Cell division in the unicellular microalga *Dunaliella viridis* depends on phosphorylation of extracellular signal-regulated kinases (ERKs)

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Abstract

In mammalian cells, MAPKs are involved in both stress response (JNK and p38 pathways) and cell proliferation and differentiation [extracellular signal-regulated kinase (ERK)] through protein kinase cascades. Exposure of *Dunaliella viridis* cell cultures to PD98059, a very specific inhibitor of the ERK signalling pathway, resulted in a total arrest of cell proliferation and a complete dephosphorylation of ERK. As shown by flow cytometry analysis of propidium iodide-stained cells, PD98059 stopped mitosis at the G2 phase after the S phase has been completed. Multiple physiological parameters such as cell motility and reducing power generation (NADPH) clearly indicate that the treated cells are wholly viable. Exposure of *D. viridis* to environmental stresses that impair cell division, such as hyperosmotic shock, nitrogen starvation, or sublethal UV irradiation, caused a marked decrease in the phospho-ERK levels as detected by western blot. Two 400 bp polynucleotides from *D. viridis* with high homologies to published sequences of ERK1 and ERK2 were cloned, sequenced, and submitted to GenBank. Northern blot analysis revealed two mRNA bands of ~1.9 kb, consistent with the expected size of ERK proteins (~40 kDa). Sequence analysis showed that they contained several mitogen-activated protein kinase (MAPK) conserved domains, including II, III, VIb, VII, and the double phosphorylation motif. Interestingly, in *D. viridis*, this motif was T*DY* instead of the canonical T*EY*. Based on this finding, ERK plant sequences can be divided into two groups, one termed the T*DY* branch and the other termed the T*EY* branch. The molecular and functional data presented here suggest that ERK is a very ancient signalling pathway and that it was already present in the last common ancestor of all eukaryotic cells.

Key words: Cell division, *Dunaliella*, ERK, environmental stress.

Introduction

Animals have three well-characterized mitogen-activated protein kinase (MAPK) cascades that participate in the cellular response to a wide variety of stress factors. These cascades consist of a series of protein kinases that phosphorylate and activate in a sequential fashion the associated downstream protein kinase. In animals, the p38 and the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) cascades are responsible for stress adaptation, while the extracellular signal-regulated kinase (ERK) cascade is involved in mitogenic stimuli and differentiation (Widmann et al., 1999). In agreement with Hirt (1997) and Tena and Renaudin (1998), MAPKs might also be involved in signal transduction of several environmental factors in plants; thus their survival in a permanently changing environment has required development of sophisticated defence and adaptation mechanisms. In a previous work (Jiménez et al., 2004), the presence of p38-like and SAPK/JNK-like MAPK signalling pathway components was demonstrated in the unicellular microalga *Dunaliella viridis*, and it was shown that operation of...
these cascades is crucial for adaptation and survival of this microalga upon hyperosmotic stress.

MAPKs are involved in signalling both cell proliferation and differentiation in mammals through the ERK phosphorylation pathway (Marshall, 1995). This pathway is structurally similar to, but functionally distinct from the other two MAPK cascades. It has been reported that in animal cells many different stimuli (e.g. growth and neurotrophic factors, cytokines, hormones and neurotransmitters, virus infection, transforming agents, and carcinogens) activate both the ERK1 and ERK2 pathways. For example, senescent cells do not phosphorylate ERK1/2 in human fibroblasts (Park et al., 2002), and activation (phosphorylation) of ERK is required for induced lens cell proliferation and fibre differentiation (Lovicu and McAvoy, 2001); moreover, inhibition of ERK signalling can block the morphological changes associated with lens fibre differentiation. Inhibition of ERK1/2 phosphorylation also reduces mitosis in fetal lung explants, diminishing branch morphogenesis and epithelial proliferation (Kling et al., 2002). Inhibition of the specific upstream MAPK kinase (MAPKK) of the ERK (MEK) reduced the serum-stimulated DNA synthesis and proliferation of Swiss 3T3 cells (Willard and Crouch, 2001). In plants, serum-stimulated DNA synthesis and proliferation of MAPK kinase (MAPKK) of the ERK (MEK) reduced the phosphorylation of ERK, or 183 and 185 of rat ERK2 at the sequence T*EY*, by a single upstream MAPKK. In this work, it is demonstrated that MAPKs of the ERK family are present in the unicellular microalga D. viridis, and that its phosphorylation is required for cell division. Moreover, for the first time, the sequence of a cDNA of an ERK from a unicellular plant is presented.

