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Strong increase in the tyrosine phosphorylation of actin upon inhibition of oxidative phosphorylation: correlation with reversible rearrangements in the actin skeleton of *Dictyostelium* cells

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SUMMARY

When oxidative phosphorylation is inhibited in cells of *Dictyostelium discoideum*, the phosphorylation of tyrosine residues on actin is strongly increased. This increase is fully reversible. Under the same conditions the amoeboid cells undergo a series of shape changes. Within three minutes the pseudopods are withdrawn and replaced by cell surface blebs. Subsequently, the cells are rounding up to become immobile. In parallel with the changes in cell shape, the distribution of actin filaments is grossly altered within the cells. The cortical network of actin filaments of normal cells is broken down, and the F-actin forms large, irregular clusters deep within the cytoplasm. In these clusters the actin is associated with myosin II and with the heterodimeric F-actin capping protein cap32/34. After restoration of oxidative phosphorylation the actin returns within less than four minutes to its normal cortical position. A causal relationship between tyrosine phosphorylation and changes in the distribution of actin remains to be established. The rearrangements in the actin system that result from the inhibition of oxidative phosphorylation indicate that the organisation of this system and its maintenance in a functional state depend on the continuous supply of energy by ATP.

Key words: tyrosine phosphorylation, actin, cytoskeleton, anoxia

INTRODUCTION

In a previous report evidence has been provided that *Dictyostelium* actin is phosphorylated in vivo at serine and tyrosine residues (Schweiger et al., 1992). Our finding, that tyrosine phosphorylation of actin transiently rises from a barely detectable level to a peak when starving cells are transferred back to nutrient medium, has indicated that the phosphorylation is regulated by a system that responds to changes in the environment of the cells. In continuing the search for conditions that affect the phosphorylation of actin, we observed that inhibition of oxidative phosphorylation in the cells causes extensive tyrosine phosphorylation. The aerobic cells of *D. discoideum* depend on oxygen to maintain normal shape and motility. They form blebs on their entire surface within a few minutes upon oxygen depletion and subsequently round up. For at least 3 hours these changes are reversible in cells gently treated with low concentrations of 2,4-dinitrophenol (DNP; Gerisch, 1962). The cells rapidly become motile again when this uncoupler of oxidative phosphorylation is removed.

In this paper we report on the marked, reversible increase in tyrosine phosphorylation of actin in response to oxygen depletion or inhibition of oxidative phosphorylation. Furthermore, we describe gross rearrangements of actin filaments in cells subjected to these conditions, and investigate the distribution of several actin binding proteins in cells showing these rearrangements.

MATERIALS AND METHODS

Culture and drug treatment of *D. discoideum* cells

Cells of *D. discoideum* strain AX2-214 were cultivated axenically in shaken suspension culture at 23˚C up to a density of not more than 5×10^6 per ml and induced to develop by washing and starvation in 17 mM Na/K-phosphate buffer, pH 6.0 (designated as phosphate-buffer), at a density of 1×10^7 cells per ml as described by Schweiger et al. (1992).

For DNP treatment starving cells were washed twice, adjusted in phosphate buffer to 1×10^7 cells per ml and incubated on a rotary shaker at 150 rpm. DNP (Sigma) was added up to a final concentration of 5×10^{-5} M from a stock solution of 1 mM in water. For the inhibition of tyrosine kinases, genistein (ICN Biochemicals, Costa Mesa CA 92626) was added from a stock solution of 5 mM in DMSO. PAO (phenylarsine oxide) (Sigma) was added from a stock solution of 35 mM in DMSO to inhibit phosphotyrosine phosphatases. The

diluted DMSO had no detectable effect on the cells (Schweiger et al., 1992).

ATP determination and immunoblotting

ATP was determined in cells starved for 4 hours before and during treatment with DNP on the shaker. A 2 ml sample of cell suspension was quickly pipetted into 1 ml of 5% trichloroacetic acid, immediately mixed and kept for 30 minutes at 23˚C. The extracts were centrifuged at 2200 ***g*** for 20 minutes at 4˚C, neutralised on ice with 1 M $NaHCO_3$, and frozen at −80˚C. The assay was performed using the ATP assay kit HS of Boehringer Mannheim in HCl-cleaned plastic tubes according to the recommendations of the manufacturer. A Lumat LB 9501 (Berthold, 75323 Wildbad, Germany) was calibrated with ATP solutions from 1×10^{-13} to 1×10^{-8}M and samples were measured in duplicate.

Proteins of total cell homogenates were separated by SDS-PAGE in 10% gels, blotted onto nitrocellulose filters and incubated with anti-phosphotyrosine mAb 5E2 (courtesy of Dr A. Ullrich) labelled with ^{125}I by the chloramine T method as described (Schweiger et al., 1990).

Fluorescence microscopy of whole-mount preparations and cryosections of cells

Suspended cells were fixed according to Stefanini et al. (1967) for 30 minutes at room temperature in a mixture containing 15% (v/v) of a saturated aqueous solution of picric acid, 2% paraformaldehyde and 10 mM PIPES adjusted with HCl to pH 6.0, essentially as described by Humbel and Biegelmann (1992). After fixation the cells were transferred through washing, embedding and labelling by pelleting in a centrifuge at each step.

For confocal microscopy, the fixed cells were transferred through phosphate buffered saline (PBS) containing 100 mM glycine, followed by PBS containing 0.5% bovine serum albumin and 0.045% fish gelatine (PBG), and were subsequently labelled for 30 minutes with TRITC-conjugated phalloidin, 50 ng/ml (Sigma). Optical sections were recorded at distances of 0.3 μm through the entire cells using a Zeiss LSM10 confocal microscope equipped with a green He-Ne laser.

