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Variations of Phosphorylation of Protein Membrane Components in Pea Root Meristems During Germination

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Abstract. A number of endogenous substrates have been found in the 27,000*g* meristematic root membrane fraction of pea seedlings. The identities of such proteins remain unknown, but their phosphorylation patterns change during germination. The presence of different protein kinase activities, or different protein phosphatase activities besides the occurrence of metabolic variations in membranes, have been indicated as key factors that could act simultaneously to give variations in protein phosphorylation pattern during germination. The absence, previously reported, of such protein kinase activity in membranes extracted from differentiated root tissue has been related to a change in protein composition. This fact leads to the hypothesis that protein phosphorylation of such membranes could have a physiological role at least in meristematic tissues. However, no clear-cut indication of which physiological role such protein phosphorylations fulfill emerges from present data. Nevertheless, the coincidence of onset of DNA synthesis and variation in protein phosphorylation pattern of these membranes suggests that these two events could have some physiological dependence.

The importance of membrane metabolism during germination has been recognized, but little is known about the activity of the enzymes concerned with membrane metabolism during the process (Mayer and Marbach 1981). Phosphodephosphorylation of protein components of membranes affects membrane permeability in animals (Rubin and Rosen 1975), but its biochemical function in plants remains largely to be determined (Ranjeva and Boudet 1987). In cultured plant cells the activity of a protein kinase associated with cell membranes is known to change during cell proliferation (Bocher et al. 1985).

We reported the presence of protein kinase activity in a 27,000*g* membrane

fraction purified from meristematic root tips (0–2 mm). Such an activity was estimated by phosphorylation of endogenous substrates (Chiatante et al. 1987). This protein kinase activity changed during germination and was absent from the same 27,000*g* membrane fraction when it was extracted from fully differentiated tissues, although its physiological role remained unknown. The membranes used in our previous work to test protein kinase activity were prepared from germinating seedlings, and it is known that membranes during germination are in a particular dynamic state (Mayer and Marbach 1981). Therefore, the reported variations in this protein kinase activity during germination and its tissue distribution could result from variations in endogenous substrate availability as a consequence of the dynamic state of this membrane system rather than regulation of enzymic activity. Before investigating the physiological role of our membrane-bound protein kinase activity, we thought it necessary to achieve a better understanding of the situation of its endogenous substrates.

In this paper we analyze the overall protein composition and the relative phosphorylation patterns of this 27,000*g* membrane system during germination and in tissues at different states of differentiation. The physiological importance of the presence of protein phosphorylations in such a membrane fraction is discussed.

Materials and Methods

Plant Material

Pea seeds (*Pisum sativum* cv. Lincoln) were surface sterilized by 1% sodium hypochlorite for 30 min, rinsed for 4 h, then grown in the dark at 25°C for variable periods of time on Agriperlite. Seeds to be utilized for in vivo embryo experiments were first surface sterilized as usual and then rinsed for 12 h before detaching the embryos from the cotyledons by hand, using a scalpel blade. Owing to the extensive handling of these seeds, embryos were again sterilized for 7 min in 0.2% sodium hypochlorite before being placed (under laminar flux) in 5 ml of the sterile basal medium of Joy and Folke (1965). The embryos in these sterile conditions showed for 4 days a substantial growth delay with respect to the control grown normally.

Membrane Preparation

Meristematic root tips (0–2 mm from the tip) were cut from 3-day-old seedlings and used for standard membrane preparation. The roots were homogenized in a glass homogenator in 50 mM Tris-HCl, pH 7.8, 1 mM DTT, and 15% w/v sucrose; 10 mM NaF and 5 MgCl were present where stated. After filtration through Miracloth (two layers), the homogenate was centrifuged at 27,000*g* for 30 min. The pellet obtained was gently resuspended in the same homogenate buffer and used as crude membrane preparation. During membrane extraction the temperature was strictly maintained at 4°C. Two hundred and fifty meristematic tips yielded 5 mg of total proteins in the pellet precipi-

tated at 27,000*g*. Protein concentration was measured according to Lowry et al. (1951).