**Materials and methods**

**Algae culture**

Dunaliella viridis Teodoresco was grown axenically at 2 M NaCl as previously described (Jiménez et al., 2004). Cell density was determined by means of counting viable cells using a haemocytometer.

**Experimental condition**

Cells in their mid-exponential growth phase were used in experiments, and were subjected to various stresses including: (i) osmotic stress; (ii) UV irradiation; and (iii) nutritional stress. Osmotic stress involved increasing the osmotic pressure of the medium by the addition of NaCl to the culture medium to a final concentration of 4 M NaCl. The influence of UV irradiation was studied by exposing cultures of D. viridis to non-lethal doses of 40 mJ cm$^{-2}$ of UV light in the range 200–400 nm using a GS Gene Linker UV chamber (Bio-Rad). Cultures were placed in Petri dishes of 14 cm in diameter, and after UV exposure they were kept under continuous orbital shaking at an irradiance of 150 μmol m$^{-2}$ s$^{-1}$ of visible light (400–700 nm). Samples were withdrawn at the described times. For nutritional stress, cells were centrifuged at 1500 g for 10 min, and resuspended in nitrate-free medium; this procedure was repeated twice.

**Treatment with inhibitors**

Appropriate volumes of concentrated PD98059 (Calbiochem, La Jolla, CA, USA) inhibitor solution in dimethylsulphoxide (DMSO) (>1000×) were added to a final concentration in the culture medium of 20 μM. This is a selective and cell-permeable inhibitor of the ERK MAPKK (MEK), that acts by inhibiting the phosphorylation of the ERK and the subsequent phosphorylation of downstream substrates.

**Western blot analysis**

At fixed times, 50 ml of culture were centrifuged at 1500 g for 10 min. The pellets were resuspended in 1 ml of MAPK lysis buffer (Capasso et al., 2001), and treated as previously described (Jiménez et al., 2004). Protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA). SDS-PAGE, electroblot to poly(vinylidene difluoride) membrane, and immuno-detection were performed as previously described (Capasso et al., 2001; Jiménez et al., 2004). Antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibody detects endogenous levels of p44 and p42 MAP kinase (ERK1 and ERK2) only when catalytically activated by phosphorylation at Thr202 and Tyr204 of human ERK, or Thr183 and Tyr185 of rat ERK2. The antibody

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Cell viability

Cell viability measurements were performed by means of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] colorimetric assay, as previously described (Capasso et al., 2003).

RNA purification and cDNA synthesis

Mid log cultures of *D. viridis* were harvested by low speed centrifugation (1500 g, 10 min) and cell pellets were lysed in 10 ml of TRIZOL (Life Technologies Inc.) with a Polytron (Dispersing and Mixing Technology); phase separation was obtained by the addition of chloroform (1:5 v/v) and centrifugation at 12 000 g for 15 min at 4 °C. The aqueous phase was collected, precipitated with isopropanol (1:2 v/v), and centrifuged. The resulting pellet was washed twice with 75% ethanol and air-dried. RNA was further purified using the RNeasy protocol (Qiagen) as per the manufacturers’ protocol. Nucleic acid quantity and purity were assessed by UV spectrometry (using the absorbance at 260 nm and the 260/232 nm ratio, respectively). RNA integrity was assessed by capillary electrophoresis using the 28S:18S rRNA ratio. Total RNA (5 μg) was reverse transcribed to cDNA with Superscript (Life Technologies Inc.) using random hexamers.

