mutant Sas proteins from the folate-repressible *discoidin I* promoter. For development, cells were resuspended at a concentration of $5{\cdot}5 \times 10^5$ to $2{\cdot}5 \times 10^6$ cells ml^{-1} in developmental buffer (DB) (5 mM $Na_2HPO_4.7H_2O$, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 0·2 mM $CaCl_2$, pH 6·2) or plated at a concentration of 1×10^6 cells cm^{-2} on DB plates containing 2 % (w/v) agar and incubated at 22 °C in the dark in a humid chamber.

Antibodies. Antibodies recognizing GFP were obtained from Zymed. Polyclonal antibodies recognizing the adhesion molecule, gp24 (gift of Dr C. H. Siu, University of Toronto, Toronto, Ontario, Canada) are described elsewhere (Brar & Siu, 1993). Polyclonal antibodies recognizing subunits of CF (countin, CF45) (gift of Dr R. H. Gomer, Rice University, Houston, TX, USA) are described elsewhere (Brock & Gomer, 1999; Brock *et al.*, 2003). Sas1-specific polyclonal

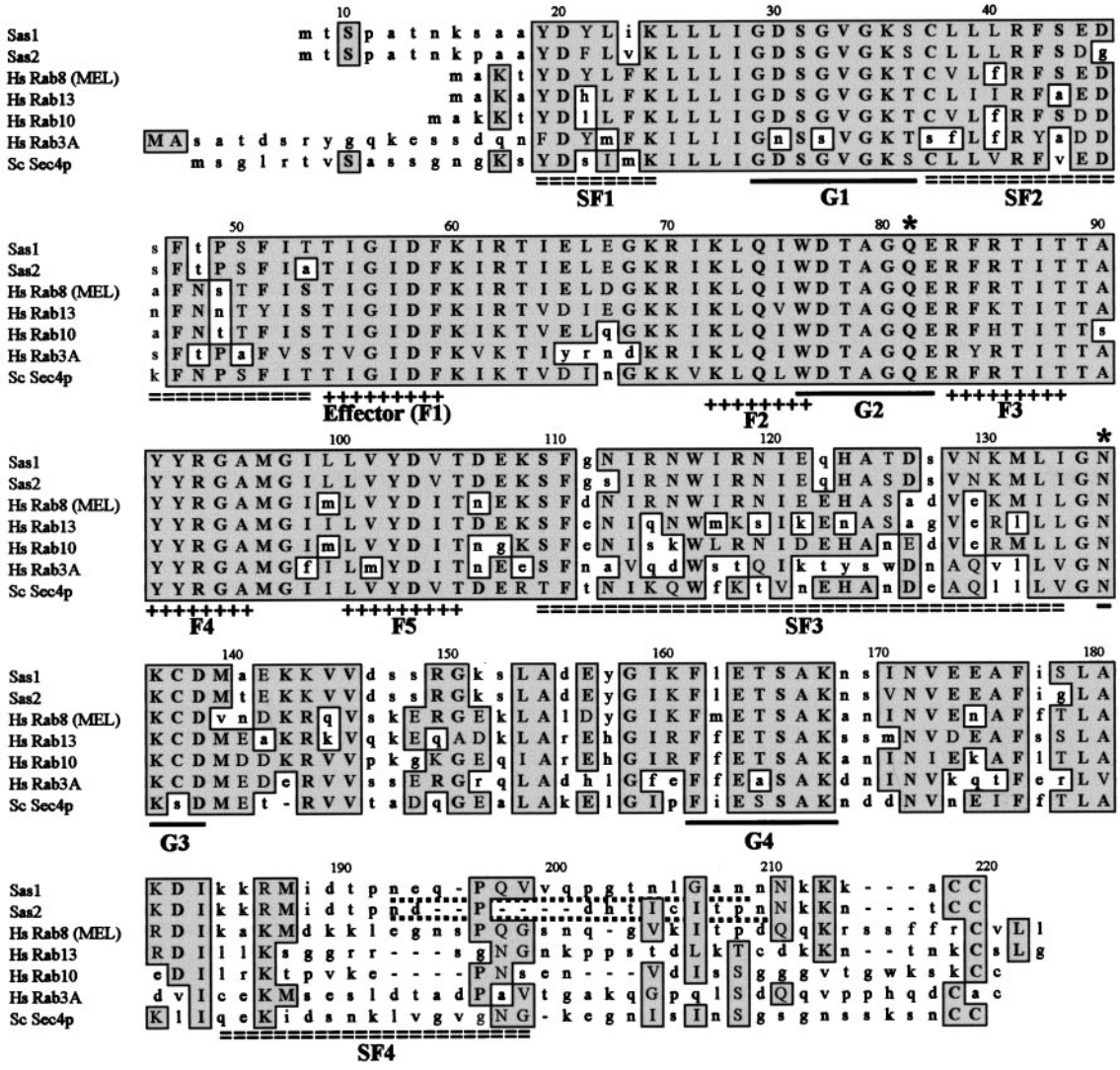

Fig. 1. Alignment of the Sas1 and Sas2 predicted amino acid sequences with Rab8-related proteins from various heterologous species. See Table 1 for gene names and accession numbers. The GTP-binding sites (G1–G4), Rab-specific functional domains (F1–F5) and Rab8 subfamily-specific domains (SF1–SF4) are indicated. Asterisks indicate the mutated amino acid residues. The dashed underlined sequences in the C-terminal region of Sas1 and Sas2 represent the peptides used for antibody production and peptide neutralization.

antibodies were produced by immunizing rabbits (Zymed) with a synthetic peptide corresponding to amino acids 185–200 in the C-terminal divergent region of Sas1 (dotted underlined sequence, Fig. 1). Antibodies were purified by affinity chromatography (Zymed) using an affinity column consisting of immobilized Sas1 peptide. The specificity of the antibodies was assessed by Western blotting (see below).

Conditioned medium and Western blot analysis. To assess expression of the mutant versions of Sas1 and Sas2, and the level of cell-associated gp24, *Dictyostelium* cells were pelleted by centrifugation at 1500 ***g*** for 4 min at 22 °C. The pellet was resuspended in 4 × LDS sample buffer (Invitrogen) and 2-mercaptoethanol (10 % v/v), heated at 70 °C for 10 min and loaded onto a NuPage Gel (10 % Bis–Tris) (Invitrogen). Electrophoresis, blotting and decoration with antibodies were performed as described by Welter *et al.* (2002). Dilutions used for the anti-Sas1, anti-GFP and anti-gp24 antibodies were 1 : 2000, 1 : 500 and 1 : 5000, respectively. To assess the specificity of the antibody, the anti-Sas1 antibody was incubated at 4 °C for 16 h with the synthetic Sas1 peptide that had been used to immunize the rabbits (100 μg ml^{-1}), or with synthetic peptide corresponding to Sas2 (100 μg ml^{-1}) (dashed underlined sequence, Fig. 1). Western blot analysis of whole-cell extracts using antibody that was peptide pre-incubated was then carried out as described above.

