Integrin and spectrin homologues, and cytoplasm-wall adhesion in tip growth

Susan G. W. Kaminskyj[1],* and I. Brent Heath[2]

[1]Department of Biological Sciences, Hansen Life Sciences Research Building, Purdue University, West Lafayette, IN 47907-1392, USA

[2]Department of Biology, York University, 4700 Keele Street, North York, Ontario, Canada M3J 1P3

*Author for correspondence

SUMMARY

Saprolegnia ferax contains an integrin homologue, identified by crossreactivity with antiserum to the consensus sequence of human/chick/*Xenopus* cytoplasmic domain β_1-integrin, which is highly conserved. In non-reduced samples, this integrin was larger than the reported size range for β_1-integrins, at 178 kDa. In reduced samples, there was a reducing agent-concentration-dependent conversion from 178 kDa to 120 kDa, well within the reported size range for β_1-integrins in other organisms. The integrin antiserum stained plasma membrane-associated patches, which had a shallow tip-high gradient. This population was reduced and its distribution perturbed in hyphae whose growth rate was reduced by half with tetrapentyl ammonium chloride. The expected integrin function in cytoplasm-cell wall attachment was shown by differential resistance to plasmolysis-induced separation, which positively correlated with integrin abundance. However, when there was separation, remnants of cytoplasm stayed attached to the wall. These were enriched in actin and integrin. *Saprolegnia* also has a spectrin homologue identified by crossreactivity with an erythrocyte spectin antibody, which has a size (246 kDa) similar to other organisms. This spectrin had a superficially similar distribution to that of integrin, but it did not participate in cytoplasm-wall anchoring. These data suggest that *Saprolegnia* hyphae have a plasma membrane which is strengthened by spectrin, and cytoplasm which is attached to the cell wall by integrin.

Key words: tip growth, hypha, actin, integrin, spectrin, cytoskeleton, *Saprolegnia*

INTRODUCTION

Tip growth is a major form of cell growth, characteristic of fungal hyphae and plant cells such as root hairs and pollen tubes. It involves localized apical extension, which produces highly polarized, tubular cells typical of species and life cycle stages. It requires coordination of apical cell wall deposition, tip extension, apex-directed cytoplasmic migration and organelle movements, the latter two being the focus of this work.

Because turgor is equivalent between the apical and increasingly vacuolate subapical regions, it cannot act as an internal motive force. Thus, with respect to cytoplasmic migration, fungal hyphae have been described as tube-dwelling amoebae (Reinhart, 1892; Isaac, 1964; Heath, 1990a), based on the similarities between amoeboid cells (Bray, 1992) and that of apical cytoplasm in tip growing cells. These include polarized distributions of cytoskeletal filaments, ion gradients and organelles (reviewed by Heath and Kaminskyj, 1989; Heath, 1990a, b; Jackson and Heath, 1993a), cytoplasmic contractility (McKerracher and Heath, 1986; Jackson and Heath, 1992; Kaminskyj et al., 1992a) and analogous responses to Ca^{2+} gradient perturbations (Kaminskyj et al., 1992a; Garrill et al., 1993).

Amoeboid cells require cytoskeleton/substratum attachments, such as focal contacts, in order to anchor the force necessary for movement (Bray, 1992). These contain plasma membrane-spanning proteins called integrins which mediate the connection between the cytoskeleton and the extracellular matrix (Hynes, 1992; Sastry and Horwitz, 1993). Recent models for some plant cell activities suggest that the cell wall is analogous to the extracellular matrix, that cytoplasm-cell wall interactions are required for normal function, and these interactions are mediated by integrin-like proteins (Kropf et al., 1988; Kropf, 1992; Saunders and Lord, 1992; Goodner and Quatrano, 1993; Wyatt and Carpita, 1993). Evidence for such models has been based on immunoblot crossreactivity with antisera to β_1-integrin (Marcantonio and Hynes, 1988; Quatrano et al., 1991) or extracellular matrix proteins (Saunders et al., 1991; Wagner et al., 1992; Zhu et al., 1993), or by inhibition of normal cell function using exogenous peptides containing integrin-extracellular matrix protein binding sequences (Schindler et al., 1989; Wayne et al., 1992; Bachewich and Heath, 1993). The detailed distribution of integrins or extracellular matrix proteins has not yet been shown in walled cells, especially hyphae, nor has there been evidence of integrins linking the plasma membrane and the hyphal cell wall.

With respect to organelle movement, both microtubules and actin can be bases for motor molecules (McKerracher and Heath, 1987; Heath, 1990a, 1994). However, as with cyto-

plasmic migration, organelle motility requires anchorage of the force-generating ensembles. It is likely that the anchorage is to the cell wall (Aist and Berns, 1981; McKerracher and Heath, 1987; Kaminskyj et al., 1989; Heath, 1990a; Aist and Bayles, 1991), but there are no reports of the distribution of appropriate candidate molecules.

In our ongoing studies on the functions of the cytoskeleton during tip growth, we have identified and localized integrin and spectrin homologues in the hyphal oomycete, *Saprolegnia ferax*. Integrin distribution and behaviour imply a role in cytoskeleton-cell wall attachment for cytoplasmic migration and organelle motility. Spectrin distribution suggests a role as a membrane skeleton.

MATERIALS AND METHODS

Culture and growth conditions

Saprolegnia ferax (Gruith.) Thuret (ATCC no. 36051) was grown on 6 mm×35 mm strips of dialysis tubing (Heath, 1987) at room temperature (~24°C) on a peptone/yeast extract medium solidified with 1.5% agar (OM; Heath and Greenwood, 1970). Colonies were also grown on OM agar containing 250 µM tetrapentyl ammonium chloride (TPAC; Kaminskyj et al., 1992b). Growth rates were calculated from colony radius at 24 hours (control) or 48 hours (TPAC). Sporangia were induced on coverslips from fibrin-enmeshed dialysis tubing colonies (Kaminskyj et al., 1992a) using the method of Holloway and Heath (1974).

Staining and microscopy

Growing colonies were anchored on coverslips (Kaminskyj and Heath, 1994) and allowed to recover in liquid OM for ≥30 minutes. Some colonies were plasmolysed in 2 M sucrose in distilled H_2O, 1 M NaCl in OM, 0.5 M polyethylene glycol-400 (PEG; Baker, Phillipsburg, NJ) in OM, or 0.5 M sorbitol in OM, for 15 minutes before fixation.

Colonies were fixed for 30 minutes in 6% formaldehyde containing 100 µM *m*-maleimidobenzoyl *N*-hydroxy succinimide ester (Molecular Probes, Eugene OR). Fixative and staining solutions were prepared in saline containing 60 mM PIPES (Calbiochem, San Diego, CA), pH 7.0, 2 mM EGTA (Sigma, St Louis, MO), 2 mM $MgCl_2$, 137 mM NaCl, 268 µM KCl. Fixed colonies were stained for actin with rhodamine-phalloidin (RP, Molecular Probes) and for other proteins by immunofluorescence (Kaminskyj and Heath, 1994).