For cryosectioning essentially as described by Humbel and Biegelmann (1992), the fixed cells were embedded in 10% gelatine in PBS, put on ice for 15 minutes and post-fixed overnight in the cold with 1% formaldehyde. The gelatine blocks were cut into pieces of 1 mm^3 and incubated with 2.3 M sucrose in PBS. Sections of 0.4 μm thickness were obtained on a Reichert-Jung FC 4D cryo-ultramicrotome (Leica, Wien, Austria).

The sections were incubated with PBS/glycine, followed by PBG, and subsequently for 1 hour with TRITC-phalloidin, 1:100 (Sigma) to label F-actin. For double-labelling of F-actin and actin-binding proteins, the sections were incubated overnight at room temperature with purified mouse mAb diluted into PBG and for 1 hour with FITC-conjugated goat anti-mouse IgG, 1: 200 (Jackson), and TRITC-phalloidin. The mAbs used were mAb 396 (Pagh and Gerisch, 1986) against myosin II heavy chains, mAb 62 (Schleicher et al., 1988) for α-actinin, mAb 454 (Brink et al., 1990) for 120 kDa gelation factor, mAb 270 and 409 against the 34 kDa subunit of the heterodimeric capping protein (Hartmann et al., 1989).

RESULTS

Reversible increase in tyrosine phosphorylation of actin upon inhibition of oxidative phosphorylation

In starving cells of *D. discoideum* that are shaken in a suspension culture, only traces of tyrosine phosphorylation are normally found in actin. In the search for conditions that affect actin phosphorylation, strong phosphorylation of tyrosine residues was observed in cells taken from a suspension culture that had been left without shaking for a couple of minutes. Without shaking, the cells settle and form a loose pellet. Consequently, we investigated whether a similar increase in tyrosine phosphorylation of actin is found when a cell suspension is allowed to sediment for 60 minutes at 23˚C in Eppendorf tubes. Fig. 1A shows that under these conditions a protein with the electrophoretic mobility of actin was strongly labelled in immunoblots with phosphotyrosine-specific antibody. This increase in tyrosine phosphorylation in the sedimented cells might be induced by cell-to-cell contact, or it might be due to the depletion of oxygen. In order to distinguish between these possibilities, cells were agitated and thus prevented from sedimenting by bubbling nitrogen through a cell suspension. Again an increase in tyrosine phosphorylation was observed (Fig. 1B), which indicates that this increase is caused by the depletion of oxygen.

If the shortening of oxygen supply acts on the cells by reducing the rate of oxidative phosphorylation, an uncoupling agent like 2,4-dinitrophenol should have a similar effect. Since DNP treatment can be easily standardised it gives highly reproducible results. Therefore, we have performed the following experiments by applying 5×10^{-5} M DNP to suspended cells. This is a mild treatment, which does not suppress respiration of the cells below the normal rate (Gerisch, 1962). Upon this treatment, the ATP concentration in the cells rapidly declined to a plateau at about 10% of its normal value (Fig. 2). Since the ATP concentration in *D. discoideum* cells is between 1 and 2 mM (Roos et al., 1977; Gerisch et al., 1979), the concentration during DNP treatment is kept in the 100 μM range.

Fig. 3A demonstrates the dramatic increase in tyrosine phosphorylation in the actin band in cells treated with 5×10^{-5} M DNP, which proved to be fully reversible (Fig. 3B). The identity of the tyrosine-phosphorylated protein as actin has been confirmed by subjecting a lysate of cells, which had been treated for 30 minutes with DNP, to a DNase I column. As described for phosphorylated actin in a previous paper

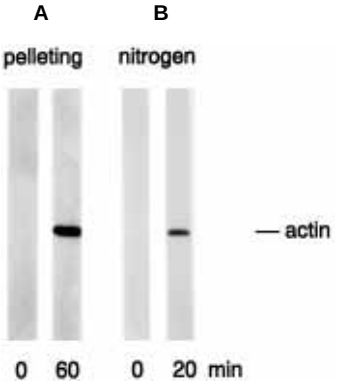

Fig. 1. Immunolabelling with anti-phosphotyrosine antibody of total cellular proteins from aerated and oxygen-depleted *D. discoideum* cells. (A) Growth-phase cells in nutrient medium before and after sedimentation for 60 minutes in Eppendorf cups at 23˚C. (B) Similar cells were kept in suspension and depleted of oxygen by bubbling nitrogen through the culture. Lysates from 2×10^6 cells per lane were subjected to SDS-PAGE and the proteins blotted onto nitrocellulose, incubated with ^{125}I-labelled anti-phosphotyrosine antibody, and autoradiographed.

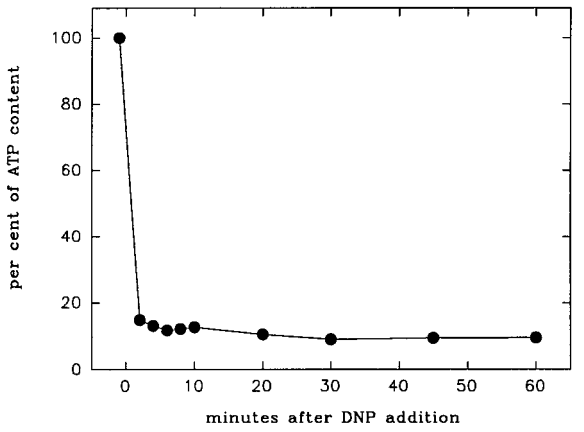

Fig. 2. Decrease in the ATP content of cells treated with 5×10^{-5} M DNP under the same conditions as in Fig. 3A. ATP content of untreated cells at 1 minute before DNP addition is put as 100%.