To assess the extracellular levels of gp24, CF45, or countin, exponential-phase *Dictyostelium* cells were resuspended at 5×10^6 cells ml^{-1} in HL5 nutrient medium or DB and shaken at 150 r.p.m. for 18 h or for 2 h, respectively. Cells and conditioned medium or DB were separated by centrifugation at 16 000 ***g*** for 4 min at 22 °C to pellet the cells. The supernatant was supplemented

with a cocktail of protease inhibitors (1 mM PMSF, 1 μg pepstatin ml^{-1}, 10 μg leupeptin ml^{-1}) and proteins >10 kDa were concentrated (approx. 10-fold) by centrifugation (2000 ***g***) using a centrifugal filter device (10 kDa cutoff, Millipore). After centrifugation, the entire volume was mixed with an equal volume of 4× LDS sample buffer (Invitrogen) and 2-mercaptoethanol (10 % v/v), heated at 70 °C for 10 min and subjected to SDS-PAGE as described above. In some instances, to verify equal loading of samples, the gels were silver stained using the GelCode SilverSNAP stain kit (Pierce) according to the manufacturer's protocol. Alternatively, resolved proteins were blotted and decorated with antibodies specific for gp24, CF45 or countin as described above. The dilution used for both the anti-countin and anti-CF45 antibodies was 1 : 3000.

Mutagenesis of the Sas cDNAs and transfection of *Dictyostelium*. To generate *Dictyostelium* mutants that conditionally overexpress mutant versions of Sas1, PCR-based site-directed mutagenesis was performed using the QuickChange Kit (Stratagene) according to the manufacturer's instructions. Site-directed mutagenesis was used to change an encoded glutamine residue (Q) to a leucine residue (L) at amino acid position 74 (region G2, Fig. 1) to generate a Sas1Q74L mutant. Alternatively, an encoded asparagine residue (N) was changed to an isoleucine residue (I) at amino acid position 128 (region G3, Fig. 1) to generate a Sas1N128I mutant. The glutamine residue participates in GTP hydrolysis and the asparagine residue participates in nucleotide binding. Comparable mutations at equivalent glutamine or asparagine residues in other small-molecular-mass GTP-binding proteins have resulted in the formation of proteins that function in a constitutively activated or dominant negative manner, respectively. As a control, corresponding mutations were also made for Sas2.

Wild-type and mutated cDNAs were subcloned behind and in-frame with a DNA element encoding the GFP in the pDJS *Dictyostelium* expression vector (gift of Dr J. A. Cardelli, LSU Health Sciences Center, Shreveport, LA, USA). The expression vector confers neomycin (G418) resistance to transfectants and the expression of heterologous proteins is controlled by a folate-repressible *discoidin I* promoter. Parental Ax2 *Dictyostelium* cells were transformed with the Sas-containing expression vectors or with the pDJS expression vector alone by electroporation as described by Kuspa & Loomis (1992). After transfection, G418-resistant clones were sorted by fluorescence-associated cell sorting (FACS) and cloned by limiting dilution.

Microscopy. In individual cells, F-actin was stained using Alexa Fluor 594 (red)-conjugated phalloidin (Molecular Probes) according to the manufacturer's protocol. Stained cells were mounted in glycerol/PBS (1 : 1) and observed using a Carl Zeiss LSM 510 confocal microscope. Measurements of aggregate size were obtained using the LSM 5.1 Image Browser software (Carl Zeiss). Images of fruiting bodies were obtained using a stereomicroscope (Wild Heerbrugg) and a Kodak DC120 digital camera.

Measurement of actin. Actin was measured according to the protocol of Gerald *et al.* (1998) with modifications. Exponential-phase cells (3×10^6) were collected by centrifugation and the pellet was resuspended in 500 μl PBS. Twenty percent of the cells was lysed in 0·5 % (v/v) Triton-X-100 and utilized for measurement of protein with the BCA Protein Assay Kit (Pierce). The remaining cells were rotated for 1 h at room temperature in the dark in actin buffer (20 mM KH_2PO_4, 10 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6·8) supplemented with 4 % (v/v) paraformaldehyde, 0·1 % (v/v) Triton-X-100 and 0·2 μM Alexa Fluor 594-conjugated phalloidin (actin stain). The stained cells were centrifuged at 16 000 ***g*** for 5 min, resuspended in 500 μl methanol, and rotated overnight in the dark at 4 °C to extract the stain. The extracted cells were then centrifuged and fluorescence of the supernatant was measured at an excitation wavelength of 590 nm and an emission of 635 nm using a FLx800 spectrofluorimeter (BioTek Instruments). Data are reported as relative fluorescence $(\mu g \text{ protein})^{-1}$.

Measurement of adhesivity. Adhesion was measured as described previously (Desbarats *et al.*, 1994). To measure adhesion during development, cells were harvested by centrifugation and resuspended at a concentration of $5{\cdot}5 \times 10^5$ cells ml^{-1} in 200 μl DB. To measure adhesion during vegetative growth, cells were harvested by centrifugation and resuspended at a concentration of $2{\cdot}5 \times 10^6$ cells ml^{-1} in 200 μl HL5 medium. This higher cell density was chosen to adjust for the lower adhesiveness that is characteristic of vegetative cells (Gao *et al.*, 2002). In some instances, to assess the contribution of Ca^{2+}-dependent CAMs, EDTA was added to a final concentration of 10 mM as described by Roisin-Bouffay *et al.* (2000). Resuspended cells were rotated vertically for 10 min at room temperature after which adhesion was estimated by counting the number of single cells and the number of aggregated cells, including doublets, using a haemocytometer. Adhesion is reported as the percentage of cells that were adhering to other cells.

Measurement of secretion of acid phosphatase. Measurement of acid phosphatase secretion was performed as described previously (Temesvari *et al.*, 1996).

