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Cell polarity and locomotion, as well as endocytosis, depend on NSF

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SUMMARY

NEM-sensitive factor (NSF) is an essential protein required during membrane transport. We replaced part of the endogenous *D. discoideum* NSF gene (*nsfA*) by a PCR-mutagenised library and isolated 11 mutants temperature-sensitive (ts) for growth. Two of these have been studied in detail. As expected, both are ts for FITC-dextran uptake by macropinocytosis, for internalising their surface membrane (monitored with FM1-43) and for phagocytosis. However, after 10-20 minutes at 28°C, they round up and cease to chemotax, move or cap ConA receptors. They fully recover when returned to 22°C. These cells carry out a normal 'cringe' reaction in response to cAMP, indicating that the actin cytoskeleton and this signal transduction pathway are still functional at 28°C. The behaviour of these mutants shows that NSF-catalysed processes are required not only for the different endocytic cycles but also for the maintenance of cell polarity. As cell locomotion depends on a cell having a polarity, the mutants stop moving at high temperature. A tentative model is proposed to explain the surprising link between membrane recycling and cell polarity revealed here.

Key words: NSF, Cell polarity, Cell locomotion, Endocytosis, *Drosophila*, *Dictyostelium*

INTRODUCTION

Cells can exhibit different kinds of polarity: the best understood of these is the separation into apical and basolateral membrane domains by specialised junctions in epithelial sheets. 'Planar polarity' exists across various epithelia and is most graphically demonstrated by the direction hairs or bristles point on the surface of the sheet. In addition, oocytes, early embryos and stem cells frequently have striking asymmetries that invariably result, after cell division, in different daughter cells. An experimentally more accessible form of polarity exists in migrating eukaryotic cells. Here, one part of the surface of the cell – its leading edge – defines the front of the cell. This polarity is intimately connected with how a cell moves. The experiments described here were initially designed to illuminate the mechanism of locomotion, but our results relate mainly on how the identity of the leading edge is maintained.

The leading edge of a cell is believed to contain the motor that drives locomotion and pulls the rest of the cell forwards, although how this motor works is debatable. In migrating fibroblasts, both exocytosis (Bretscher, 1983; Bretscher and Thomson, 1983) and actin polymerisation occur here (Wang, 1985), whereas crosslinked antigens form a cap at the trailing end of the cell (Taylor et al., 1971). This polarity is presumed to be a reflection of the existing arrangement of the actin and/or microtubule cytoskeletons. In a migrating cell, this internal polarity can be extremely flexible, as demonstrated by the ease and speed with which a cell can reorientate in the face of a changing chemotactic signal (Parent and Devreotes, 1999). Understanding how external signals cause a cell to reorientate may provide a route to unravel the internal machinery which defines the polarity of a cell and how the motor works. Much progress is being made in the highly motile amoeba of *Dictyostelium discoideum* to define those genes whose products guide the processes which lie between chemoattractant and chemotaxis (Chung et al., 2001). However, such genetic studies are limited at present to the disruption of non-essential genes by homologous recombination (De Lozanne and Spudich, 1987; Manstein et al., 1989). Many genes whose products might have been expected to be required to function in the motor of the cell can be inactivated, usually giving a surprisingly normal cell (Noegel and Schleicher, 2000). It therefore seemed desirable to develop methods for making conditional mutants in essential *Dictyostelium* genes, as is routinely done in yeast.

Dictyostelium amoebae have a haploid genome; this makes it possible to replace an individual gene with a disrupted copy by homologous recombination. We hoped to adapt this procedure to replace an essential gene with a copy containing a mutation that would make the encoded protein temperature sensitive (ts). For this, we chose the gene for NSF for a variety of reasons. First, NSF is required to dissociate snare complexes after membrane fusion has occurred (Beckers et al., 1989; Malhotra et al., 1988; Whiteheart et al., 2001). As such, it would be expected to provide an essential function and, unsurprisingly, null mutants in yeast (*Sec18*) and *Drosophila* (*comatose*) are lethal. In *Dictyostelium*, there is a single gene for NSF (*nsfA*), the cDNA sequence of which has been determined (Weidenhaupt et al., 1998). Second, the amino acid

sequence of NSF is highly conserved, allowing point mutants in one animal to be transplanted into another. In the *comatose* gene several ts mutants exist whose amino acid exchanges are known (Pallanck et al., 1995; Siddiqi and Benzer, 1976; Tolar and Pallanck, 1998). Third, the behaviour of a ts mutant in *nsfA* might shed light on the role of membrane circulation in cell locomotion (Abercrombie et al., 1970; Bretscher, 1984).

This straightforward approach of exchanging an amino acid in *nsfA* to make a ts mutant did not succeed. However, the high level of homologous recombination we found at this locus made it feasible to replace the endogenous *nsfA* with a PCR mutagenised *nsfA* library and so generate a panel of ts mutants. We analysed two NSF ts mutants and find that they are unable to carry out normal endocytic processes at the restrictive temperature. In addition, they appear to have lost their cell polarities and are consequently unable to move.

