Plant Physiol. (1991) 95, 822–831
0032-0889/91/95/0822/10/$01.00/0

Received for publication July 30, 1990
Accepted October 27, 1990

A 150 Kilodalton Cell Surface Protein Is Induced by Salt in the Halotolerant Green Alga *Dunaliella salina*[1]

Avi Sadka, Stanley Himmelhoch, and Ada Zamir*

Biochemistry Department (A.S., A.Z.) and Biological Services (S.H.), Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT

***Dunaliella salina* is an extremely halotolerant, unicellular, green alga lacking a rigid cell wall. Osmotic adaptation to high salinities is based on the accumulation of glycerol. To uncover other functions responsible for halotolerance, protein profiles of algae continuously grown in different salinities were compared. A 150 kilodalton protein (p150) increased in amount with salt concentration. Furthermore, when the cells were subjected to drastic hyperosmotic shocks, p150 started to rise long after completion of the osmotic response but coincident with reinitiation of cell proliferation. Cells with an initially higher level of p150 resumed growth faster than cells with a lower level of the protein. Addition of cycloheximide early after hyperosmotic shock prevented the rise in p150, indicating this rise was due to *de novo* synthesis of the protein. These observations suggest that p150 is a salt-induced protein required for proliferation of the cells in saline media. p150 was purified to homogeneity and found to be a detergent-soluble glycoprotein. Polyclonal antibodies against p150 recognized a single protein component in *D. salina* crude extracts. A high M_r cross-reacting protein was also observed in another *Dunaliella* strain, *D. bardawil*. Immunoelectron microscopy localized p150 to the cell surface.**

The genus *Dunaliella* includes halotolerant, unicellular, motile green algae with exceptional morphological and physiological properties (1, 5). The algae lack a rigid polysaccharide cell wall and are capable of growth in salinities as different as 0.05 M and 5.0 M NaCl, maintaining throughout this range a relatively low intracellular sodium concentration. When the cells, which are surrounded by only a plasma membrane, are subjected to hypo- or hyperosmotic shocks they first undergo rapid volume and shape changes. Subsequent synthesis or elimination of glycerol to an intracellular level osmotically compatible with the external salinity then permits the cells to return to their normal volume and attain an appropriate turgor pressure (1, 3, 4, 7, 17). Cell volume and glycerol content adjustments in response to hypo- or hyperosmotic shocks are insensitive to protein synthesis inhibitors added prior to the shock (8, 27), indicating that the reactions involved are not controlled at the level of gene expression.

Investigations of the mechanism of halotolerance in *Dunaliella* by biochemical and physiological approaches have focused so far on the osmoregulated changes in glycerol content (1, 8). A crucial, yet hitherto unaddressed question is whether these responses alone are sufficient to permit growth and proliferation of the algae under such extremely varied salinities. Additional functions, possibly regulated at the level of gene expression, could be required for example to modify membrane properties or to control ion fluxes.

As an approach to uncover additional functions related to halotolerance and their regulation, we have compared the protein profiles of *Dunaliella salina* continuously grown under extremely different salinities. These analyses have revealed a striking, salt-related difference in the abundance of a protein of approximately 150 kD (p150): it was barely detectable in cells grown in 0.5 M NaCl, but became a major protein in cells grown in 3.0 M salt and above. To further characterize this response, we have first focused on the protein itself, its structural and functional characteristics and subcellular localization. The protein was purified to homogeneity, shown to be a detergent-soluble glycoprotein, and used to raise anti-p150 polyclonal antibodies. Further analyses provided evidence for the salt induction of p150, its potential role in restoring cell proliferation after hyperosmotic shocks, and its exclusive localization to the cell surface.

MATERIALS AND METHODS

Algae and Growth Conditions

The source of the *Dunaliella salina* strain used in this study and growth conditions were as previously described (4). The NaCl concentration in the medium was as indicated for each experiment. The axenic cultures studied were started from single cells, as described elsewhere (21) and all manipulations were carried out under aseptic conditions.

Two-Step Hyperosmotic Shock

To a 500 mL culture (in a 2 L Erlenmeyer flask) containing approximately 5×10^5 cells/mL, in a medium with 0.5 M NaCl, were added 142.8 mL of 5 M NaCl in growth medium, bringing the NaCl concentration to 1.5 M. The cells were incubated for 2 to 3 h under standard growth conditions to allow osmotic recovery, and to 300 mL of this culture were added 262 mL of 5 M NaCl in growth medium, to give a final concentration of 3.5 M salt, and the cells were immediately returned to growth conditions.