Primer design

To design the ERK1 amplification primers, protein and nucleotide sequences from the following organisms were retrieved from GenBank and analysed by protein CrustalW alignment: *Medicago sativa* (accession no. J024336), *Blumeria graminis* (accession no. AF301165), *Giardia intestinalis* (accession no. YI49274), and *Mus musculus* (accession no. NM_011952). Several conserved regions were detected, in particular, the amino acid sequences VAIKKI and YVATRWYR were used to design the following degenerated PCR amplification primers, the following GenBank data were utilized: (accession no. X82646), and (accession no. NM_139021). The conserved amino acid sequences TDAQRTFREI were designed: ERK1-F1 5′-GAGCGAGCAAGACCATAGTC-3′ and ERK1-R1 5′-GGSGCCCKRTACCACRGHTG-3′. For the ERK2 amplification primers, the following GenBank data were utilized: *Giardia intestinalis* (accession no. YI49275), *Plasmodium falciparum* (accession no. X82646), and *Homo sapiens* (accession no. NM_139021). The conserved amino acid sequences TDAQRTFREI were reverse transcribed to cDNA with Superscript (Life Technologies Inc.) using random hexamers.

PCR and cloning

cDNA samples were amplified using the above-described primers and the Advantage cDNA PCR kit (Clontech) in a Mastercycler gradient apparatus (Eppendorf). Products were analysed by 2% agarose gel electrophoresis, and bands of interest were detected by ethidium bromide staining and excised. DNA was extracted with the RNeasy protocol (Qiagen) and transfected into JM109 competent cells (Promega). Insert-containing clones were selected by ampicillin resistance and blue/white test. Plasmid DNA from white colonies was obtained using a QIAprep system (Qiagen) and analysed by agarose gel electrophoresis upon digestion with *Sal*I. Relevant clones were sequenced with the T7 and SP6 primers.

Northern blot analysis

RNA isolated as described above was separated by agarose gel electrophoresis as described by Liu and Chou (1990) and transferred to a nylon membrane by using the Genie blotter (Idea Scientific) as suggested by the manufacturer.

The primer DERK1Sp-R 5′-GTCCTGTCAGAAGATGCGG-GATTTGTCGG-3′ was used as an ERK1-specific probe; this sequence is complementary to the partially cloned *D. viridis* ERK1 mRNA (see Results). The primer DERK2-Pb 5′-CTGGGACACT-GAGCGACGAGACATAGTC-3′ was used as an ERK2-specific probe; this sequence is complementary to the partially cloned *D. viridis* ERK2 mRNA (see Results). Primers were radiolabelled with [γ-32P]ATP using the enzyme polynucleotide kinase and purified by spin desalting on Sephadex G-25 columns. Prehybridization and hybridization were carried out in rapid hybridization buffer (Amersham) at 42 °C for 2 h. Blots were washed three times in 1.5× sodium chloride–sodium citrate–EDTA (SSPE)–0.1% SDS at room temperature, and three times in 0.75× SSPE–0.1% SDS at 42 °C, exposed to X-ray film, and the results were analysed as described above for western blot. Equal loading in northern blots was verified by staining with ethidium bromide and analysis of RNA band intensity.

Detection of cell cycle phase by means of flow cytometry

The cell cycle phase of *D. viridis* was detected by means of propidium iodide staining and flow cytometry analysis. Cells were harvested by centrifugation (1500 g, 10 min) and the pellets were suspended in 4% paraformaldehyde prepared in 2 M NaCl. Cells were then resuspended in 0.5 ml of Na-citrate (50 mM)+200 mg of RNase+saponin (0.1% v/v) and incubated for 2 h at 37 °C. After this period, 0.5 ml of Na-citrate (50 mM)+2 mg of propidium iodide+saponin (0.1% v/v) were added, and incubated for 30 min at room temperature. Flow cytometry analysis was performed as described in Sazer and Sherwood (1990) using a Becton Dickinson FAC-Scan system.

