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A Salt-Induced 60-Kilodalton Plasma Membrane Protein Plays a Potential Role in the Extreme Halotolerance of the Alga *Dunaliella*[1]

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The halotolerant alga *Dunaliella salina* grows in saline conditions as varied as 0.5 and 5 M NaCl, maintaining throughout this range a low intracellular ion concentration. To discover factors potentially involved in ionic homeostasis, we grew cells in media with different salinities or osmolarities and compared their protein profiles. The comparisons indicated that the amount of a 60-kD protein, p60, greatly increased with an increase in salinity and was moderately enhanced when NaCl was substituted with iso-osmotic glycerol. Cells transferred from low to high NaCl or from high glycerol to iso-osmotic NaCl media transiently ceased to grow, and resumption of growth coincided approximately with an increase in p60. The protein, extracted from a plasma membrane fraction, was purified to homogeneity. Anti-p60 antibodies cross-reacted with a 60-kD protein in *Dunaliella bardawil*. Immunoelectron microscopy of *D. salina* cell sections indicated that p60 was exclusively located in the plasma membrane. Its induction by salt, the correlation between its accumulation and growth resumption in high concentrations of salt, and its plasma membrane localization suggest the possibility that p60 could play a role in ionic homeostasis in conditions of high salinity, although different types of function could also be considered.

Because of increasing salinization of agricultural soils and water resources, considerable attention has been drawn to mechanisms enabling plants to cope with osmotic and salt stresses. Responses to high levels of ionic or nonionic solutes and decreased water potential have been studied in a variety of plants and derived cell cultures, as well as in photosynthetic and nonphotosynthetic microorganisms. These studies addressed such physiological adaptive processes as the biosynthesis of intracellular compatible solutes (e.g. Sadka et al., 1989; Delauny and Verma, 1993) and changes in ion transport and metabolic functions (e.g. Adams et al., 1992; Perez-Prat et al., 1992; Curti et al., 1993; Gaxiola et al., 1992; Niu et al., 1993). Another widely pursued approach has aimed to identify components involved in osmotic and salt-stress tolerance by identifying mRNAs and proteins selectively induced under such conditions (e.g. Hurkman et al., 1988; Claes et al., 1990; Holland et al., 1993; Vernon et al., 1993) or in response to ABA, a hormonal mediator of the osmotic stress response in plants (Skriver and Mundy, 1990).

Useful models for such studies are plants and derived cell lines showing outstanding salt tolerance (Adams et al., 1992). A model of particular interest is provided by green algae of the genus *Dunaliella* (Avron, 1986; Sadka et al., 1989). These unicellular algae, possibly the most salt tolerant among eukaryotic photosynthetic organisms, are able to proliferate in extremely varied salinities, e.g. 0.05 and 5 M NaCl, while maintaining a relatively low internal sodium concentration (Karni and Avron, 1988). Lacking a rigid cell wall, the algal cells respond to increases or decreases in the external salinity by immediate shrinking or swelling, respectively. Subsequent synthesis or elimination of glycerol to an intracellular concentration osmotically balancing the external salinity permits the cells to regain their original volume (Sadka et al., 1989).

The glycerol-mediated osmotic responses probably constitute only one facet of the salt tolerance capacity of *Dunaliella*. Additional mechanisms may exist for the control of ion fluxes across the plasma membrane to maintain intracellular ionic homeostasis under different salinities (Pick, 1992). In an effort to discover components potentially involved in such mechanisms, we set out to identify proteins preferentially induced in high salt-grown *Dunaliella salina*. Comparing protein patterns in cells grown in different salinities, we previously detected a protein of 150 kD, p150, whose level increased dramatically with the external salinity (Sadka et al., 1991). The coincidence between resumption of cell division after a drastic hyperosmotic shock and p150 induction suggested that p150 might be involved in sustaining algal growth in high salt concentrations.

Here we describe another salt-induced protein in *D. salina*. The newly discovered 60-kD protein, designated p60, accumulated in response to high NaCl and, to a lower extent, high nonionic osmoticum. The conditions and kinetics of p60 induction were determined, and the protein was purified and characterized. Immunoelectron microscopy localized p60 in the plasma membrane, similarly to p150, distinguishing both proteins from most other subcellularly localized, salt-induced plant proteins described so far. Embedded in the plasma membrane, p60 and p150 could fulfill a variety of roles, among them the control of intracellular ionic levels.