[1] This study was supported in part by the Forchheimer Center for Molecular Genetics, Weizmann Institute of Science.

Cell Density, Glycerol, and Cell Volume Determinations

The methods used were as described previously (4).

Preparation of Cell Extracts

Culture samples containing 10^7 cells were centrifuged at 3000 rpm for 10 min 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 centrifugation in a microfuge and were resuspended in 0.1 mL H_2O, immediately followed by the addition of 50 μL of 3× loading buffer (18) and incubation at 85°C for 4 min.

Gel Electrophoresis and Immunoblot Analysis

Crude protein extracts, or purified fractions were analyzed by SDS-PAGE (19); resolving gels were 7.5% or 7.5 to 15% polyacrylamide. Two-dimensional PAGE was performed as described elsewhere (24). The gels, loaded with an equivalent of 2.5×10^6 cells per lane, were stained with Coomassie blue, or electroblotted and probed with antibodies, followed by ^{125}I-labeled protein A, as previously described (6).

Analysis of Glycoproteins

Gel blots were analyzed by a nonradioactive, avidin-biotin based assay (2). Periodate oxidation was followed by a reaction with streptavidin hydrazide, binding of biotinylated alkaline phosphatase, and final detection with a phosphatase substrate.

Purification of p150

The purification scheme is summarized in "Results." In a standard procedure, algae grown in 3.5 M NaCl to approximately 8×10^5 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 [New Brunswick] 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-pyrophosphate containing, as protease inhibitors [32], 5 mM ϵ-caproic acid and 1 mM benzidine) to give a density of 10^8 cells/mL. The cells were homogenized manually in 10 mL batches, in a Ten Broeck type homogenizer with a Teflon pestle, and incubated for 20 min in ice. The lysates were centrifuged at 5000 rpm (Sorvall, rotor SS-34) for 8 min at 4°C, and the pooled supernatants were distributed into 8 to 12 polypropylene 16 mL centrifuge tubes (Sorvall) and centrifuged in a Sorvall SS-34, or SM-24 rotor, at 18,500 rpm (40,000*g*) for 90 min, at 4°C. The supernatants were removed and the pellets were resuspended by vortexing in cold lysing solution or in Tris solution (1 mM EDTA, 10 mM Tris-HCl [pH 7.8] containing ϵ-caproic acid and benzidine as in the lysing solution), to a give a density of 1.25×10^6 cell equivalents/μL. The suspension was distributed into six to eight microfuge tubes, Triton X-100 or Chaps[2] were added to a final concentration of 0.2%, and the mixtures were thoroughly mixed. After 10 min incubation in ice, the suspensions were centrifuged at 18,500 rpm (Sorvall, rotor SS-34 equipped with adaptors) for 1 h at 4°C. The supernatants (referred to as the S40 fraction), containing a total of approximately 5 mg protein enriched in p150, could be stored at −20°C for at least several months. S40 fractions from 2 to 3 fractionations (12–15 mg 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 a XM-100 membrane, in ice. The concentrate was loaded onto a 1.8 cm diameter by 30 cm high column of DEAE-Sephadex (A-25), equilibrated in the cold with lysing or Tris solution containing 0.2% Triton X-100 or Chaps. The column was washed with approximately 250 mL of the solution used for equilibration and then eluted with 200 mL of a 0.05 to 0.4 M NaCl linear gradient made in the buffer used for equilibration of the column. Fractions of approximately 3 mL were collected, and 40 μL samples were analyzed by SDS-PAGE. The elution of p150 started at approximately 0.16 M NaCl. The eluted protein was sometimes further purified by preparative SDS-PAGE or by separation in a gel permeation HPLC system (16).

Protein Microsequencing

Determination of the N-terminal sequence of SDS-PAGE or HPLC purified p150 was performed on Applied Biosystems Inc. model 475A automatic pulsed liquid gas phase protein microsequencer equipped with a model 120A on-line HPLC PTH amino acid analyzer and a model 900A data acquisition and processing unit (20).

Raising of Antibodies

A DEAE-Sephadex purified preparation of p150, containing 150 μg protein, was electrophoresed on 21 lanes of an SDS gradient gel of 5 to 10% polyacrylamide. The gel was stained with Coomassie blue and the p150 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 broken by passage through a series of syringes with decreasing diameter. An equal volume of incomplete Freund adjuvant was mixed with the crushed gel suspension by repeated passage through a syringe and the mixture was injected subcutaneously into a rabbit. A second, identical injection was given 12 d later. The immune serum used in the present experiments was collected 12 d after the second immunization.