(Schweiger et al., 1992), the protein bound to the column was eluted by formamide, and subsequently recognised in immunoblots by the phosphotyrosine-specific antibody.

As one would expect, the DNP-induced increase in tyrosine phosphorylation and the reversal of this effect depend on the interplay of tyrosine kinases and phosphatases within the cells. In the presence of genistein, an inhibitor of tyrosine kinases (Akiyama et al., 1987; Akiyama and Ogawara, 1991), the DNP-induced increase in actin phosphorylation was lower than in control cells (Fig. 3C). In the presence of PAO, an inhibitor of tyrosine phosphatases (Bernier et al., 1988; Fallon, 1990), the dephosphorylation after removal of DNP was prevented (Fig. 3D). When cycloheximide was added together with DNP, no change in the rate or extent of the increase in actin phosphorylation was observed, which argues against a requirement of de novo protein synthesis for the response. At the concentration of 250 μg per ml used, cycloheximide reduces the rate of protein synthesis in *D. discoideum* by 90% (Müller-Taubenberger et al., 1988).

Rearrangements of actin filaments upon inhibition of oxidative phosphorylation

D. discoideum cells rapidly change their shape and become immobile if oxygen is lacking (Gerisch, 1962). First, the irregularly shaped pseudopods are replaced at the entire cell surface by rounded blebs. A few minutes later these blebs fuse together so that the cells round up and become surrounded by a smooth surface. Finally, vesicles, mitochondria and other organelles agglomerate in the center of the cells, separated from the plasma membrane by a hyaline zone of cytoplasm. Only the contractile vacuole, which becomes arrested in a dilated state, remains in contact with the plasma membrane. The same changes are observed when DNP is applied. If the concentration of DNP is carefully calibrated, the cells remain in this paralysed state for at least 3 hours without being lysed. Within a few minutes after removal of DNP the cells resume their irregular shape and recover motility (Gerisch, 1962).

The increase in tyrosine phosphorylation of actin and the redistribution of intracellular particles have prompted us to investigate the organisation of the actin system in the DNP-treated cells. Whole-mount preparations and cryosections of fixed cells were labelled with phalloidin for F-actin. Fig. 4 shows the sequence of changes in F-actin distribution upon addition of DNP and after its removal. Untreated cells showed the typical accumulation of F-actin in the cortex of the cells. At 3 minutes after DNP addition the actin was split at the site of the blebs into two layers: an inner one that followed the initial outline of the cell cortex below the blebs, and a thin outer layer beneath the surface of the blebs. Visualisation of the

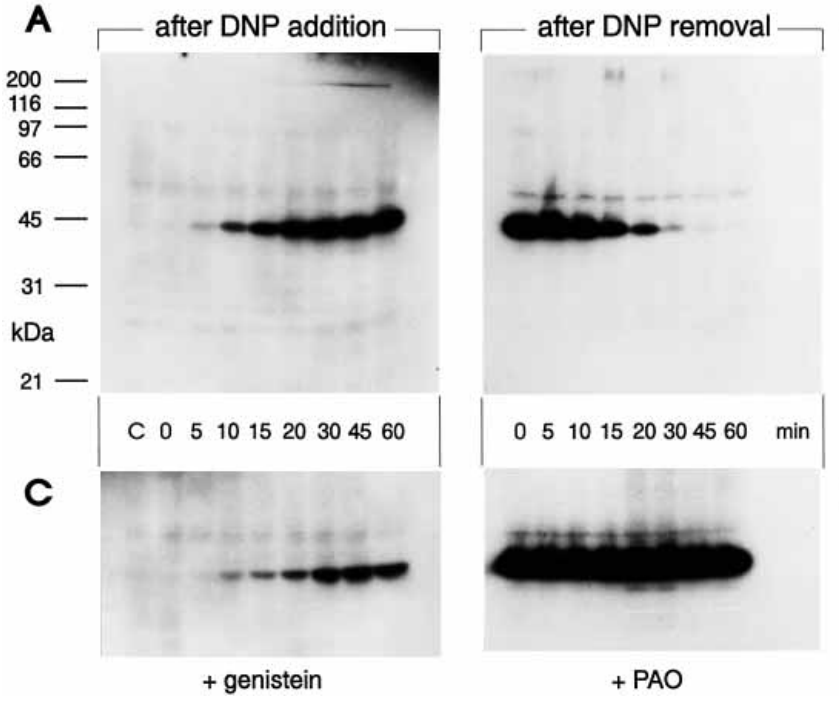

Fig. 3. Immunolabelling with anti-phosphotyrosine antibody of total cellular proteins from a shaken suspension of *D. discoideum* cells incubated with DNP. Cells starved for 4 hours were washed, resuspended at a density of 1×10^7 cells per ml, and divided into four samples to which 5×10^{-5} M DNP was added. (A) The cells were incubated with DNP with no other additions. (B) After 60 minutes of DNP treatment the cells were washed once and incubated in phosphate buffer for another 60 minutes. (C) In addition to DNP, 80 μM of the tyrosine kinase inhibitor genistein was added. (D) After 50 minutes of DNP treatment a sample of the cell suspension was supplemented with 35 μM of the phosphotyrosine phosphatase inhibitor PAO and shaken for another 10 minutes before the cells were washed as in B. Subsequently, PAO was re-added and shaking continued. Equivalents of 2×10^6 cells per lane were subjected to SDS-PAGE and immunoblotting as in Fig. 1. For the control (C), cells from the same suspension before the addition of DNP were used, for the 0 time point cells were taken immediately after DNP addition. In cells shaken in phosphate buffer with no DNP for 60 minutes no increase in tyrosine phosphorylation was detectable (data not shown).