In Vitro Membrane Phosphorylation

Membranes were routinely phosphorylated by incubations in a mixture containing, in a final volume of 100 µl, variable amounts of total proteins, 0.5 mM ATP, 2×10^6 cpm (γ-^{32}P) ATP and appropriate concentrations of $MgCl_2$, $MnCl_2$, EDTA in 50 mM Tris HCl, pH 7.8. NaF was included in the incubation mixture. Incubation was carried out at 37°C for variable periods of time and stopped by adding appropriate volumes of buffer to give a final mixture containing 4% SDS, 6.6% 2-mercaptoethanol, 15% glycerol, 80 mM Tris HCl, pH 7.8, and traces of bromophenol blue. Aliquots of the final mixture were SDS-electrophoresed, and gel strips were used to estimate the phosphorylation of endogenous substrates by liquid scintillation.

In Vivo Membrane Phosphorylation

After appropriate periods of time, the culture medium was removed and substituted with 3 ml of fresh sterile medium containing 0.12 mCi/ml of γ-^{32}Pi in which the phosphate concentration had been reduced to 10^{-5} M. The embryos were incubated for an additional 6 h at 25°C, then rinsed (three times within 30 min) with homogenation buffer. After incubation the meristematic root tips were cut with a scalpel and used as fresh material for routine membrane preparation. Seedlings normally grown for 3 days were also used alternatively to embryos for protein phosphorylation pattern analysis in vivo. In these cases batches of 100 cotyledon-free seedlings were placed in a standing position in a slim glass beaker with their roots deeply submerged in labeling solution throughout the incubation. The incubation mixture was similar to the one used for embryos, and the other conditions were similar. At the end of the incubation, the seedlings were rinsed and treated as usual for membrane preparation from root tips.

Electrophoresis of Phosphorylated Proteins

SDS gel electrophoresis was based on methods of Weber and Osborn (1969) and Davies and Stark (1976). Electrophoresis was carried out at 4°C with constant current of 50 mA until the tracking dye (bromophenol blue) had reached the bottom of the gel (17 h). A 10% polyacrylamide gel slab ($20 \times 15 \times 0.1$ cm) was routinely used, and molecular weight markers were placed in the same slab together with the sample. Gels were stained for 4 h with 0.025% of Coomassie blue in 25% isopropanol and 10% acetic acid, destained in acetic acid (10%).

Radioactivity Detection

Gel strips including electrophoresed proteins were bottled in vials containing a scintillation cocktail and counted in a Packard model 3320 liquid scintillation spectrometer.

Cytofluorimetry

Cytofluorimetric determinations were carried out as described previously (Sgorbati et al. 1986). Briefly, after fixation of the roots in 4% formaldehyde in Tris buffer, nuclei were released by crushing the terminal 2 mm and stained in suspension with DAPI (final concentration 5.6 μM in buffer). The fluorescence intensity of each single nucleus was measured by a Leitz MPV compact microscope photometer. Three hundred nuclei were scored for each sample.

Results

Since protein composition and relative phosphorylation patterns of 27,000*g* membranes should be examined at different times of germination and in tissues at different states of differentiation, it seemed opportune to us to investigate the physiological state of the material used as membrane source. We used a rapid cytofluorimetric procedure (Sgorbati et al. 1986) to determine DNA content in meristematic nuclei stained with DAPI (Fig. 1). This analysis was repeated on nuclei extracted from root tips (0–2 mm) of pea seedlings after 8, 24, 48, and 72 h of germination. After 8 h of germination, the fluorescence histogram showed two distinct classes of nuclei with 2C and 4C DNA content, which represented meristematic cells still blocked (as in the quiescent embryo) in the G1 and G2 phases of the cell cycle, respectively.

After 24 h some nuclei with intermediate DNA content appeared; they were the first nuclei progressing from the G1 phase to the S phase of the cell cycle. These data showed that in our material, as in other pea cultivars (Schatt et al. 1985), DNA synthesis in the root tip started only after 24 h of germination. In later hours, the normal condition of a proliferating meristem with a considerable fraction of S cells was established (intermediate part of the histogram).