MATERIALS AND METHODS

DNA constructs and sequences

The entire *nsfA*-coding sequence and intron (2342 base pairs), plus extra-NSF 5′ and 3′ regions (305 and 1700 bp, respectively) were assembled by PCR and a blasticidin resistance cassette inserted 220 base pairs beyond the end of the *nsfA*-coding sequence. The *Com4* (AKQI**G**TMLN to AKQI**E**TMLN) and *6* (EISL**P**NEQ to EISL**S**NEQ) mutations in *D. melanogaster* were incorporated into the *D. discoideum* sequences (ARQI**G**KMLN and EISL**P**DEH) using synthetic primers and PCR. The checked sequences contained the following changes:

pC4

The DNA sequence around the mutated site was changed from **GCC.AGA.**CAA.ATC.**GGT**.AAG.ATG.TTA.AAT to **.GCG.CGC.** CAA.ATC.**GAA**.AAG.ATG.TTA.AAT, introducing a unique GCGCGC (*Bss*HII) site.

pC6

Likewise, the sequence was altered from GAG.ATC.TC**A. TTA.CCC.**GAT.GAA.CAT to GAG.ATC.TC**C**.**TTA.AGT.**GAT. GAA.CAT, introducing a unique CTTAAG (*Afl*II site).

pC6N

A nonsense codon was placed in frame with the *Com6* mutation so that the relevant sequence was GAG.ATC.TC**C.TTA.AGT. *TAA***.GAA.CAT

Positions of all sites are given from the 5′ end of the construct, defined by a *Pst*I site. Two libraries were used in which the DNA had been mutagenised between bases 1140-1700 (coding largely for the D1 domain of the protein; library size ~60,000) and bases 1700-2860 (coding for the last 60 residues of D1 and the whole of D2; library size ~6000). These were incorporated into pC6 and yielded the mutant lines ts4, ts5 and ts7, and ts1-3, ts6 and ts8-11, respectively.

The mutagenised regions from the two mutants described here were copied out of genomic DNA and two independent clones of each were sequenced. The mutations are: in *nsfA1*, A1847G (giving amino acid change K472E), A1939T (E502D), T1977C (V515A), T2005G (N524K), A2025T (K531M) and A2366T (I645F); and in *nsfA2*, A1854T (E474V), T1984A (D517E), C2131A (F566L), A2398T (K655M) and T2574A (M714K). In addition, these lines also carry the *Com6* mutation.

Cell transformation and mutant selection

DNA was linearised and introduced into Ax2 amoebae by electroporation (Howard et al., 1988). Cells were then diluted into medium at 3×10^5/ml and dispensed into flat-bottomed 96-well plates at 0.1 ml/well and held at 20°C. After 1 day, 0.05 ml 30 μg/ml blasticidin in medium was added/well and the cells grown until colonies were apparent. During this selection process, all transfected cells start with a complement of wild-type NSF and, as such, would initially be expected to grow normally. If the endogenous gene were replaced by one encoding a poorly functional NSF, the clone might grow more slowly at the permissive temperature once the native NSF had become depleted; indeed, it might grow only by phagocytosis (of its dead neighbours) and not axenically, and thus give rise only to small clones. Although likely to be clonal, the content of each well was recloned: the clone size was guessed (between 50-3000 cells) and an aliquot of ~30 cells plated out on bacteria at 20°C. Two colonies from each plate were tested for growth on bacteria at 20°C and at 26.5°C. Temperature-sensitive clones were saved. The strain harbouring the *Com6* mutation is HM1058; the original *nsfA 3, 4-9* and *10,11* lines are HM1059, 1061-1066, 1068, 1069. The recreated *nsfA 1* and *2* lines are HM1060 and HM1067, respectively. HM1060 can only be grown on bacterial plates, whereas HM1067 will grow on plastic or in suspension in axenic medium.

Endocytosis assays

FITC-dextran

Cells (2.5×10^7/ml) were preincubated in KK2, FITC-dextran (to 4 mg/ml) added and aliquots removed at the times indicated, washed with cold buffer containing 1% FCS, dissolved in detergent and the fluorescence measured (Aguado-Velasco and Bretscher, 1999).

FM1-43

Cells (2.5×10^7/ml) were preincubated in KK2, FM1-43 added to 10μM and dye uptake measured (Aguado-Velasco and Bretscher, 1999) but in the absence of added sorbitol, as some cell lysis occurs with sorbitol with both ts1 and ts2 at 28°C; this results in a high background and poor recovery of cells. The rate of uptake of FM1-43 by bacterially grown cells is unaffected by sorbitol. Unlike measurements on FITC-dextran uptake, FM1-43 uptake is extremely sensitive to cell damage as the dye binds tenaciously to intracellular components, presumably nucleic acids. It is possible that some of the apparent uptake of dye at 28°C is caused by cell damage, although inspection of labelled cells suggests this is not the case.

Phagocytosis

This was measured using fluorescent 1 μm beads (Witke et al., 1992).

Microscopy

To examine the effect of high temperature on the behaviour of mutant cells, a slide was constructed whose temperature could be rapidly changed. It had two chambers: an upper one on that an inverted coverslip with attached cells is held over a drop of buffered salt solution (KK2) by 1 mm high glass posts; and a lower sealed chamber separated from the upper chamber by a coverslip. This lower chamber, with a thickness of 2 mm, has attached entrance and exit tubes through which water can flow. This allows the temperature of the upper chamber, monitored with a small thermistor, to be changed from 20°C to 28°C within about 15 seconds. The slide was set up on a BioRad Radiance confocal microscope with a 10× air objective and DIC images collected.