Statistical analysis

All data are present as mean ±SD. One-way analysis of variance (ANOVA) test (SPSS 11.5) was performed to evaluate statistically significant changes in metabolites between the study groups. *P*-values <0.5 were recognized as statistically significant.

Results

Cell division is abolished in the presence of ERK inhibitor

The first objective of this study was to evaluate the possible physiological implications of an active ERK cascade in *D. viridis*. For this, cultures of the microalga were inoculated in either the presence or absence of the specific inhibitor of ERK phosphorylation, PD98059. Growth of *D. viridis* was almost completely inhibited in the presence of 20 μM of the inhibitor (Fig. 1), both when it was added to the growth medium upon dilution (Fig. 1A), and when it was added 48 h after inoculation of the cultures, at the onset of the exponential phase of growth (Fig. 1B). As can be seen, only one division cycle of the cells occurred after the addition of the inhibitor. Similar results were obtained at different initial culture densities (not shown). Cell density was much lower in the cell
cultures treated with the ERK inhibitor. However, the inhibitor did not affect cell viability, since cell motility (not shown) and cell capacity for generating reducing power (in the form of NADH and NADPH) were not affected (Fig. 2). Interestingly, while *Dunaliella* cells were not dividing in the presence of the ERK inhibitor, they continued to generate reducing power. A significant drop in cell viability was detected after 4–5 d in the presence of the inhibitor, that might be related to the average life span of non-dividing *Dunaliella* cells. It is noteworthy that PD98059 is a very selective and cell-permeable inhibitor of the ERK pathway and at the concentration used does not appear to affect alternative signalling pathways.

Propidium iodide staining was employed for DNA-specific fluorescence by means of flow cytometry and showed that cells incubated in the presence of 20 μM of the ERK inhibitor developed twice the amount of DNA as compared with the control cells (Fig. 3). The analysis of control cultures of *D. viridis* demonstrates that the majority of the cells contain a single copy of DNA and indicates that the G₁ phase is the longest duration in the cell cycle. *Dunaliella* cells exposed to ERK inhibitor double their DNA content during S phase, and cell cycle progression is arrested at G₂ phase and does not progress further into mitosis. Therefore, ERK phosphorylation is crucial for the continuation of cell division in *Dunaliella* cells.

**p44 and p42 proteins are phosphorylated in dividing cells**

Once the possible implication of an ERK-type MAPK in *Dunaliella* cell division was shown, the second objective of this work was to detect the presence of ERK-like proteins in this microalga, and to investigate its relationship to cell division. For this purpose, cell extracts were assayed with specific antibodies that detect endogenous levels of p44 and p42 MAPK (ERK1 and ERK2) only when catalytically activated by phosphorylation at the threonine and tyrosine in the amino acid sequence T*E*/DY*. This sequence is absolutely conserved in all organisms in which ERK has been cloned and sequenced. Figure 4A shows two proteins of 42 kDa and 44 kDa that were phosphorylated in cells of *D. viridis* while actively dividing. The apparent molecular weights of these bands are similar to those of the phosphorylated ERK1/2 from mammals. Band analysis indicated that maximal phosphorylation occurred during days 1–5, representing the exponential phase of growth of the cultures. Phosphorylation was highly reduced at the stationary phase of growth and when cultures entered a clear decline including cell death (days 9 and 12, respectively). Specific antibodies against the non-phosphorylated form of ERK1/2 (p42 and p44) did not cross-react with any proteins from *D. viridis* extracts (data not shown).