MATERIALS AND METHODS

Algae and Growth Conditions

The source of the *Dunaliella salina* strain used in this study and growth conditions were as described by Ben Amotz and

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Avron (1983). The NaCl concentration in the medium was as indicated for each experiment. A medium containing 0.5 M NaCl supplemented with 1.5 or 4 M glycerol is referred to as low or high glycerol medium, respectively. The axenic cultures studied were started from single cells, as described elsewhere (Lers et al., 1990), and all manipulations were carried out under aseptic conditions.

NaCl-Induced Two-Step Hyperosmotic Shock

A 500-mL culture of 5×10^5 cells/mL (in a 2-L Erlenmeyer flask) grown in a medium with 0.5 M NaCl was centrifuged at 2000*g* for 10 min at room temperature, and the pellet was resuspended in 500 mL of 1.5 M NaCl medium. The cells were incubated for 2 to 3 h under standard growth conditions to allow osmotic recovery and subsequently centrifuged as above. The cell pellet was resuspended in 500 mL of a 3.5 M NaCl medium, and the culture was immediately placed under growth conditions.

Glycerol-Induced Two-Step Hyperosmotic Shock

The starting culture was the same as that used for the salt-induced hyperosmotic shock. The cells were collected by centrifugation as described above and resuspended in 500 mL of the low glycerol medium. The cells were incubated for 2 to 3 h under standard growth conditions to allow osmotic recovery and subsequently centrifuged as above. The cell pellet was resuspended in 500 mL of high glycerol medium, and the culture was immediately placed under growth conditions.

Iso-Osmotic Cell Transfer from High Glycerol to High Salt Medium

Cells growing in high glycerol medium were collected by centrifugation as described above and resuspended in 3.5 M NaCl medium.

Cell Density and Volume Determinations

The methods used were as described previously (Ben Amotz and Avron, 1983).

Preparation of Cell Extracts

Culture samples containing 10^7 cells were centrifuged as described above, and the cells were resuspended in 1.0 mL of growth medium. The suspensions were transferred to microfuge tubes. The cells were collected by 30 s of centrifugation in a microfuge and were resuspended in 0.1 mL of 1 mM PMSF, immediately followed by the addition of 50 μL of 3× loading buffer (Laemmli, 1970) and incubation at 100°C for 3 min.

Gel Electrophoresis and Immunoblot Analysis

Crude protein extracts, plasma membrane fractions, or purified preparations were analyzed by SDS-PAGE (Laemmli, 1970; Sadka et al., 1991); resolving gels were 8.75% polyacrylamide. Two-dimensional PAGE was performed as described by O'Farrell (1975). Each lane was loaded with an extract made from 1×10^6 cells or an aliquot of a plasma membrane preparation containing 50 μg of protein. The gels were stained with Coomassie brilliant blue or were electroblotted and probed with antibodies that were detected by chemiluminescence (Renaissance, DuPont).

Purification of p60

Following a standard procedure (essentially similar to that reported by Sadka et al., 1991), algae grown in 3.5 M NaCl to approximately 1×10^6 cells/mL were pooled from three 1.5-L cultures in 2.5-L Low Form flasks (alternatively, a 10-L culture was grown in a 12-L fermentor illuminated with eight 20-W fluorescent lamps, with constant stirring) and collected by centrifugation at 2000*g*, for 10 min at 4°C. The cells were gently resuspended in a total of 100 mL of growth medium and sedimented by centrifugation at 2000*g* for 8 min at room temperature. The tubes containing the pelleted cells were cooled in ice and gently resuspended in cold, freshly made lysing solution (10 mM tetra Na PPi containing as protease inhibitors 5 mM ε-caproic acid and 1 mM benzidine) to a density of 10^8 cells/ml. The cells, in 10-mL batches, were homogenized manually in a Ten Broeck-type homogenizer with a Teflon pestle and incubated for 20 min on ice. The lysates were cleared by centrifugation at 5000 rpm (Sorvall, rotor SS-34) for 8 min at 4°C, and the pooled supernatants were spun at 200,000*g* for 1 h at 4°C. The supernatants were removed and the pellets were resuspended by vortexing in Tris solution (1 mM EDTA, 10 mM Tris-HCl [pH 7.8], 5 mM ε-caproic acid, and 1 mM benzidine) to a density of 1×10^6 cell equivalents/mL. The suspension was distributed into six to eight microfuge tubes, Triton X-100 was added to a final concentration of 1%, and the samples were thoroughly mixed. After 10 min of incubation on ice, the suspensions were again centrifuged at 200,000*g* for 1 h at 4°C. The supernatants (referred to below as the plasma membrane fraction) could be stored at −20°C for at least several months. Plasma membrane fractions from two to three preparations (12–15 mg of protein in 8–10 mL) were pooled and concentrated to 1 to 2 mL in an Amicon cell model 12 using a PM-10 or an XM-100 membrane in ice. The concentrate was loaded onto a 1.8- × 30-cm column of DEAE-Sephadex (A-25), equilibrated in the cold with the Tris solution containing 0.2% Triton X-100. The column was washed with approximately 250 mL of the same Tris solution as used for equilibration and then eluted with 200 mL of 0.05 to 0.4 M NaCl linear gradient made in the same buffer. Fractions of approximately 3 mL were collected, and 40-μL samples were analyzed by SDS-PAGE. The eluted protein was further purified by preparative SDS-PAGE.