With a better understanding of the physiological state of the tissues we used as membranes source, we investigated at different times of germination the protein composition and the relative pattern of phosphorylation of the 27,000*g* membrane pellet prepared from meristematic root tips. Batches of 120 meristematic root tips were cut from seedlings at various intervals. Each batch was treated separately for preparation of a 27,000*g* membrane pellet, which was assayed for endogenous protein kinase activity. Electrophoresed protein components of these membrane pellets were stained with Coomassie blue to analyze their overall pattern (Fig. 2) and were subsequently autoradiographed to examine their relative phosphorylation pattern (Fig. 3). The overall protein pattern in 27,000*g* membrane pellets did not change during germination (Fig. 2), although two bands in the range of 205 kDa disappeared after 17 h. The

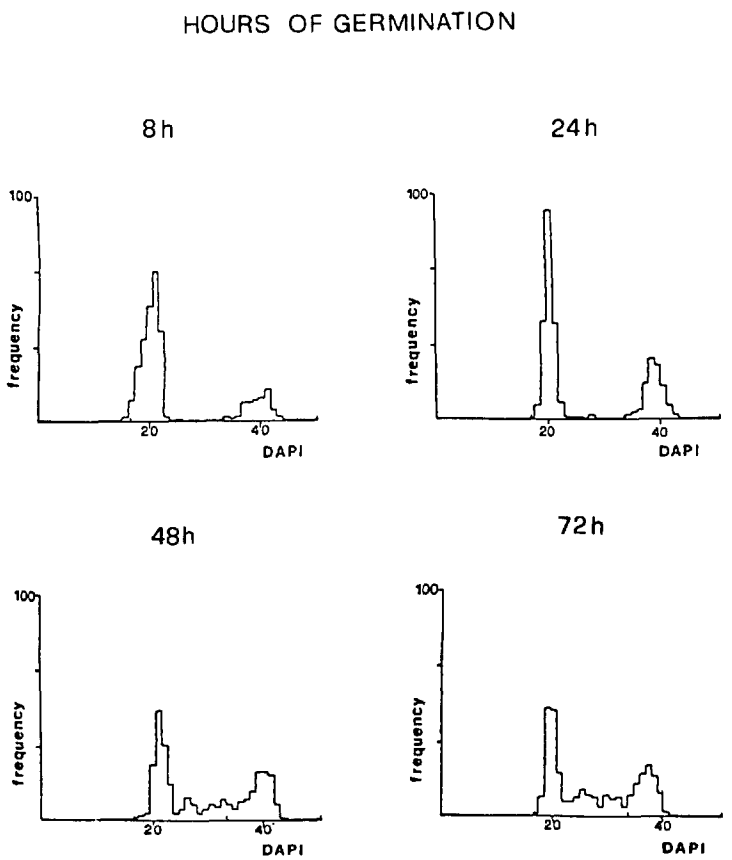

Fig. 1. DAPI fluorescence distributions of nuclei from pea root meristems at different hours from the beginning of imbibition. Abscissa: DAPI fluorescence (mV).

amount of material available from each of the many individual polypeptide bands was too low to permit any detailed quantitative comparison. However, the total protein content values found in the 27,000*g* membrane pellets after 8 and 17 h were similar and were higher than those obtained after 28, 53, 72, and 96 h; after 28 h this value remained constant (data not shown).

We suggested previously that the reason for such a protein concentration decrease could be the decrease in the number of cells per tip during early hours of germination (Chiatante et al. 1987). The correspondent autoradiograms of ^{32}P-labeled proteins in Fig. 3 showed that enormous differences existed between protein phosphorylation patterns at different times of germination. After 8 h of germination, the number of ^{32}P-labeled proteins was very high. They spanned across a molecular weight range of more than 205 kDa to around 14 kDa; at least 20 heavily labeled bands could be counted. A similar distribution of labeled proteins was also found after 17 h of germination, although the relative amount of phosphorylation of individual bands decreased. An interesting change in phosphorylation patterns was found with endogenous substrates ex-

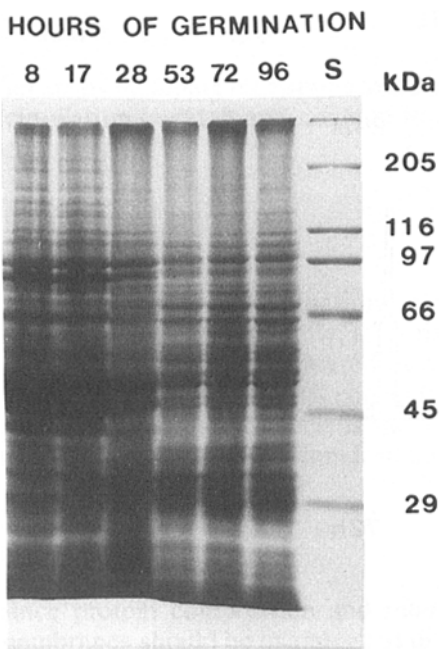

Fig. 2. SDS-PAGE of proteins from a 27,000*g* membrane fraction obtained from root meristematic tips of seedlings at different times of germination. S lane indicates molecular weight markers.

tracted from meristematic tips of seedlings germinated for 28 h. In fact, a considerable fall in the number of total bands and a general decrease in the level of phosphorylation were found in all the bands but one at an apparent molecular weight of 62 kDa. This protein seemed to increase slightly its relative level of phosphorylation in respect to the one shown by the same protein at earlier hours of germination (Table 1).