**Phosphorylation of p44 and p42 proteins is abolished in the presence of ERK inhibitor**

Cell extracts of cultures of *D. viridis* inoculated in either the presence or absence of the specific inhibitor of
mammalian ERK phosphorylation—PD98059, were assayed with the antibodies against the mammalian phosphorylated form of ERK1 and ERK2. It should be noted that the inhibitor PD98059 acts to inhibit the upstream kinase (MKK1/2) from ERK1/2 and thereby prevents phosphorylation of ERK1/2. Figure 4B demonstrates that in the presence of the ERK inhibitor, dephosphorylation of both p44 and p42 proteins occurs in cell cultures. These ERK-like proteins were strongly phosphorylated at day 1 (after inoculation with cultures in their mid-log phase of growth); however, no phosphorylated protein was detected in Dunaliella cell extracts at day 4. In contrast, increasing phosphorylation occurred between day 1 and day 5 in control cultures, coinciding with the exponential growth phase (Figs 1A, 4A). Thus, there is clearly a direct correlation between the inhibition of cell division and the level of phosphorylation of ERK-like proteins in D. viridis.

Dephosphorylation of the ERK-like proteins is induced by stress

Several kinds of environmental stresses are known to inhibit cell division transiently in Dunaliella (e.g. hyper-osmotic stress). In this study, the influence of specific stresses on phosphorylation of p44 and p42 in D. viridis was evaluated. Figure 5A shows that the level of...
phosphorylation of both proteins was significantly reduced after hyperosmotic stress (2 M → 4 M NaCl) and in response to non-lethal UV stress (Fig. 5B). Moreover, nitrogen starvation resulted in an almost total dephosphorylation of both proteins over a more extended period of time (Fig. 6). Dephosphorylation of the p44 and p42 ERK-like proteins coincided with cell division arrest.

Cloning of ERK1- and ERK2-like mRNAs

When total RNA extracted from Dunaliella cultures was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with the ERK1-specific primers described in the Materials and methods, an amplicon of the expected size (~450 bp) was detected upon agarose gel electrophoresis. Similarly, an amplicon of ~400 bp was detected after RT-PCR with the ERK2-specific primers. These fragments were extracted from the gel, ligated in the pGEM-Teasy vector, and cloned. Multiple plasmid preparations were obtained and those containing the expected size insert were sequenced from both ends using T7 and SP6 primers. A 446 bp and a 395 bp polynucleotide sequence were obtained that unambiguously code for a 148 polypeptide and a 131 polypeptide sequence. These sequences have been submitted to GenBank under accession numbers AY628422 and AY630341 for ERK1 and ERK2, respectively. As shown in Fig. 7, these sequences have a high degree of homology with ERKs from a wide array of organisms. As depicted in Fig. 8, several canonical serine, threonine, or tyrosine protein kinase domains (Hanks and Quinn, 1991) were identified, in particular, domains II, III, VIb, and VII. Most importantly, the Dunaliella ERK sequences contain the double phosphorylation motif characteristic of MAPKs in general and of ERK in particular T=E/DYVATRW (JM Capasso, unpublished results). To verify the existence of these mRNAs, RNA was extracted from algal cultures and analysed by northern blot using specific D. viridis ERK1 and ERK2 oligonucleotide probes. As shown in Fig. 9, both probes detected bands of similar size (1.9±0.2 kb) that are consistent with a 400 amino acid protein. However, the band intensity of ERK1 is at least one order of magnitude stronger than that of ERK2, corroborating the western blot results.

Fig. 5. Time dependence of ERK phosphorylation level and cell density following hyperosmotic and UV stress. Both (A) hyperosmotic stress (2 M → 4 M NaCl) and (B) non-lethal UV stress (40 mJ cm⁻²) clearly reduced the level of phosphorylation of ERK-like proteins in D. viridis cultures. This reduction in phosphorylated ERK proteins is correlated with cell division arrest. Data points represent the mean and SEM, n=6. Shown is a representative western blot (50 mg of protein per lane), and densitometry measurements of two independent experiments are also depicted.