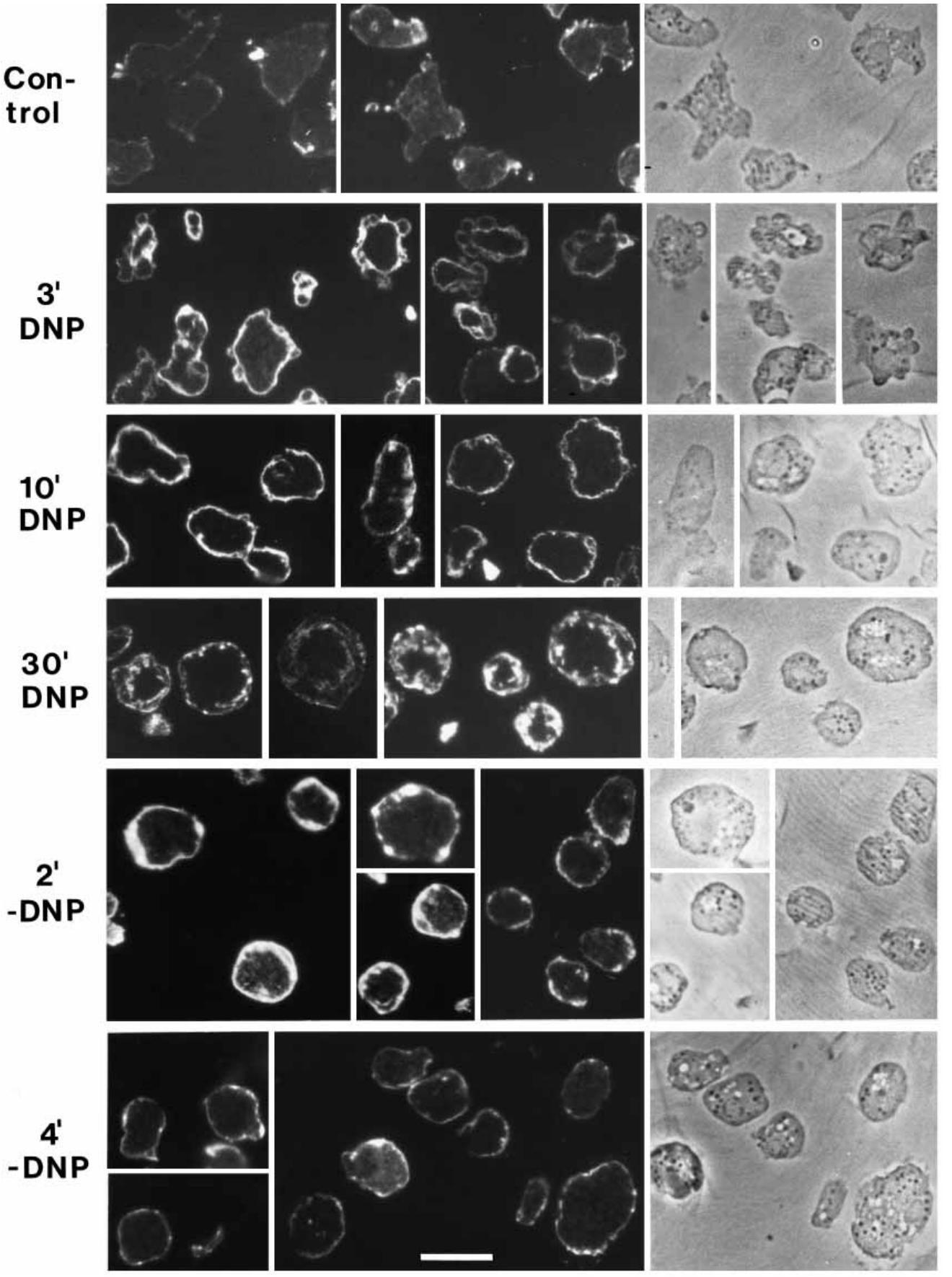

Fig. 4

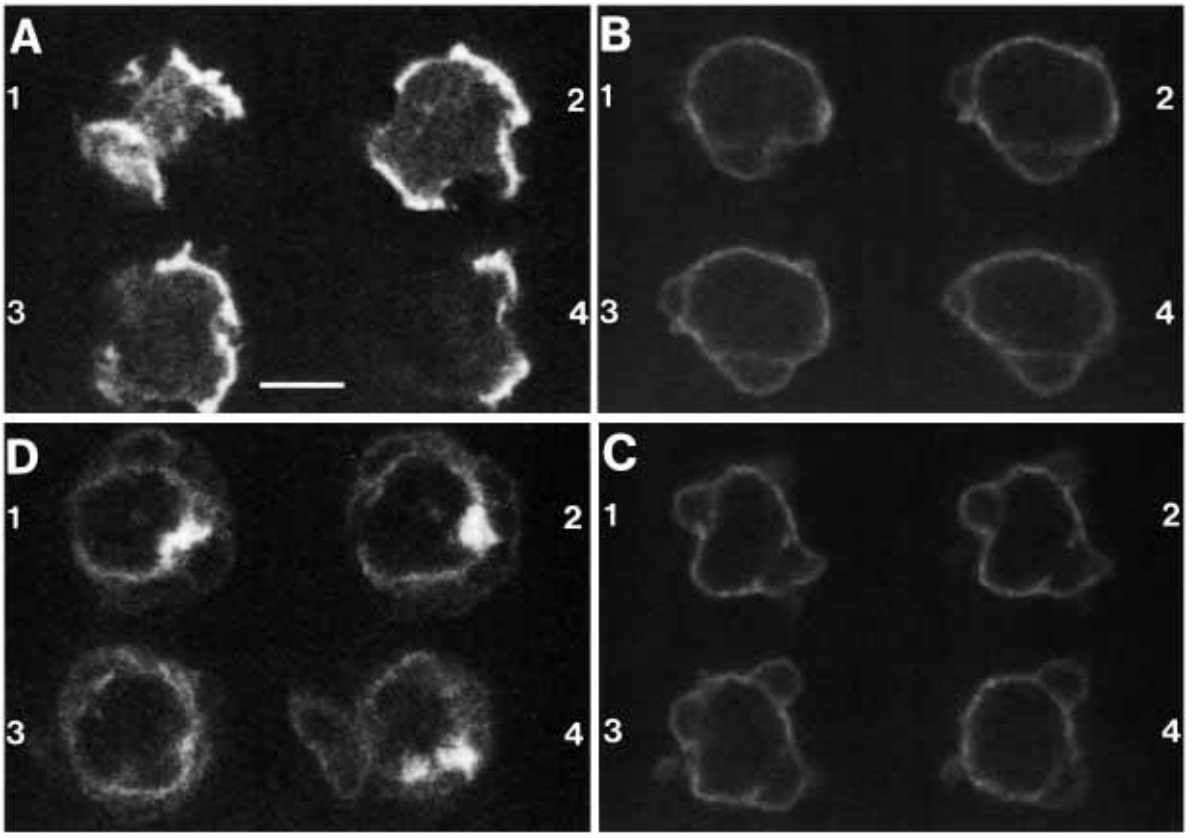

Fig. 5. Confocal sections showing F-actin distribution in untreated cells (A), or cells treated for 3 minutes (B,C) or 30 minutes (D) with DNP. Growth-phase cells, freshly washed in phosphate buffer were fixed with picric acid/formaldehyde in suspension, and labelled with TRITC-phalloidin. Numbers 1 refer to sections close to the free surface of the cells, numbers 4 next to the substratum. (A) An untreated cell showing the normal actin cortex, which is thickened at the leading edge. The distance between the optical sections was 1.5 μm. (B and C) Two cells showing discontinuities in the actin cortex where blebs have broken through. In (B) the distances from 1 to 4 were 0.9, 0.3 and 0.9 μm, respectively; in (C) the distances were 0.3, 0.6 and 0.9 μm. (D) Final stage of actin assembly into irregular clusters. Section distances were 1.5 μm throughout. Bar, 5 μm.

three-dimensional distribution of F-actin by confocal imaging indicated that the actin cortex is first ruptured and cytoplasm is then squeezed through the gap to form the bleb (Fig. 5). At 10 minutes, when the blebs were fused to form a smooth boundary of the cells, the remnants of the initial actin cortex, which had been separated from the plasma membrane, were almost completely dispersed and most of the F-actin was accumulated in a layer beneath the cell surface (Fig. 4). This accumulation turned out to be only an intermediate state of actin redistribution.