Immunoelectron Microscopy

Sectioning and staining procedures were essentially as described (29, 30). Fifteen milliliters of a 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 fresh growth medium and resuspended in 10 mL of the same medium. Gluteraldehyde and acrolein were added to final concentra-

[2] Abbreviation: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

tions of 2% and 0.1%, 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 sucrose 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. Immunolabeling was with anti-p150 antibodies (1:150, or 1:300 diluted immune serum) or preimmune serum (1:150 dilution) followed by gold-conjugated goat anti-rabbit IgG (Janssen). Positive staining of the immunolabeled sections was as described. Sections were examined in a Philips model 410 transmission electron microscope operated at 80 kV.

RESULTS

Protein Profiles of Low and High Salt-Grown Cells

It is possible to envisage two classes of salt-induced changes in the protein profiles of the algae. Similarly to some stress responses (*e.g.* ref. 13), changes of one class could be transient in nature and therefore detectable only soon after the shock. Other changes could be long term and therefore discernible in comparisons of cultures growing continuously in different salinities. Directing our attention to changes of the second type, we compared the protein patterns of cells grown for at least several weeks in media containing 0.5, 3.0, or 4.0 M NaCl (Fig. 1). The gels revealed a number of differences in protein levels with the most pronounced change an increase in a protein of approximately 150 kD (p150). This protein was present in the extracts of cells grown in 3.0 M or 4.0 M salt, but was barely detectable in the extract of the cells grown in 0.5 M NaCl.

Although these results suggested that p150 was salt-induced, we considered two additional possibilities. Since, by several criteria, high salt concentrations were somewhat stressful to the cells, p150 could represent a persistently synthesized, stress-inducible protein. To test this possibility, cells grown continuously in 0.5 M NaCl at the optimal temperature of 26°C were incubated for 24 h at 37°C, a temperature at which the cells practically ceased to proliferate and were halted in their motility. When compared with cells growing at 26°C (not shown), no meaningful difference between the two protein patterns was evident, leading to the conclusion that p150 was not induced by heat stress. Because in early experiments cells grown in high salt were close to the stationary phase, we also considered it possible that p150 accumulated in nondividing cells, independently of the medium salinity. Cultures growing in 0.5 or 3.5 M NaCl were therefore compared during early log phase as well as at the beginning of the stationary phase of growth. The results (not shown) revealed the consistent, growth phase-independent presence of p150 in the cells growing in 3.5 M NaCl, but not in the cells growing at the lower salt concentration. Hence, the appearance of p150 is not growth-phase related.

Purification of p150

To extend our studies of p150, we set out to purify the protein from cells grown in 3.5 M NaCl. Following cell lysis in hypotonic medium and removal of cell debri, centrifugation of the cleared extract at 40,000*g* yielded a pellet highly enriched in p150. Solubilization of p150 from this pellet was achieved by using a buffer containing 0.2% Triton X-100, or Chaps, another nonionic detergent. Very little protein was released into the supernatant in the absence of detergent. Chromatography of the detergent-soluble fraction on DEAE-Sephadex (with detergent present in the buffers used for both equilibration and elution of the column) led to the purification of p150 to apparent homogeneity as judged by one-dimensional PAGE (Fig. 2, lane 6).

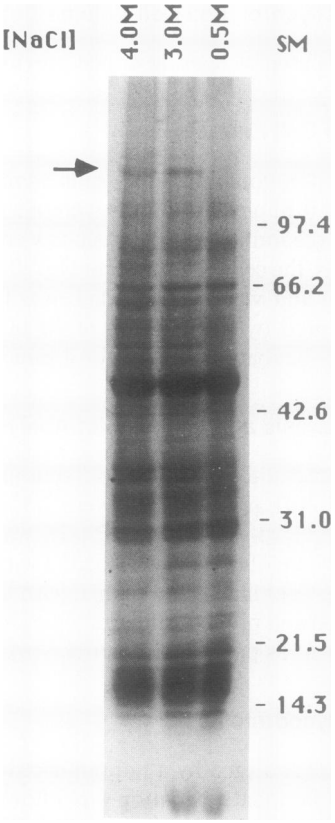

Figure 1. Protein profiles of *D. salina* cells grown in different salinities. Extracts of cells from approximately 10^6 cells/mL cultures in media with the indicated NaCl concentrations were electrophoresed and stained with Coomassie blue as described in "Materials and Methods." SM, position of molecular weight standards; arrow, position of p150.

