

# Specific changes to the mechanism of cell locomotion induced by overexpression of $\beta$ -actin

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## SUMMARY

Overexpression of  $\beta$ -actin is known to alter cell morphology, though its effect on cell motility has not been documented previously. Here we show that overexpressing  $\beta$ -actin in myoblasts has striking effects on motility, increasing cell speed to almost double that of control cells. This occurs by increasing the areas of protrusion and retraction and is accompanied by raised levels of  $\beta$ -actin in the newly protruded regions. These regions of the cell margin, however, show decreased levels of polymerised actin, indicating that protrusion can outpace the rate of actin polymerisation in these cells. Moreover, the expression of  $\beta^*$ -actin (a G244D mutant, which shows defective polymerisation *in vitro*) is equally effective at increasing speed and protrusion. Concomitant changes in actin binding proteins show no evidence of a consistent mechanism for increasing the rate of actin polymerisation in these actin overexpressing cells. The increase in motility is confined to poorly spread cells in both cases and the excess motility can be abolished by blocking myosin function with butanedione monoxime (BDM).

Our observations on normal myoblasts are consistent with the view that they protrude by the assembly and cross linking of actin filaments. In contrast, the additional motility shown by cells overexpressing  $\beta$ -actin appears not to result from an increase in the rate of actin polymerisation but to depend on myosin function. This suggests that the additional protrusion arises from a different mechanism. We discuss the possibility that it is related to retraction-induced protrusion in fibroblasts. In this phenomenon, a wave of increased protrusion follows a sudden collapse in cell spreading. This view could explain why it is only the additional motility that depends on spreading, and has implications for understanding the differences in locomotion that distinguish tissue cells from highly invasive cell types such as leucocytes and malignant cells.

Key words: Cell movement, Cell polarity, Cell adhesion, Interference microscopy, Microfilament

## INTRODUCTION

In order to crawl forward, a cell must protrude the leading regions of its margin over the substratum and either simultaneously or asynchronously retract its trailing regions. To understand how the cell does this, we need to know how protrusions are formed. Protrusions can take the form of filopodia, lamellipodia or blebs, and lamellipodia may represent an intermediate state between filopodia and blebs. The leading margin of cells has long been known to be a site of actin filament assembly (Svitkina et al., 1986; Wang, 1985), and numerous studies have demonstrated that the polymerisation of actin can drive protrusion (see reviews by Condeelis, 1993; Mitchison and Cramer, 1996; Mogilner and Oster, 1996; Small et al., 1993).

In view of this evidence that actin polymerisation is important for protrusion and motility, it might be expected that overexpression of actin would increase cell motility. The mRNA and protein for the  $\beta$ -actin isoform are known to localise to the cell periphery and this isoform is therefore more

likely than  $\gamma$ -actin to affect motility (Hooek et al., 1991; Hill and Gunning, 1993; Kislauskis et al., 1993; Kislauskis et al., 1997). However, although overexpression of  $\beta$ -actin has been shown to have a marked effect on myoblast morphology (Schevzov et al., 1992), its effect on cell motility has not previously been investigated. Here we have used the DRIMAPS (Digitally Recorded Interference Microscopy with Automatic Phase Shifting) system of computer-assisted microinterferometry (Dunn and Zicha, 1995) to analyse the speed, spreading and rates of protrusion and retraction of myoblasts overexpressing  $\beta$ -actin. This was complemented by fluorescence microscopy to localise  $\beta$ -actin and polymerised actin in fixed cells. In order to investigate further the role of actin polymerisation, we analysed the locomotion of myoblasts that overexpressed  $\beta^*$ -actin, a mutant  $\beta$ -actin (G244D) that is known to be defective in its ability to polymerise *in vitro* (Leavitt and Kakunaga, 1980; Millonig et al., 1988; Taniguchi et al., 1988). We also measured how the expression levels of several actin binding proteins were affected by overexpression of  $\beta$ - or  $\beta^*$ -actin; these include three that are known to

influence actin polymerisation:  $\beta$ -thymosin, profilin and ADF/cofilin.

The role of myosin in protrusion is more problematical. The large family of myosin isoforms presents a complex array of single and double-headed motor proteins that must have countless interactions, motor functions and transport functions within the moving cell. The best studied of these functions is the interaction of aggregates of non-muscle myosin II with actin filaments in meshworks to produce contraction. One result of a generalised contraction of the actomyosin meshwork pervading the cell body is to set up a pressure gradient that tends to force fluid flow towards weaker regions of the actin cortex. The blebbing mode of protrusion is thought to occur in this way (Keller and Egli, 1998; Stossel et al., 1999) and can be prevented by counterbalancing the internal hydrostatic pressure of the cell using reverse osmotic pressure (Harris, 1973). It is known that actin polymerisation is not needed for the initial formation of blebs (Cunningham, 1995; Stossel et al., 1999) but that filaments form only when the bleb is beginning to collapse.

Blebbing tends to be the main mode of locomotion in rapidly moving, invasive types of cells that show poor adhesion to the substratum. Most normal tissue cells, on the other hand, move by extending lamellipodia. Despite the evidence that myosin is involved in the protrusion of blebs, there is little evidence that it is needed for other types of protrusion. Nevertheless, it has been proposed that the fluid-driving force is likely to be present even when the cell is not blebbing (Cunningham, 1995; Bereiter-Hahn and Luers, 1998) and thus it may make a contribution to the protrusion of lamellipodia even though it may not be the main driving force. Here we examine how much of the protrusion depends on myosin activity by measuring the effect on the protrusion rate of blocking myosin function.

There is also another respect in which myosin activity may influence the protrusion of normal tissue cells. Early observations that spontaneous or microneedle-induced retractions of the tails of fibroblasts could lead, a fraction of a minute later, to greatly enhanced protrusive activity at the leading cell margin revealed that the two marginal activities can be closely linked (Chen, 1979; Dunn, 1980). It is known that the tail retraction involves a phase of active contraction (Chen, 1981) and that the enhanced protrusive activity can often take the form of blebbing (Brown and Dunn, 1989), which suggests that the fluid drive mechanism may account for this coordination of the two activities. However, time-series analysis of the rates of protrusion and retraction of steadily moving fibroblasts, showing no blebbing, has revealed that these two activities can still be correlated during normal locomotion (Dunn and Zicha, 1995). Assuming that the normal retraction of the margin requires myosin function, this suggests another way in which myosin activity may influence protrusion. Here we have looked at the effect of overexpressing actin and of blocking myosin function on the coordination of these two activities.

## MATERIALS AND METHODS

### Transfection and recovery of permanently transfected clones overexpressing $\beta$ -actin or expressing $\beta^*$ -actin

Conditionally immortal *H2k<sup>b</sup>-tsA58* myogenic cells were isolated

from 1-2 day old *H2k<sup>b</sup>-ts<sup>6</sup>* mice, and cultured as described (Clark et al., 1997; Morgan et al., 1994). A single clone (clone 3) was transfected, either by electroporation or by calcium phosphate precipitation. For electroporation, we trypsinised the cells and resuspended them in DMEM (Gibco) supplemented with 10% fetal calf serum at  $1 \times 10^7$  cells per ml. We then added 50  $\mu$ g of DNA to 0.5 ml of the cell suspension in an electroporation cuvette, and pulsed at 250 V, 1500  $\mu$ F and  $\infty \Omega$ , using an EQUIBIO electroporator (FLOWGEN). The cells were immediately plated out in fresh growth medium. For calcium phosphate precipitation, we used the Cellfect kit (Pharmacia) and followed the manufacturer's instructions, adding 10  $\mu$ g of DNA to  $5 \times 10^5$  cells. 2 days after transfection, we selected for permanently transfected clones by adding G418 to the growth medium (1 mg/ml) for the next 10 days. After 10-14 days, we picked individual clones from the plates and expanded them for further analysis. Samples were frozen at  $-80^\circ\text{C}$  for long-term storage.