Raising of Antibodies

A DEAE-Sephadex-eluted fraction enriched in p60 was electrophoresed on SDS-PAGE, the gel was stained with Coomassie brilliant blue, and the p60 bands were excised and washed three times with PBS, with the last wash lasting for several hours. The gel slices, in a minimal volume of the wash buffer, were homogenized, and an equal volume of complete Freund's adjuvant was mixed with the homoge-

nized gel suspension by repeated passage through a syringe. The 4-mL mixture containing approximately 120 μg of protein was injected subcutaneously into two rabbits. Additional injections of similarly prepared p60 mixed with an equal volume of incomplete Freund's adjuvant were administered 24 and 44 d after the first injection. The immune serum used in the present experiments was collected 10 d after the third injection.

Immunoelectron Microscopy

A 15-mL suspension of algae grown in 3.5 M NaCl to a density of 5×10^5 cells/mL was centrifuged, and the cells were washed once with and resuspended in 10 mL of growth medium. Glutaraldehyde, acrolein, and paraformaldehyde were added to final concentrations of 2, 0.1, and 4%, respectively, and the suspensions were gently shaken for 1 h at room temperature. One-milliliter samples were transferred to microfuge tubes, and the cells were pelleted by centrifugation at maximal speed for 30 min. The tips of the tubes containing the cell pellets were cut into 0.5-mm^3 cubes, infiltrated with 2.3 M Suc in PBS for 2 h at room temperature, and rapidly frozen in liquid nitrogen. Ultrathin frozen sectioning was performed with a Reichert-Jung microtome fitted with a FC4D cryo-attachment (Sadka et al., 1991). Immunolabeling was with anti-p60 antibodies (1:70 diluted immune serum), or similarly diluted preimmune serum, followed by gold-conjugated goat anti-rabbit IgG. Staining of the immunolabeled sections and examination in a Philips model 410 transmission electron microscope operated at 80 kV were as described by Sadka et al. (1991).

RESULTS

D. salina cells were grown in media containing 0.5 or 3.5 M NaCl. Another, largely nonionic medium (iso-osmotic with the 3.5 M NaCl) contained 0.5 M NaCl and 4 M glycerol (high glycerol medium). Glycerol was chosen as the nonionic solute because even at very high concentrations it did not inhibit cell growth. Moreover, *Dunaliella* plasma membranes were previously demonstrated to be practically impermeable to glycerol (Brown et al., 1982), as may be expected from the ability of the cells to retain high concentrations of glycerol, which provides the intracellular osmoticum.

In the analysis shown in Figure 1, plasma membrane fractions were analyzed for their protein composition by SDS-PAGE. A comparison of the protein profiles showed the presence of an intensely stained band, corresponding to a 60-kD protein, in the 3.5 M NaCl-grown cells that was barely detected in the cells grown in 0.5 M salt. This band was present, albeit at a lower level, in cells grown in the 4 M glycerol medium. In addition to p60 accumulation, the analysis reconfirmed the increase in p150 level in the high salt-grown cells and indicated a considerable accumulation of p150 also in cells grown in the high glycerol medium. Similar analyses of whole cell extracts (data not shown) also revealed the changes in p60 but on the background of a far more complex protein composition. Judged by the intensity of staining, the 3.5 M salt-grown cells contained comparable amounts of p60 and p150.