To ascertain that the major p150 band represented a single protein, to determine its approximate pI, and to test its relationship to a minor, sometimes coeluted protein of lower M_r, a fraction containing both proteins (analyzed in Fig. 2, lane 2) was subjected to two-dimensional PAGE (Fig. 3). Isoelectric focusing did not resolve additional components, indicating p150 represented a single polypeptide. Their identical acidic isoelectric points as well as chromatographic and electrophoretic resemblance strongly suggest that p150 and the minor accompanying protein are interrelated (see also below).

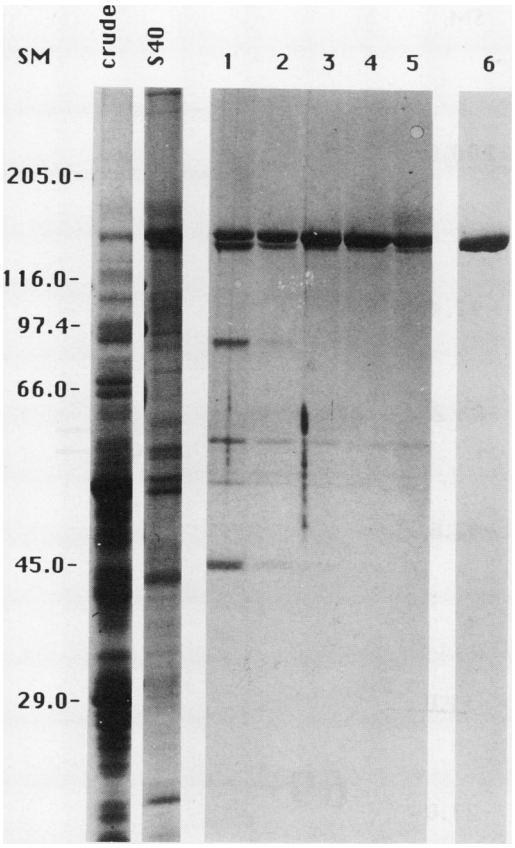

Figure 2. Gel-electrophoretic analysis of fractions at different stages of p150 purification. The initial crude extract, S40 fraction, and fractions eluted from DEAE-Sephadex were analyzed by gel electrophoresis and Coomassie blue staining as described above. Lanes 1 to 5, DEAE-Sephadex eluted fractions containing p150 (every second fraction was analyzed); lane 6, a fraction from a separate DEAE-Sephadex chromatography containing apparently homogeneous p150.

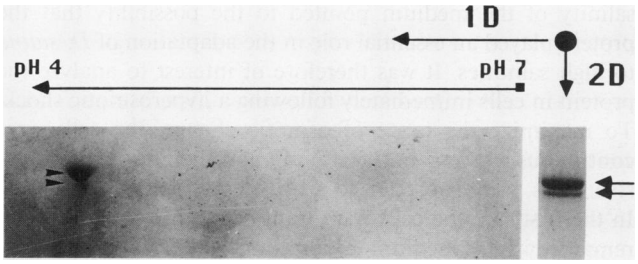

Figure 3. Two-dimensional gel electrophoretic analysis of partially purified p150. A sample from the DEAE-Sephadex eluted fraction shown in Figure 2, lane 2, was analyzed by electrofocusing in the first dimension and by electrophoresis with SDS in the second dimension. As a standard, another aliquot was run only in the second dimension. The proteins were detected by Coomassie blue staining. Arrows, position of p150, and minor accompanying protein.

Evidence that p150 Is a Glycoprotein

The presence of p150 in a high speed sedimented cell fraction together with its detergent solubility suggested that p150 was a membrane protein and hence a potential glycoconjugate. To determine whether p150 was a glycoprotein, column-purified fractions were electrophoresed on polyacrylamide-SDS gels and blots were analyzed using a nonradioactive, avidin-biotin based method to detect carbohydrates. The analyses included a homogeneous fraction of p150 (analyzed in Fig. 2, lane 6) as well as a fraction including the faster moving protein (analyzed in Fig. 2, lane 2). The results (Fig. 4) indicate that p150 (and the accompanying, faster-mobility component) reacted positively in this assay. A control (not shown) with unmodified streptavidin gave no signal. In parallel to p150 we analyzed avidin, a glycoprotein which reacted positively in this assay and bovine serum albumin, a nonglycosylated protein which reacted negatively (data not shown). These results are consistent with p150 and its accompanying component being glycoproteins.