Three transfections were carried out. One set of cells was transfected with the plasmid PG4, which contains a 14 kbp *EcoRI* fragment encoding the wild-type human  $\beta$ -actin gene isolated from HuT14T cells in the pSV2 neo vector (Leavitt et al., 1984). This included the  $\beta$ -actin promoter and 3' untranslated region (UTR) sequences, as well as the neo gene that confers resistance to the antibiotic G418 to the cells. A second set of cells was transfected with the plasmid PG5, which contains a 14 kbp *EcoRI* fragment encoding  $\beta^*$ -actin: a single mutant form (Gly-244 to Asp) of the human  $\beta$ -actin gene isolated from HuT14T cells in the pSV2 neo vector. Again, this includes the  $\beta$ -actin promoter, 3' UTR and neo gene. The PG4 and PG5 vectors were kind gifts of Dr Peter Gunning (CMRI, Australia). Finally, as a control for the process of transfection itself, a third set was cotransfected with a PCMV5 vector, which does not have any gene insert and does not express any protein in mammalian cells, and the pgkneo vector at 1/10th concentration to confer resistance to G418. We recovered 33 PG4 clones, 29 PG5 clones and 20 sham-transfected clones.

To determine which clones expressed the human  $\beta$ - or  $\beta^*$ -actin gene, we used reverse transcription-polymerase chain reaction (RT-PCR). RNA was isolated and cDNA prepared from each clone as described (Wells et al., 1997). The cDNA was used in an RT-PCR reaction with primers specific for either mouse or human  $\beta$ -actin cDNA. A single forward primer (GACAGGATGCAGAAGGAGAT, from the  $\beta$ -actin gene sequence in Accession #X00351) was used, together with either a human  $\beta$ -actin gene reverse primer (TGTGTGGACTTGGGAGAGGACT) that specifically amplified human  $\beta$ -actin cDNA, or a mouse  $\beta$ -actin reverse primer (GCCATGCCAATGTTGTCTCTTA, designed from Accession #X07365) that specifically amplified mouse  $\beta$ -actin cDNA. The predicted sizes for the amplified products were 614 bp for the human and 273 bp for the mouse  $\beta$ -actin cDNA.

Several clones had strong RT-PCR bands, suggesting that they expressed the human  $\beta$ - or  $\beta^*$ -actin. For further analysis, we chose two PG4 clones that expressed human  $\beta$ -actin and one PG5 clone that expressed  $\beta^*$ -actin. These are referred to below as  $\beta$ -actin overexpressers and  $\beta^*$ -actin expressers, respectively. Neither the wild-type clone (henceforth called Wt) nor the sham-transfected clones (henceforth called Null) showed a band for human actin by RT-PCR, demonstrating the specificity of the primers for human  $\beta$ -actin mRNA (data not shown). These four sets of clones provided our four experimental groups that were analysed by time-lapse phase-shifting interference microscopy, by protein gels and western analysis, and by immunofluorescence and confocal microscopy.

### Analysis of expression by western analysis

Protein samples were made by directly scraping cells, growing in flasks, into 200-500  $\mu$ l of solution A (50 mM Tris, 2% SDS). A sample was removed for estimation of the protein concentration, using the Pierce micro BCA assay (Pierce, UK), following the manufacturer's instructions. An equal volume of 2 $\times$  Laemmli buffer (Laemmli, 1970)

was added to the remainder of the sample, which was heated to 95°C for 5 minutes and then stored at -20°C. Samples were analysed by one-dimensional gel electrophoresis using 7.5% or 12% polyacrylamide slab gels. Equivalent amounts of protein were loaded for each sample. For analysis of low molecular mass proteins, we used 10%-20% gradient Tris Tricine ready gels (Bio-Rad Laboratories, UK) or 16.5% Tris Tricine gels (Schagger and von Jagow, 1987). To visualise the protein bands, the gels were stained either with Coomassie Blue or with silver stain (Bio-Rad Laboratories, UK). For western analysis, the proteins were transferred to nitrocellulose (Amersham, UK) or PVDF (Millipore), as described (Wells et al., 1997). PVDF gave the best results for proteins with lower molecular mass, such as profilin.

The relative intensities of specific bands on the western blots were analysed by imaging the blots with a CCD camera (Biorad, gel doc system) and using gel analysis software (Scion) to quantify the intensity of the bands. At least four gels were run for each protein investigated, using protein samples from at least four different cultures. The primary antibodies used were: anti-profilin (a kind gift of M. F. Carlier, France), anti- $\beta_4$ -thymosin (a kind gift of V. Nachmias, Penn University, USA), anti- $\gamma$ -actin (a kind gift of Dr Gabbiani, Italy), anti mutant  $\beta$ -actin (a kind gift of U. Aebi and C. Schoenberger, Switzerland), anti-ADF (a kind gift of Jim Bamburg, Colorado State University) and anti-ezrin (a kind gift of Dr P. Mangeat, France).

#### Measurement of G/F actin ratios

G/F actin ratios were measured as described (Heacock and Bamburg, 1983). Cells growing on 10 cm<sup>2</sup> tissue culture plates were washed in PBS (Mg<sup>2+</sup>, Ca<sup>2+</sup> free), and lysed in 150  $\mu$ l of cold (-10°C) lysis buffer (2 mM Tris, 2 mM MgCl<sub>2</sub>, 0.2 mM DTT, 15% glycerol and 1% Triton-X 100, pH 7.4) containing 40  $\mu$ g of myosin. The cells were scraped off into an Eppendorf tube, a further 150  $\mu$ l of lysis buffer was used to rinse the scraper into the tube and the cells were spun for 1 minute in a microcentrifuge at 10000 g. Laemmli buffer was added to the supernatant. The pellet was resuspended in 300  $\mu$ l of ice-cold actomyosin buffer (2 mM Tris, 1 mM ATP, 0.2 mM CaCl<sub>2</sub>, 0.05 M DTT, pH 8) and Laemmli buffer was added. Samples were analysed by 12% SDS-PAGE and stained by Coomassie Blue (Biosafe, Biorad), with pure skeletal actin loaded into separate lanes for comparison. The gels were scanned and the relative intensities of the bands that corresponded to actin were calculated using Scion image.

#### Digitally recorded interference microscopy with automatic phase shifting (DRIMAPS)

Cells were trypsinised and plated onto acid-cleaned glass coverslips coated with 0.01% gelatin at a density of  $2 \times 10^4$  cells per cm<sup>2</sup>. They were incubated in CO<sub>2</sub>-independent medium (Gibco) containing 20% FCS, 2% CEE, 4 mM glutamine and 100  $\mu$ g/ml penicillin/streptomycin at 37°C for approximately 4 hours prior to filming to allow the cells to spread. The coverslip was then sealed onto a chamber, with a small air bubble to buffer the medium, on the stage of a Horn-type transmitted-light interference microscope modified for automatic phase shifting, and examined using a CCD video camera connected to a frame grabber and processor board in a PC. Further details of the DRIMAPS system and interference recordings have been described (Dunn and Zicha, 1995; Dunn and Zicha, 1998; Zicha and Dunn, 1995).