Statistical analysis. All values are given as a mean ± SD. Statistical analyses were performed using GraphPad Instat V.3 with One Way ANOVA and a Tukey-Multiple Comparison test. *P* values less than 0·01 were considered highly statistically significant and *P* values between 0·01 and 0·05 were considered statistically significant.

RESULTS

Sas1 and Sas2 are related to Rab8-like proteins

The Sas1 and Sas2 cDNAs encode putative proteins of 23·2 and 22·6 kDa, respectively, that share at least 41 % identity and at least 60 % similarity with other Rab8-like proteins (Fig. 1 and Table 1). The Sas proteins appeared to be most related to human Rab8 (75 % similarity; Table 1). Sas1 and Sas2 possess all of the domains that are characteristic of Rabs, including the four domains that are proposed to participate in the binding of GTP/GDP (Pai *et al.*, 1989) (regions G1–G4, Fig. 1) and five Rab-specific functional domains (Pereira-Leal & Seabra, 2000; Stenmark & Olkkonen, 2001) (regions F1–F5, Fig. 1). The effector domain (F1) and four subfamily-specific domains (SF1–SF4) provide the signature sequences that differentiate the various subclasses of Rab proteins (Pereira-Leal & Seabra, 2000; Stenmark & Olkkonen, 2001). For Sas1 and Sas2, high homology to other Rab8-like proteins was demonstrated for all but one of these domains, SF4, further confirming their relatedness to this group (Fig. 1). The limited homology in the SF4 domain is consistent with a recent report describing low homology in this domain for the Rab8 subfamily (Pereira-Leal & Seabra, 2000).

Generation of cell lines expressing mutant versions of Sas1

Given its pattern of expression, Sas1 provides an opportunity to study a Rab8-like protein that may play an important role in the early development of *Dictyostelium*. To define its

Table 1. Percentage identities (similarities) among members of the Rab8-related group of GTPases

Sas1, *Dictyostelium discoideum* Sas1 (GenBank M34457); Sas2, *Dictyostelium discoideum* Sas2 (M34456); HsRab8, human Rab8 (NM005370); HsRab13, human Rab13 (NM002870); HsRab10, human Rab10 (NM016131); HsRab3A, human Rab3A (NM002866); ScSec4p, *Saccharomyces cerevisiae* Sec4p (M16507).

Name	Sas2	HsRab8	HsRab13	HsRab10	HsRab3A	ScSec4p
Sas1	87 (91)	62 (75)	55 (72)	55 (73)	41 (60)	50 (71)
Sas2		62 (75)	55 (73)	54 (72)	43 (61)	51 (71)
HsRab8			60 (77)	66 (76)	45 (61)	48 (68)
HsRab13				60 (77)	40 (57)	52 (68)
HsRab10					42 (61)	49 (66)
HsRab3A						41 (59)

function, we generated mutant cell lines that express wild-type (Sas1WT) or one of several mutant versions (Sas1Q74L, Sas1N128I) of Sas1 tagged N-terminally with GFP. The N-terminal fusion was chosen to avoid interference with the C-terminal elements responsible for membrane association (Schafer & Rine, 1992). Expression of mutant proteins was under the control of the folate-repressible *discoidin I* promoter. For overexpression studies involving mutant Rab proteins, it is important to confirm that phenotypes do not result from cross-talk with irrelevant signalling pathways. Given the relatedness of Sas1 and Sas2, we also generated cell lines expressing GFP-tagged Sas2WT or Sas2Q74L as controls. While G418-resistant cell lines were obtainable by transfection with the pDJS expression vector harbouring a cDNA encoding GFP-tagged Sas2N128I, expression of this chimeric protein could not be confirmed by Western blot analysis. Therefore, these cell lines were excluded from the studies. A control cell line that expressed only GFP (GFP-control) was also generated.

To assess the expression of mutant Sas proteins, polyclonal antibodies were generated using a peptide corresponding to the C-terminal divergent region of Sas1 (dotted underline, Fig. 1). Affinity purified antiserum recognized a band on Western blots of whole-cell lysates consistent with the predicted molecular mass of the protein (Fig. 2a, lane 1). A similar sized band was not recognized by pre-immune serum nor by secondary antibody (Fig. 2a, lanes 2 and 3, respectively). Occasionally, an unidentified protein species of approximately 40 kDa was recognized by immune and pre-immune serum (Fig. 2a, lanes 1 and 2). As an additional control, the anti-Sas1 antibody was pre-incubated with Sas1 and Sas2 synthetic peptides prior to Western blot analyses. Binding of antibody to the putative Sas1 protein species on Western blots was abolished by pre-incubating the antibody with the Sas1 synthetic peptide (Fig. 2b, lane 2), but not with a Sas2 synthetic peptide (Fig. 2b, lane 3).

After transfection, G418-resistant clones were sorted by FACS and cloned by limiting dilution. Maximum expression of mutant Sas1 proteins was achieved by removal of folate from the culture medium for 72 h. Western blot

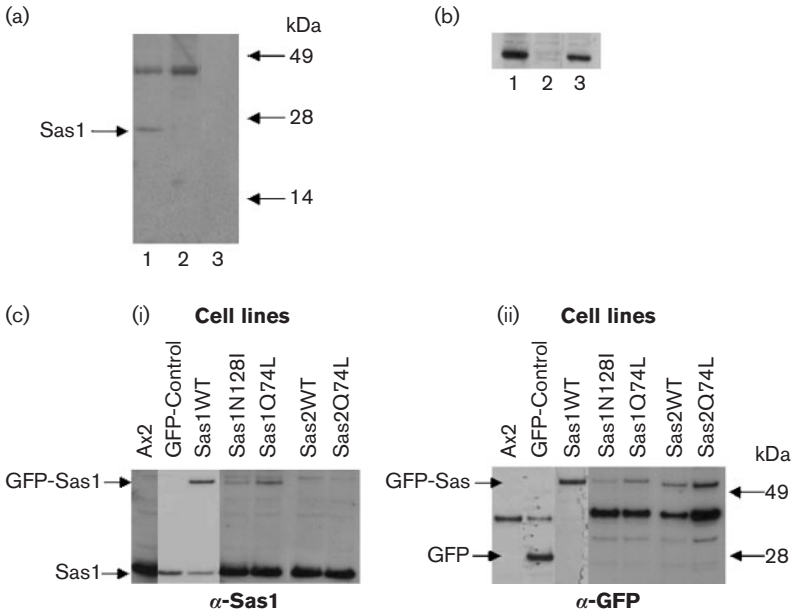

Fig. 2. Western blot analysis of control and mutant cell lines. (a) Blotted whole-cell extracts were decorated with the anti-Sas1 antibody (lane 1), pre-immune serum (lane 2) or goat anti-rabbit secondary antibody alone (lane 3). (b) To assess the specificity of the anti-Sas1 antibody, affinity-purified anti-Sas1 antibody was pre-incubated with the Sas1 peptide or with a Sas2 peptide. Blotted whole-cell extracts were decorated with anti-Sas1 antibody that had not been pre-incubated with any peptide (lane 1), or with anti-Sas1 antibody that had been pre-incubated with Sas1 (lane 2) or Sas2 (lane 3) peptides. (c) Blotted whole-cell extracts from wild-type or mutant cell lines were decorated with anti-Sas1 (i) or anti-GFP (ii) antibodies.