For immunofluorescence, fixed colonies were rinsed for 2× 5 minutes in saline, the wall was degraded for 5 minutes in 10 mg/ml Driselase (Kyowa Hakko Kogyo, Tokyo, Japan), and membranes were permeabilized in 0.05-0.1% (v/v) Triton X-100 (Baker) for 10 minutes. Nonspecific binding was blocked for 20 minutes before incubating in primary antisera (60-90 minutes at room temperature), and again before incubating in secondary antisera (as above) and RP. Blocking solution contained 5% (v/v) fresh egg albumin (Grey Ridge Farms, Toronto, ON) and 2.5% (w/v) skim milk powder (Carnation, Toronto, ON). Colonies were rinsed for 3× 10 minutes in saline after each staining step.

Polyclonal primary antisera, from rabbits, were raised against: (1) the consensus peptide for the cytoplasmic domain of β_1-integrin (Marcantonio and Hynes, 1988); (2) chicken erythrocyte spectrin (ICN, St Laurent, PQ (no longer available), and Sigma); (3) chicken gizzard tropomyosin (ICN); (4) chicken gizzard α-actinin (ICN); (5) yeast actin binding proteins: ABP1, cofilin and fimbrin; (6) peroxidase-conjugated anti-goat IgG (Sigma). Antiserum dilutions were 1:100 for integrin and spectrin, and 1:10 otherwise. Immunoblots using whole anti-spectrin serum showed a band with similar mobility to actin, and immunofluorescence with whole antiserum showed staining patterns which were congruent with RP. Thus, for spectrin immunofluorescence, the antiserum was adsorbed against rabbit myofibrils, 24 hours at 4°C on a rotating tilt table, which were then removed by centrifugation at 20,000 *g* for 20 minutes at 4°C. Myofibrils were prepared from glycerinated rabbit psoas muscle in 6 mM Na/K phosphate buffer, pH 6.8, containing 100 mM KCl and 5 mM $MgCl_2$. Using myofibril-adsorbed spectrin antiserum, there was no subapical congruence between spectrin and actin patterns.

Monoclonal antibodies were raised in mice against chicken gizzard vinculin, clones C19 and VIN-11-5 (ICN). These antibodies were used diluted 1:10.

Secondary antisera (Amersham, Chicago, IL) were used diluted 1:50-1:100. They were: (1) FITC-conjugated anti-rabbit, raised in goat; (2) FITC-conjugated anti-mouse, raised in sheep; and (3) Texas Red-conjugated anti-rabbit, raised in donkey. Anti-rabbit sera were adsorbed against lyophilized *Saprolegnia* powder to reduce nonspecific wall adherence (5 mg powder/200 µl diluted antibody) for 90 minutes at room temperature. The powder was removed before use by centrifugation at 20,000 *g* for 20 minutes at 4°C.

Phase-contrast and epifluorescence microscopy used an NA 1.25, 100× objective. Confocal microscopy used an MRC600 scanner, krypton-argon laser, and K1 (FITC) and K2 (rhodamine) filter sets (Bio-Rad, Mississauga, ON) and an NA 1.4, 60× phase-contrast objective. Image analysis used 'area intensity' software from Image 1 (Empix Imaging, Mississauga, ON) to analyze dual-wavelength, near-median confocal images which had not been contrast enhanced; for each experiment, images were at the same magnification. RP-stained apical remnants were outlined (using 'trace'), and compared with the same region on the FITC (anti-integrin or -spectrin) image. Once chosen, the selected area was fixed in size and shape, and could be dragged. Areas are expressed as number of pixels $\times 10^3$; intensities as sum of grey scale values within the area. Fluorescence intensity ratios were calculated for each hypha as:

$$[RP(tip) - RP(background)] \div [FITC(tip) - FITC(background)].$$

Equally bright images would have a ratio of 1; relatively faint FITC images would have a ratio greater than 1. Data are presented for cells prepared and examined in one session, since variations in confocal settings and sample preparation affected the amount and apparent staining intensity of apical remnants. Statistically comparable ratios were obtained in replicate experiments. Statistical analyses used Statview SE+Graphics 1.02 (Abacus Concepts, Inc., Berkeley, CA). Figures are contrast enhanced to facilitate visual comparison.

Electrophoresis and immunoblotting

Saprolegnia colonies were grown for 3 days on 35 mm×75 mm sheets of dialysis tubing on solid OM. To reduce protease activity during collection/freezing and sample preparation, 40 µl of protease inhibitor solution containing 50 mM AEBSF (Calbiochem) and 500 µM leupeptin (Sigma) was added to each mycelial mat (~200 mg fresh weight) before harvest. Mycelia were collected rapidly and frozen (~10 seconds, total) in liquid nitrogen, ground in a mortar at −80°C, transferred to cold preweighed Eppendorf tubes, and stored at −80°C until use.

Fertilized chicken eggs (Brampton Chick Hatchery, Brampton, ON) were incubated for 4 days at 37°C and 86% relative humidity in a TX6 incubator (Marsh Farms, Garden Grove, CA). Embryos were isolated (~20 seconds), frozen (~20 seconds), then prepared as above. Unlike *Saprolegnia* colonies, the protease inhibitor solution would not wet the embryos. Protease inhibitor was added to the frozen tissue during grinding, using 60 µl solution per embryo.

For electrophoresis, 2 µl/mg of 100°C sample buffer (double strength buffer:distilled water, 3:1) was added to frozen samples, which were briefly vortexed and placed in a boiling water bath. Sample buffer contained 2% SDS (BDH specially pure, Toronto, ON) in 62 mM Tris-HCl, pH 6.8 (BDH), or 5% SDS and 10 mM EDTA (Fisher) in 100 mM Tris-HCl, pH 6.8 (Marcantonio and Hynes, 1988).

Thiol-reducing buffers contained 2-100 mM dithiothreitol (DTT; Bethesda Research Labs, Gaithersburg, MD) or 5% β-mercaptoethanol (Sigma). Samples were boiled for 5 minutes, chilled in ice-water, centrifuged at 20,000 *g* for 20 minutes at 4°C, and filtered through an Acrodisc (0.2 μm pore, Gelman Sciences, Rexdale, ON). Some samples were Centricon-30 or -100 fractionated (Amicon, Beverly, MA) according to the manufacturer's instructions.

Protein concentrations were determined using a modification of the enhanced copper (Lowry) method, since sample buffer components interfere with standard protein assays (Stoscheck, 1990). SDS was replaced with distilled water in preparing the copper reagent; bovine serum albumin standards and 5 μl samples of unknowns (with ≤2 mM DTT) were diluted in 2% SDS in 62 mM Tris-HCl, pH 6.8. Under these conditions there was a linear relationship between A_{750} and protein for 10-250 μg bovine serum albumin per 400 μl. Protein content of samples extracted with >2 mM DTT was taken to be equivalent to similar buffer with ≤2 mM DTT. Typically, whole protein extracts from *Saprolegnia* contained 2 μg protein/μl; chick extracts contained 5 μg protein/μl.