#### BDM treatment

To determine the effect of inhibiting myosin on the motility of the Wt clone and  $\beta$ -actin overexpressing clones, two cultures of each type of clone were trypsinised, plated onto glass coverslips and incubated as described above. Just prior to filming, the coverslip was sealed into a chamber containing CO<sub>2</sub> independent medium as described above, containing 10 mM butanedione monoxime (BDM), which had been freshly made. This concentration of BDM was the same as that used

previously to inhibit myosin in re-spreading cells (Cramer and Mitchison, 1995).

#### Analysis of cell motility

Data from isolated cells were gathered as time series for each parameter (see below), with a sampling interval of 5 minutes. Although data obtained at 1-minute intervals were available in the DRIMAPS recordings, the choice of a 5-minute sampling interval has some advantages. Since the path of a cell is a form of random walk, too long a sampling interval gives an underestimate of the 'true' speed of the cell whereas noise begins to dominate the measurement and give an overestimate of speed if the sampling interval is too short. There is evidence that much of this noise is not due to instrumental error but arises from microtubule activity at the cell margin (Dunn et al., 1997b) and we have found that a 5-minute sampling interval is generally the best compromise for fibroblast-like cells.

For each measured parameter and each cell, the time series generally consisted of more than one continuous sequence since values were missing during the time that the specific cell was not isolated from other cells or from the boundary of the recording field. The protrusion and retraction regions were as defined previously (Dunn et al., 1997a).

The parameters used in this analysis were: **mass** (pg), total dry mass of cell at time  $t$ ; **area** ( $\mu\text{m}^2$ ), total spread area of cell at time  $t$ ; **spreading index** ( $\mu\text{m}^2 \text{pg}^{-1}$ ), area  $\div$  mass at time  $t$ ; **speed** ( $\mu\text{m} \text{minute}^{-1}$ ), displacement of the centroid of cell mass during interval  $\{t, t + 5 \text{ minutes}\} \div 5$ ; **protrusion area** (%),  $100 \times$  area of protrusion region during interval  $\{t, t + 5 \text{ minutes}\} \div$  area at time  $t$ ; **retraction area** (%),  $100 \times$  area of retraction region during interval  $\{t, t + 5 \text{ minutes}\} \div$  area at time  $t$ ; **protrusion mass** (%),  $100 \times$  mass of protrusion region during interval  $\{t, t + 5 \text{ minutes}\} \div$  mass at time  $t$ ; **retraction mass** (%),  $100 \times$  mass of retraction region during interval  $\{t, t + 5 \text{ minutes}\} \div$  mass at time  $t$ ; **polarity** ( $\mu\text{m}$ ), distance between area centroids of protrusion and retraction regions at time  $t$ .

As a preliminary to plotting, the data were first gathered into groups. The control group (black squares in Figs 2, 3 and 4) consists of the pooled data for the Wt clone and the two Null clones. The overexpresser group (open squares in Figs 2, 3 and 4) consists of the pooled data for the  $\beta$ -actin overexpressers and the  $\beta^*$ -actin expressers. In some cases the  $\beta$ -actin overexpressers (open triangles in Fig. 2) and the  $\beta^*$ -actin expressers (open stars in Fig. 2) are treated separately. The data for each plot were sorted within each group according to the  $x$ -variable of the plot and then partitioned into eight equal size subgroups. Each point represents the mean  $x$  value and mean  $y$  value for each subgroup with the error bar being 3 standard errors (approximately 99% confidence interval) for the mean  $y$  values. There are 960 ( $x, y$ ) values in each of the control subgroups; 757 in each of the overexpresser subgroups and 119 in each of the mutant subgroups – a total of 14,688 sampled values for each parameter. In the BDM experiments, which were analysed separately, there are only 51 values in each of the Wt subgroups and 62 in each of the overexpresser subgroups – a total of 904 values.

These data were not entirely independent since several measurements were generally taken from each cell throughout the 20-hour recording period and several cells were sampled from each culture; at least six cultures were recorded for each of the six clones studied. For critical tests of significance, therefore, all possible sources of variation were taken into account using a full analysis of variance (ANOVA). For this test, all the parameter values belonging to a cell were nested together at the *cells* level; all the cells belonging to one culture were nested at the *cultures* level and all the cultures derived from one clone were nested at the *clones* level. The uppermost level of nesting – the *actin* level – distinguished between the control group (pooled Wt and Null) and the overexpresser group (pooled  $\beta$ - and  $\beta^*$ -overexpressing cells). The number of data values are not generally the same within each group at any nesting level and so unbalanced ANOVA tests were used (Milliken and Johnson, 1992).

The mutual interdependency of protrusion and retraction were examined using the cross-correlation function (Dunn et al., 1997a) on the original data collected at 1-minute intervals. Trends in the data due to the cells increasing in size were removed by dividing by cell mass and significant autocorrelations were removed by fitting an autoregressive (AR[1]) model to each series using the Yule-Walker method (Dunn et al., 1997a). The residuals from this procedure formed pre-whitened series, which were then used to calculate the final cross-correlograms.

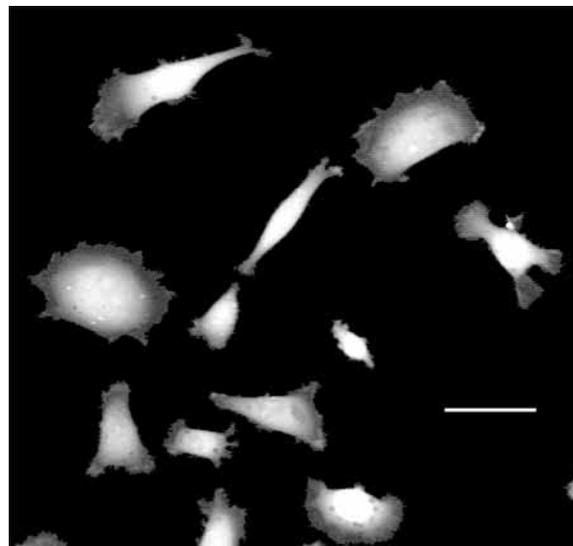
### Combined fluorescence and DRIMAPS microscopy

Myoblasts were plated at a density of  $2 \times 10^4$  cells  $\text{cm}^{-2}$  on acid-washed glass coverslips coated with 0.01% gelatin (Sigma). After 24 hours, cells were fixed in 4% paraformaldehyde dissolved in cytoskeletal buffer (Small, 1981), for 20 minutes. Cells were then permeabilised using cytoskeletal buffer containing 0.2% Triton X-100 for 5 minutes and used immediately for immunostaining. Rhodamine-phalloidin actin (Molecular Probes) diluted 1/20 in TBS/1% BSA/1% FCS was added to the permeabilised cells and incubated for 20 minutes. The coverslips were washed in TBS, and then incubated for 40 minutes with anti- $\beta$ -actin antibody (Sigma) diluted in TBS/1% BSA/1% FCS. The slides were washed a third time, and incubated for 40 minutes in anti-mouse Alexa 488 (Molecular Probes) diluted in TBS/1% BSA/1% FCS. After washing in TBS, slides were washed and mounted in water, sealing the coverslips with nail varnish to prevent them drying out. The slides were imaged using an inverted Nikon microscope, a Hamamatsu Orca CCD camera and Kinetic Imaging software. The positions of each field were recorded using an England Finder. The same fields were subsequently identified using the England Finder coordinates on the DRIMAPS system to image the dry mass of the cells. The two fluorescence images were then each ratioed by dividing by cellular dry mass on a pixel-by-pixel basis and combined as the red (polymerised actin) and green ( $\beta$ -actin) channels in a single image. The final image can thus be interpreted as a qualitative map of actin concentration relative to total cellular material, although no attempt has been made yet to calibrate these images.