analysis of mutant cell lysates demonstrated that anti-Sas1 antibody recognized two forms of the Sas proteins in transformed cells: a 23 kDa protein representing endogenous Sas1 and a unique 50 kDa species representing the GFP-tagged mutant version (Fig. 2c, i). The Sas1 antibody did not recognize a comparable 50 kDa protein species in cell lines expressing mutant versions of Sas2 suggesting that this antibody does not cross-react with this highly related Rab (Fig. 2c, i). Although an anti-GFP antibody recognized several non-specific bands in some of the cell lines, GFP-tagging was confirmed by decoration with GFP-specific antibodies (Fig. 2c, ii). All subsequent studies were performed with two independent clones of each mutant.

Expression of Sas1 mutant proteins alter the actin cytoskeleton

Rab8-like proteins have been shown to regulate the formation of membrane extensions by altering the actin cytoskeleton (Peranen *et al.*, 1996). Therefore, to explore the functional relatedness of Sas1 to Rab8-like proteins we examined these features in the mutant cell lines. Vegetative *Dictyostelium* cells are generally amoeboid in shape and possess a cortical actin cytoskeleton localized at the cell periphery. Overexpression of GFP-alone (Fig. 3A) or Sas1WT (Fig. 3B) had no effect on cell shape or the arrangement of actin as evidenced by F-actin staining. While the plasma membrane of cells expressing Sas2WT or Sas2Q74L (Fig. 3C, D) appeared slightly more 'ruffled' than control cells, the F-actin cytoskeleton was enriched in the cortical regions of the cell. Sas1N128I mutants, although appearing slightly less amoeboid, demonstrated little apparent change in the localization of the actin cytoskeleton (Fig. 3E–H). In contrast, expression of Sas1Q74L induced the formation of membrane extensions that were concentrated in one or several locations on the cells (Fig. 3I–L). The formation of these extensions was accompanied by a change in the localization of actin whereby most of the F-actin in the cell was now concentrated in the polarized cellular protrusions. Although the arrangement of actin was altered in the Sas1Q74L cell line, the level of F-actin was relatively constant among the cell lines (Fig. 3M). Together, these data suggest that, like mammalian Rab8, Sas1 may regulate the arrangement of the actin cytoskeleton and the formation of cellular extensions.

Sas1 mutants display defects in aggregation and adhesion

Since mammalian Rab8 and Rab13 also function in the formation of cell adhesions (Sheth *et al.*, 2000; Marzesco *et al.*, 2002; Lau & Mruk, 2003), we tested the possibility that Sas1 may have a similar function by examining developmental structures, the formation of which depend on cell–cell interactions. Control cells expressing GFP alone (Fig. 4B, G) were identical to the parental cell line, Ax2 (Fig. 4A, F), with respect to timing of development and morphology of developmental structures. In contrast, the Sas1N128I-expressing mutant initially formed aggregates that were significantly larger (Fig. 4C) than those of control cell lines, suggesting that these cells may be hyper-adhesive. As development proceeded, these large aggregates dissociated into smaller groups of cells (data not shown), giving rise to a large number of very small fruiting bodies (Fig. 4H). This phenotype is similar to that of a hyper-adhesive *Dictyostelium* mutant that overexpresses gp80 (Faix *et al.*, 1992). Interestingly, cells expressing Sas1Q74L did not aggregate, nor did they form fruiting bodies (Fig. 4D). Unlike cells expressing Sas1Q74L, cells expressing a wild-type version of Sas2 (data not shown) or Sas2Q74L (Fig. 4E, I) displayed large but loosely organized aggregates and were able to complete development.

To determine if Sas1Q74L cells could indeed be associated with aggregates, we mixed equal numbers of parental and Sas1Q74L-expressing cells and initiated development. Aggregation (Fig. 5) and fruiting body formation (data not shown) were normal in this mixed population of cells and confocal microscopic examination of aggregates demonstrated that the Sas1Q74L-expressing cells (green) were found in aggregates (Fig. 5). Although we cannot rule out the possibility that these mutant cells may be defective in chemotaxis and the possibility that they end up in aggregates passively, videomicroscopy suggests that these cells display random vegetative motility similar to that of wild-type cells (data not shown).

To explore the possibility that the lack of aggregation observed for cells expressing Sas1Q74L may be the result of reduced cell–cell adhesion, we measured adhesivity in the Sas1 mutants during development. Cells were collected at regular intervals during the first 8 h of development and adhesion was measured as described previously (Desbarats *et al.*, 1994). Sas1Q74L-expressing cells were less adhesive than parental wild-type cells throughout the first 6 h of development, reaching a statistically significant lower level of adhesivity by 8 h of development (Fig. 6a). Sas1N128I-expressing cells were more adhesive than wild-type cells over the same time period, reaching a statistically significant higher level of adhesivity by 8 h. This supports the notion that this mutant is hyper-adhesive (Fig. 6a). This suggests that, like other Rab8-related proteins, Sas1 may directly or indirectly regulate cell–cell adhesion.