Samples were mixed 4:1 with sample buffer containing 50% glycerol and 0.001% Bromophenol Blue (BDH) before loading. Proteins (25-100 μg/4× 1.5 mm well) were separated by SDS-PAGE using methods of Hames (1981), transferred to nitocellulose (Towbin et al., 1979), and stained with 0.2% Ponceau S (Allied Chemical, Morristown, NJ) in 3 mM trichloroacetic acid (Fisher) to visualize the lanes. Some gels were silver-stained for protein (Merrill et al., 1980) or carbohydrate (Tsai and Frasch, 1982). For the latter, periodic acid was replaced (w/w) with sodium periodate. Nitrocellulose blocking was in TBS-T (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween-20 (Fisher) containing 5% milk powder, for 45 minutes at 23°C, during which the Ponceau S was eluted.

The blots were probed with the same polyclonal primary antibodies at the same dilutions as used for immunofluorescence. Monoclonal anti-(chicken gizzard) actin, clone N350 (Amersham), raised in mouse, was used at 1:1000. Monoclonal anti-(rabbit myosin heavy chain) was used at 1:50. Incubations were 1-3 hours in a moist box at room temperature. Peroxidase-conjugated secondary antisera (Sigma) were used at 1:200. Incubations were 1-2 hours as above. Peroxidase was developed with H_2O_2 and 4-chloro-l-naphthol (Sigma).

RESULTS

Immunoblotting

Saprolegnia proteins (from mycelial extracts, Fig. 1a,b) with the expected molecular masses crossreact with antisera to actin (42 kDa; Fig. 1c), myosin (205 kDa; Fig. 1d), and spectrin (246 kDa; Fig. 1e). Under nonreducing conditions there is a β$_1$-integrin-reactive band at 178 kDa (Fig. 1f). With thiol-reducing agents (2-100 mM DTT or 5% β-mercaptoethanol) this band appears to be converted into one of 120 kDa, which occurs in a DTT concentration-dependent manner (Fig. 1g-i). Both of these proteins appear to be glycosylated, since bands with similar masses and relative intensities to those on immunoblots are also found on carbohydrate-stained gels (Fig. 1j).

Proteins in walled cells are very susceptible to proteolysis (Quatrano et al., 1991), and this appears to be the case for *Saprolegnia* also, because samples prepared without the addition of protease inhibitors gave multiple lower mass spectrin and integrin crossreactive bands (not illustrated). Similarly, chick embryo proteins showed smaller than expected integrin and spectrin crossreactive bands, in addition to the predicted bands at 116 kDa and 230/240 kDa, respectively (not illustrated).
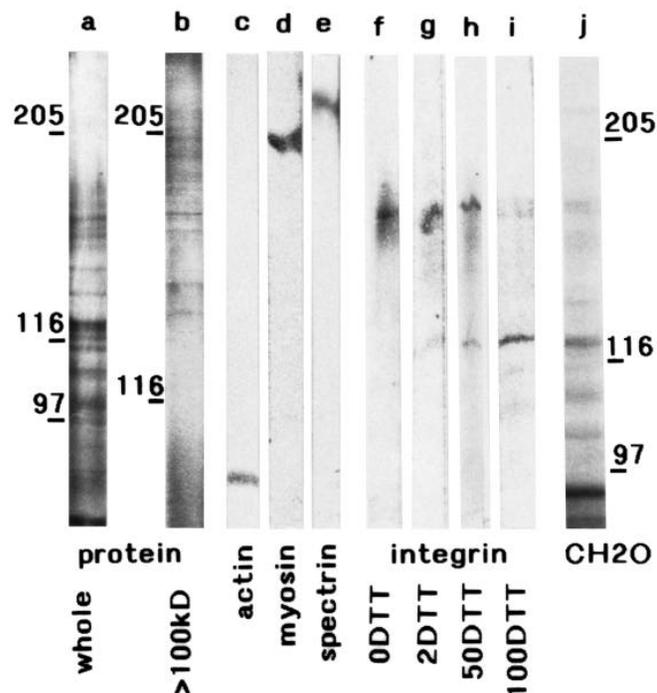
No *Saprolegnia* proteins crossreacted with antisera to α-

Fig. 1. (a,b) SDS-polyacrylamide gels silver-stained for protein, with 25 μg of whole (a), or >100 kDa-enriched (b) *Saprolegnia* proteins, extracted with 2% SDS and 2 mM DTT. (c-e). Immunoblots of whole (c, d) or (>100 kDa)-enriched (e) *Saprolegnia* proteins (100 μg/lane, extracted as above) showing crossreactive bands to actin at 42 kDa (c), myosin at 205 kDa (d), and spectrin at 246 kDa (e). The actin band in (c) was run on a 12.5% acrylamide gel (compared with 7.5 or 8.75% for the other gels), thus its location in the figure is approximate. (f-i) Immunoblots of 100 μg loadings of whole *Saprolegnia* proteins extracted with 5% SDS and 10 mM EDTA containing 0 (f), 2 (g), 50 (h) and 100 (i) mM DTT, showing integrin crossreactive bands at 178 kDa shifting to 120 kDa as the concentration of DTT increases. Molecular mass markers (in kDa) for lanes f-j are indicated at the right. (j) SDS/polyacrylamide gel of 50 μg of whole *Saprolegnia* protein, extracted as in h, and silver-stained for carbohydrate, showing bands at 178 kDa and 120 kDa which comigrate with integrin crossreactive bands in h and i. Two other carbohydrate bands, >205 kDa and <97 kDa, do not have corresponding integrin crossreactive proteins.

actinin, tropomyosin, vinculin, yeast ABP1, cofilin or fimbrin, or peroxidase-conjugated anti-goat IgG, when these were used as primary antibodies for immunoblotting. Omitting primary antibodies gave no crossreaction.

Distribution of integrin and spectrin in *Saprolegnia* hyphae and sporangia

In hyphae, the integrin antiserum most prominently stained peripheral patches (integrin patches) (Fig. 2b,d), with diffuse, central staining which was excluded from large organelles. The granularity of the immunofluorescence patterns may be technique-related, or may indeed reflect discontinuous distributions, possibly adhesion complexes in the case of integrin staining.