Two-dimensional gel electrophoresis was conducted to ascertain that the intense band corresponding to 60 kD seen in the SDS-PAGE analysis of plasma membranes represented a single protein species (Fig. 2). The results indicated that the band consisted of a single major polypeptide whose isoelectric point of approximately 4.6 closely resembled that of p150.

A plasma membrane fraction of *D. salina* cells grown in 3.5 M NaCl served as a source for p60 purification. The plasma membrane fraction was extracted with 1 or 2% Triton X-100, and the extract was fractionated on a DEAE-Sephadex column by elution with a NaCl gradient (Fig. 3). Although eluting ahead of the peak fraction of p150, the peak fraction of p60 still contained p150. A complete purification of p60 was attained by preparative SDS-PAGE of the column fraction(s) most enriched in p60.

Purified p60 was used to raise antibodies in rabbits. The specificity of the polyclonal antibodies and the distribution of p60 antigens in crudely fractionated cell extracts are shown in Figure 4. The immune serum recognized mostly a protein corresponding to p60. Several other weakly cross-reacting proteins, partly recognized also by the preimmune serum, did not accompany p60 into the plasma membrane fraction. This analysis also showed that the anti-p60 antibodies do not cross-react with p150. As shown previously and confirmed in the present study, the anti-p150 antibodies also do not cross-react with p60. Hence, p60 is structurally distinct from p150, the other major plasma membrane protein identified in *Dunaliella*.

Immunoblot analysis of *D. bardawil*, another salt-tolerant strain of *Dunaliella* grown in 3.5 M NaCl, clearly showed the presence of an intensely cross-reacting protein migrating similarly to p60 (Fig. 5). Thus, p60, similarly to p150, is conserved in at least these two salt-tolerant strains of *Dunaliella*.

Immunoblot analysis was also used to compare the level of p60 in *D. salina* continuously grown in media with different salinities and osmolarities (Fig. 6). In agreement with the

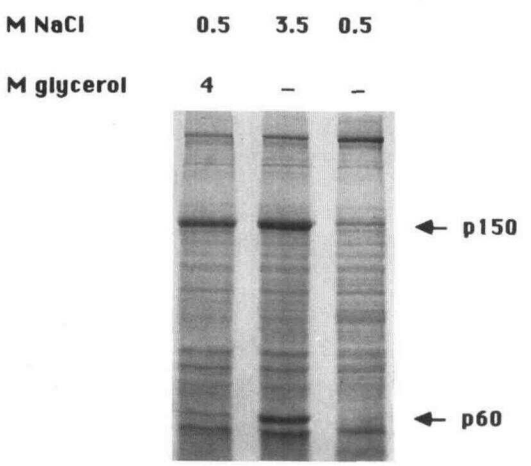

Figure 1. Effect of salt and high osmoticum on the protein composition of plasma membrane fractions. *D. salina* cells grown in the indicated media were fractionated and analyzed by SDS-PAGE and Coomassie brilliant blue staining as described in "Materials and Methods." Positions of p60 and p150 are indicated by the arrows.

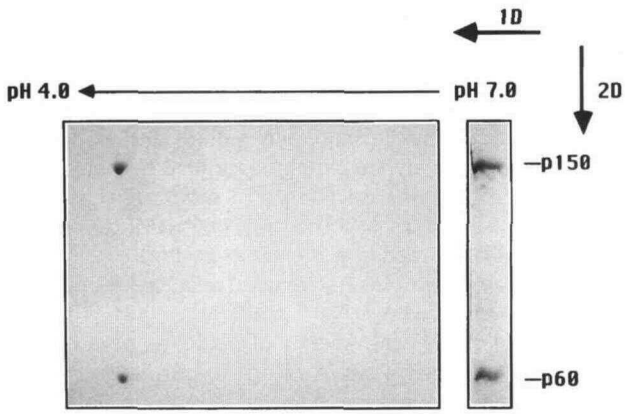

Figure 2. Two-dimensional gel electrophoresis of a plasma membrane fraction from 3.5 M NaCl-grown *D. salina*. One aliquot of the sample was fractionated by isoelectrofocusing in the first dimension (1D) and by SDS-PAGE in the second dimension (2D, right). Another aliquot was run only in the second dimension (left).