## RESULTS

### Expression of human $\beta$ -actin and G/F actin ratios

Transfection of either the human  $\beta$ -actin gene or the human  $\beta^*$ -actin gene into mouse myoblasts increased the levels of  $\beta$ -actin expression by about 60% compared to wild-type (Wt) cells while not affecting the levels of  $\gamma$ -actin expression (Table 1). A sham transfection (Null cells) did not change the levels of  $\beta$ -actin or  $\gamma$ -actin expression. These findings are broadly similar to those described previously (Lloyd et al., 1992). Consistent with the impaired ability of  $\beta^*$ -actin to polymerise



**Fig. 1.** Part of a frame from a DRIMAPS recording of a  $\beta$ -actin-overexpressing clone. The grey scale represents dry mass density (as described by Dunn and Zicha, 1995). Scale bar, 50  $\mu\text{m}$ .

in vitro, we found that the relative amount of unpolymerised actin in  $\beta^*$ -actin expressers was nearly 20% greater than in the three other groups (Table 1). This suggests that the polymerisation of this mutant form is also defective when it is expressed in cells.

### Analysis of cell speed and spreading

The motile behaviour of Wt, Null,  $\beta$ -actin overexpressers and  $\beta^*$ -actin expressers was investigated by quantitative processing of the time-lapse sequences of DRIMAPS images. All the clones showed a wide variety of cellular morphologies (Fig. 1) and it was difficult to detect any differences in cell behaviour by visual inspection of the time-lapse sequences.

In unbalanced ANOVA tests (see Materials and Methods), we found that the mean speed of cells in the overexpresser group was almost double that of the control group and this was highly significant (mean speed  $\pm$  s.e.m.: Controls,  $0.540 \pm 0.015$   $\mu\text{m minute}^{-1}$ ; Overexpressers,  $0.936 \pm 0.025$   $\mu\text{m minute}^{-1}$ ; ANOVA,  $P < 0.001$ ). At the clones level, the mean cell speed of the Null clones did not differ significantly from that of the Wt clone and, more importantly, the much higher mean speed of the overexpressing cells did not differ significantly between the

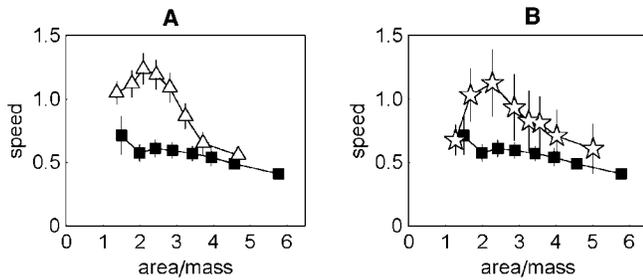
**Table 1. Expression levels of  $\beta$ -,  $\gamma$ - and total actin and G/F ratios in control and transfected cells**

Clones	$\beta$ -actin	$\gamma$ -actin	Total actin	G/F actin ratio
Wt	$1.00 \pm 0.07$ (4)	$1.00 \pm 0.21$ (5)	$1.00 \pm 0.14$ (4)	$0.928 \pm 0.110$ (5)
Null	$1.30 \pm 0.13$ (6)	$1.15 \pm 0.12$ (8)	$0.83 \pm 0.16$ (4)	$0.956 \pm 0.049$ (5)
$\beta$ -actin overexpressers	$1.58 \pm 0.12$ (7)**	$1.03 \pm 0.11$ (10)	$1.53 \pm 0.21$ (7)*	$0.918 \pm 0.056$ (11)
$\beta^*$ -actin expressers	$1.65 \pm 0.14$ (4)**	$0.85 \pm 0.15$ (5)	$1.85 \pm 0.44$ (4)	$1.103 \pm 0.075$ (6)*

Mean expression levels of  $\beta$ -actin,  $\gamma$ -actin and total actin were normalised to the mean expression level for the same protein in wild type cells (Wt). The values were obtained by analysing several different cultures in several blots in which the protein samples had been equally loaded. The two sham transfected clones are pooled in the second row (Null). The two human  $\beta$ -actin transfected clones are pooled in the third row and the fourth row represents the clone transfected with  $\beta^*$ -actin.

Values are means  $\pm$  s.e.m. and the number of blots analysed is shown in parentheses.

For  $\beta$ -actin,  $\gamma$ -actin and total actin, each data sample in each of the three lowest rows was compared with the Wt sample in an unpaired, two-tailed *t*-test and the asterisks summarise the resulting probability values of significance as follows: \*\* $P < 0.01$ , \* $P < 0.05$ . See text for the special interpretation of the asterisk in the G/F actin ratio column.



**Fig. 2.** (A) Mean cell speed plotted against spreading index for the control (■) and for the  $\beta$ -actin-overexpressing clones ( $\Delta$ ). (B) Comparison of the  $\beta^*$ -actin-expressing clone ( $\star$ ) with the control clones (■). Error bars show the 99% confidence interval for the mean.

$\beta$ -actin overexpressers and the  $\beta^*$ -actin expressers. This result was surprising since we expected the poor polymerisation of  $\beta^*$ -actin to lead to considerable functional defects.

Another striking feature of the analysis emerged when we examined cell speed in relation to cell spreading by measuring the area-to-mass ratio or spreading index of the cells (Dunn and Zicha, 1995). In the cases of both the  $\beta$ - and  $\beta^*$ -overexpressing cells, the increase in speed was confined to poorly spread cells with a spreading index of less than about  $3.0 \mu\text{m}^2 \text{pg}^{-1}$  (Fig. 2). The control cells, on the other hand, did not show much change in motility with spreading. This was a very consistent effect. All three of the control clones, when examined individually, showed very little variation in cell speed with spreading yet all three of the clones overexpressing actin showed a much increased speed in the poorly spread cells. The overall levels of cell spreading appeared slightly reduced in overexpressers compared to controls but this difference was not quite significant (mean spreading index  $\pm$  s.e.m.: Controls,  $3.34 \pm 0.06 \mu\text{m}^2 \text{pg}^{-1}$ ; Overexpressers,  $2.79 \pm 0.05 \mu\text{m}^2 \text{pg}^{-1}$ ; ANOVA,  $0.05 < P < 0.1$ ).

There are two possible ways in which the relationship between speed and spreading of the overexpressing cells could have arisen. Either there are distinct populations among the overexpressing cells that show different spreading and motility characteristics or the speed of individual cells is directly related to their spreading. To decide between these two possibilities, we analysed continuous runs of data from single cells (Table 2). This demonstrated that individual cells moved more rapidly when poorly spread and this effect was much more striking in the overexpressers. The data for the  $\beta$ -actin overexpressers were again very similar to those for the  $\beta^*$ -actin expressers.

We conclude that the relationship between speed and spreading is not due to cell-to-cell differences such as might have arisen from differences in expression levels.

**Analysis of protrusion**

We next demonstrated that the increased speed of the overexpressing cells was due to increased areas of protrusion and retraction of the cell margin. This step was necessary because it has been shown theoretically that the area of protrusion and retraction is not the only factor determining cell speed: cell polarity, which we have defined as the distance separating the centroids of the protrusion and retraction regions, is equally effective (Dunn et al., 1997a). In the analysis shown (Fig. 3), the data for the two overexpressing groups are pooled together, but in separate analyses (not shown) we found no essential differences between  $\beta$ - and  $\beta^*$ -overexpressers. Both protrusion and retraction areas were strongly increased in the overexpressing cells compared to controls and this increase was largely confined to poorly spread cells (Fig. 3C,D) as with cell speed (Fig. 3A). Polarity, in contrast, was unchanged compared to controls and stayed quite constant at about  $20 \mu\text{m}$  over the whole range of cell spreading (Fig. 3B).