The phosphorylation pattern of labeled proteins after 53 h of germination showed a further reduction in the number of labeled bands with three major bands at 62, 50, and 32 kDa, respectively. The band at 62 kDa was the most pronounced one. This kind of phosphorylation pattern remained constant after 72 and 96 h of germination.

We reported previously that the protein kinase activity extracted from meristematic root tips of 3-day-old seedlings showed a maximum of activity after 45 sec of incubation and was activated in the presence of high concentrations of Mg^{2+} and Mn^{2+} in the incubation mixtures. Again it seemed important to look for pattern variation in the endogenous substrates of this protein kinase activity. In this regard, we found that a number of bands in the range of 62–32 kDa always phosphorylated (Fig. 4), although their phosphorylation increased during the first 45 sec of incubation before it started to decrease. Specifically, the bands at 59, 43, and 37 kDa disappeared after 8 min (Fig. 4). As regards the metal ion effect, we found an unspecific increase in all the bands with respect to the control when 2 mM Mn^{2+} and Mg^{2+} were present in the incubation mixtures (data not shown). More interestingly, we found that by increasing the concentration of these metal ions to 5 mM, the phosphorylation of bands at molecular weights of 59, 54, and 50 kDa (Fig. 5) increased specifically.

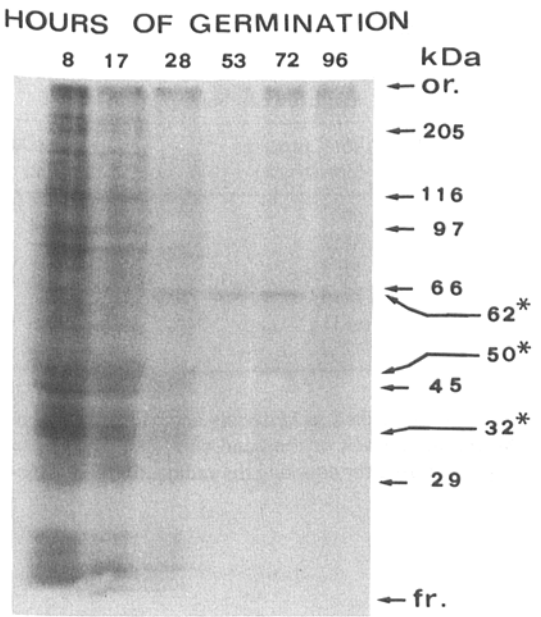

Fig. 3. Autoradiography of SDS-PAGE of phosphorylated proteins from 27,000*g* membrane fractions obtained from root meristematic tips of seedlings at different times of germination. The conditions of phosphorylation are described in Materials and Methods. On the right, the positions of molecular weight markers. Longest arrows indicate proteins phosphorylating at the latest time of germination.

This work also compared the protein phosphorylation patterns obtained with 27,000*g* membranes prepared from meristematic tissues (0–2 mm from the tip) with the 27,000*g* membranes prepared from adult tissue (2–20 mm). The rationale was again that the absence of protein kinase activity in membranes from adult tissues could be due to lack of endogenous substrates rather than enzymatic activity. In this regard, we did not find any labeled band in 27,000*g* membranes prepared from adult tissues. Moreover, analysis of the Coomassie pattern showed a very substantial difference between the protein composition of these membranes and the correspondent ones prepared from meristematic tissues (data not shown).