At 30 minutes a final stage of actin assembly in DNP-treated cells was obtained. A fraction of the actin filaments remained associated with the plasma membrane, but most of the F-actin was now clustered at the inner boundary of the zone of hyaline cytoplasm (Fig. 4). Three-dimensional imaging showed large actin clusters surrounding the central portion of the cells in irregular arrangements. It became clear from these images that the actin did not form a closed cage around the agglomerated organelles (Fig. 5).

The changes in actin distribution are reversible within a surprisingly short time when DNP is removed. Already within 2 minutes after DNP removal, the F-actin clusters became fuzzy, indicating mobilisation of the actin. At 4 minutes the normal accumulation of actin filaments in the cortex of the cells was restored (Fig. 4), and the cells were ready to move and change their shape.

Fig. 4. Sequence of changes in the distribution of F-actin upon DNP addition to *D. discoideum* cells and during their recovery from DNP treatment. Cells treated for 3, 10 or 30 minutes with 5×10^{-5} M DNP are shown in comparison to untreated control cells. After 30 minutes of incubation DNP was removed by centrifugation of the cell suspension, and the cells were shaken in phosphate buffer for 2 or 4 minutes. For comparison with the fluorescent labelling of F-actin, the right half of each field is also shown in phase contrast. The photographs demonstrate cell surface blebbing at 3 minutes, transient reassembly of actin at the plasma membrane at 10 minutes, and final clustering of actin in the interior of the cells at 30 minutes. During recovery of the cells, dispersal of the actin clusters at 2 minutes and the reestablished actin cortex at 4 minutes is seen. The cells were fixed with picric acid/formaldehyde and cryosections were labelled with TRITC-phalloidin. Aggregation-competent cells harvested at 6 hours of starvation were used for the experiment shown; similar results were obtained with growth-phase cells. Bar, 10 μm.