The increased protrusion and retraction of the overexpressing cells were more remarkable when we examined the dry masses of these regions (Fig. 3E,F). These results indicate that up to three times as much of the total cellular material is involved in protrusion and retraction in the overexpressers compared with the controls. Again, this difference occurs only in the case of cells that are not fully spread.

**Effects of actin overexpression on actin binding proteins**

The increased motility that we have observed is unlikely to be due to increased rates of actin polymerisation, or to increased levels of polymerised actin, since there is no diminution of the effect in the  $\beta^*$ -actin expressers compared to  $\beta$ -actin expressers. Nevertheless, the genes that are under regulation by actin levels include proteins that control actin polymerisation, and there is a possibility that changes in the expression levels of these could account for the effects.

Of the main proteins that are important in controlling actin polymerisation dynamics,  $\beta$ -thymosin buffers actin monomers, profilin exchanges ATP for ADP on actin monomers and promotes polymerisation, and ADF/cofilin severs filaments releasing monomers for polymerisation (Loisel et al., 1999). Ezrin is another actin binding protein whose function is obscure but is included since it is thought to bind preferentially

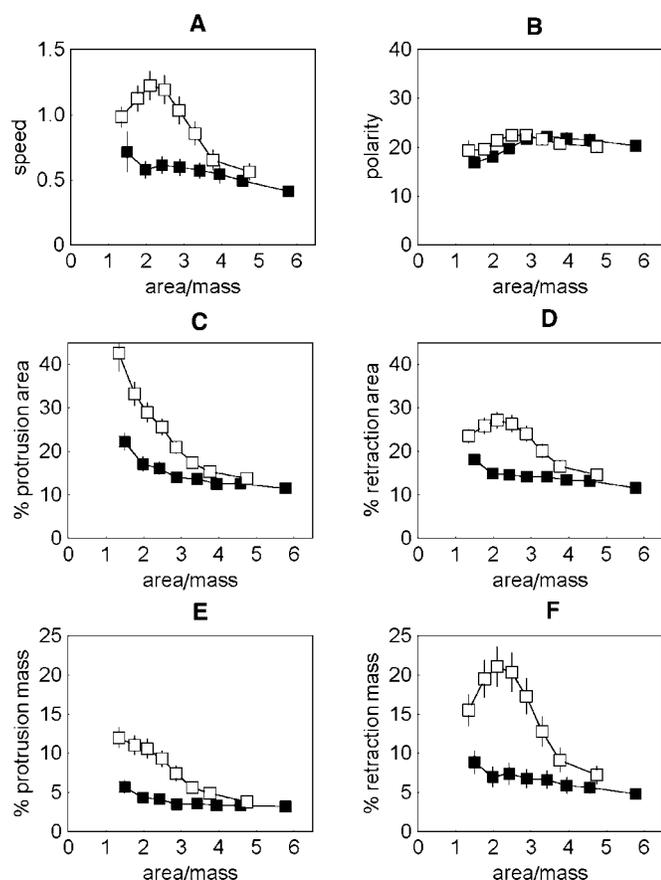
**Table 2. Mean speed in relation to spreading in individual cells**

Clone	n	Speed ( $\mu\text{m minute}^{-1}$ )	
		2<spreading index<3.5	spreading index>3.5
Controls (Wt+Null)	109	0.815 $\pm$ 0.051	0.630 $\pm$ 0.039
$\beta$ -actin overexpressers	106	1.131 $\pm$ 0.065** (+0.316)	0.787 $\pm$ 0.058* (+0.157)
$\beta^*$ -actin expressers	25	1.140 $\pm$ 0.179** (+0.325)	0.776 $\pm$ 0.077 (+0.146)

This table shows an analysis of continuous runs of data from single cells in which the cell had been both poorly spread and well spread during the run. This selection procedure eliminated about half the total number of runs and the mean duration of those remaining was 2.63 hours. The table shows two mean speeds, one for those times when the cell was poorly spread (2<spreading index<3.5) and the other for when it was well spread (spreading index>3.5).

Values are means  $\pm$  s.e.m. The mean differences in speed between the overexpressers and the controls are given in parentheses.

Each sample of speeds from overexpressers was compared with the corresponding control sample in an unpaired, two-tailed *t*-test and the asterisks summarise the resulting probability values of significance as follows: \*\* $P < 0.01$ , \* $P < 0.05$ .



**Fig. 3.** Various measures of cell motility plotted against spreading index for the control (■) and for the pooled overexpressing clones (□). The measures are: mean cell speed (A), mean polarity (B), mean protrusion area (C) and mean retraction area (D), expressed as percentages of total cell area, mean protrusion mass (E) and mean retraction mass (F), expressed as percentages of total cell mass. Error bars show the 99% confidence interval for the mean.

to  $\beta$ -actin. When we examined the expression levels of these proteins in  $\beta$ - and  $\beta^*$ -cells compared to the control group (Table 3), we did not find a consistent pattern that would explain our results.

In  $\beta$ -actin overexpressing cells,  $\beta$ -thymosin increased, profilin levels did not change, and ADF/cofilin increased. The  $\beta^*$ -actin expressers showed a similar increase in  $\beta$ -thymosin but also showed a significant increase in profilin and a fall in ADF/cofilin (although not significant, this observation of a 46% decrease in ADF is consistent with a previous report (Minamide et al., 1997); also, the difference in ADF/cofilin levels between  $\beta$ -actin

overexpressers and  $\beta^*$ -actin expressers was highly significant at the 1% level). Ezrin showed no significant changes between the three groups.

### Effects of BDM on motility

If an increased rate of actin polymerisation is not responsible for the elevated motility of the overexpressing cells, then it is still possible that the delivery of excess actin to the leading edge could increase protrusion regardless of whether or not the actin is competent to polymerise. This would be consistent with the increased mass of material that we have found in the protrusions of the overexpressers. As discussed in the Introduction, there are several possible mechanisms by which the rate of delivery of material to the leading edge of the cell might depend on myosin function. BDM, a known inhibitor of muscle myosin II, is also an inhibitor of non-muscle myosin II and myosin V adenosine triphosphatases, and has been used to inhibit postmitotic cell spreading (Cramer and Mitchison, 1995). We therefore decided to test its effects on two cultures of Wt cells and two cultures of  $\beta$ -actin overexpressers (Fig. 4).

We found that BDM abolished the excess speed of the  $\beta$ -actin overexpressers compared to the controls (Fig. 4). BDM did not abolish the motility of control cells, though it did partially suppress the motility of well spread cells (Fig. 4). The greatest effect of BDM was on protrusion and retraction masses (comparing Fig. 4E,F with Fig. 3E,F). This indicates that the excess motility of the overexpressing cells, and only the excess motility, is dependent on myosin function.

### Actin polymerisation in protrusions

The codistribution of polymerised actin and  $\beta$ -actin lends further support to the view that the excess motility is not due to increased rates of actin polymerisation (Fig. 5). In the Null culture, we found the highest concentration of  $\beta$ -actin in peripheral structures where the margin was convex and obviously protruding. This was usually colocalised with polymerised actin staining to give a yellow colour (Fig. 5B). In contrast, the  $\beta$ -actin and  $\beta^*$ -actin overexpressing cells (Fig. 5D,F) showed a high concentration of  $\beta$ -actin only in these regions, to give a green staining. This suggests that the bulk of  $\beta$ -actin was unpolymerised in these regions. The high densities of  $\beta$ -actin in these regions suggest that unpolymerised  $\beta$ -actin is responsible for the large excess mass that we measured in protrusive regions of overexpressing cells in the analysis of DRIMAPS recordings.