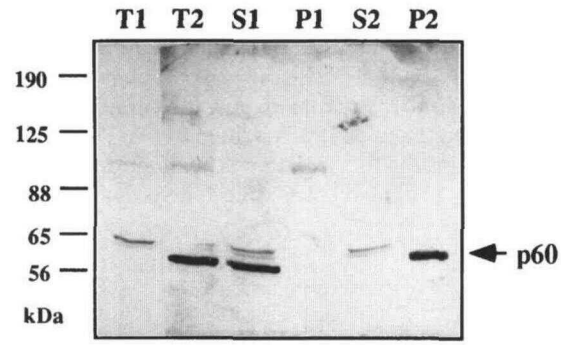

Figure 4. Immunoblot analysis of different fractions of 3.5 M NaCl-grown *D. salina*. SDS-PAGE and immunoblot analyses with anti-p60 immune or preimmune serum were as described in "Materials and Methods." T1 (T2), Total cell extract analyzed with preimmune (or immune) serum; S1 (P1), supernatant (pellet) after the 5,000 rpm centrifugation of the lysates; S2 (P2), supernatant (pellet) after the 200,000g centrifugation (centrifugations as in the p60 purification procedure described in "Materials and Methods").

conclusion based on gel staining (Fig. 1), the level of p60 increased with external salinity. (In the experiment shown, the p60 level is not strictly proportional to the medium salinity apparently because of an exceptionally high signal observed for the cells grown in 1 M NaCl.) The addition of 4 M glycerol to a medium with 0.5 M NaCl induced accumulation of p60 but to a lower level than that observed in the iso-osmotic, 3.5 M NaCl medium, indicating that high salt is more effective than iso-osmotic glycerol in induction of p60. It is important to note that subjecting the algae to general stress conditions such as heat shock or exceedingly high light intensities did not result in p60 induction (data not shown).

In addition to analyses of p60 in cells growing continuously in media of different osmolarities, p60 was also analyzed during the recovery of cells from a drastic hyperosmotic shock generated by adding NaCl (Fig. 7). In agreement with our previous observations, the cells regained their original volume, i.e. completed osmotic adjustment, long before proliferation was resumed 20 to 28 h after the shock. The p60 level, assayed by immunoblot analysis, remained below detection until 8 to 17 h after the shock. A progressive increase was observed at later times.

As shown in Figure 6, high osmolarity, contributed mainly by glycerol, was less effective in inducing p60 than an iso-osmotic concentration of NaCl. The experiment shown in Figure 8 examined changes in growth and p60 accumulation in cells transferred from the high glycerol medium to the iso-osmotic 3.5 M NaCl medium. As expected, the transfer had virtually no effect on cell volume. Yet, cell division was halted, or strongly retarded, for approximately 10 h after the transfer. Immunoblot analyses for p60, performed at different times after the change of medium, indicated that the starting cells already contained an elevated level of the protein (cf. Fig. 6). This level remained unaltered for several hours and started to increase only 11 to 20 h after the change of medium. Thus, transfer from a high osmoticum contributed mainly by a nonionic solute to an equivalent osmoticum generated by salt resulted in a marked induction of p60. The increase in p60 approximately coincided with the renewal of cell proliferation.

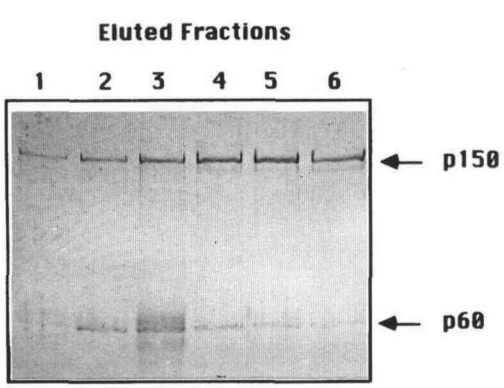

Figure 3. Partial purification of p60 by chromatography on DEAE-Sephadex. An extract of a plasma membrane fraction from 3.5 M NaCl-grown *D. salina* was chromatographed, and eluted fractions were analyzed as described in "Materials and Methods." The gel was stained with Coomassie brilliant blue. Peak fractions of p60 and p150 are in lanes 3 and 5, respectively.