Clusters of actin filaments in DNP-treated cells are associated with myosin II and heterodimeric capping protein

In an attempt to identify proteins that form complexes with the actin in cells reversibly immobilized by the inhibition of

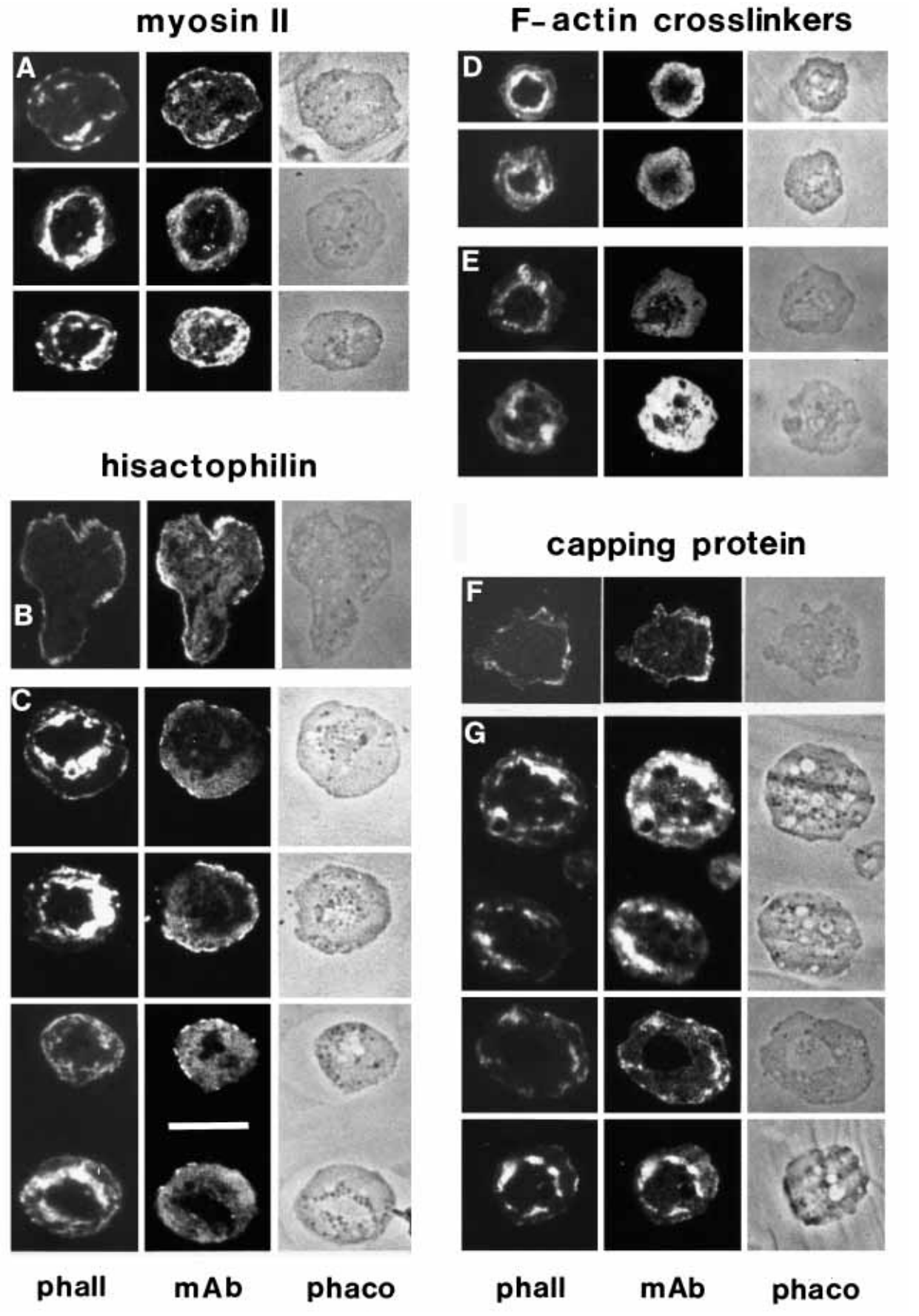
myosin II
F-actin crosslinkers
A
D
E
hisactophilin
capping protein
B
C
F
G
phall
mAb
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Fig. 6

oxidative phosphorylation, we have labelled together with F-actin the major motor protein, myosin II, and four other proteins that are known to regulate in vitro the polymerisation of actin or crosslink its filaments. Cryosections of DNP-treated cells were double-labelled by incubation with TRITC-conjugated phalloidin and with antibodies specific for single proteins followed by FITC-conjugated second antibodies. Myosin II proved to be associated with the actin clusters in DNP-treated cells. In addition, myosin II was found in the hyaline outer layer of the cells where only minor amounts of F-actin were detected (Fig. 6A).

The two major F-actin crosslinking proteins of *D. discoideum* cells, α-actinin and 120 kDa gelation factor (ABP 120), were included in this study because it seemed reasonable that the clustering of actin in DNP-treated cells is due to crosslinkage of the filaments. According to previous reports from our group (Schleicher et al., 1988; Brink et al., 1990), both crosslinking proteins are distributed in the cytoplasm of untreated cells, with no marked enrichment in the cell cortex where F-actin is accumulated. Similarly, in DNP-treated cells no obvious enrichment of the two crosslinking proteins within the actin clusters was observed. Both α-actinin and gelation factor were distributed throughout the cytoplasm (Fig. 6D and E). The weaker label seen in the central portion of the cells is explained by the dense agglomeration of vesicles, mitochondria and other organelles, which leave little space between them to be filled with cytoplasm. On the basis of these fluorescence images we cannot rule out that a small fraction of α-actinin or gelation factor is associated with the clustered actin and crosslinks the filaments. However, the data do not provide proof for a crosslinking activity of these two proteins in DNP-treated cells.