Integrin patches were abundant in the apical, i.e. 0-10 μm, segment of hyphal tips and declined through the 10-70 μm zone to lower levels in the most distal regions measured, 150-250 μm from the tip (Fig. 4A, Table 1). However, all hyphal
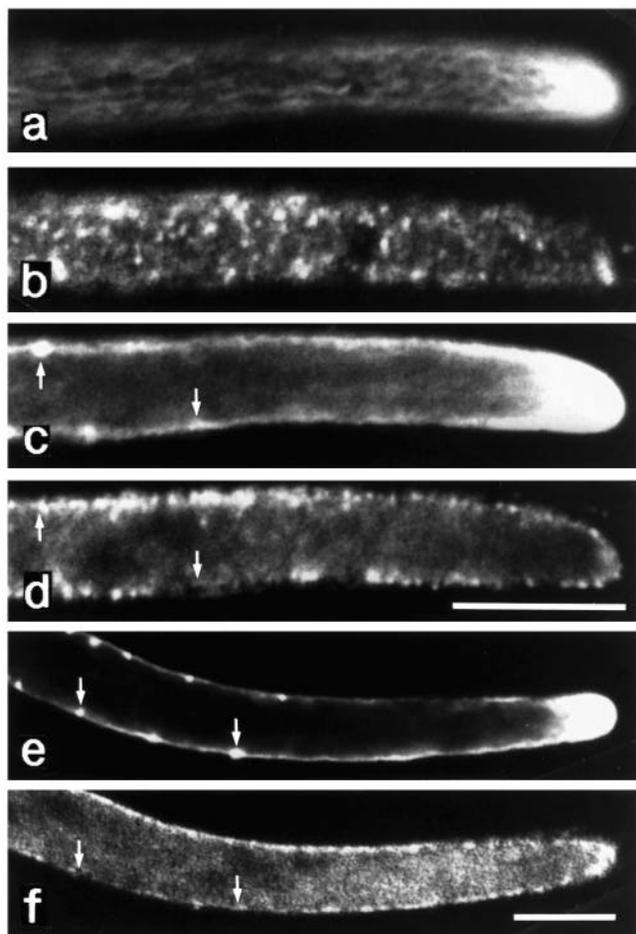
Fig. 2. Peripheral (a,b) and near-median (c-f) confocal images of two hyphae (a-d and e-f) dual stained for F-actin (a,c,e) and integrin (b,d) or spectrin (f). F-actin filaments (a, the most apical plaque in this plane of optical section was further back than the field of view) and plaques (c-d, arrows), do not colocalize with integrin patches (a vs b, c vs d). Spectrin staining was not restricted to regions of subapical cortical actin abundance (e vs f). Bar, 10 μm.

Fig. 3. Near-median confocal images of integrin (a,b) and spectrin (c,d) staining in expanding sporangia. The cytoplasm in b and d, but not a and c, separated from the wall (arrows in d) during immunofluorescence processing, revealing that much of the integrin (b), but very little of the spectrin (d) remained attached to the wall. Bar, 50 μm in a, c, d; 10 μm in b.

regions contained integrin patches. In the 0-10 μm segment of hyphal tips, F-actin and integrin patches were so abundant that colocalization was inevitable. Subapically, where the density of both proteins was lower, the integrin patches did not colocalize with either actin filaments or plaques (Fig. 2a-d), confirmed by colour merge analysis (not shown).

The spectrin antisera also stained prominent peripheral patches (spectrin patches) in hyphae. These were superficially similar to the integrin patches (Fig. 2f), and diffuse central staining was again present. Spectrin patches were enriched at the tip (0-10 μm value: 0.548±0.013, *n*=46; >0.5 with 1-tailed *t*-test, *P*=0.0002), and were seen in all regions examined. F-actin and spectrin patches were both so abundant at the tips that they colocalized, but subapically many spectrin patches did not colocalize with actin (Fig. 2e,f).

We could not dual-label for spectrin and integrin as these antisera were both raised in rabbits. However, different masses of crossreactive proteins on immunoblots, and different staining patterns in plasmolysed hyphae (cf. Fig. 6c,d and e,h) and sporangia (cf. Fig. 3b and d), discussed below, suggest that these antisera do not crossreact.
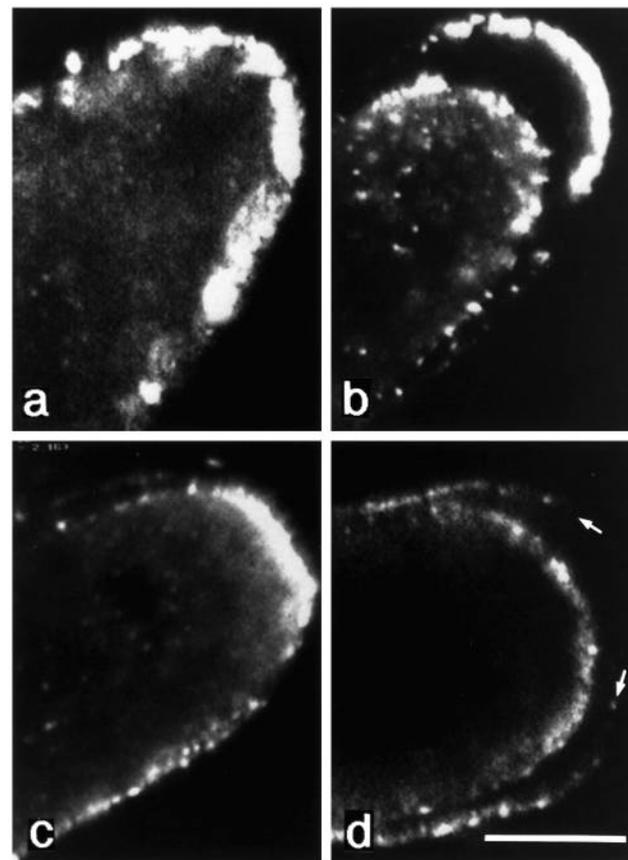
During asexual reproduction, hyphae destined to become sporangia cease extending, expand and accumulate cytoplasm by apex-directed cytoplasmic migration (Gay and Greenwood, 1966). During this stage, sporangia contained integrin (Fig. 3a,b) and spectrin (Fig. 3c,d) patches, which were so abundant in the tips that they could not be resolved for counting. In sporangia whose cytoplasm separated from the cell wall during immunofluorescence processing, integrin remained associated with the wall (Fig. 3b; 11 of 13 sporangia), whereas spectrin segregated with the cytoplasm (Fig. 3d; 5 of 5 sporangia).

Saprolegnia hyphae did not stain with antisera to α-actinin, tropomyosin, vinculin, yeast ABP1, cofilin or fimbrin, and omitting primary antibodies gave no staining. In contrast, actin and tubulin antibodies gave detailed patterns which were very different from integrin or spectrin immunofluorescence (Kaminskyj and Heath, 1994).