### Coordination of protrusion and retraction

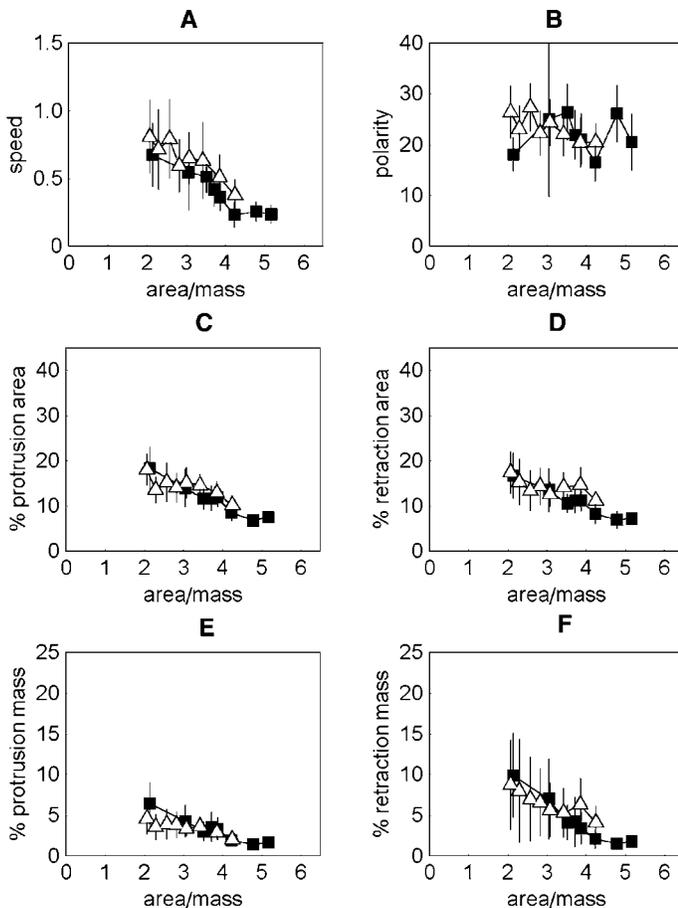
We found that in the control cells (Figs 6A, Wt and 6C, Null) the largest positive cross-correlation occurs at a lag of  $-1$  minute, which suggests that fluctuations in protrusion tend to be followed by compensatory fluctuations in retraction 1

**Table 3. Expression levels of  $\beta$ -thymosin, profilin, ezrin and ADF in control and transfected cells**

Clones	$\beta$ -thymosin	Profilin	Ezrin	ADF
Controls (Wt+Null)	1.00 $\pm$ 0.16 (10)	1.00 $\pm$ 0.13 (10)	1.00 $\pm$ 0.07 (13)	1.00 $\pm$ 0.18 (10)
$\beta$ -actin overexpressers	2.34 $\pm$ 0.18 (7)**	1.13 $\pm$ 0.17 (8)	0.94 $\pm$ 0.05 (10)	1.72 $\pm$ 0.25 (8)*
$\beta^*$ -actin expressers	2.22 $\pm$ 0.47 (4)**	1.75 $\pm$ 0.30 (4)**	0.90 $\pm$ 0.08 (5)	0.54 $\pm$ 0.31 (4)

Mean expression levels of  $\beta$ -thymosin, profilin, ezrin and ADF normalised to the mean expression level for the same protein in the pooled wild-type cells and sham transfected clones (Wt+Null).

Details as for Table 1 except that each data sample in each of the two lowest rows was compared with the Wt+Null sample in an unpaired, two-sided *t*-test.



**Fig. 4.** A series of the same plots that were used in Fig. 3, with identical scaling of the axes, except that the cells here are from a small sample of four cultures treated with 10 mM BDM during the 20 hour recording period. Two of the cultures were from control clones (■) and two from  $\beta$ -actin-overexpressing clones ( $\Delta$ ). Error bars show the 99% confidence interval for the mean. It should be noted that these data show much longer error bars simply as a result of the much smaller sample sizes.

minute later. In contrast, in the overexpressing cells (Fig. 6B,  $\beta^*$ -actin and D,  $\beta^*$ -actin) the levels of cross-correlation were reduced at all time lags. BDM treatment appeared to have remarkably little effect on either control (Fig. 6E, BDM-treated Wt; compare to 6A) or overexpressing cells (Fig. 6E, BDM-treated Wt; compare to 6B). In summary, regardless of BDM treatment, the control cells (left hand column) show a very similar pattern of correlation with the largest peak at a lag of  $-1$  minute, and these correlations were consistently reduced in the overexpressing cells (right hand column).

## DISCUSSION

To summarise, we have found an increased speed of locomotion in myoblasts overexpressing  $\beta$ -actin. This is characterised by increased areas of protrusion and retraction and highly increased masses of protruded and retracted material. There is no clear evidence that the increased protrusion is due to, or is accompanied by, increased rates of

actin polymerisation. On the other hand there is evidence that the excess motility is dependent on myosin function and only occurs when the cells are poorly spread. While coordination of protrusion and retraction is reduced by overexpressing  $\beta$ -actin, this appears not to be influenced by blocking myosin function.

We interpret these results to mean that the overexpression of  $\beta$ -actin not only increases cell locomotion but also produces a qualitative change in its mechanism, particularly in that of protrusion. Dealing firstly with normal myoblasts, we have shown that their speed of locomotion does not depend very much on their state of spreading, and that protrusion and retraction are moderately well coordinated. Further observations are consistent with the view that these cells protrude by the assembly and cross linking of actin filaments. Not only does the localisation of  $\beta$ -actin at the active cell margin coincide with a high concentration of polymerised actin, but there is little effect on locomotion of disrupting myosin function except in very well spread cells.

In contrast, the additional motility shown by cells overexpressing  $\beta$ -actin appears to have quite different characteristics. Their speed of locomotion is highly dependent on spreading and the increased motility is confined to poorly spread cells. The coordination between protrusion and retraction appears to be much reduced, with no correlation coefficient exceeding 0.2. Moreover, there is evidence that the additional motility does not result from an increase in the rate of actin polymerisation. Firstly, the high concentration of  $\beta$ -actin at the active cell edges is no longer colocalised with a high concentration of polymerised actin. This indicates that the protrusion of the margin can outpace the rate of actin polymerisation in these cells. Secondly, despite its reportedly defective polymerisation *in vitro*, expressing  $\beta^*$ -actin has very similar effects in all respects to overexpressing  $\beta$ -actin. Thirdly, blocking myosin function with BDM totally abolishes the additional motility of overexpressing cells, leaving a basal level that is indistinguishable from that of BDM-treated wild-type cells at all states of spreading.

Since the rate of actin polymerisation remains to be measured directly in the control and overexpressing cells, we will examine in more detail the evidence that the increase in motility of the overexpressing cells does not result from an increase in the rate of actin polymerisation. The reports that  $\beta^*$ -actin is defective in its ability to polymerise are based on experiments *in vitro* (Millonig et al., 1988; Taniguchi et al., 1988) and it is possible, though we think unlikely, that this defect is somehow masked when the mutant is expressed *in situ* in the cell. Against this possibility, it has been shown that when  $\beta^*$ -actin is expressed in cells it does have several specific effects that are different from those of  $\beta$ -actin. For example, its incorporation into the cytoskeleton of human fibroblasts is much reduced compared to normal  $\beta$ - and  $\gamma$ -actins (Leavitt and Kakunaga, 1980) and its effects in regulating the expression of other proteins differ from those of expressing  $\beta$ -actin (Minamide et al., 1997). In addition, we have also detected a small though significant increase in the G/F actin ratio when it is expressed.