A protein thought to regulate the association of actin filaments with the plasma membrane is hisactophilin. This extremely histidine-rich protein nucleates actin polymerisation and binds along actin filaments in a pH-dependent manner. In untreated cells about half of the hisactophilin exists as a soluble protein in the cytoplasm and the other half is bound to the inner surface of the plasma membrane (Scheel et al., 1989). In DNP-treated cells no extensive changes in the distribution of hisactophilin were found. Hisactophilin remained associated with the plasma membrane of the DNP-treated cells where it often formed small patches, and was also detected throughout the cytoplasm. There was definitely no enrichment of hisactophilin observed within the intracellular clusters of actin, indicating that the hisactophilin is dissociated from actin filaments under these conditions, probably because of a low intracellular pH.

Fig. 6. Distribution of actin-binding proteins relative to F-actin. Aggregation-competent cells treated for 30 minutes with DNP or control cells were fixed and cryosectioned as in Fig. 4. The sections were double-labelled with TRITC-phalloidin for F-actin, and monoclonal antibodies plus FITC-conjugated anti-mouse IgG for the proteins indicated on top of the panels. The crosslinker labelled was α-actinin in (D) and 120 kDa gelation factor (ABP 120) in (E). The anti-myosin antibody used for (A), mAb 396, recognises monomeric as well as filamentous myosin II (Pagh and Gerisch, 1986). Except for (B) and (F), which refer to sections of control cells, all photographs were taken from DNP-treated cells. Phalloidin (phall) and antibody (mAb) label of each section are shown in parallel with phase-contrast images (phaco). Bar, 10 μm.

The heterodimeric capping protein cap32/34 of *D. discoideum* is a homologue of cap Z in the muscle cells of vertebrates (Hartmann et al., 1989, 1990; Caldwell et al., 1989; Casella et al., 1989). In untreated cells of *D. discoideum* cap32/34 was accumulated in the cortex, but substantial labelling was also found in the cytoplasm. The sites of strong cap32/34 labelling in the cell cortex did not always coincide with the sites most intensely labelled for F-actin (Fig. 6F). In DNP-treated cells cap32/34 was strictly colocalised with F-actin, indicating that the actin clusters of these cells consist of capped filaments (Fig. 6G). Because of the nucleating activity of cap32/34 it is possible that this protein is involved in the polymerisation of actin within the cytoplasm of DNP-treated cells.

DISCUSSION

In DNP-treated cells actin appears to be tyrosine phosphorylated at an increased rate

In this paper we have reported on two reversible changes in the actin system that occur in cells that are immobilised by the inhibition of oxidative phosphorylation: (1) a strong increase in the tyrosine phosphorylation of actin, and (2) redistribution of actin filaments into clusters in the interior of the cells. In the following we first discuss these two effects separately and then outline possibilities of a link between them.

What is the mechanism of the increase in tyrosine phosphorylation of actin and how is this increase connected to the inhibition of oxidative phosphorylation? A key observation has been that a blocker of tyrosine phosphatases, PAO, does not produce an increase in tyrosine phosphorylation in the starved cells that have been used for the treatment with DNP (Schweiger et al., 1992). PAO has been found to cause an increase in tyrosine phosphorylation only during growth of the cells in nutrient medium. These results indicate that in the starved cells used, inhibition of tyrosine phosphatases is not sufficient for a significant increase in actin phosphorylation. It can be deduced then that DNP does not act by inhibiting the dephosphorylation of actin but by increasing the rate of its phosphorylation. One possibility would be that a tyrosine kinase is induced or activated. De novo synthesis of a new kinase is unlikely, since cycloheximide does not significantly reduce the DNP response. Activation of a kinase is conceivable, although it seems paradoxical to assume that a kinase becomes more active when the ATP concentration in a cell declines to about 10% of its normal level. Nevertheless, considering cascades of kinases and phosphatases that are regulated by phosphorylation and may have different affinities for ATP, activation of an actin kinase in DNP-treated cells remains a reasonable possibility. Another possibility is that in these cells actin as a kinase substrate is altered in a way that makes it accessible to phosphorylation. A change in the phosphorylatability of actin by protein-protein interaction has been exemplified by Gettemans et al. (1992). These authors have demonstrated that *Physarum* actin becomes phosphorylatable at two threonine residues in a complex with fragmin (Maruta and Isenberg, 1983), an actin capping and severing protein from this slime mould (Hasegawa et al., 1980; Hinssen, 1981). It is conceivable that during reorganisation of the cytoskeleton caused by the inhibition of oxidative phosphorylation, the asso-

ciation of actin with other proteins is altered in *D. discoideum* cells and phosphorylation sites become exposed. These sites may no longer be masked by a protein bound to actin, or they may be exposed by a conformational change in the actin when it interacts with a particular binding protein. In this context it is relevant that an 18mer peptide corresponding to the carboxy-terminal sequence of *Dictyostelium* actin (Vandekerckhove and Weber, 1980) is an excellent substrate for tyrosine kinases of *D. discoideum* (Schweiger et al., unpublished data). Since this sequence encompasses Tyr362 of actin, this residue is a candidate for the phosphorylation site.