Influence of growth rates on integrin abundance

To assess whether integrin patch number and distribution in hyphae correlated with growth, we reduced growth rate by half with TPAC (Table 1; Kaminskyj et al., 1992b) and determined the corresponding effect on integrin abundance. The tip-high integrin gradient of control hyphae was perturbed in the extreme apex of TPAC-treated hyphae (Fig. 4B; Table 1) and the abundance of integrin patches per segment was reduced in
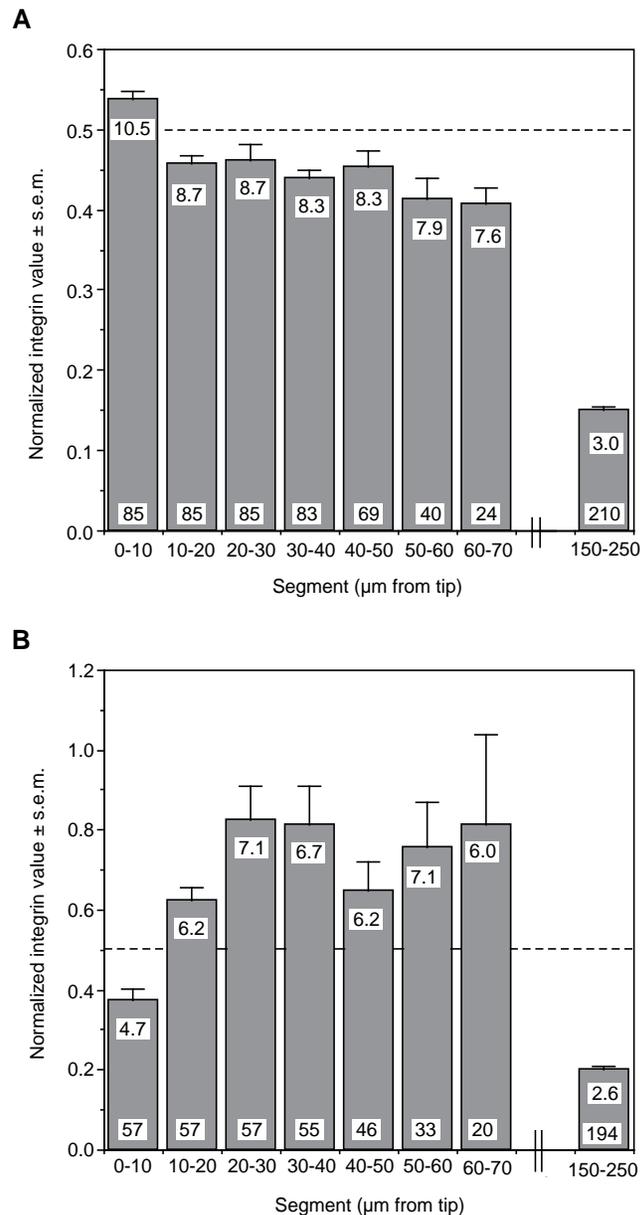
Fig. 4. Abundance of integrin patches in control (A) and TPAC-treated (B) hyphae. Near-median confocal sections were divided into 10 μm segments and the patches were counted in each. Data for each hypha were normalized by dividing the number of patches per segment by the total for the first two segments. Subapical (150-250 μm) segments were normalized using average 0-20 μm values. An even distribution would give a normalized value of 0.5 (the broken lines). The upper number in each bar is the average number of integrin patches per segment; the lower is the number of different hyphae (0-70 μm) or segments (150-250 μm) scored. For control and TPAC, numbers of integrin patches and normalized integrin values were similar in the 10-70 μm segments (ANOVA, $P \geq 0.2823$). Control normalized integrin values were higher (0-10 μm) or lower (10-70 μm and 150-250 μm) than 0.5 at $P<0.005$ (1-tailed t-test); TPAC values were lower (0-10 μm, 150-250 μm) or higher (10-70 μm) than 0.5 at $P<0.0005$ (1-tailed t-test).

the 0-10 μm (ANOVA, $P=0.0001$), 10-40 μm ($P \leq 0.01$), and subapical (ANOVA, $P=0.001$) segments.

Growth rates in hyphae vary erratically under constant

Table 1. Effect of growth conditions on extension rate, cortical actin, distributions of integrin patches, and apical attachment after plasmolysis

		Hyphae		
	Treatment:	Control	TPAC	Sporangia
Extension rate (μm/min)†		11.6±0.3*	6.5±0.4	0
Actin cap length (μm)		36.1±1.3	17.0±1.9	7.6±2.1
[n]		[51]	[56]	[15]
Percentage of tips with apically attached cytoplasm after plasmolysis‡				
Solute:	Sucrose	90±0.5	72±0.1	48±0.5
	[$n1,n2$]	[2,127]	[2,129]	[2,102]
	Sorbitol	37±1.0	21±0.4	77±0.2
	[$n1,n2$]	[4,318]	[3,259]	[2,70]
	NaCl	34±0.1	17	46±7.0
	[$n1,n2$]	[3,153]	[1,102]	[2,80]
	PEG	1±0	1±0	50±6.5
	[$n1,n2$]	[3,114]	[2,66]	[2,70]

*All values are mean ± s.e.
†Determined from colony radius.
‡Mean of percentages from $n1$ colonies, for a total of $n2$ tips scored.

culture conditions, although the average rate remains steady (Kaminskyj et al., 1992b). However, the length of the filamentous cap of F-actin in the tips correlates with changes in growth rate (Jackson and Heath, 1990), especially in hyphae growing slower than 12 μm/min ($r^2=0.742$; Kaminskyj et al., 1992b). We used F-actin cap length to look for finer correlations between growth rate and integrin patch abundance. As expected, control hyphae had longer actin caps than TPAC-treated hyphae (Table 1, ANOVA, $P=0.0001$). Control hyphae with longer actin caps had more integrin patches in the 0-10 μm segment; however, the correlation was low ($r^2=0.06$; $P=0.0802$).

Integrin, but not spectrin, apparently mediates cytoplasm-wall adhesion

We used the clear difference between control and TPAC-treated hyphae to assess the role of integrins in cytoplasm-wall adhesion in *Saprolegnia*. If integrins are involved, then adhesion should be stronger in integrin-rich control hyphal tips, i.e. control hyphae should have more apical wall-attached cytoplasm after plasmolysis than TPAC treated hyphae. Colonies were plasmolysed with hypertonic solutions, and scored for apical-attached cytoplasm (Table 1; Fig 5). All hyphae were plasmolysed either apically or subapically with each treatment. However, apical cytoplasmic attachment was greater in control than TPAC-treated hyphae plasmolysed with sorbitol, sucrose and NaCl (factorial ANOVA, $P=0.0312$), although not with PEG.

Plasmolysed tips with apical separation had phase dark, wall-attached remnants (Figs 5c,d, 6a), especially prominent after PEG plasmolysis. These appeared to be regions where cytoplasm-to-wall attachment was stronger than cohesion to the adjacent cytoplasm.

Sporangia were more likely than hyphae to have tip-attached cytoplasm after plasmolysis, when all plasmolysing solutions including PEG were compared (factorial ANOVA, $P=0.0279$; Table 1).

Characterization of apical attachment sites

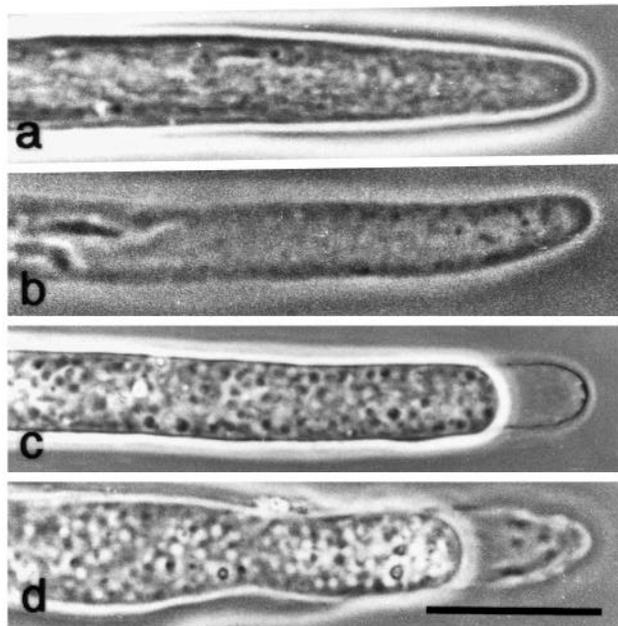If integrins mediate actin-wall interactions in *Saprolegnia*,