Anti-p150 Antibodies

A sample from the column fraction analyzed in lane 4 of Figure 3 was electrophoresed under conditions which permitted complete separation of the major band from the faster, minor band. The major band was excised from the gel and used to immunize rabbits. The immune serum was first tested in immunoblots of crude extracts of *D. salina* cells grown in different salinities (Fig. 5A). The antibodies recognized only a single protein band of the expected mobility, with the intensity of staining increasing with the salinity of the growth medium. This assay demonstrated the presence, albeit at a low level, of a cross-reacting protein in cells grown in 0.5 M

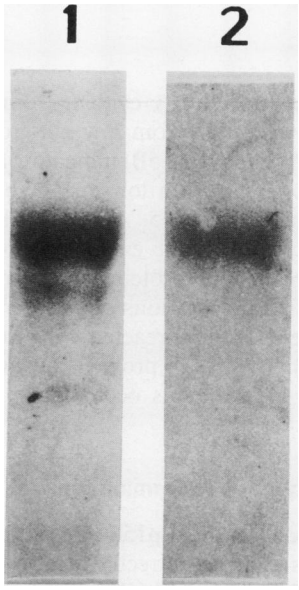

Figure 4. Carbohydrate analysis of p150. The DEAE-Sephadex eluted fractions shown in Figure 2, lanes 2 and 6, were gel-electrophoresed in lanes 1 and 2, respectively. A gel blot was analyzed for carbohydrates as described in "Materials and Methods."

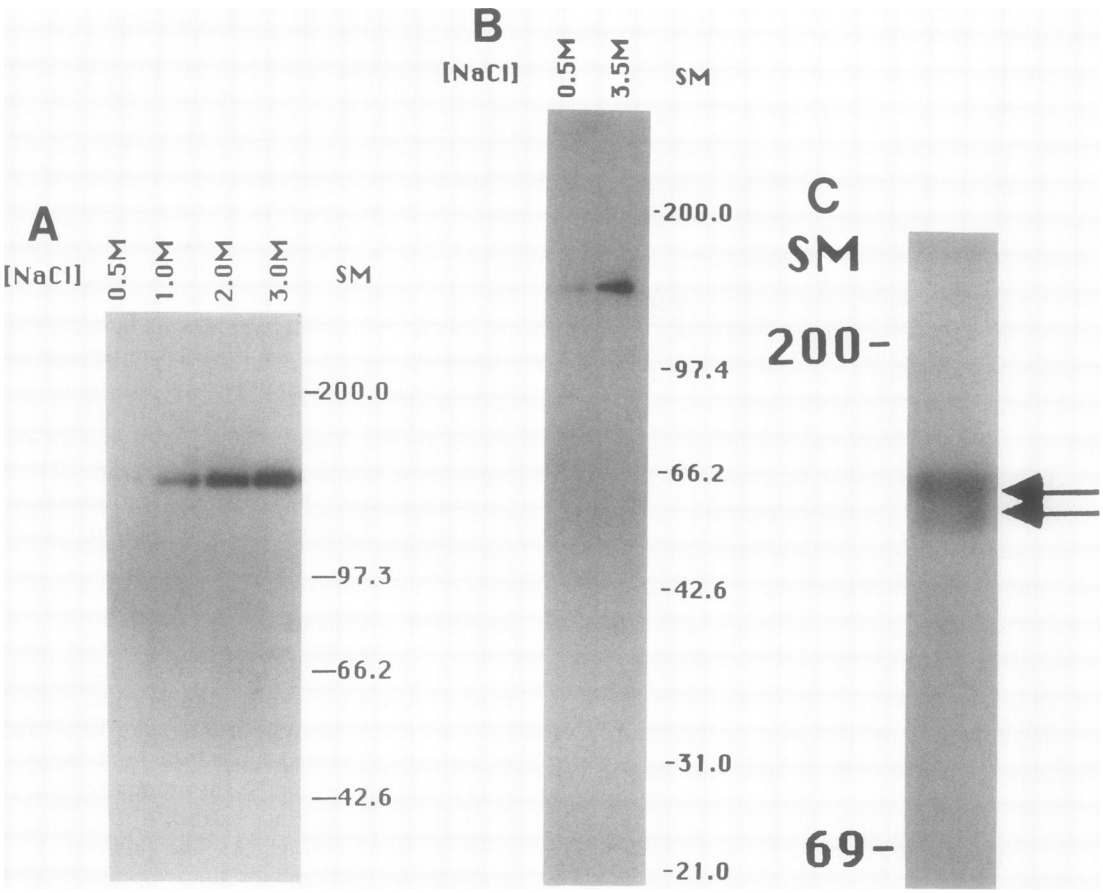

Figure 5. Immunoblot analyses with polyclonal anti-p150 antibodies. Immunoblot analyses using a 1:1000 diluted immune serum of: A, crude extracts of cells continuously grown in media with the indicated salt concentrations; B, the S40 fraction from cells grown in media with the indicated salt concentration. (SM, position of molecular weight standards); C, the DEAE-Sephadex eluted fraction shown in Figure 2, lane 2. Arrows indicate position of p150 and minor accompanying protein.