Although vegetative cells are generally less adhesive than developing cells, alterations in adhesion can occasionally be detected in vegetative cells at high densities (Gao *et al.*, 2002). Therefore, we also characterized adhesivity in the cell lines during this phase of the life cycle. Since the Ca^{2+}-dependent CAM, gp24, is expressed in vegetative cells (Yang *et al.*, 1997), adhesion was also measured in the presence and absence of EDTA to assess the contribution of such Ca^{2+}-dependent CAMs. First, the higher level of adhesion observed for the vegetative cells as compared to the developing cells is a reflection of the increased cell number used for these assays and is consistent with a previous report (Gao *et al.*, 2002). Second, there were no differences

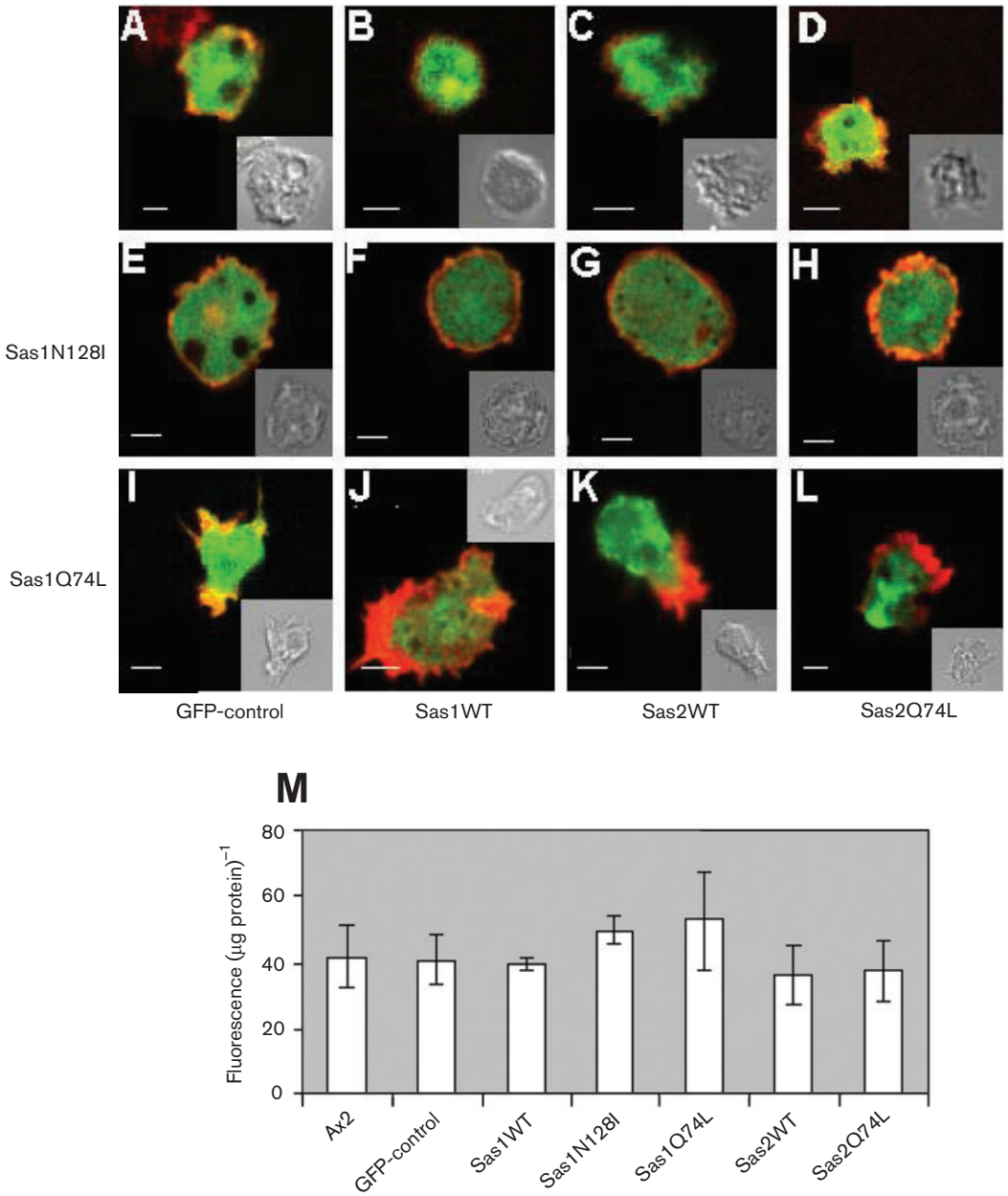

Fig. 3. Localization and level of F-actin in control and mutant cell lines. Differential interference contrast (insets) and fluorescence images of actin-stained [Alexa Fluor 594 (red) phalloidin] GFP-expressing (A), Sas1WT-expressing (B), Sas2WT-expressing (C), Sas2Q74L-expressing (D), Sas1N128I-expressing (E–H) and Sas1Q74L-expressing (I–L) mutants. Expression of GFP or GFP-tagged Sas1 mutant proteins is apparent (green). Bars, 5 μm. (M) Relative level of F-actin in control and mutant cell lines. Level of F-actin is given as relative fluorescence standardized (μg protein)$^{-1}$.

in the adhesiveness of parental (Ax2) and GFP-control cells (data not shown). Finally, it was observed that during vegetative growth cells expressing Sas1Q74L were significantly less adhesive than GFP-control cells, whereas cells expressing Sas1N128I were significantly more adhesive than GFP-control cells (Fig. 6b). Unlike the cell line expressing Sas1Q74L, cells expressing Sas2WT or Sas2Q74L were slightly more adhesive than control cells. The differences, however, were not statistically significant.

Importantly, there were no detectable differences in adhesivity among the cell lines in the presence of EDTA (Fig. 6b), suggesting that Sas1 may regulate cell–cell adhesions via a Ca^{2+}-dependent CAM such as gp24. We therefore performed a Western blot analysis of cell lysates from control and mutant cells using antibodies specific for gp24. Since gp24 may also be released into the extracellular milieu, we also performed a similar Western blot analysis of conditioned medium. Consistent with the observed alterations in