RESULTS

Introducing mutant NSF gene sequences

The genomic sequence of *D. discoideum nsfA* and the region around it was assembled from the Jena database (Fig. 1A). Changes to the *nsfA* sequence were engineered to encode the mutations found in *Drosophila* Com4 or 6 (Muller et al., 1999b;

Pallanck et al., 1995; Tolar and Pallanck, 1998) and, adjacent to them, unique restriction sites included to help identify these sites. Gene-replacement vectors were constructed by insertion of a blasticidin-resistance cassette 220 bp beyond the *nsfA* termination codon, so generating the vectors pC4 and pC6. Linearised DNA was introduced into Ax2 amoebae and blasticidin-resistant clones isolated at 20°C and tested for ts growth. None was ts. Southern blots revealed that ~95% clones had arisen by homologous recombination, a surprisingly high proportion; this is shown for a single clone, from pC6 (Fig. 1C,D). All pC6-derived homologous recombinants (17 in toto) contained the *Com6* mutation, but none of the pC4 clones the *Com4* mutation (M. S. B., unpublished). These results suggest that *Com6* has no evident phenotype and that *Com4* is lethal. This is supported by the additional finding that when amoebae are transformed with a pC6 vector having an adjacent in-frame UAA codon (vector pC6N) all 12 transformants examined had arisen by homologous recombination, but none now had the *Com6* site (M. S. B., unpublished). We concluded two things from this: first, that *nsfA* is essential for viability and that a similar approach of gene replacement by a marked copy, with or without an in-frame stop codon, may provide a general method to discover whether any given gene is essential; second, because of the high efficiency of homologous recombination observed, a new vector library in which part of *nsfA* is randomly mutagenised could be used to generate a panel of homologous recombinants that might contain some ts alleles.

ts NSF mutants

PCR mutagenesis was used to make two mutagenic libraries that covered different regions of NSF, both in a pC6 background. Ax2 amoebae were transformed and resistant clones selected in growth medium at 20°C. Small and large clones – about 1500 in all – were recloned on bacterial plates and then screened for growth at 20°C and 26.5°C on plates. Eleven ts mutants, *nsfA1-11*, were isolated, all of which had arisen by homologous recombination and contained the *Com6* site.

We decided to focus on just two mutants, ts1 (*nsfA1*) and ts2 (*nsfA2*), because, on warming to a restrictive temperature of 28°C, both showed early and similar phenotypic changes, yet only one (ts2) was able to grow in liquid culture (‘axenic growth’). That they are indeed true gene replacements is shown by Southern blots (Fig. 1C): both lines also carry the *Com6* site (Fig. 1D). To prove that the temperature sensitivity of each of these two lines is caused by mutation in *nsfA*, we cloned each mutated region (*Nsi*I to *Bam*HI, Fig. 1A) by PCR. Their sequences showed that ts1 and ts2 contained, respectively, six and five induced mutations that affect the coding region. These mutated regions were reassembled into the standard pC6 vector (replacing the wild-type *Nsi*I/*Bam*HI sequence) to generate two new mutagenic vectors. These were transformed into Ax2: both vectors gave rise to a high proportion of ts clones. This shows that it is possible to recreate these ts mutants with mutated DNA, and therefore that the mutations that cause temperature sensitivity are indeed in *nsfA*.

As mutations in NSF would be expected to affect membrane processes, we first examined endocytosis. Recreated lines were used and, unless otherwise stated, were harvested from bacterial plates.