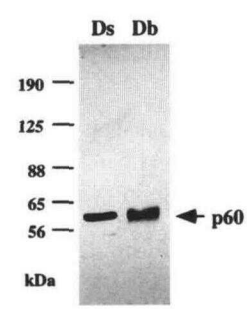

Figure 5. Analysis of *D. bardawil* with anti-p60 antibodies. Total extracts of *D. salina* (Ds) and *D. bardawil* (Db) cells grown in 3.5 M NaCl were analyzed by SDS-PAGE and immunoblotting.

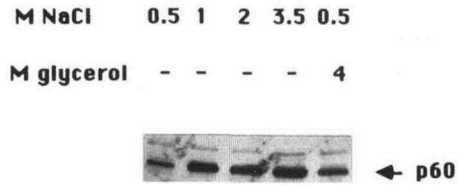

Figure 6. Immunoblot analysis of *D. salina* cells grown in different media. Cells were grown in media with the indicated salt concentrations or in high glycerol medium. SDS-PAGE and immunoblotting were as described.

The localization of p60 in the plasma membrane was first indicated by subcellular fractionation. Furthermore, typical of membrane proteins, p60 is insoluble in aqueous solutions unless a detergent is present. Definitive subcellular localization of p60 was achieved by immunoelectron microscopy of cryosectioned whole cells using gold-conjugated goat anti-rabbit IgG to visualize the bound antibodies (Fig. 9).

Preliminary tests with membrane-blotted p60 indicated that treatment with 2% glutaraldehyde severely reduced the efficacy of antibody binding. Nevertheless, this concentration of glutaraldehyde was used for fixation of cryosectioned cells to preserve morphological features. The micrographs clearly demonstrate the plasma membrane localization of the p60 antigens. The cytoplasm, chloroplast, and nucleus were rarely labeled. Of special interest is the section shown in Figure 9B, which permits the comparison of the plasma membrane with the nonlabeled nuclear membrane and chloroplast envelope.

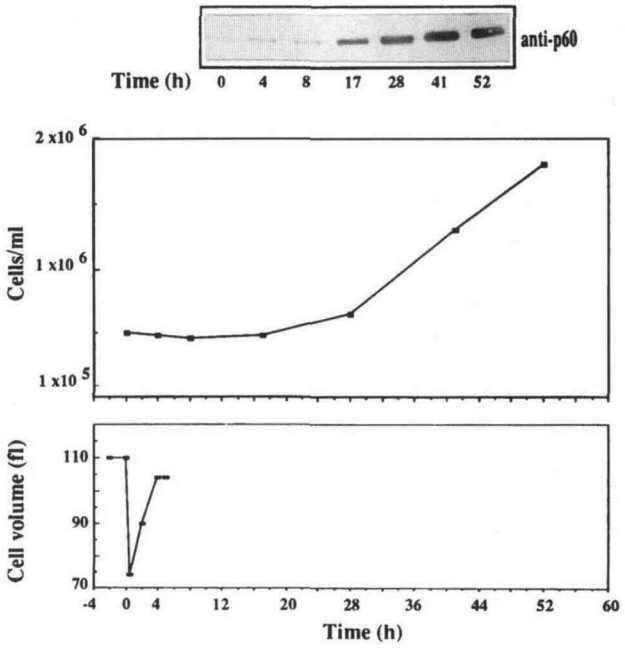

Figure 7. Time course of recovery and p60 induction after salt-induced hyperosmotic shock. Cells grown in 0.5 M NaCl were transferred in two steps to a medium with 3.5 M NaCl as described in "Materials and Methods." Cell number and volume, as well as p60 antigens, were monitored at different times after the second increase in salt concentration.