An obvious question concerning the tyrosine phosphorylation of actin is whether there are functional consequences that make this regulation relevant to the survival of cells under adverse conditions. The analysis of functional consequences requires a system for efficient phosphorylation in vitro by which phosphorylated and non-phosphorylated actin can be compared. It will also be necessary to separate the effects of tyrosine and serine phosphorylation, which occur together in vivo (Schweiger et al., 1992). Until such a system is established, a working hypothesis can be proposed assuming that the non-tyrosine-phosphorylated actin is the functional form that supports cell movement, and that the phosphorylation of tyrosine residues turns the actin into a resting state. In this state the actin might be better protected against adverse conditions as they exist in cells whose ion pumps and osmoregulatory system are paralysed by the lack of ATP. This working hypothesis is based on the observation that under the three conditions known to increase tyrosine phosphorylation of actin, the cells tend to round up. This occurs in the presence of the phosphatase blocker PAO (Schweiger et al., 1992), upon transfer of starving cells to nutrient medium (Howard et al., 1993), and in response to the inhibition of oxidative phosphorylation as shown in the present paper.

Changes in cell shape and rearrangements in the actin system

The shape changes in DNP-treated cells are initiated by cell surface blebbing. Three-dimensional analysis by confocal microspcopy has indicated that the blebs are formed by rupture of the cortical layer of actin filaments. This may result from an alteration in the actin layer itself by which its rigidity is reduced, but also from an increased internal pressure due to the inactivation of the contractile vacuole, the osmoregulatory organelle of *Dictyostelium* cells. The final stage of rearrangements in the actin skeleton is characterised by large clusters of actin filaments. The proteins that crosslink actin filaments in these clusters have still to be identified. There are several candidates, for instance the 30 kDa bundling protein characterised by Fechheimer et al. (Fechheimer and Taylor, 1984; Fechheimer et al., 1991).

The finding that in the presence of both PAO and DNP a small portion of F-actin remains in contact with the plasma membrane suggests that the changes in the actin skeleton, induced by these drugs, occur within the cortical network of actin filaments rather than at the boundary between actin and the membrane. In order to avoid oversimplification we should point out, however, that the structural changes induced by PAO and DNP are not the same. In the presence of PAO little phalloidin-labelling is observed, and the labelled F-actin forms a shallow rim along the plasma membrane (Schweiger et al., 1992). In the presence of DNP much F-actin is detected by phalloidin-labelling (Fig. 4). Abundant formation of filaments in DNP-treated cells may be caused by the conversion of ATP-actin into ADP-actin. This has a lower critical concentration for polymerisation in the presence of thymosin β_4, which is present in high concentrations in mammalian cells (Carlier et al., 1993). This is consistent with the observation that in epithelial cells ATP depletion causes polymerisation of actin (Hinshaw et al., 1993). However, thymosin β_4 has not been detected in *D. discoideum* cells (M. Schleicher, personal communication). The possibility might be considered that in these cells the phosphorylation of actin has an influence on the equilibrium between G- and F-actin.

The actin clusters in DNP-treated cells are labelled with antibodies against myosin II and against the heterodimeric capping protein cap32/34 (Fig. 6). The myosin probably forms a rigor complex with actin at the low ATP concentration in these cells. The presence of cap32/34 indicates that the actin filaments are in a capped state, which limits their elongation. The strict colocalisation with actin suggests that the capping protein is highly active in DNP-treated cells. Interaction of this protein with actin has recently been shown to be enhanced in vitro by hsc70, a constitutively expressed protein of the heat-shock family (Haus et al., 1993). It will be a goal of future work to find out whether hsc70 plays a role in the rearrangements of the cytoskeleton observed in cells suffering from reduced oxidative phosphorylation.

Relationship between tyrosine phosphorylation of actin and rearrangements in the actin system

The increase in tyrosine phosphorylation of actin in DNP-treated cells overlaps in its time course with the rearrangements of the actin skeleton. The question is whether tyrosine phosphorylation and actin redistribution are coupled to a common effector produced by the inhibition of oxidative phosphorylation, or whether one of them depends on the other. If the structural alteration in the actin skeleton is the primary event, phosphorylatable tyrosine residues might be exposed on actin by changes in its association with other proteins, as discussed before. If activation of a tyrosine kinase is the primary event, the phosphorylation of actin could influence its association with other proteins and thus cause the structural changes observed in the cytoskeleton. However, close comparison of the time required for tyrosine phosphorylation with the changes in actin distribution makes it unlikely that all these changes are initiated by the phosphorylation of actin. The onset of cell surface blebbing after DNP addition is rapid, and an actin cortex is almost instantaneously re-established when DNP is removed (Fig. 4). This contrasts with the periods of time elapsing before actin is substantially tyrosine phosphorylated or, respectively, dephosphorylated (Figs 3 and 4). Further analysis of the sequence of events in DNP-treated cells will require a combination of experiments on actin phosphorylation in vitro with studies on mutants that lack specific actin-binding proteins (Schleicher et al., 1988; Brink et al., 1990; Witke et al., 1992).

Our results show that actin changes its localisation within a few minutes when a cell turns from a motile to an immobile state and vice versa. This means that the spatial organization of the actin system depends on the continuous supply of an energy source. In this respect the actin skeleton in a motile cell

resembles a dissipative structure (Nicolis and Prigogine, 1977) that is built in a chemical system far from equilibrium under dissipation of energy.

The reversible changes described in this paper may be a basis for analysing the energy-dependent protein-protein interactions by which the actin system is kept functional in a state appropriate for mediating cell movement and chemotaxis.

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