Endocytosis

Three assays have been used to measure different aspects of

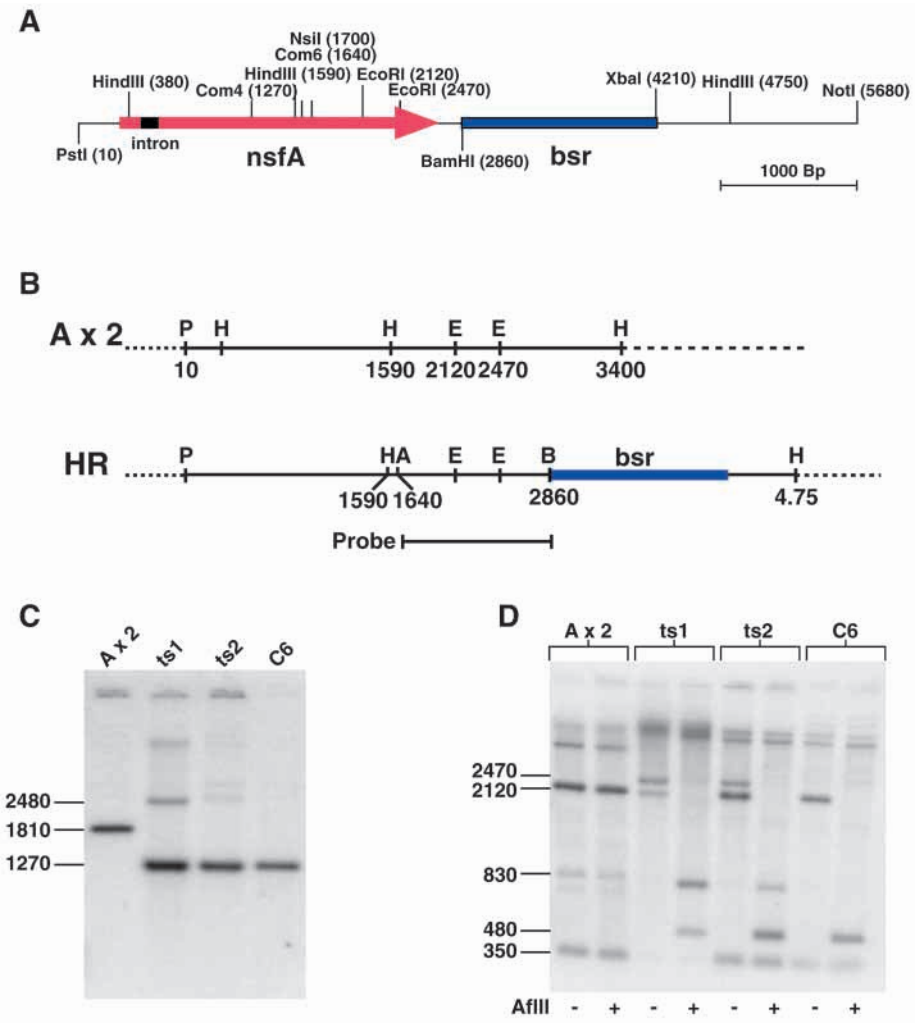

Fig. 1. Southern blots of genomic DNA establish homologous gene replacement with *Com6* site included. (A) Structure of the transforming DNA used. (B) Arrangement of restriction sites around the *nsfA* gene in Ax2 and in a homologous recombinant (HR) containing the *Com6* site (marked by A). Restriction sites: P (*Pst*I); H (*Hin*dIII); A (*Afl*II); E (*Eco*RI); B (*Bam*HI). The probe covers bases 1700-2860. (C) Blot of Ax2, ts1, ts2 and C6 (a pC6-derived clone) cut with B+H. (D) As in C, but cut with P and E±A. The main band at 2120 in Ax2 is insensitive to A; the HR lines are sensitive, showing that they each contain *Com6*. Additional bands are partial cleavages.

surface uptake by amoebae: medium (fluid phase) uptake, cell-surface uptake using the dye FM1-43 and phagocytosis.

For fluid phase uptake, intracellular accumulation of FITC-dextran from the medium was measured (Hacker et al., 1997; Kayman and Clarke, 1983; Vogel et al., 1980). Cells were preincubated at either 22°C or 28°C for 20 minutes, the fluorescent tracer added and uptake followed with time (Fig. 2A). At 22°C, Ax2 and the ts lines have similar rates of fluid uptake; by contrast, at 28°C, this uptake is severely depressed in the ts lines and enhanced in Ax2. This shows that macropinocytosis in the mutants is temperature sensitive. However, the apparent similarity in the rate of FITC-dextran uptake in each line at 22°C was somewhat surprising, as ts2, but not ts1, is able to grow axenically. As axenic growth results in, and requires, a five- to tenfold increase in the rate of fluid phase uptake (Aguado-Velasco and Bretscher, 1999; Kayman and Clarke, 1983), we also determined the rate of FITC-dextran uptake with cells grown in axenic medium (Fig. 2B). Cells were harvested from bacterial plates, washed free of bacteria and incubated overnight in axenic medium. Under these conditions, both Ax2 and ts2 show a similarly enhanced rate of fluid phase uptake at 22°C, whereas ts1 does not: ts1 appears unable to be upregulated, the low levels of uptake being the same as when the cells are grown on bacteria. As macropinocytosis is used to take up nutrients for axenic growth, this observation provides a simple explanation for the lack of growth of ts1 in axenic medium, although how an apparently inductive process can be affected by mutation in NSF is intriguing.

Surface uptake was determined by the internalisation of the dye FM1-43, which binds reversibly to the outer lipid monolayer of the cell. When vesicles bud into the cell, the dye is trapped and can no longer be washed away; dye uptake can thus be used to measure the rate of total surface uptake by cells (Betz and Angleson, 1998; Betz et al., 1996; Lagnado et al., 1996). We have previously used this method to determine the rate at which surface is taken up by vegetative *Dictyostelium* amoebae (Aguado-Velasco and Bretscher, 1999); this turns out to be the same for cells that take up large amounts of fluid phase or almost none – about one cell surface equivalent every ~4 minutes. When a similar protocol is used to measure surface uptake in mutant cells at 22°C (Fig. 2C), we find that both behave like Ax2. At 28°C, the rate of FM1-43 uptake by Ax2 is enhanced about twofold; conversely, in both mutants it is reduced by half (compared with the rate at 22°C). This shows that both lines are temperature sensitive for FM1-43 uptake. However, unlike fluid phase uptake, which is essentially eliminated, there does appear to be some residual internalisation at the restrictive temperature.

Phagocytosis was measured by the uptake of fluorescent microbeads (Fig. 2D) (Vogel et al., 1980; Witke et al., 1992). At 22°C, both ts lines accumulate beads at about half the rate seen with Ax2; at 28°C, the rate of accumulation of beads by