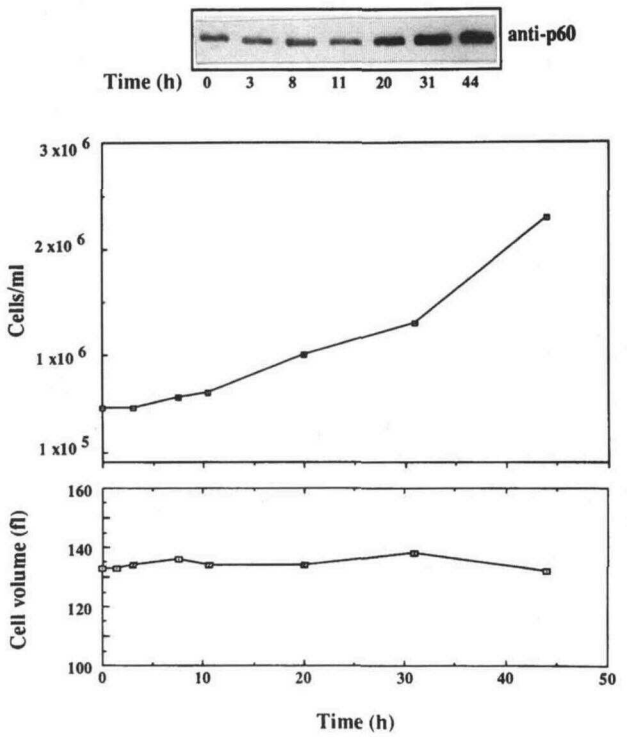

Figure 8. Time course of recovery and p60 induction after an iso-osmotic replacement of glycerol with NaCl. Cells grown in high glycerol medium were transferred to 3.5 M NaCl medium. Cell number and volume, as well as p60 antigens, were monitored at different times after the change of medium.

No significant labeling was evident with preimmune serum, verifying the specificity of antibody binding.

DISCUSSION

Cellular levels of p60 are strictly dependent on external salinity: the protein is barely detectable in cells grown in low salt but becomes a major plasma membrane component in cells growing in high salt. In cells subjected to a drastic hypersaline osmotic shock, the early phase of glycerol accumulation is followed by a second, longer phase encompassing still undefined, adaptive processes enabling resumption of cell proliferation. Because p60 is induced close to the restoration of cell division, it is possible that this protein could be involved in maintaining conditions permitting unhindered growth in high salt, although other possibilities cannot be disregarded.

The induction of p60 is more effective in high NaCl than in iso-osmotic glycerol, suggesting a quantitative distinction in sensing of osmotic stresses generated by ionic or nonionic solutes. The selectivity of *Dunaliella* with regard to the medium composition prevented us from conducting a more detailed comparison of different solutes. *Dunaliella* generally cannot tolerate high concentrations of salts other than NaCl. Similarly, nonionic solutes other than glycerol were found to be toxic to the algae.

The quantitative difference in p60 induction by high salt and high glycerol provided us with a useful tool to examine