NaCl, which was not clearly evident from Coomassie blue staining. An S40 fraction from low salt-grown cells was enriched with this protein (Fig. 5B) indicating a similar fractionation pattern for p150 from low and high salt grown cells. The last two analyses (Fig. 5, A and B) did not reveal the faster migrating component evident in previous analyses. Overall, the level of this protein varied greatly in different crude and purified preparations of p150. When present, the minor component immunoreacted with the anti-p150 antibodies (Fig. 5C). This result provides further support for the close similarity between this occasionally observed protein and p150.

N-Terminal Sequence Determination

DEAE-Sephadex-purified p150 free of the higher mobility component was analyzed directly or after passage on a gel permeation HPLC system. Similar results were obtained with the two preparations establishing the sequence of the first 13 amino acid residues as: ATQVKASGTVKNV. A comparison to DNA and protein sequence libraries did not reveal any meaningful similarities to this sequence.

Kinetics of p150 Induction after Hyperosmotic Shock and Dependence on *de Novo* Protein Synthesis

The dependence of the intracellular level of p150 on the salinity of the medium pointed to the possibility that the protein played an essential role in the adaptation of *D. salina* to high salinities. It was therefore of interest to analyze the protein in cells immediately following a hyperosmotic shock. To maximize the range of osmotic change, the cells were continuously grown in 0.5 M NaCl and then, to avoid excessive stress, were subjected to a two-step hyperosmotic shock. In the first step the cells were transferred to 1.5 M NaCl and remained in this medium until resumption of normal volume and cell motility indicated that osmotic adjustment had occurred. Following this 2 to 3 h step, the salinity of the medium was adjusted to 3.5 M NaCl. By the criteria of cell motility and glycerol contents (not shown) as well as by cell volume, osmotic readjustment was complete by 4 h, but cell proliferation was not reinitiated until after 24 h following the second shock (Fig. 6). Thus, under these conditions, resumption of cell proliferation did not coincide with osmotic recovery, but started only after an extended, additional lag period.