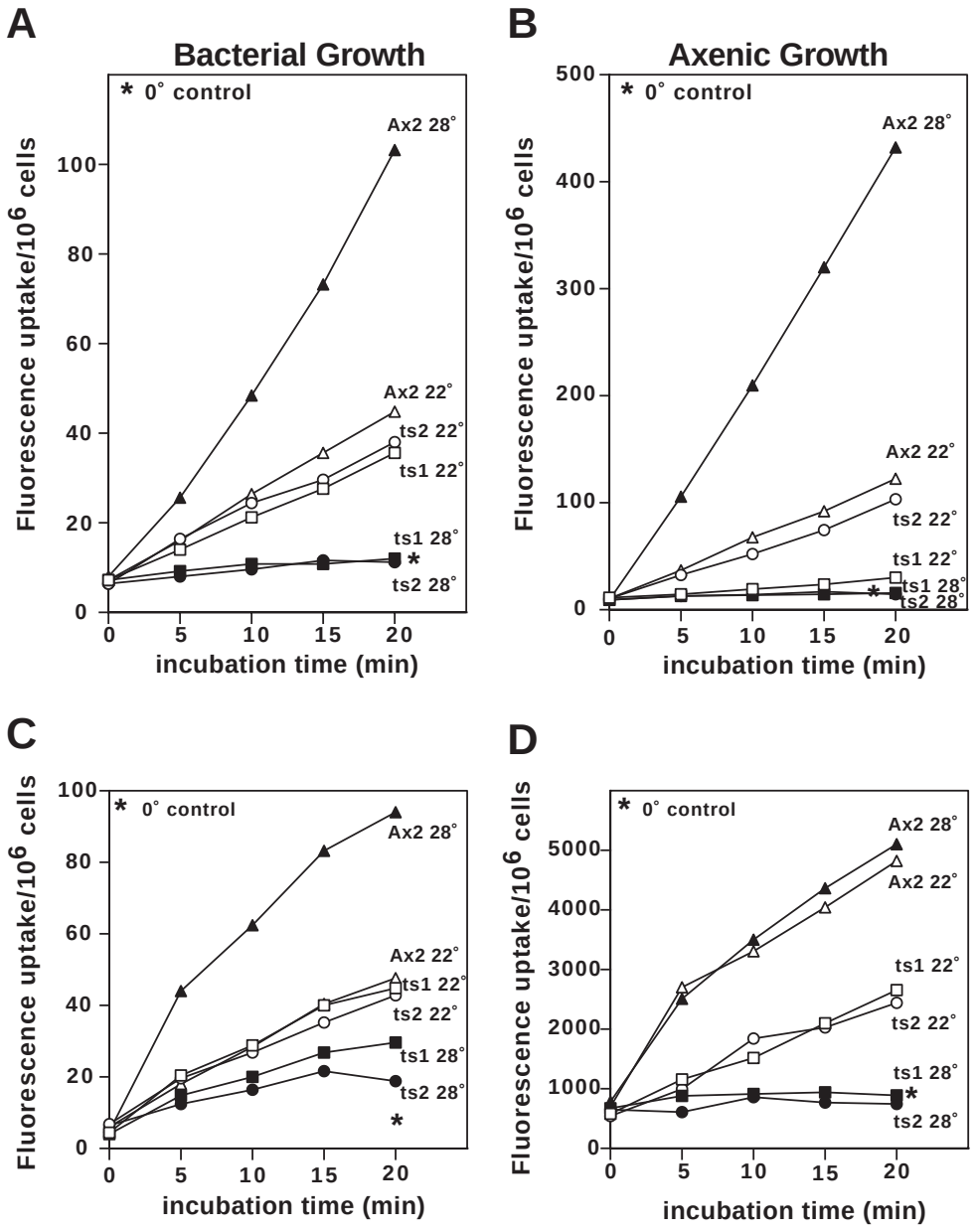

Fig. 2. Endocytosis by Ax2 and ts mutants after a 20 minutes preincubation at 22°C or 28°C. (A,B) Fluid phase uptake measured with FITC-dextran with cells harvested from bacterial plates (A) or held overnight in axenic medium at 5×10^6 cells/ml (B). (C) Cell surface uptake measured with FM1-43. (D) Phagocytosis of fluorescent beads. Each experiment was carried out at least five times, all with similar results; a representative example of each experiment is shown.

Ax2 remains about the same, but that of the ts lines drops at least 20 times to levels indistinguishable from background. As the ts lines grow more slowly than Ax2 on a lawn of bacteria at 22°C, this is consistent with the decreased rates of phagocytosis observed at the permissive temperature compared with Ax2. Furthermore, as both ts lines do not phagocytose at the restrictive temperature, this may explain why they do not grow on a bacterial lawn at 28°C.

The endocytic phenotypes of the two mutants, when bacterially grown, are very similar. All the membrane processes we examined – fluid phase uptake, internalisation of cell surface area and phagocytosis – are temperature sensitive. Although this is anticipated if NSF is required in at least one membrane fusion step in these different membrane cycles (Beckers et al., 1989), the extent of sensitivity does not seem to be the same for each cycle and this was not anticipated. In particular, fluid phase internalisation and phagocytosis are inhibited by about 95-100% after 20 minutes uptake at 28°C, yet surface uptake, measured with FM1-43, is only inhibited by about 75% (both normalised with respect to Ax2) and even less when shorter times are compared (Fig. 2C). We believe that this difference is significant and may mean that the cycle in which surface uptake occurs – the molecular basis of which is unknown – may not be directly dependent on NSF. By contrast, the endocytic cycles responsible for fluid phase uptake and for phagocytosis presumably do depend on NSF and at 28°C are blocked quickly. The uptake cycle by which FM1-43 accumulates inside the cell may depend on an NSF homologue, such as p97 (Muller et al., 1999a), and slows down at 28°C as NSF-dependent trafficking processes cease.

Cell locomotion and capping

With the assurance that these mutants are defective in endocytosis, we next sought to discover how cell locomotion may be affected and therefore what the role of endocytosis may be in motile processes. Initially, we examined the ability of Ax2 and the mutants to chemotax towards cAMP; this was examined in a drop assay on an agar surface performed over 3 hours. Although all three lines chemotaxed at 22°C, only Ax2 did so at 28°C; cells in both mutants became round and, over the period of the experiment, no noticeable taxis occurred (C. R. L. T. and M. S. B., unpublished). Ax2 cells continue to move normally and ts1 cells behave like ts2.