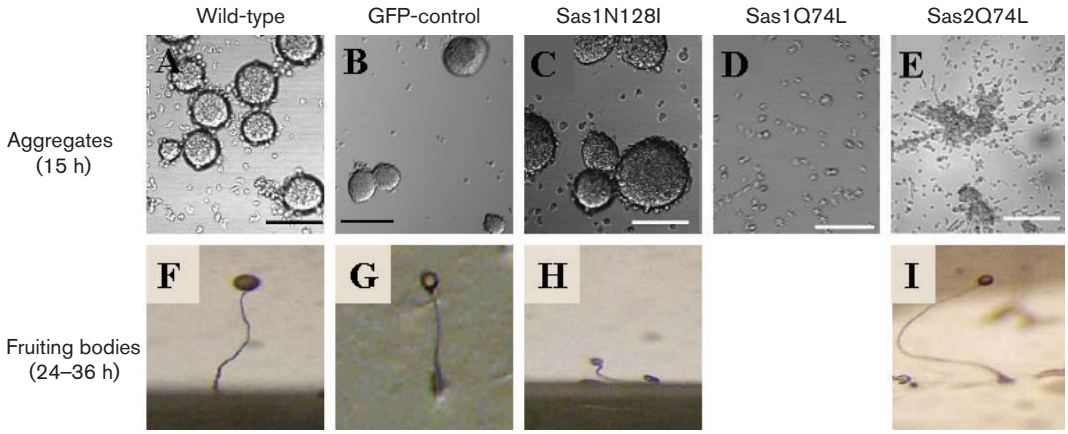

Fig. 4. Aggregates and terminal fruiting bodies of parental (Ax2, Wild-type; A, F), GFP-control (B, G), Sas1N128I-expressing (C, H), Sas1Q74L-expressing (D) and Sas2Q74L-expressing (E, I) cell lines. All images of aggregates (A–E) or fruiting bodies (F–I) were taken at the same magnification. Bars (A–E), 100 μm. The mean diameter of an aggregate of the Sas1N128I-expressing mutant was 70·3 ± 17·4 μm ($n = 20$) which was significantly higher ($P < 0·05$) than that of control cells which was 54·1 ± 17·4 μm ($n = 17$).

adhesivity, Western blot analysis demonstrated that total (cell-associated and extracellular) gp24 was less abundant for Sas1Q74L cells and more abundant for Sas1N128I cells as compared to the other cell lines (Fig. 6c).

Expression of Sas1Q74L alters the extracellular level of countin

Given that the Sas1N128I mutant had a 'gain of function' adhesion phenotype, and that the Sas1Q74L mutant had a 'loss of function' adhesion phenotype, we hypothesized that the role of Sas1 in adhesion was indirect. Several members of the Rab8 family of GTPases, including mammalian Rab8 (Chen *et al.*, 2001), Rab3A (Vadlamudi *et al.*, 2000) and *Saccharomyces* Sec4p (Guo *et al.*, 1999), have been shown to regulate secretion. Therefore, one possible explanation may be that the Sas1Q74L cell line oversecretes a signalling molecule or molecules that in turn regulate adhesion. By deduction, the Sas1N128I cell line would undersecrete

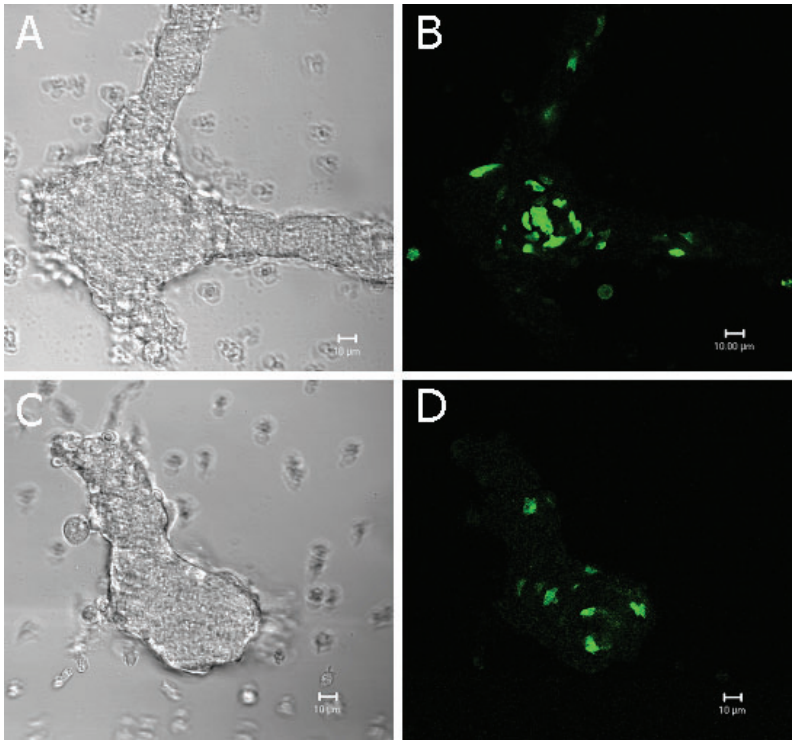

Fig. 5. Differential interference contrast (A, C) and fluorescence images (B, D) of two examples of chimeric aggregates of wild-type Ax2 and Sas1Q74L-expressing cell lines. Equal numbers of wild-type Ax2 and mutant cells were mixed, allowed to aggregate, and examined by confocal microscopy (only the middle optical section of the entire aggregate is shown). Microscopic examination of aggregates demonstrated that Sas1Q74L-expressing cells (green) are found in the aggregates. Bars, 10 μm.