Changes in the regulation of other proteins, however, introduce another complication that is difficult if not impossible to discount entirely. In particular, specific changes in the levels of the various actin-binding proteins may be able to compensate for the defective polymerisation of  $\beta^*$ -actin.

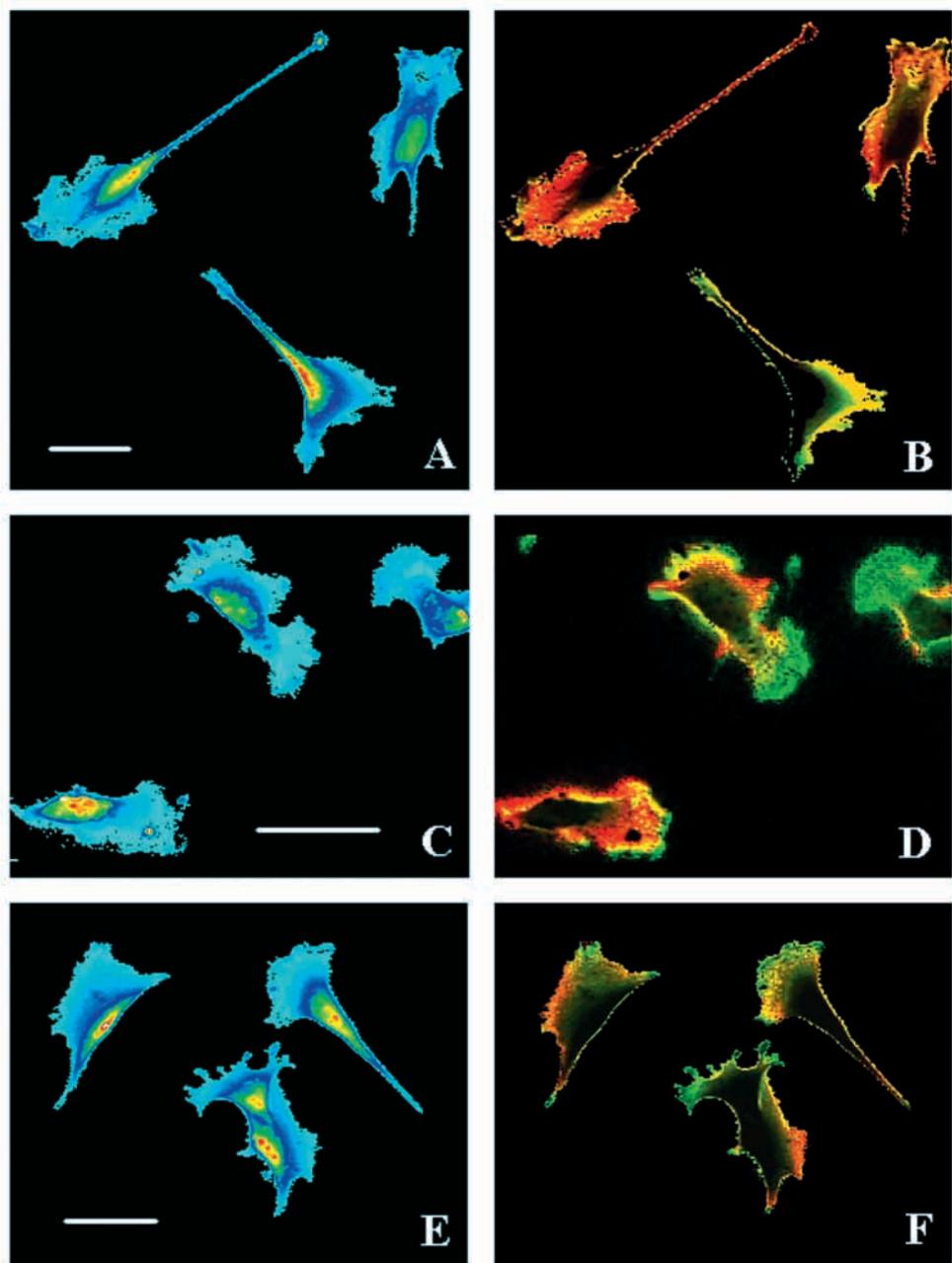
Here we have tried to cover some of the more likely possibilities.

Profilin, for example, promotes nucleotide exchange on actin monomer (Goldschmidt-Clermont et al., 1992) and thus its increase in  $\beta^*$ -actin expressing cells could promote actin assembly of endogenous monomer. However, any increase in the rate of endogenous monomer assembly must be accompanied by a similar or even greater increase in its rate of depolymerization in order to account for the small increase in G/F actin ratio. Such an increase in actin depolymerization rate is improbable in view of the 46% reduction in the level of the actin-depolymerizing protein, ADF/cofilin, that we found in the  $\beta^*$ -actin expressers. Although only bordering on significance when compared with Wt, this observation that ADF/cofilin is downregulated in myoblasts expressing  $\beta^*$ -actin is supported by an earlier report (Minamide et al.,

1997) and is very significant in comparison with  $\beta$ -actin overexpressers.

In other cases, the absence of compensatory changes in regulation of actin-binding proteins could lead to an increased rate of actin polymerisation on expression of  $\beta^*$ -actin. For example, increasing actin monomer concentration could effectively provide a sink for sequestration proteins such as  $\beta$ -thymosin and thus drive an increase in the rate of endogenous actin polymerization. However, we have presented evidence that the levels of  $\beta$ -thymosin in the  $\beta^*$ -actin expressing cells (and in the  $\beta$ -actin overexpressing cells) are increased to a greater extent than the increase in actin, thus making it unlikely that the rate of actin polymerisation could be increased by this mechanism.

Although these results, taken together, strongly indicate that the additional motility induced by overexpressing actin is not



**Fig. 5.** Myoblasts from one of the Null clones (A,B), from one of the  $\beta$ -actin overexpressing clones (C,D) and from the  $\beta^*$ -actin expresser (E,F). (A,C,E) Pseudocoloured DRIMAPS images show the distribution of cellular material in sample cells from the Null,  $\beta$ -actin and  $\beta^*$ -actin overexpressing clones. (B,D,F) Fluorescence images for polymerised actin and  $\beta$ -actin in the same cells as in A,C,E that were ratioed by dividing by cellular dry mass on a pixel-by-pixel basis and combined as the red (polymerised actin) and green ( $\beta$ -actin) channels in single images. These images therefore show only those regions where the respective actin concentration was high (see Materials and Methods for details). Scale bars, 50  $\mu$ m.