As this deficiency could lie in the inability of the cells either to recognise chemotactic signals or to move, we examined cell locomotion in a slide chamber whose temperature could be controlled. This revealed that cells of both lines appear to move normally at 22°C; however, about 10 minutes after a temperature shift to 28°C, cells of both lines (unlike Ax2) start to become rounder and largely cease moving after a further 10 minutes (Fig. 3; see Movie 1 at http://www.mrc-lmb.cam.ac.uk/PAL/Sup_Material_MSB). Rounding up of ts cells might be caused by different steps in membrane circulation stopping at different times after a temperature shift. For example, if endocytosis continued after exocytosis had ceased, the net loss of surface area might round the cells up. To examine this, ts1 cells were held at 28°C, and thin sections were prepared and examined by electron microscopy. This showed that the surfaces of these cells are far from taut: they usually have a scalloped appearance, which suggests that the rounding up was not caused by a lack of surface area (not shown). Although these cells do not translocate, they are not frozen in shape: some residual motion remains due to the frequent protrusion of bulges, giving the cells a 'rabbit in a sack' appearance, as seen in Movie 2 (http://www.mrc-lmb.cam.ac.uk/PAL/Sup_Material_MSB). These bulges may reflect nuclear motion or exocytosis of that membrane associated with the remaining surface uptake that still seems to occur at the restrictive temperature. Our observations indicate that membrane circulation and cell locomotion are connected processes, although they do not show what that connection is.

A readily measured property of all motile cells is their ability to cap crosslinked receptors (Abercrombie et al., 1972; Taylor et al., 1971). We used fluorescent ConcanavalinA (ConA) to crosslink surface ConA receptors (Aguado-Velasco and Bretscher, 1997) to see whether the capping process is affected in the ts mutants. We found that at 22°C Ax2 and the mutants capped surface-attached ConA readily; however, after cells had been preincubated at 28°C, although capping proceeded normally with Ax2, it did not occur with either mutant (Fig. 4).

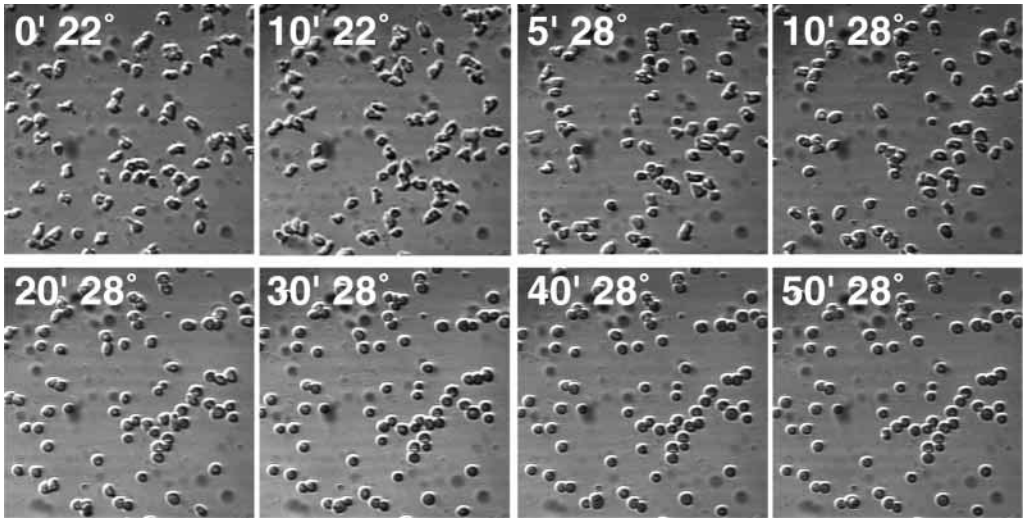

Fig. 3. Behaviour of ts2 cells during temperature shift. Cells from the growth zone on a bacterial plate were allowed to attach to a coverslip, rinsed and placed above a temperature-controlled chamber at 22°C and observed (top two left-hand panels). The temperature was then raised to 28°C (top two right-hand panels and lower panels); the cells became rounder by 10 minutes and stopped migrating at about 20 minutes.

Cringe reaction and cell survival

To discover whether the actin cytoskeleton functions normally in these mutants at 28°C, we studied the 'cringe' reaction. When starved amoebae are pulsed with cAMP over a period of 3-4 hours, they become hypersensitive to stimulation by a uniform high concentration of this hormone: they react by undergoing an initial rapid, transient and extensive polymerisation of G-actin (peaking at ~10 seconds), followed by a slower increase in F-actin over a period of 60 seconds that, in wild-type cells, correlates with pseudopod extension (Hall et al., 1988). It therefore provides both a measure of the function of the cAMP signal transduction pathway and of the assembly, and its control, of the actin cytoskeleton. After pulsing with cAMP, cells were preincubated at either 22°C or 28°C for 20 minutes before stimulating with 1 μM cAMP. Even though both ts lines have rounded up under these conditions, when stimulated with cAMP they respond with the same dynamics and similar magnitude as does Ax2 (Fig. 5A). This demonstrates that, not only does the regulation and assembly of the actin cytoskeleton still function normally at 28°C, but the cAMP signalling system is unimpaired.