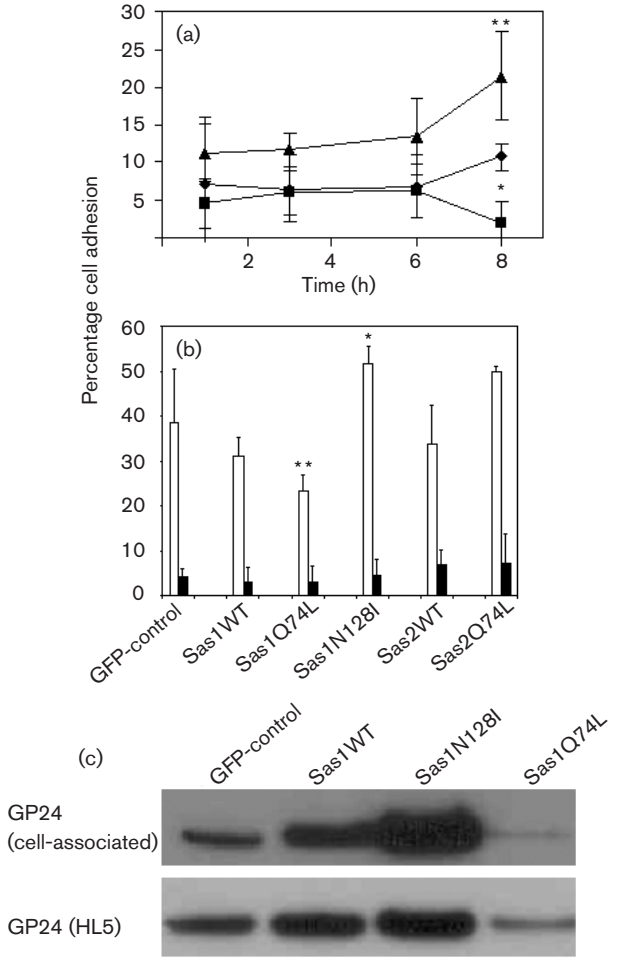

Fig. 6. Cell–cell adhesion and the level of gp24. (a) Total adhesion during development of parental (Ax2; diamonds), Sas1Q74L-expressing (squares) and Sas1N128I-expressing (triangles) cell lines during the first 8 h of development. Values presented are the mean (±SD) of four experiments. Adhesivity of the Sas1N128I and Sas1Q74L mutants was significantly different from that of the control at 8 h (*$P<0{\cdot}05$; **$P<0{\cdot}01$). (b) Adhesion of control and mutant vegetative cells in the absence (open bars) or presence (solid bars) of EDTA. Values presented are the mean (±SD) of four experiments. Adhesivity of the Sas1N128I and Sas1Q74L mutants compared to the control was significantly different (*$P<0{\cdot}05$; **$P<0{\cdot}01$). (c) Western blot analysis of control and mutant cell lysates (cell-associated) and conditioned nutrient medium (HL5) using anti-gp24 antibody.

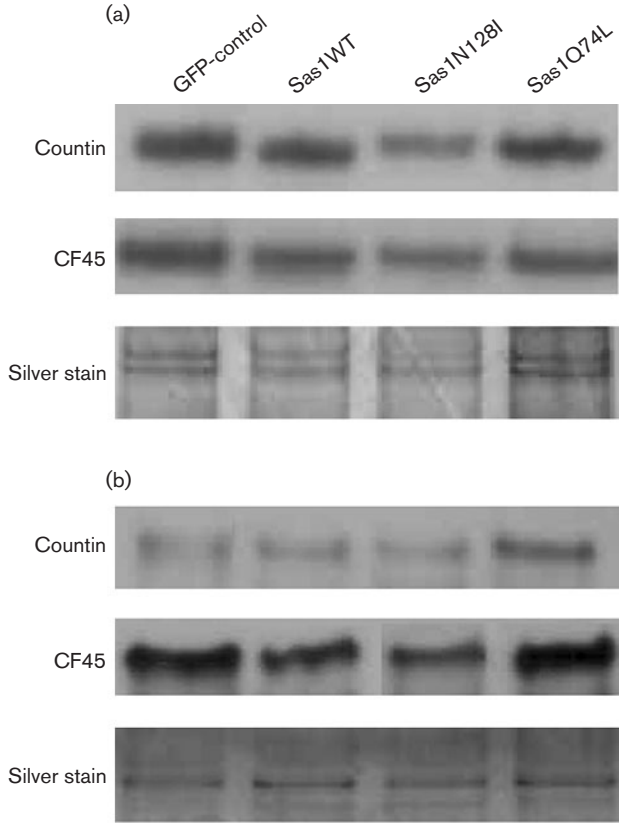

Fig. 7. Western blot analysis of conditioned nutrient medium (a) and DB (b) from control and mutant cell lines using anti-countin and anti-CF45 antibodies. Silver stain of a prominent protein band is shown to demonstrate equality of protein loads.

such signalling molecules. Given the increased expression of Sas1 early in development, it is conceivable that this Rab may regulate secretory events that are important during this phase of the life cycle. One candidate signalling molecule that would account for the observed phenotypes would be CF, which is secreted when cells reach high density and during starvation. One subunit, countin, negatively regulates adhesion by regulating the expression of gp24 and other CAMs (Roisin-Bouffay *et al.*, 2000).

To test the possibility that Sas1 regulates the extracellular levels of countin, control and mutant cells were incubated at high density in nutrient medium or starved in DB and the level of extracellular countin was examined by Western blot analysis. In nutrient medium, there were minimal detectable differences in the level of extracellular countin among the cell lines (Fig. 7a). However, during starvation the level of extracellular countin was higher for the Sas1Q74L cell line as compared to the other cell lines (Fig. 7b). Scanning densitometry indicated that the level of extracellular countin was 1·7-fold higher for the Sas1Q74L cell line as compared to control cells. This suggests that Sas1 may regulate the release of countin during starvation and the increased level of extracellular countin may account for the decrease in the level of the gp24 observed for this cell line.

Although an increased level of gp24 was observed for the Sas1N128I cell line, the extracellular level of countin was

not decreased when cells were grown to high density or starved in DB. One possible explanation might be that only slight changes in countin are required to influence the level of gp24 and that the level of extracellular countin is slightly decreased for the Sas1N128I cell line, but undetectable by Western analysis. This would also explain the decrease in the level of gp24 observed in vegetative Sas1Q74L cells even though extracellular countin is not obviously altered for this cell line under these conditions. Another possible explanation might be the Sas1N128I cell line undersecretes another subunit of CF that would similarly disable the complex. We therefore examined the extracellular level of another subunit of CF, CF45, by Western blot analysis. The extracellular levels of CF45 were not dramatically altered for the mutant cell lines under both conditions (Fig. 7). Therefore, it is likely that Sas1 does not regulate the extracellular levels of this subunit but we cannot rule out the possibility that another subunit of CF is undersecreted from the Sas1N128I cell line. Together, the data indicate that Sas1 may regulate the release of at least one subunit of CF, countin.

Expression of Sas1Q74L alters the extracellular level of acid phosphatase

Given the role of Rab8-like GTPases in secretion (Chen *et al.*, 2001; Vadlamudi *et al.*, 2000; Guo *et al.*, 1999), we also performed standard secretion assays to measure the rate of secretion of a lysosomal hydrolase, acid phosphatase. Exponentially growing cells were harvested and resuspended in growth medium, and at 2 h the activity of acid phosphatase was measured in both cells and the medium (Fig. 8). In growth medium, acid phosphatase was secreted from all of the cell lines to the same extent (approx. 2–5·7 % of the total enzyme activity was found in the extracellular medium at 2 h) (Fig. 8, open bars). In addition to initiating development, starvation also induces slight increases in the secretion of lysosomal hydrolases (Cardelli, 1993; Temesvari *et al.*, 1996). This response was examined in the Sas mutants by performing standard secretion assays, as described above, using cells that had been resuspended in DB (Fig. 8, closed bars). All of the cell lines responded to starvation by slight increases in the secretion of acid phosphatase. However, the response was significantly pronounced in the Sas1Q74L-expressing mutant line which displayed a level of secretion that was 6·3-fold higher than that of the Sas1Q74L-expressing mutant in nutrient medium and 2·3-fold higher than that of the GFP-control in starvation conditions. This suggests that Sas1 may play a role in secretion during development.