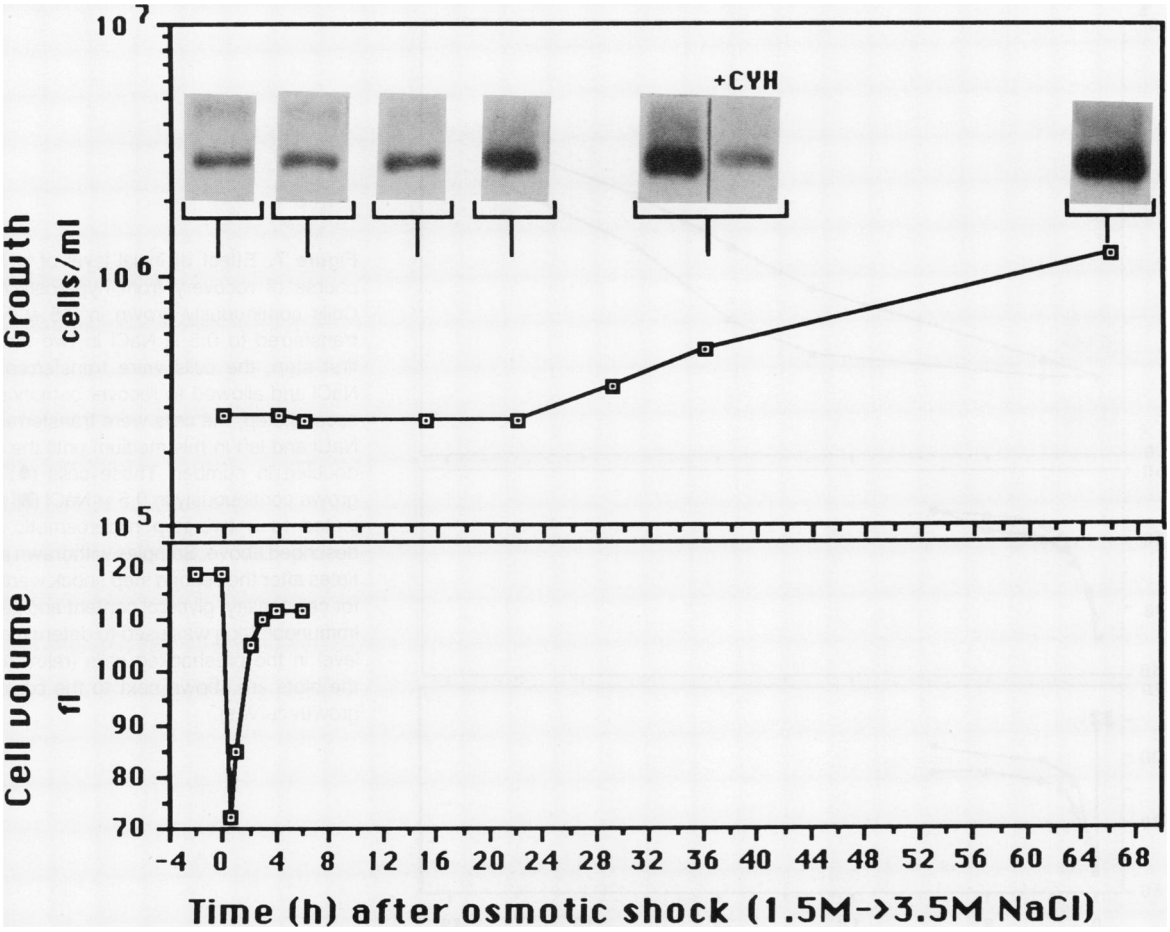

Figure 6. Course of recovery and p150 induction after hyperosmotic shock. Cells grown in 0.5 M NaCl were subjected to a two step hyperosmotic shock as described in "Materials and Methods." Cell density and volume were determined at different times after the second shock. Samples withdrawn from the preshocked cells (shown next to the zero time point) and at the indicated times after the shock were also analyzed for p150. To a sample removed 14 h after the shock, cycloheximide was added to 1 μg/mL, and 22 h later (36 h postshock), the treated cells were analyzed for p150.

Extracts made from cells at different times during and following osmotic recovery were also analyzed for p150 by immunoblot analysis with anti-p150 antibodies (Fig. 6). The initial level of p150 (somewhat higher in this experiment as compared with the results shown in Fig. 5A) remained unchanged until after 15 h following the shock, but a distinct increase was evident at 22 h, a time just preceding the reinitiation of cell division. A further increase was observed in the actively growing cells.

The experiment shown in Figure 6 was also used to determine whether the increase in the level of p150 represented *de novo* synthesis of the protein. For this purpose, cycloheximide was added to part of the culture 14 h after the shock. The concentration of the inhibitor used in this experiment was previously shown to completely inhibit ^{35}S incorporation into protein but not to totally disable the cells, as indicated by their normal responses to hypo- or hyperosmotic shocks (27). Analysis of the cycloheximide-treated sample 36 h after the shock indicated no increase in the level of p150. Thus, p150 induction was due to the *de novo* synthesis of the protein.

Effect of p150 on the Growth Lag following Hyperosmotic Shock

When cells grown continuously in 3.5 M NaCl were transferred to 0.5 M salt and allowed to recover from the hypoosmotic shock, they retained a relatively high level of p150 as compared to cells grown continuously in low salt. It was thus possible to compare the two types of cells for the course of their recovery from an identical hyperosmotic shock. The results (Fig. 7) show the two types of cells do not differ significantly in the rate of their osmoregulatory response, as expressed in volume and glycerol content readjustment. However, the cells enriched in p150 increase in number already 3 h after the shock, whereas a similar increase in the control cells is evident only 20 h after the shock. Although somewhat different growth kinetics were observed in different experiments, the correlation between p150 and growth resumption was observed in all cases. These results rule out a requirement for p150 induction during the early osmoregulatory response and suggest that an elevated level of the protein was required for cell proliferation in high salt.