We showed previously that this membrane system presented protein phosphorylations also in vivo (Chiatante et al. 1987), but it is known that protein phosphorylation patterns obtained in vitro might not reflect the phosphorylation events that actually occur within the tissue (Veluthambi and Poovaiah 1986). Therefore, we decided to analyze the phosphorylation patterns of membranes phosphorylated in vivo. Two different approaches were adopted to study the phosphorylation pattern of endogenous proteins in vivo. The first (Fig. 6A) used as material source for meristematic membrane preparations the root tips (0–2 mm) of embryos grown for 2 days in liquid medium and pulsed with ^{32}Pi (see Materials and Methods). We found that a number of protein components of the 27,000*g* membrane pellet in a range from 205 kDa to 26 kDa were labeled. To exclude the unbound ^{32}Pi, the proteins with a molecular weight smaller than 26 kDa were removed together with the dye front. The bulk of radioactivity found at the origin of the resolving gel was due to difficulties in the complete solubilization of 27,000*g*-labeled membrane pellets and

Table 1. Radioactivity bound to the total proteins present in the gel track or to the protein at an M_r of 62 kDa during germination.

Hours of germination	Protein conc. loaded in the gel (mg/track)	Radioactivity bound	
		Total proteins (cpm/track)	62 kDa (cpm)
8	0.43	6046	ND
17	0.43	3852	ND
28	0.36	1757	111
53	0.19	1216	120
72	0.19	811	137
96	0.14	780	100

ND: Not determined.
The incubation conditions of 27,000*g* membranes are described in Materials and Methods and in the legend to Fig. 3. After electrophoresis the complete track or the band corresponding to the protein at an M_r of 62 kDa was cut off from the gel and used for counting the radioactivity incorporated by liquid scintillation.

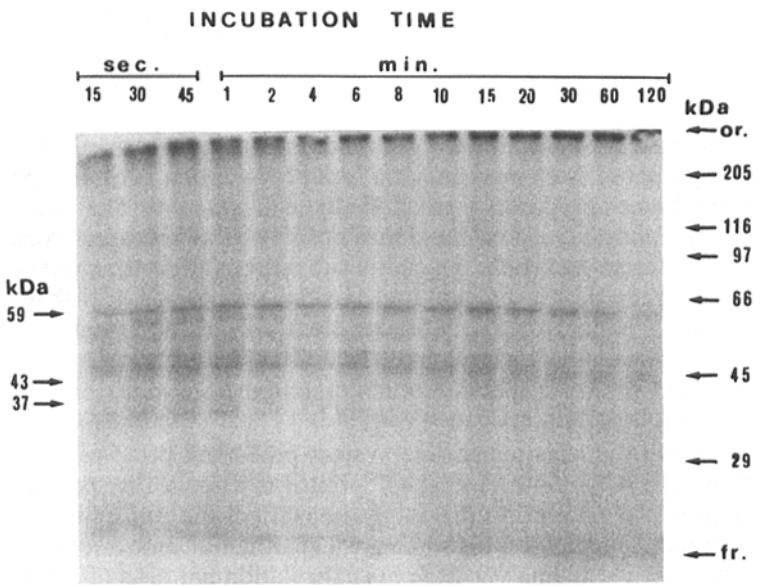

Fig. 4. Protein phosphorylation time course. Proteins were present in 27,000*g* membrane fractions obtained from root meristematic tips of 3-day-old seedlings. Incubation was carried out as described in Materials and Methods. On the right, position of molecular weight markers; on the left, some proteins that dephosphorylate during incubation after 6–8 min. NaF was routinely included in the incubation mixtures.

to the fact that, in order to increase the visibility of the weakly labeled bands, the autoradiogram was overexposed (3 weeks).

By comparing Fig. 6A with Fig. 3, we found that a good level of similarity existed between the pattern of protein phosphorylation obtained with mem-