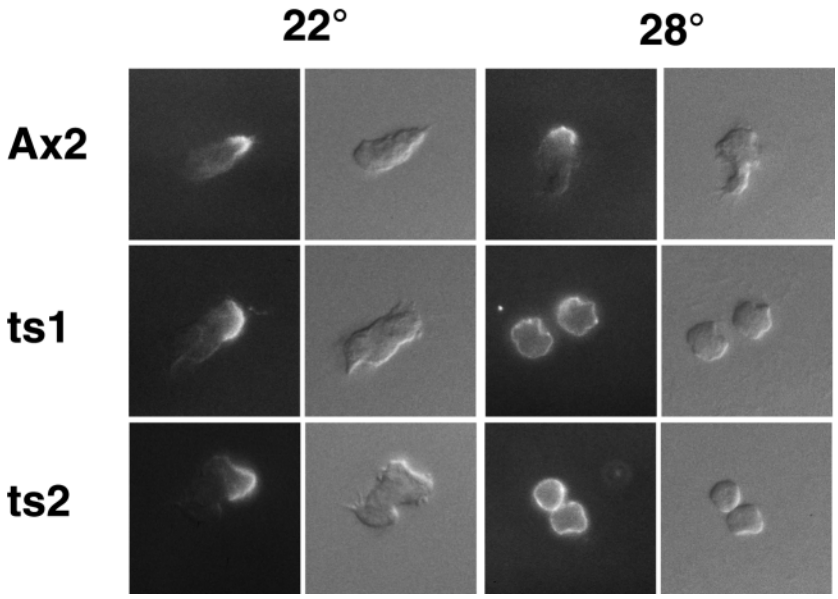

Fig. 4. Capping of ConA receptors. Amoebae, preincubated either at 22°C or 28°C, were labelled with Fl-ConA for 5 minutes at the appropriate temperature, washed and incubated a further 5 minutes, fixed with formaldehyde and viewed according to Aguado-Velasco and Bretscher (Aguado-Velasco and Bretscher, 1997). The fraction of capped cells, measured as those with the fluorescence confined to one half of the cell surface, was: at 22°C, Ax2, 76±5%; ts1, 68±2%; ts2, 77±4%; and at 28°C, Ax2, 79±2%; ts1, 0%; ts2, 0%.

Further evidence that these mutant cells do not just suffer from a general malaise at 28°C is provided by their recovery when cooled. After 2 hours at 28°C, all the rounded cells return to their usual morphology when held at 20°C for ~1 hour. Furthermore, when a toothpick-load of either mutant is placed on a bacterial plate, no sign of a colony arises after 3 days at 26.5°C. However, when these plates are returned to 22°C and left for 3-4 days, colonies of the mutants appear (C. R. L. T. and M. S. B., unpublished). It is clear that these cells are fully viable at the restrictive temperature for at least the short periods of time employed here.

Cell polarity and the cytoskeleton

The mutant lines described here represent the first molecularly defined mutations which block cell locomotion. They show that cap formation, like cell migration, is dependent on the continued activity of NSF. But how these motile processes are linked to NSF is unclear. As locomotory processes clearly depend on the actin cytoskeleton, we examined the distribution of F-actin in mutant cells under normal and restrictive conditions. ts2 cells were held at either 20°C or 28°C before fixing, permeabilising and staining with fluorescent phalloidin to locate F-actin (Fig. 5B). This shows that, at 20°C, F-actin is localised at the leading edge and in foci. However, in the spherical cells seen at 28°C, although the amount of F-actin is unaffected by the temperature change (normalised to Ax2; see legend to Fig. 5), it is rearranged so that the inner surface appears fairly uniformly covered, although randomly placed foci still exist (Fig. 5C; see Movie 3 at http://www.mrc-lmb.cam.ac.uk/PAL/Sup_Material_MSB).

DISCUSSION

Our initial aim was to examine whether membrane recycling is required for cell migration in *Dictyostelium.* In motile fibroblasts, the surface membrane internalised by coated pits is returned to the plasma membrane at the front of the cell where it may assist in extending the cell forwards and thereby be essential for locomotion. By contrast, this exocytosis occurs randomly over the cell surface in stationary cells (Bretscher, 1984). We hoped to examine the role of membrane recycling in *Dictyostelium* by generating ts mutants in NSF, a gene essential for many steps of membrane transport and hence also for viability. The success of our novel method, which yielded 11 ts mutants in NSF, depends to a great extent on having a high rate of homologous recombination at the *nsfA* locus. To what extent this method can be adapted to other genes remains to be discovered; we believe that if the rate of homologous recombination is around 30% or higher and results in a substantial region of the coding sequence of the gene under study being replaced, the method should be viable. However, as the effective level of homologous recombination falls below 30%, the number of clones that need to be screened to find a ts mutant grows so rapidly that it becomes impractical. Of the *nsfA* mutants isolated, only two have been studied in detail. They show, as expected, that membrane cycling processes become interrupted at the restrictive temperature. These results fully support the prevailing view that NSF is required in many different membrane fusion steps. They also reveal that NSF activity is required for cells to move.