Fig. 6. Comparison of the effect of nitrogen starvation stress on the phosphorylation of ERK-like proteins and cell density in D. viridis as a function of incubation time. Nitrogen depletion from the medium resulted in an almost total dephosphorylation of both ERK proteins, coinciding with total cell division arrest. Data points represent the mean and SEM, n=6. Shown is a representative western blot (50 mg of protein per lane), and densitometry measurements of three independent experiments are also depicted.
Levels of ERK1 mRNA do not change

Even though it was not possible to establish potential variations in the non-phosphorylated ERK proteins in Dunaliella as a result of a lack of cross-reactivity with the mammalian derived antibodies, the data resulting from cloning of both MAPKs in Dunaliella clearly show that the levels of ERK mRNA do not change in batch cultures over a period of 21 d (Fig. 10); this is found in the face of a clear decline in culture viability in the latter stages.

Discussion

In this study, it was demonstrated that cell division in the unicellular microalga D. viridis is almost completely inhibited by a very specific and selective inhibitor of ERK phosphorylation in mammals. Under these conditions, cell viability was not affected. These results clearly indicate that an ERK-type MAPK pathway is involved in cell proliferation in D. viridis. Moreover, the use of specific antibodies against the dual-phosphorylated form of human ERK (ERK1 and ERK2) reveals the presence of two proteins of 44 kDa and 42 kDa in actively dividing cells of D. viridis similar in molecular weight to ERK1 and ERK2 from mammals. Maximal phosphorylation occurred during the exponential phase of growth of the cultures. Moreover, the ERK inhibitor clearly eliminated additional phosphorylation of ERK, resulting in dephosphorylation of existing ERK proteins during continued incubation of D. viridis. Therefore, it may be concluded that phosphorylation of an ERK-type MAPK in the unicellular microalga D. viridis is crucial for cell division. In addition, it was demonstrated that various stress conditions, which inhibit cell division, also significantly reduce the level of phosphorylation of ERK proteins. To our knowledge, this is the first report that relates cell division and MAPK phosphorylation in photosynthetic unicellular organisms.
ERKs have been reported to be involved in proliferation and differentiation in different mammalian cells (Marshall, 1995), and in plants there is ample evidence for their role in stress response (Coronado et al., 2002). Based on sequence analysis, the presently known plant MAPKs are most similar to ERKs (Mishra et al., 2006; Zhang et al., 2006), even though increasing evidence indicates that those kinases are also involved in various forms of biotic and abiotic stress responses, and not only in cell proliferation and division control. In a previous work (Jiménez et al., 2004), the involvement of p38-like and JNK-like MAPKs in stress response in D. viridis was demonstrated; however, no sequence analysis of those kinases has yet been reported. A variety of genes encoding MAPKs have been identified in plants (e.g. alfalfa, Arabidopsis, parsley, pea, petunia, and tobacco), and all amino acid sequences of the presently known plant homologues are most similar to the ERKs. However, some expressed sequence tags and sequences derived from the Arabidopsis genome project are clearly distinct from ERKs (Ligterink, 2000). From an analysis of the sequence homology of the predicted amino acid sequences, plant ERK-like MAPKs can be further divided into four distinct subgroups (Ligterink, 2000), and at least some of the MAPKs of subgroup III are involved in cell cycle regulation (Bögø et al., 1999, 2000). The first evidence that protein phosphorylation regulates mitosis in plant cells was the finding that metaphase was prolonged when cells were treated with the protein kinase inhibitor K252-a (Wolniak and Larsen, 1995). Several recent works provide evidence of the activity of ERK-like MAPKs in plants also involved in cell division and differentiation. Jonak et al. (1993) found that a plant homologue of MAPK is expressed in alfalfa proliferating cells, while Duerr et al. (1993) reported the recovery of a full-length cDNA clone encoding a MAPK from alfalfa of 44 kDa with characteristics of ERK that may play a role in the mitogenic induction of symbiotic root nodules on alfalfa by Rhizobium signal molecules. Treatment of Arabidopsis roots with auxin (a plant hormone involved in plant growth and development) transiently induced increases in protein kinase activity with characteristics of mammalian ERK-like MAPKs (Mockaitis and Howell, 2000). In addition, several studies were aimed at investigating whether MAPKs are involved in G1 phase control. Mizoguchi et al. (1994) found that cell cycle progression in cultured tobacco cells was blocked by omitting auxin from the medium, while Wilson et al. (1997) reported that cultured tobacco BY2 cells were arrested in the G1 phase of the cell cycle by omitting phosphate from the medium. After adding back auxin or phosphate, the cells resumed proliferation. In this work, it has been shown that ERK phosphorylation is crucial for cell cycle progression in D. viridis, and that non-dividing cells have their cell cycle blocked in G2 phase, and demonstrate twice the amount of DNA as compared with control, dividing cells. Interestingly, other researchers have shown that activation of an ERK-type MAPK in plants occurs in response to stress;
however, no direct correlation of phosphorylation of those proteins and cell proliferation was shown. Samuel et al. (2000) reported that brief exposure to ozone led within minutes to activation of an ERK-type MAPK (~46 kDa) in tobacco. Novikova et al. (2000) found increased MBP phosphorylation in wild type A. thaliana treated with ethylene (a plant hormone involved in fruit ripening and in stress response), and that a polypeptide band of 47 kDa cross-reacted with both ERK1 and phosphotyrosine antibodies. Huang et al. (2002) report isolation of a MAPK from rice that may function both in the stress signalling pathway and in panicle development. All these works help to understand that ERK phosphorylation is crucial for plants’ response to stress.