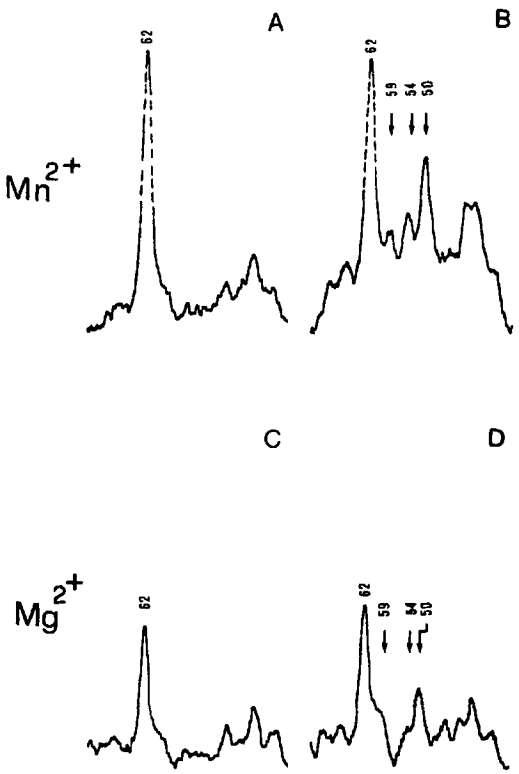

Fig. 5. Effect of different concentrations of metal ions on protein phosphorylations. The 27,000*g* membrane fraction was obtained from meristematic tips of 3-day-old seedlings. Incubation was carried out for 10 min as described in Materials and Methods. $MgCl_2$ and $MnCl_2$ were assayed in the presence of EDTA (0.5 mM). In each incubation mixture 0.1 mg of proteins was present, and aliquots of those mixtures (0.025 mg prot.) were electrophoresed and autoradiographed as described in Materials and Methods. The autoradiogram was scanned by a Beckman DU 8 spectrophotometer. A and C indicate incubations with 2 mM; B and D indicate incubation with 5 mM of each metal ion. Arrows indicate the M_r in kDa of some proteins which phosphorylate only in the presence of higher metal ion concentrations.

branes of 2-day-old cultured embryos pulsed in vivo and the one obtained with membranes extracted from seedlings germinated for 8 and 17 h and phosphorylated in vitro. The similarity of phosphorylation patterns between membranes extracted from seedlings of different ages could be due to the fact that cultured embryos germinated more slowly than seedlings of a correspondent age grown in "normal" conditions (data not shown).

The second approach for the analysis of phosphorylation patterns of endogenous substrates labeled in vivo used as source of 27,000*g* membranes the root tips of seedlings grown normally on moist Agriperlite for 3 days before pulsing (Fig. 6B). In this case, the number of labeled bands was smaller than in Fig. 6A, with the most pronounced band at a molecular weight of 100 kDa. When this second phosphorylation pattern obtained by in vivo labeling was compared with the correspondent pattern of phosphorylation of 3-day-old mem-

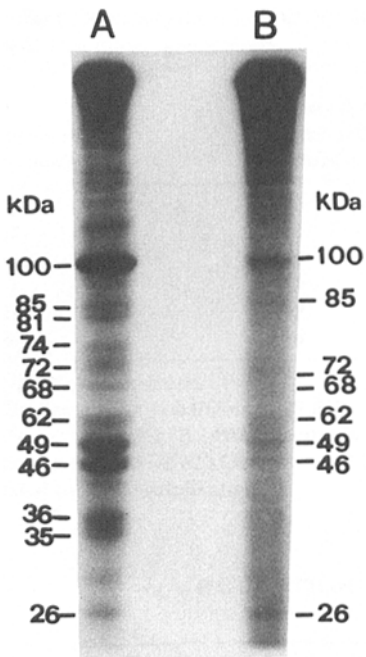

Fig. 6. Analysis of in vivo protein phosphorylation patterns. In vivo conditions are described in Materials and Methods. (A) Autoradiography of SDS electrophoresed phosphorylated proteins from embryos grown in liquid medium sterile culture for 2 days. (B) Autoradiography of SDS electrophoresed phosphorylated proteins from seedlings grown 3 days in normal conditions.

branes labeled in vitro, some differences were revealed, but the reasons for their presence was not known.

Discussion

The presence of protein kinase activity, previously reported in a 27,000*g* membrane fraction prepared from meristematic root tips of pea seedlings, has been confirmed in this work. A number of endogenous substrates of such activity has been found in the same membrane fraction by in vivo and in vitro assays, although the phosphorylation patterns of these substrates in vivo and in vitro were not identical.

Data presented here indicate that the number of protein classes present in the 27,000*g* membrane pellet obtained from meristematic pea root tips (0–2 mm from the tip) during the first 4 days of germination does not change, although nothing is known about protein turnover of these membranes. In germinating embryos of wild oat it was found that the number of protein classes extracted from total membrane preparations did not change even after 72 h of germination (Cuming and Osborne 1978), although a rapid turnover of the protein components of the cellular membranes occurred. In our case, the evident stability of protein composition of these 27,000*g* meristematic membranes during germination contrasts strikingly with the changes in their phosphorylation patterns. At present, three hypotheses could explain these changes: (1) the occurrence of conformational modifications in protein components of these membranes leading to a consequent loss of their phosphorylation sites; (2) oc-

currence of more than one protein kinase activity, each with different substrate affinity and present or active at different germination times; (3) variations during germination of the activities of specific phosphatases. These three hypotheses fit the present results and also agree with the fall in total and specific protein kinase activity during germination that we reported previously in the same membrane system (Chiatante et al. 1987).