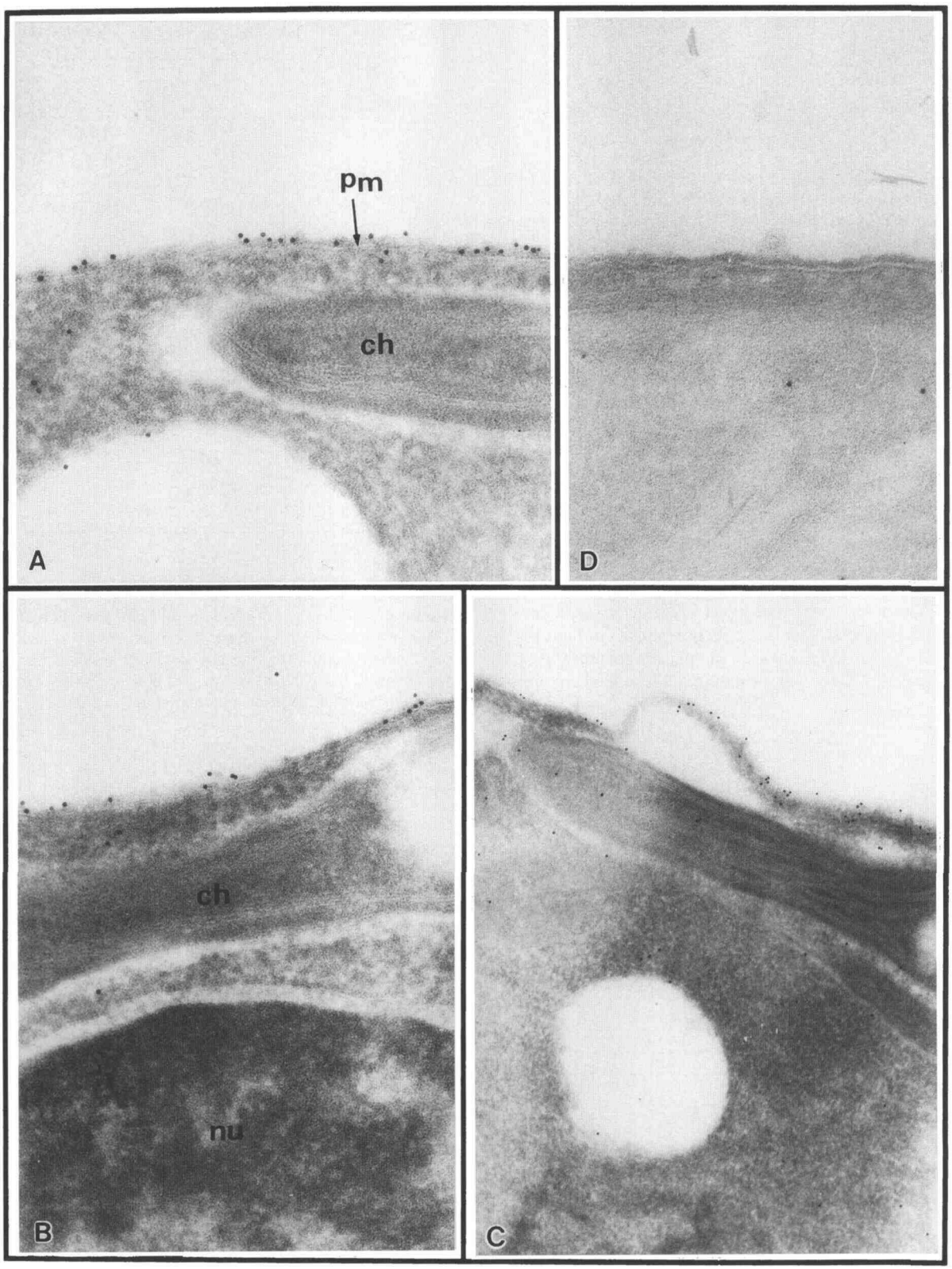

Figure 9. Subcellular localization of p60 in whole cell sections. Cryosectioning and immunoelectron microscopy of cells grown in 3.5 M NaCl was as described in "Materials and Methods." A, B, and C, Anti-p60 immune serum; D, preimmune serum. pm, Plasma membrane; ch, chloroplast; nu, nucleus.

the possibility that a decrease in volume could serve as the primary signal for osmotic stress responses in *Dunaliella*. In cells subjected to an iso-osmotic replacement of glycerol with NaCl, cell volume, a sensitive measure of external osmoticum, remained unaltered; yet, cell division was arrested for several hours and resumed in conjunction with p60 induction. These results clearly show that the signal for p60 induction does not merely emanate from a change in volume. Differences in the nature or extent of responses to different osmotic stresses are generally considered to reflect a difference in adaptation requirements.

Based on theoretical considerations and experimental observations, several types of mechanisms may underlie the adaptation of plants to osmotic stresses. Among others, these mechanisms include the accumulation of compatible osmolytes (Sadka et al., 1989; Delauny and Verma, 1993), biochemical changes in photosynthesis (Vernon et al., 1993), altered expression of H^+-ATPase and Ca^{2+}-ATPase (Perez-Prat et al., 1992; Niu et al., 1993), and induction of proteins that were thought to enable internal water retention and protection of cellular structures (Espeland et al., 1992; Close and Lammers, 1993). Other highly important and yet less understood mechanisms are responsible for the prevention of cytoplasmic accumulation of toxic ions, e.g. Na^+. By its pattern of accumulation and subcellular localization, p60 could play a role in ion homeostasis, but a definitive functional assignment must await further investigation.

Our studies have now revealed two salt-induced plasma membrane proteins in *D. salina*, both conserved also in *D. bardawil*. In high salt-grown cells, p150 and p60 are the most abundant among the plasma membrane proteins. Despite a similarity in isoelectric point, the proteins represent structurally distinct entities as evidenced by the absence of immunological cross-reactivity. Although the two proteins exhibit similar kinetics of induction by high salt, p150 is induced to a relatively higher level than p60 by the nonionic osmoticum. This difference does not necessarily imply dissimilar mechanisms of induction or functions for the two proteins but may, rather, reflect nuances in the sensing or regulatory mechanisms operating in each case. It should be remembered that in its natural habitats *Dunaliella* encounters mostly high concentrations of NaCl rather than nonionic solutes. Therefore, both p60 and p150 would be normally induced. The similarity of their induction course and final level may indicate that these two major plasma membrane proteins could functionally interact with each other.

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