In contrast, in the unicellular microalga D. viridis, ERK1 and ERK2 were rapidly dephosphorylated in response to stress, coinciding with cell division arrest. Other authors have demonstrated that ERKs are also mostly responsible for cell proliferation control in plant cells. The present data reinforce this hypothesis, since phosphorylation of ERK-type proteins is necessary for cell division in a unicellular microalga D. viridis. ERK1 and ERK2 were rapidly dephosphorylated in response to stress, coinciding with cell division arrest. Other authors have demonstrated that ERKs are also mostly responsible for cell proliferation control in plant cells. The present data reinforce this hypothesis, since phosphorylation of ERK-type proteins is necessary for cell division in a unicellular microalga D. viridis.

Cloning of ERKs from D. viridis showed a high degree of identity with those from a very wide variety of organisms, ranging from protozoa to human. The sequence of ERK1 from Dunaliella indicates that the characteristic TEY motif is not universal among all organisms. In fact, the TEY motif is found in all animals, protozoa, fungi, and most plants in which this MAPK has been cloned and sequenced. However, a group of plants shows the TDY motif, among them Dunaliella, Zea mays, Selaginella, Arabidopsis, Triticum, and Oryza. The present data enable a cladogram to be proposed based on the sequence of ERK1 (Fig. 11), in which two different branches for plants appear, one containing the characteristic motif TEY and including the microalga Chlamydomonas (as well as animals, fungi, and protozoa) and another plant branch characterized by the presence of the TDY motif that includes Dunaliella. The apparent evolutionary divergence between the two closely related green algae, Chlamydomonas and Dunaliella, is surprising. However, since this comparison is based on about one-third of the ERK1 or ERK2 coding sequence (even thought they are the most conserved part of the mRNAs), it is likely that the complete sequence of the
corresponding mRNAs may change the cladogram. On the other hand, the database has been searched and the sequence TEY in Arabidopsis and TDY in Chlamydomonas sequences was not found. Since the complete genome of these organisms is not available, the possibility cannot be discarded that there is another set of ERK-like genes with this motif (i.e. TEY versus TDY). One explanation may be that the switch from TEY to TDY involves only a single nucleotide substitution in the third position of the codon. Since that mutation will lead to a conserved amino acid substitution, it is possible that this single nucleotide substitution may have occurred during plant evolution.

In conclusion, cell division in the microalga D. viridis is crucially dependent on phosphorylation of ERK MAPKs. Cell division control seems to follow similar patterns of signal transduction in unicellular and multicellular organisms. Analysis of ERK genes indicates that ERK sequences are strongly conserved from unicellular microalgae to higher plants and mammals, suggesting that the origin of the ERK pathway pre-dates the divergence of the plant and the fungi/animal lineages.

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