At present, we do not have any information about the occurrence of conformational modifications in the protein components of our 27,000*g* membrane system during germination. Moreover, changes in activity of protein kinases and phosphatases rather than availability of substrates have been suggested to be responsible for the phosphorylation of specific proteins in proliferative conditions of yeast cells (Tripp et al. 1986). We cannot exclude that more than one protein kinase enzyme exists in our membrane system, as is the case with the membrane fraction prepared from pea shoots (Hetherington and Trewavas 1984). The occurrence of phosphorylation of specific proteins in presence of Mn^{2+} and Mg^{2+} in the incubation mixtures reported in this paper seems to suggest that this could also be the case in our root membrane system. In fact, the phosphorylation of specific proteins in the presence of metal ions could result from activation of a metal ion-dependent enzymatic form or forms. During germination of barley and wheat embryos, the level of a particular protein kinase decreased to 50% of its original value after 3 h (Sathyanarayana et al. 1987). If several enzymes are present in our membrane system, then it is likely that the respective levels do not remain constant during germination and that it would fit well with the variation in phosphorylation pattern that we observed. As regards the hypothesis of the presence of differential activities of protein phosphatases, we have already suggested their presence in this meristematic membrane system (Chiatante et al. 1987). Here the dephosphorylations of particular proteins at different times shown by time-course experiments confirm such previous findings and suggest that more than one protein phosphatase could be present. The presence of different protein phosphatases active at different times of germination could also explain the loss of protein phosphorylation bands during the germination. It would be interesting to repeat the time-course experiments with membranes extracted after 8 and 28 h of germination, when the biggest changes in kinase activity have been observed.

We cannot single out the predominant factor responsible for the changes in protein kinase activity observed in our membrane system during germination. The situation remains complex, and it is likely that two or more factors are active simultaneously to regulate the phosphorylation of this membrane system during germination. Furthermore, these data indicate that more attention should be given to membrane biochemistry when studying variations in membrane-bound protein kinase activity during germination.

We previously reported that no protein phosphorylation was detected in this 27,000*g* membrane pellet when it was prepared from adult tissue (Chiatante et al. 1987). Here we show that the Coomassie blue protein pattern of 27,000*g* membranes extracted from adult tissue is completely different from the one shown by 27,000*g* membranes of meristematic tissue. This result indicates that a different protein composition of these 27,000*g* membranes occurs in tissues fully differentiated. Therefore, the absence of protein kinase activity in 27,000*g*

membranes extracted from adult tissue could also be due to the lack of its endogenous substrates. However, comparison of phosphorylation of meristematic and differentiated tissue at several different stages of germination would help to assess changes.

The physiological relevance of the presence of protein phosphorylation in these membranes of meristematic tissue still needs to be clarified. The absence of protein phosphorylation in 27,000*g* membranes prepared from differentiated tissue supports the hypothesis that in meristematic tissues the phosphorylation of such membranes must have a physiological role. However, what this physiological role is remains to be demonstrated. Changes in protein kinase activities and phosphorylation pattern during cell growth and differentiation have been reported (Bocher et al. 1985, Heim et al. 1985).

Our data from cytofluorimetric analysis of the amount of DNA content in DAPI-stained nuclei, extracted from the meristematic tissue we use as membrane source, indicate that the first DNA syntheses start in our root meristems after 24 h of germination. That is more or less the same period of time during which the very striking changes in phosphorylation patterns have been observed (Figs. 1, 3). Consequently, the question arises whether the changes in phosphorylation patterns in these 27,000*g* meristematic membranes, observed more or less contemporaneously to the onset of DNA synthesis, are occasional or could be indicative of any physiological reciprocal dependence between these two events. If this is the case, then the reason for the presence of such activity only in meristematic tissues will become clear.

This hypothesis will be tested in the future by investigating the presence of this protein kinase activity and its relative pattern of protein phosphorylations in seedlings grown in the presence of aphydicolin (a strong inhibitor of DNA polymerase alpha). The rationale will be to study protein phosphorylations in a meristematic tissue in which cell proliferation activity has been blocked. The subcellular location of these membranes and the identities of their major phosphorylating components will be also actively pursued.

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