For a cell to translocate across a substrate, two conditions need to be satisfied: the cell must have a functional motor and

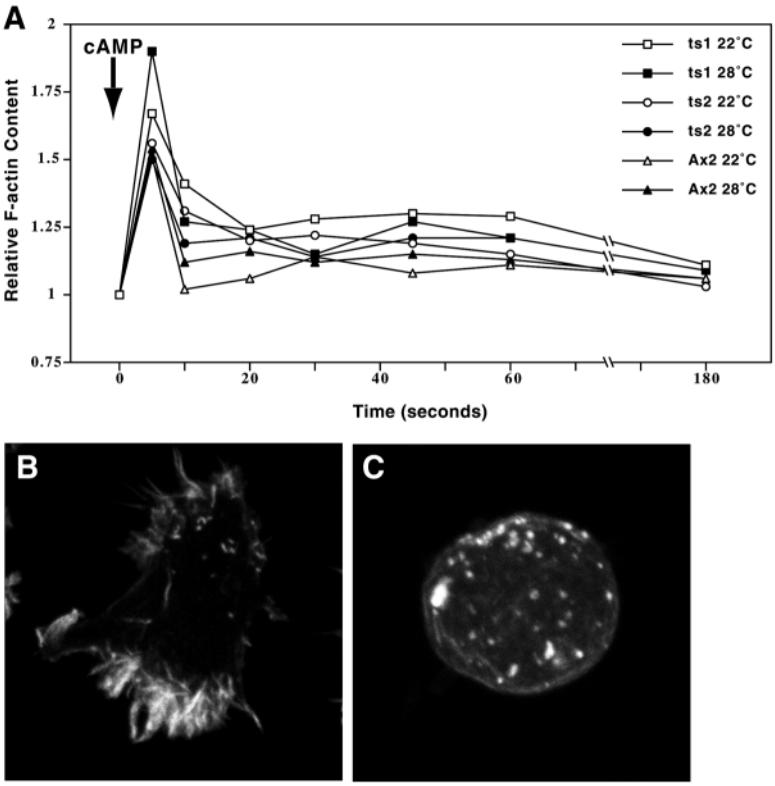

Fig. 5. Actin polymerisation in sensitised amoebae. (A) F-actin levels in sensitised amoebae after addition of cAMP – known as the 'cringe' reaction (Hall et al., 1988). (B,C) ts2 amoebae held at 22°C (B) or 28°C (C), fixed with formaldehyde and stained with Alexa 488-phalloidin to reveal the intracellular distribution of F-actin. The levels of F-actin at 28°C, based on phalloidin binding (as in A) and normalised to that at 22°C, were measured as Ax2, 0.68; ts1, 0.57; ts2, 0.68.

the motor must have a polarity. Our results show that, at high temperature, a mutant cell stops translocating and this implies that either the motor or the polarity of the cell (or both) is defective. The overwhelming impression we have of these mutant cells, whether one looks at their spherical shape, their uniform distribution of ConA receptors under capping conditions or, more especially, their even distribution of surface-associated filamentous actin, is that they have lost their polarity at the restrictive temperature. Assuming that this is so, our observations indicate that NSF, like clathrin (Wessels et al., 2000), is required not only for some membrane uptake processes, but that its activity is essential to help establish, or maintain, the polarity of the cell. Without this, the cells could not move or cap surface receptors, whatever the mechanism of locomotion.

A migrating cell – whether mammalian or amoeboid – has a polarity defined by its leading front. How this external feature relates to the internal organisation of the cell is unclear; however, an internal polarity is likely to include a cytoskeleton primed in such a way that molecular components required for the advance of the leading edge are transported up to it. This polarity is, however, flexible. In the absence of any external signals, cells usually continue in the same direction for a while – their movement has a persistence, which suggests that there is a positive feedback loop that gives the leading front an inherent stability. As the cell advances, the cytoskeleton is continually maintained or rebuilt so as to bring components for lamellar extension up to that front. However, many cells can change direction extremely quickly given an external cue: when this happens, a new front is established and the old one loses its activity within seconds. This means that, in the absence or presence of external cues, the leading edge and cytoskeleton communicate with one another to reinforce the existing polarity (Weiner et al., 2000).

But how can this polarity depend on NSF? In mammalian cells, the leading edge is the site at which recycling membrane is added to the cell surface (Bretscher, 1996), and this may also occur in amoebae (Aguado-Velasco and Bretscher, 1999), although there is no direct evidence for it. We propose that the addition of this membrane to the front of the cell causes a transient and internal 'orienting' signal to be emitted that helps organise the cytoskeleton for continued vesicle delivery in that direction. In this way, a continuation of the polarity would be achieved with the cytoskeleton itself being continuously replenished. However, this homeostasis could be over-ridden by an external hormonal signal. In this situation, the region of the cell surface most highly activated by hormonal receptors would emit a stronger internal signal, with the consequential formation of a reoriented cytoskeleton delivering vesicles to a new front. Once again, this would be stabilised by the orienting signal. If vesicle flow were stopped, this signal would cease and the cytoskeleton, and hence the cell, would lose its polarity. In this way, the maintenance of polarity would depend on a continuation of membrane recycling pathways and thus depend on NSF.

It may be that the cell polarities observed in different contexts can be subdivided into two broad classes. The first would include epithelial cell polarity where a comparatively stable arrangement exists with apical and basolateral domains of the plasma membrane separated by permanent barriers. The other class might include the polarity that exists in dividing stem cells and oocytes, or that of migrating cells or epithelial planar polarity. This latter class of polarities appears to have a more kinetic character: during the period in which they have their influence, there may be a continuing process to maintain them. Our present ignorance of the underlying molecular mechanisms that generate them means that we cannot be certain whether some, or all, depend on basically similar mechanisms. For example, it seems possible that the polarities seen in chemotaxis and planar polarity are related at a molecular level: both can be oriented by external chemicals such as a chemoattractant [fMLP or cAMP (Weiner et al., 2000)] or a morphogen [possibly a Wnt protein acting on the frizzled receptor (Adler and Lee, 2001)]. An understanding of the basic machinery used to determine the polarity of a cell should enable one to see how those specific components used in a particular process function, and therefore what they do. Whether NSF is required to maintain all these more dynamic polarities remains to be established.

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