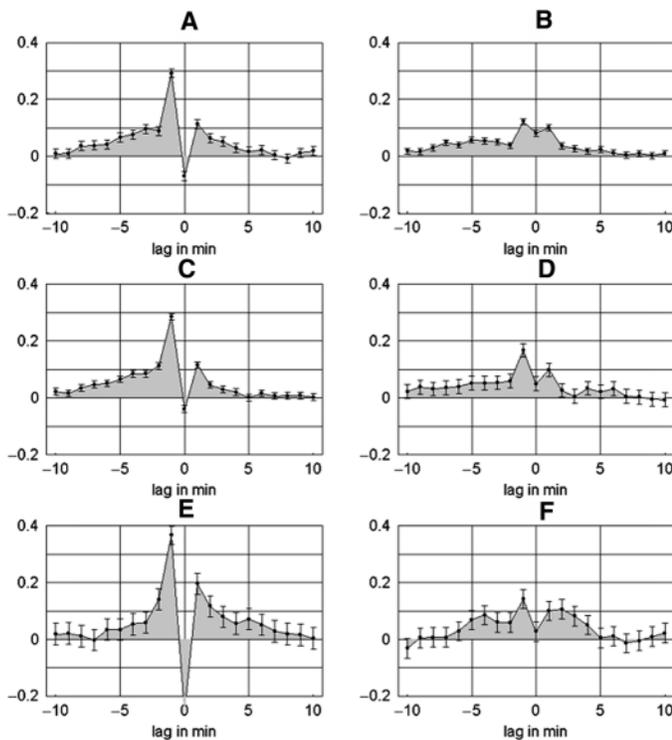
due to increased rates of actin polymerisation, they have not eliminated this possibility. It may not even be possible within a finite set of experiments to eliminate all pathways by which actin binding proteins might be involved in the increased motility of the cells. However, even if there were a general increase in the rate of actin polymerisation in the overexpressing cells, it is unlikely to have occurred at sites of protrusion, in view of the decreased presence of filamentous actin at the active cell margin. Moreover, our proposal that the mechanism of the additional motility differs from that of normal motility is further supported by its dependency on myosin function.

The mechanisms by which protrusion could depend on myosin activity have been relatively little explored. Several myosin isoforms are thought to have a transport function and it is thus possible that they could participate in the delivery of material to the leading edge of the cell. Furthermore, it is conceivable that this form of transport could be increased by overexpressing actin, since more actin filaments could well provide more 'tramlines' to support and guide these motor proteins. This is unlikely to explain our results, however, since it does not explain why only the additional motility is sensitive to blocking myosin function. Alternatively, the influence of myosin on protrusion might depend on actomyosin contraction.

We have shown that there is some coordination of protrusion and retraction activities during myoblast locomotion. If retraction depends on actomyosin contraction, this coordination offers a route by which protrusion might also depend on actomyosin contraction. The flaw in this argument is that this coordination is not increased but reduced by overexpressing actin. Furthermore, blocking myosin function by BDM treatment does not affect the coordination of the control cells nor does it affect the reduced coordination of the  $\beta$ -actin overexpressing cells. Thus, whatever the explanation of the coordination of protrusion and retraction, it seems to depend only on actin levels, not on myosin function, and does not therefore offer a pathway by which protrusion could be influenced by myosin function.

As discussed in the Introduction, there is another mechanism by which actomyosin contraction could influence protrusion, although this has previously been associated with quite different cell types that protrude by blebbing, move very rapidly and tend to be invasive. These include leukocytes (Stossel et al., 1999) and Walker carcinosarcoma cells (Keller and Egli, 1998). The mechanism proposed for their protrusion is that a generalised (possibly isometric) contraction of the actomyosin meshwork pervading the cell body sets up a pressure gradient, which tends to force fluid flow towards weaker regions of the actin cortex where it erupts in blebs at the cell margin. This fluid carries a high density of monomeric actin, which does not begin to polymerise until after the bleb has protruded and begins to collapse (Cunningham, 1995; Stossel et al., 1999). This mode of protrusion thus appears to share the characteristics of the additional motility of myoblasts overexpressing  $\beta$ -actin in that it depends on myosin activity but not on actin polymerisation. It also shares the characteristic that participating cells are poorly spread since leukocytes have a mean spreading index of only  $2.01 \mu\text{m}^2 \text{pg}^{-1}$  (our unpublished observation) and Walker carcinosarcoma cells have an even lower mean spreading index of  $1.33 \mu\text{m}^2 \text{pg}^{-1}$  (our unpublished observation). One possible explanation of this is that well-spread cells cannot generate sufficient hydrostatic pressure, since the free contraction of their actomyosin meshwork is resisted by firm adhesions to the substratum.

One problem with proposing that this mechanism of enhanced, fluid-driven protrusion operates in the actin-overexpressing myoblasts is that the actin overexpression does not commonly result in blebbing. However, a possibly closely related phenomenon, which also does not invariably result in blebbing, is that of retraction-induced protrusion, discussed in the Introduction. As mentioned there, a large wave of increased protrusion can be induced in a normal fibroblast by detaching its tail from the substratum (Chen, 1979; Dunn, 1980). It is only when the tail retraction, and the subsequent contraction of the cell body, are particularly large that this protrusion takes the form of blebbing. Measured flow rates of nonaqueous material into these blebs can be as high as  $1 \mu\text{m second}^{-1}$  or higher (Brown and Dunn, 1989), and there can be little doubt that the bleb formation is fluid-driven as in the leukocytes and Walker carcinosarcoma cells described above. Nevertheless, smaller tail retractions, including most of those that occur spontaneously during normal locomotion, still result in a wave of increased protrusion, but this generally takes the form of lamellipodia rather than blebs. One feature that retraction-



**Fig. 6.** Cross-correlograms showing the cross-correlation at different lag times between the 1-minute protrusion areas and the 1-minute retraction areas after detrending and pre-whitening (see Materials and Methods for more details). Significant cross-correlations at positive time lags indicate that fluctuations in protrusion lag behind fluctuations in retraction whereas significant cross-correlations at negative time lags indicate the reverse. Error bars show the 95% confidence interval for the correlation coefficient. (A) Wt, (B)  $\beta$ -actin, (C) Null, (D)  $\beta^*$ -actin, (E) BDM-treated Wt, (F) BDM-treated  $\beta$ -actin.

induced protrusion has in common with the increased protrusion resulting from actin overexpression is that both are associated with reduced spreading. Another is that both are probably myosin-dependent. Although this has not yet been shown directly in the case of retraction-induced protrusion, it is thought that the later phase of retraction is due to an active contraction (Chen, 1981). Also, it is perhaps significant in this respect that the respreading of rounded cells following mitosis has been shown to depend on myosin function (Cramer and Mitchison, 1995).

Our view is therefore that the overexpression of actin may increase motility by sustaining a mode of enhanced protrusion that occurs only occasionally during the normal locomotion of fibroblasts and fibroblast-like cells. This happens when a fibroblast suddenly decreases its spread area by spontaneously releasing several adhesions to the substratum. The ensuing contraction and increase in internal pressure forces an increased flow of fluid towards weak regions of the actin cortex at the leading margin. Although this fluid is packed with monomeric actin, the increased delivery of fluid to the margin may force the membrane forward too fast for actin polymerisation to catch up and, in extreme cases, blebbing will result. In less extreme cases, some actin polymerisation may occur simultaneously and lamellipodial protrusion will result even though the main driving force is still the fluid flow. Within a minute or two the cell settles down to a fairly steady state and actin polymerisation takes over as the driving force for the lowered rate of lamellipodial protrusion.

In cells overexpressing actin, it is possible that the excess amounts of available actin can maintain this mode of increased protrusion for much longer periods by increasing the fluid phase of the cytoplasm. Such an increase in the cytosolic component would be expected since actin is a relatively small protein with a high osmotic effect. However, the overexpression of other small proteins might not be equally effective since it is probable that some actin polymerisation is subsequently required to consolidate the newly formed protrusions. Thus, our observation that the excess actin need not be entirely polymerisable, as in the case of the  $\beta^*$ -actin, does not suggest that the polymerisation of actin is not required for this mode of locomotion but only that it is not a rate-limiting factor.

As with the phenomenon of retraction-induced protrusion, the effects on motility of overexpressing actin will require a lot more work before they are fully understood. Our purpose here is to draw attention to a possible link between the two phenomena. More importantly, our results may help to shed some light on the problem of whether the fluid-driven mode of protrusion is confined to highly invasive, blebbing cell types, such as some leucocytes and malignant cells, or whether it can occasionally supplement the locomotory mechanism of fibroblast-like tissue cells.

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