Fig. 5. Phase-contrast images of living *Saprolegnia* hyphae growing in OM (a), or after plasmolysis with hypertonic sucrose (b, with apically attached cytoplasm), sorbitol (c, with apically separated cytoplasm and minute cytoplasmic remnants; NaCl gave similar results) and PEG (d, with apical separation and prominent wall-attached remnants, here in peripheral section to show their typical elongated shape). Bar, 10 μm.
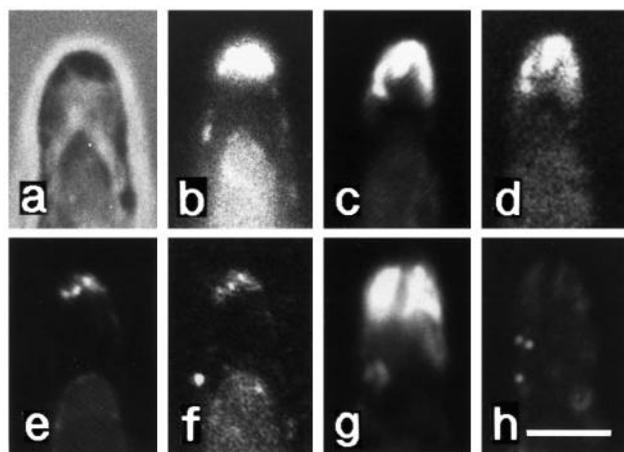

Fig. 6. Near-median optical sections of cytoplasmic remnants attached to the apical walls of 3 control (a-d, g,h) and 1 TPAC-treated (e,f), PEG-plasmolysed hyphae stained for actin (b,c,e,g), integrin (d,f) and spectrin (h). The phase-dark remnants (a) contain actin (b,c,e,g) and are also rich in integrin (d,f), but not spectrin (h).TPAC-treated hyphae (e,f) had significantly less apical remnant material than control hyphae, but similar intensity ratios between actin and integrin staining (c:d vs e:f). Bar, 5 μm.

then: (*a*) the cytoplasmic remnants after plasmolysis-induced tip separation should contain integrin; (b) control hyphae (with more apical integrin patches) should have more remnant material than TPAC-treated ones; and (c) integrin staining should be brighter than spectrin staining in the remnants, since spectrin does not participate directly in transmembrane

Table 2. Staining intensities of apical remnants in PEG-plasmolysed *Saprolegnia* hyphae

A. Actin and integrin-staining in control and TPA-treated hyphae

	Control	TPA	ANOVA
Remnant area†	169.9±14.2*	48.6±5.6	*P*=0.0001
Intensity ratio‡	3.7±0.5	3.2±0.4	*P*=0.4858
n	25	28	

B. Actin and integrin or spectrin staining in control hyphae

	Integrin	Spectrin	ANOVA
Remnant area†	231.4±28.9*	302.4±34.5	*P*=0.16
Intensity ratio‡	1.9±0.2	4.3±0.4	*P*=0.0003
n	16	26	

Data are presented for images (not contrast enhanced) of hyphae prepared and examined in one session, since variations in confocal settings and sample preparation affected the amount and apparent staining intensity of apical remnants.

*All values are mean ± s.e.

†Area is number of pixels $\times 10^3$ in region of interest, which was chosen on the RP-stained image.

‡Intensity is sum of pixel grey scale values within the chosen area. Statistically comparable intensity ratios (actin:integrin or spectrin; see Materials and Methods) were obtained in replicate experiments.

linkages (Bennett, 1990). These experiments, summarized in Table 2, used PEG-plasmolysis, since apical separation was consistently high, and similar between control and TPAC-treated hyphae.

Plasmolysed hyphae were strikingly similar in their actin and integrin staining patterns (Fig. 6c-f), with both being concentrated in the remnants. There were significantly more apical remnants in control than TPAC-treated hyphae, but in both treatments the fluorescence intensity ratios were similar (Table 2; cf. Fig. 6c,d and e,f). In control hyphae (TPAC-treated hyphae were not examined), the apical remnants were significantly enriched in integrin over spectrin (Table 2; cf. Fig. 6c,d and g,h).

DISCUSSION

This is the first cytological study of cytoplasmic anchorage in hyphae. We have identified a novel integrin homologue using immunoblotting, shown with immunofluorescence that it is concentrated at the plasma membrane in a non-uniform pattern, experimentally manipulated the integrin distributions and correlated them with cytoplasm-cell wall attachment. We also identified and localized a spectrin homologue, again with a polarized distribution.

Presence of integrin and spectrin homologues

Saprolegnia protein extracts contain an integrin homologue which crossreacts on immunoblots with cytoplasmic domain β1-integrin antiserum. This domain is conserved in vertebrates, invertebrates and fungi (Marcantonio and Hynes, 1988), as well as algae (Quatrano et al., 1991). Reported sizes of β1-integrins show considerable variation, from 76 to 130 kDa (Hynes, 1987; Marcantonio and Hynes, 1988; Quatrano et al., 1991), spanning that of the reduced *Saprolegnia* β1-integrin

(120 kDa). β_1-Integrins have conserved *intrachain* disulphide bridges, so the reduced protein normally has lower mobility (Marcantonio and Hynes, 1988). The unexpected increase in mobility of the *Saprolegnia* integrin after reduction suggests the presence of an *interchain* disulphide bond, as reported for the $\alpha_{IIb}\beta_3$-integrin (Hynes, 1987), glycoprotein IIb (Calvete and González-Rodriguez, 1986). The *Saprolegnia* integrin homologue appears to be glycosylated, like other integrins (Hynes, 1992): carbohydrate-staining bands in protein extracts comigrated and varied in relative intensity after sample reduction in the same way as the integrin crossreacting bands on immunoblots.

Saprolegnia also contains a spectrin homologue similar in size, 246 kDa, to those of animals (Bennett, 1990), plants (Michaud et al., 1991; de Ruijter and Emons, 1992; Faraday and Spanswick, 1993), and protists (Choi and Jeon, 1989). Finding a single crossreactive band after probing with an erythrocyte antispectrin, rather than a doublet, is likely because erythrocytes contain α and β spectrins, whereas nonerythroid cells contain α and γ subunits (Bray, 1992). Spectrin is a membrane skeleton protein (Bennett and Gilligan, 1993) whose enrichment in *Saprolegnia* hyphal apices might contribute to membrane stability during extension.

Integrin functions

Integrins appear to mediate attachment between the cytoskeleton and the cell wall, which is analogous to an extracellular matrix (Kropf et al., 1988; Kropf, 1992; Saunders and Lord, 1992; Goodner and Quatrano, 1993; Wyatt and Carpita, 1993). In *Saprolegnia* this model is supported by: (a) the peripheral location of the integrin patches; (b) the correlation between their abundance and the likelihood of the plasma membrane to separate from the wall during plasmolysis; (c) the greater resistance to plasmolysis in the integrin-rich sporangial tips relative to hyphal tips; (d) the formation of integrin- and actin-rich cytoplasmic remnants attached to cell walls following plasmolysis; and (e) the colocalization of actin and integrin in hyphal and sporangial tips.