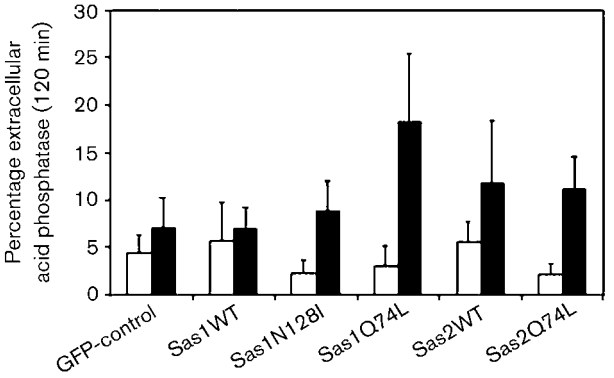

Fig. 8. Secretion of acid phosphatase from control and mutant cell lines. Medium was removed from exponentially growing cells and replaced with fresh nutrient medium (open bars) or DB (closed bars). At 120 min, cells were pelleted and solubilized, and both the cell samples and supernatants were assayed for acid phosphatase. Data are given as the mean ± SD ($n=4$). All cell lines display an increase in secretion under starvation conditions. The Sas1Q74L-expressing cell line displayed a highly statistically significant ($P<0·01$) increase in acid phosphatase secretion as compared to GFP-control under starvation conditions and a statistically higher increase in secretion ($P<0·05$) when compared to the Sas1Q74L-expressing cell line in nutrient medium.

DISCUSSION

We have shown that overexpression of Sas1Q74L resulted in the appearance of actin-rich membrane protrusions, inhibition of aggregation, a reduced level of a CAM, gp24, and increased levels of extracellular countin, and acid phosphatase during starvation. These phenotypic changes were specific to Sas1, as expression of a comparably mutated related Rab, Sas2, did not induce the same alterations. Since similar phenotypic changes were not observed in the mutant overexpressing Sas1WT, the role of Sas1 in the regulation of these phenotypic changes may not be rate-limiting. Overexpression of Sas1N128I resulted in enhanced aggregation and increased levels of the CAM, gp24. The putative role of Sas1 in the regulation of the actin cytoskeleton, cell adhesion and secretion supports the relatedness of this Rab with other Rab8-like proteins. In addition, the data demonstrate that the unique multifunctionality of this subfamily of Rabs may be evolutionarily conserved. Finally, expression of mutant Sas2 did not appear to alter early developmental events, which is consistent with its late developmental pattern of expression.

Regulation of tissue size is critical during development and one of the ways in which *Dictyostelium* cells sense group size is through an extracellular protein complex, namely counting factor, CF (Brock & Gomer, 1999; Roisin-Bouffay *et al.*, 2000; Brock *et al.*, 2002, 2003; Jang *et al.*, 2002; Tang *et al.*, 2002). Since Rab8-related proteins regulate secretory events in a variety of cell types (Huber *et al.*, 1993, 1995; Guo *et al.*, 1999; Chen *et al.*, 2001), it is not surprising that Sas1, a developmentally regulated Rab8-like protein, may control similar processes in *Dictyostelium*. Interestingly, the extracellular levels of CF45 were not altered for the Sas1Q74L mutants, suggesting that the mutant does not have a generalized secretion defect and that the release of the various CF subunits may rely on different mechanisms.

This is consistent with a previous report (Brock *et al.*, 2003) that demonstrates that the subunits of CF are not secreted as a stoichiometric complex.

Although the Sas1N128I cells formed large aggregates, the aggregates became fragmented at later developmental time points. It has been shown that overexpression of the CAM, gp80, results in aggregates that are less flexible and unable to withstand shearing forces generated during coordinated cell motility (Faix *et al.*, 1992). This situation leads to dissociation of aggregates and the formation of smaller fruiting bodies. Therefore, the inability of the Sas1N128I cell line to remain adherent during development may be due to the inappropriately high levels of gp24; however, we cannot rule out the possibility that the levels of other CAMs are also increased in this cell line. Interestingly, several of the Rab8-related proteins have also been implicated in adhesion (Sheth *et al.*, 2000; Marzesco *et al.*, 2002; Lau & Mruk, 2003).

Cells in which the gene encoding gp24 is disrupted are able to complete development; however, these mutants display delayed culmination and abnormal slug and fruiting body morphology (Wong *et al.*, 2002). In contrast, blocking gp24-mediated cell–cell interactions using anti-gp24 antibodies or purified gp24 completely inhibits aggregation (Knecht *et al.*, 1987; Brar & Siu, 1993; Wong *et al.*, 1996). This raises the question of why the developmental outcome of loss of gp24 through genetic disruption is different from that of functional loss through interference. It is possible that other uncharacterized CAMs compensate for gp24 in the null cell line. In support of this, gp24 null cells exhibit precocious expression of gp80 (Wong *et al.*, 2002). Moreover, disruption of gp24 resulted only in a 50 % reduction in EDTA-sensitive adhesions, indicating the existence of additional Ca^{2+}-dependent CAMs (Wong *et al.*, 2002). Surprisingly, although significantly reduced in gp24, Sas1Q74L-expressing cells displayed a non-aggregative phenotype similar to that of gp24-blocked. The severity of this defect may suggest that multiple CAMs may be affected in the Sas1Q74L-expressing mutant.

It is indeed interesting that, in addition to their role in vesicle trafficking, Rab8-like proteins regulate the actin cytoskeleton and filopodia formation (Peranen *et al.*, 1996; Imamura *et al.*, 1998; Hattula *et al.*, 2002). During *D. discoideum* development, membranes of streaming cells first make contact through filopodia that are rich in gp24 (Sesaki & Siu, 1996). It is intriguing that a developmentally regulated Rab8-like protein, Sas1, might regulate both the formation of these filopodia and the level of gp24. Since the observed changes in gp24 are not sufficient to explain the phenotype of the Sas1Q74L-expressing mutant, the severity of the aggregation defect observed may be the result of combined defects in filopodia formation, secretion of counting factor, and levels of gp24.

This is the first report describing the function of a developmentally regulated Rab8-like protein of *Dictyostelium*. During development, secretion, actin cytoskeletal changes and cell–cell adhesion are important in controlling multicellular structure formation. These studies identify for the first time a Rab protein that may regulate several of these processes simultaneously. Identification of Sas1-interacting proteins and secreted proteins from Sas1 mutants will provide further insight into the mechanism by which Sas1 and other Rab8-related proteins function.

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