Integrins may anchor hyphal cytoplasm for apex-directed contractions (McKerracher and Heath, 1986; Jackson and Heath, 1992; Kaminskyj et al., 1992a), which may be an extreme form of the manner in which cytoplasm moves forward with the growing tip (Kaminskyj et al., 1992a). Contraction polarity implies differential anchorage of the contractile system, consistent with the gradients of integrin patches in hyphae and sporangia.

As with cytoplasmic migration, organelle motility requires anchorage, possibly to the cell wall (Aist and Berns, 1981; Aist and Bayles, 1991). The apical and subapical integrin patches are well placed for a role in this process, and their distribution along hyphae accords with similarly distributed organelle movements.

Apically, integrin and spectrin may function in tip morphogenesis, since recent observations indicate a role for the actin cap found in oomycete hyphal tips (Jackson and Heath, 1990, 1993b; Kaminskyj et al., 1992a,b; Money and Harold, 1992, 1993). Peptides which compete for integrin-extracellular matrix interactions in animal cells interfere with growth and morphology of *Saprolegnia* hyphae (Bachewich and Heath, 1993), implying a linkage between the cytoskeleton and the forming wall.

Relationship between integrins, spectrins and actin arrays

In apical regions, *Saprolegnia* integrin appears to associate with both actin arrays and the cell wall. In hyphal tips, actin and integrin (but not spectrin) colocalize in cytoplasmic remnants after plasmolysis; in sporangial tips, integrin (again, not spectrin) stays attached to the apical wall even if the cytoplasm is detached during immunofluorescence preparation. These results are consistent with integrin being a transmembrane link between wall proteins and actin. In contrast, spectrin seems to be more independent of the predominant peripheral actin arrays, as also seen in nonwalled cells (Bennett, 1990; Bennett and Gilligan, 1993).

The apparent lack of coincidence between subapical actin plaques and integrin patches seems contrary to the apical linkages assumed above. However, the nature of the labile actin plaques is unknown (Heath, 1987, 1988, 1990a; Harold and Harold, 1992; Kaminskyj and Heath, 1994). They may form artefactually and separate from integrin if interfilament interactions are stronger than those between actin and integrin.

Integrins may contribute to hyphal polarity

Integrins mediate signalling as well as anchorage (Hynes, 1992; Sastry and Horwitz, 1993). In *Saprolegnia*, the integrin distribution correlates with stretch-activated Ca^{2+} channels (Garrill et al., 1992, 1993; Levina et al., 1994). In fibroblasts, integrin-mediated adhesion regulates ion channel activity (Schwartz et al., 1991). Channel function (Garrill et al., 1993) and integrin-extracellular matrix interaction (Bachewich and Heath, 1993), and both are required for growth in *Saprolegnia*, suggesting that they may be interdependent. Similarly, stretch-activated Ca^{2+} channels and an integrin-like protein are codistributed in characean internode cells and both are required for gravisensing (Staves et al., 1992; Wayne et al., 1992).

REFERENCES

Aist, J. R. and Bayles, C. J. (1991). Ultrastructural basis of mitosis in the fungus *Nectria hematococca* (sexual stage of *Fusarium solani*). I. Asters. *Protoplasma* **161**, 111-122.

Aist, J. R. and Berns, M. W. (1981). Mechanics of chromosome separation during mitosis in *Fusarium* (*Fungi imperfecti*): new evidence from ultrastructural and laser microbeam experiments. *J. Cell Biol.* **91**, 446-458.

Bachewich, C. and Heath, I. B. (1993). Effects of cell wall-cytoskeleton linkage inhibition on growth and development in *Saprolegnia ferax*. *Inoculum* **44**, 25.

Bennett, V. (1990). Spectrin: a structural mediator between diverse plasma membrane proteins and the cytoskeleton. *Curr. Opin. Cell Biol.* **2**, 51-56.

Bennett, V. and Gilligan, D. M. (1993). The spectrin-based membrane skeleton and micron scale organization of the plasma membrane. *Annu. Rev. Cell Biol.* **9**, 27-66.

Bray, D. (1992). *Cell Movements*. Garland Publishing, New York.

Calvete, J. J. and González-Rodriguez, J. (1986). Isolation and biochemical characterization of the α- and β-subunits of glycoprotein IIb of human platelet plasma membrane. *Biochem. J.* **240**, 155-161.

Choi, E. Y. and Jeon, K. W. (1989). A spectrin-like protein present on membranes of *Amoeba proteus* as studied with monoclonal antibodies. *Exp. Cell Res.* **185**, 154-165.

de Ruijter, N. C. A. and Emons, A. M. C. (1992). The occurrence and localization of spectrin in plant cells(ed. M. M. A. Sassen, J. W. M. Derksen, A. M. C. Emons and A. M. C. Wolters-Arts. *Sixth Cell Wall Meeting. Nijmegen University Press*.

Faraday, C. D. and Spanswick, R. M. (1993). Evidence for a membrane skeleton in higher plants: a spectrin-like polypeptide co-isolates with rice root plasma membranes. *FEBS Lett.* **318**, 313-316.

Garrill, A., Lew, R. R. and Heath, I. B. (1992). Stretch-activated Ca^{2+} and Ca^{2+}-activated K^+ channels in the hyphal tip plasma membrane of the oomycete *Saprolegnia ferax. J. Cell Sci.* **101**, 721-730.

Garrill, A., Jackson, S. L., Lew, R. R. and Heath, I. B. (1993). Ion channel activity and tip growth: tip-localized stretch-activated channels generate an essential Ca^{2+} gradient in the oomycete *Saprolegnia ferax. Eur. J. Cell Biol.* **60**, 358-365.

Gay, J. L. and Greenwood, A. D. (1966). Structural aspects of zoospore production in *Saprolegnia ferax* with particular reference to the cell and vacuolar membranes. In *The Fungus Spore*, *Colston Papers*, vol. 18 (ed. M. F. Madelin), pp. 93-110. Butterworths, London.

Goodner, B. and Quatrano, R. S. (1993). *Fucus* embryogenesis: a model to study the establishment of polarity. *The Plant Cell* **5**, 1471-1481.

Hames, B. D. (1981). An introduction to polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins: a Practical Approach* (ed. B. D. Hames and D. Rickwood), pp. 1-91. IRL Press, Oxford.

Harold, R. L. and Harold, F. M. (1992). Configuration of actin microfilaments during sporangium development in *Achlya bisexualis*: comparison of two staining prototcols. *Protoplasma* **171**, 110-116.

Heath, I. B. and Greenwood, A. D. (1970). The structure and formation of lomasomes. *J. Gen. Microbiol.* **62**, 129-137.

Heath, I. B. (1987). Preservation of a labile cortical array of actin filaments in growing hyphal tips of the fungus *Saprolegnia ferax. Eur. J. Cell Biol.* **4,** 10-16.

Heath, I. B. (1988). Evidence against a direct role for cortical actin arrays in saltatory organelle motility in hyphae of the fungus *Saprolegnia ferax. J. Cell Sci.* **91**, 41-47.

Heath, I. B. (1990a). The roles of actin in tip growth of fungi. *Int. Rev Cytol.* **123**, 95-127.

Heath, I. B. (1990b). *Tip Growth in Plant and Fungal Cells.* Academic Press, New York, Toronto.

Heath, I. B. (1994). The cytoskeleton. In *The Growing Fungus* (ed. N. A. R. Gow and G. M. Gadd), pp. 99-134. Chapman and Hall, London (in press).

Heath, I. B. and Kaminskyj, S. G. W. (1989). The organization of tip-growth-related organelles and microtubules revealed by quantitative analysis of freeze-substituted oömycete hyphae *J. Cell Sci.* **93**, 41-52.

Holloway, S. A. and Heath, I. B. (1974). Observations on the mechanism of flagellar retraction in *Saprolegnia terrestris. Can. J. Bot.* **52**, 939-942.

Hynes, R. O. (1987). Integrins: a family of cell surface receptors. *Cell* **48**, 549-554.

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Isaac, P. K. (1964). Cytoplasmic streaming in filamentous fungi. *Can. J. Bot.* **42**, 787-792.

Jackson, S. L. and Heath, I. B. (1990). Evidence that actin reinforces the extensible hyphal apex of the oomycete *Saprolegnia ferax. Protoplasma* **157**, 144-153.

Jackson, S. L. and Heath, I. B. (1992). UV microirradiations elicit Ca^{2+}-dependent apex-directed cytoplasmic contractions in hyphae. *Protoplasma* **170**, 46-52.

Jackson, S. L. and Heath, I. B. (1993a). Roles of calcium ions in hyphal tip growth *Microbiol. Rev.* **57**, 367-382.

Jackson, S. L. and Heath, I. B. (1993b). UV microirradiation implicates F-actin in reinforcing growing hyphal tips. *Protoplasma* **175**, 67-74.

Kaminskyj, S. G. W., Yoon, K. S. and Heath, I. B. (1989). Cytoskeletal interactions with post-mitotic migrating nuclei in the oyster mushroom fungus, *Pleurotus ostreatus*: evidence against a force-generating role for astral microtubules. *J. Cell Sci.* **94**, 663-674.

Kaminskyj, S. G. W., Jackson, S. L. and Heath, I. B. (1992a). Fixation induces differential polarized translocations of organelles in hyphae of *Saprolegnia ferax. J. Microsc.* **167**, 153-168.

Kaminskyj, S. G. W., Garrill, A. and Heath, I. B. (1992b). The relation between turgor and tip growth in *Saprolegnia ferax*: turgor is necessary but not sufficient to explain apical extension rates. *Exp. Mycol.* **16**, 64-75.

Kaminskyj, S. G. W. and Heath, I. B. (1994). A comparison of techniques for localizing actin and tubulin in hyphae of *Saprolegnia ferax. J. Histochem. Cytochem.* **42**, 523-530.

Kropf, D. L. (1992). Establishment and expression of cellular polarity in fucoid zygotes. *Microbiol. Rev.* **56**, 316-339.

Kropf, D. L., Kloareg, B. and Quatrano, R. S. (1988). Cell wall is required for fixation of the embryonic axis in *Fucus* zygotes. *Science* **239**, 187-190.

Levina, N. N., Lew, R. R. and Heath, I. B. (1994). Cytoskeletal regulation of ion channel distribution in the tip-growing organism *Saprolegina ferax. J. Cell Sci.* **107**, 127-134.

Marcantonio, E. E. and Hynes, R. O. (1988). Antibodies to the conserved cytoplasmic domain of the integrin β_1 subunit react with proteins in vertebrates, invertebrates and fungi. *J. Cell Biol.* **106**, 1765-1772.

McKerracher, L. J. and Heath, I. B. (1986). Fungal nuclear behaviour analyzed by ultraviolet microbeam irradiation. *Cell Motil. Cytoskel.* **6**, 35-47.

McKerracher, L. J. and Heath, I. B. (1987). Cytoplasmic migration and intracellular organelle movements during tip growth of fungal hyphae. *Exp. Mycol.* **11**, 79-100.

Merrill C. R., Goldman, D., Sedman, S. A., Ebert, M. H. (1980). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**,1437-1438.

Michaud, D., Guillet, G., Rogers, P. A. and Charest, P. M. (1991). Identification of a 220 kDaa membrane-associated plant cell protein immunologically related to human β-spectrin. *FEBS Lett.* **294**, 77-80.

Money, N. P. and Harold, F. M. (1992). Extension growth of the water mold *Achlya*: interplay of turgor and wall strength. *Proc. Nat. Acad. Sci. USA.* **89**, 4245-4249.

Money, N. P. and Harold, F. M. (1993). Two water molds can grow without measureable turgor pressure. *Planta* **190**, 426-430.

Quatrano, R. S., Brian, L., Aldridge, J. and Schultz, T. (1991). Polar axis fixation in *Fucus* zygotes: components of the cytoskeleton and extracellular matrix. *Development Suppl. 1*, 11-16.

Reinhardt, M. O. (1892). Das Wachsthum der Pilzhyphen. *Jb. Wiss. Bot.* **23**, 479-566.

Sastry, S. K. and Horwitz, A. F. (1993). Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signalling. *Curr. Opin. Cell Biol.* **5**, 819-831.

Saunders, L. C., Wang, C. S., Walling, L. L. and Lord, E. M. (1991). A homolog of the substrate adhesion molecule vitronectin occurs in four species of flowering plants. *The Plant Cell* **3**, 629-635.

Saunders, L. C. and Lord, E. M. (1992). A dynamic role for the stylar matrix in pollen tube extension. *Int. Rev. Cytol.* **140**, 297-316.

Schindler, M., Meiners, S. and Cheresh, D. A. (1989). RGD-dependent linkage between plant cell wall and plasma membrane: consequences for growth *J. Cell Biol.* **108**, 1955-1965.

Schwartz, M. A., Lechene, C. and Ingber, D. E. (1991). Insoluble fibronectin activates the Na^+/H^+ antiporter by clustering and immobilizing integrin $\alpha5\beta1$, independent of cell shape. *Proc. Nat. Acad. Sci. USA* **88**, 7849-7853.

Staves, M. P., Wayne, R. and Leopold, A. C. (1992). Hydrostatic pressure mimics gravitational pressure in characean cells. *Protoplasma* **168**, 141-152.

Stoscheck, C.M. (1990). Quantitation of proteins. *Meth. Enzymol.* **182**, 50-68.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Tsai, C.-M. and Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**, 115-119.

Wagner, V. T., Brian, L. and Quatrano, R. S. (1992). Role of a vitronectin-like molecule in embryo adhesion of the brown alga *Fucus. Proc. Nat. Acad. Sci. USA* **90**, 3644-3648.

Wayne, R., Staves, M. P. and Leopold, A. C. (1992). The contribution of the extracellular matrix to gravisensing in characean cells *J. Cell Sci.* **10,** 611-623.

Wyatt, S. E. and Carpita, N. C. (1993). The plant cytoskeleton - cell-wall continuum. *Trends Cell Biol.* **3**, 413-417

Zhu, J.-K., Shi, J., Singh, U., Wyatt, S. E., Bressan, R. A., Hasegawa, P. M. and Carpita, N. C. (1993). Enrichment of vitronectin- and fibronectin-like proteins in NaCl-adapted plant cells and evidence for their involvement in plasma membrane-cell wall interaction. *The Plant Journal* **3**, 637-646.