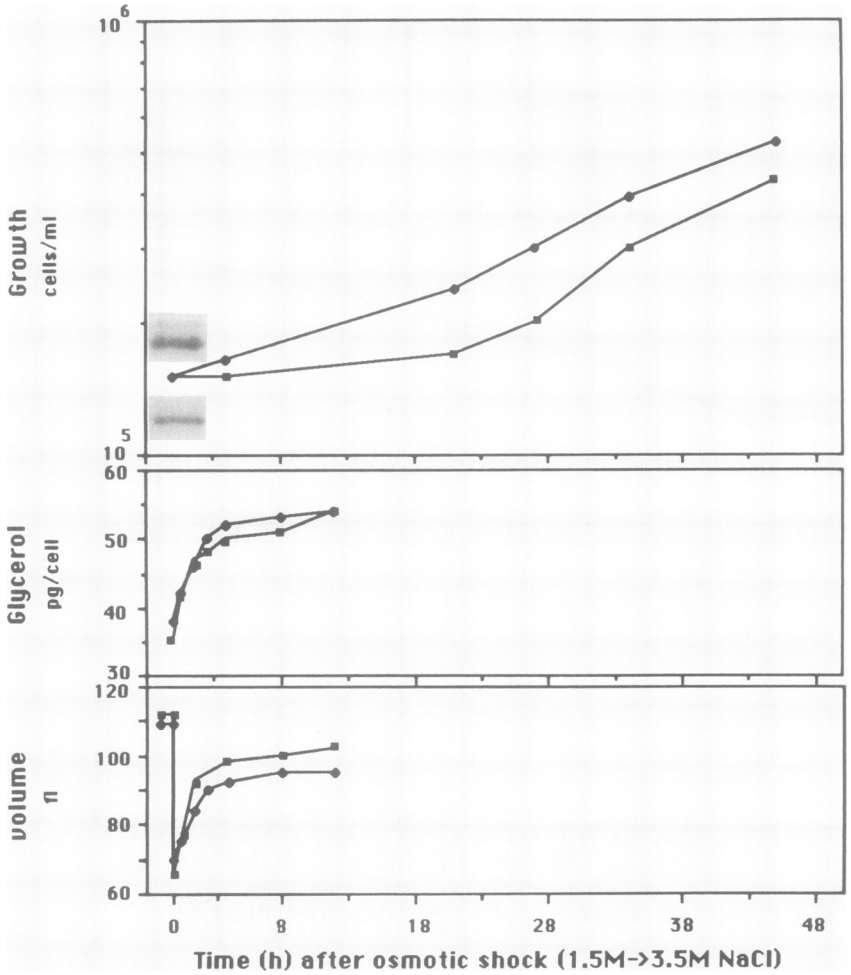

Figure 7. Effect of initial level of p150 on the course of recovery from hyperosmotic shock. Cells continuously grown in 3.5 M NaCl were transferred to 0.5 M NaCl in two steps: in the first step, the cells were transferred to 1.5 M NaCl and allowed to recover osmotically; in the second step, the cells were transferred to 0.5 M NaCl and left in this medium until the cells have doubled in number. These cells (♦) and cells grown continuously in 0.5 M NaCl (■) were subjected to a two-step hyperosmotic shock as described above. Samples withdrawn at different times after the second step shock were analyzed for cell density, glycerol content and cell volume. Immunoblotting was used to determine the p150 level in the preshocked cells (relevant parts of the blots are shown next to the corresponding growth curves).

Subcellular Localization of p150

Immunoelectron microscopy was used to determine the subcellular localization of p150 in cells grown in 3.5 M NaCl. The gluteraldehyde-acrolein fixed algae were ultrathin frozen sectioned and immunolabeled with rabbit anti-p150 antibodies, followed by gold-conjugated goat anti rabbit IgG. Preimmune serum from the same rabbit was used as a control for nonspecific labeling (Fig. 8D). A survey of large cell sections (Fig. 8A) indicates the protein is predominantly located at the cell surface where it is most probably embedded in the plasma membrane (which was partly detached from the body of the cells during sectioning). A larger magnification is shown in Fig. 8C. In a dividing cell, p150 was seen in the outer membrane, but not in the septum separating the daughter cells (Fig. 8B).

P150 in *Dunaliella bardawil*

The anti-p150 antibodies were also used to detect the presence of cross-reacting proteins in a related species, *D. bardawil*. The results (Fig. 9), indicate that *D. bardawil* contains a cross-reacting protein of an apparently larger molecular mass than p150.

DISCUSSION

Dunaliella provides the most striking example for osmoregulation based on the intracellular accumulation of a compatible, organic solute (31). However, when viewed more broadly, salt adaptation does not end with the glycerol-mediated osmotic balancing, but must also encompass the ability of the cells to proliferate under high salinities. The present experiments provide evidence that these two aspects are indeed mechanistically distinct and suggest a role of a salt-induced 150 kD glycoprotein in sustaining growth of *D. salina* in high salinities.

In a previous attempt to distinguish between osmotic and growth regulation, we showed that *D. salina* cells exposed to moderate hyperosmotic shocks, *i.e.* 0.5 M to 1.0 M or 1.5 M NaCl, started to divide soon after the cells had recovered osmotically (27). But, if immediately after recovery the cells were transferred to 3.0 M or 3.5 M salt, osmotic readjustment preceded by several hours the resumption of cell division. Therefore under these conditions osmotic recovery *per se* was clearly insufficient and additional requirements had to be fulfilled to permit reinitiation of cell division and to sustain growth at the elevated salinity.

The accumulation of a suitable amount of p150 could be

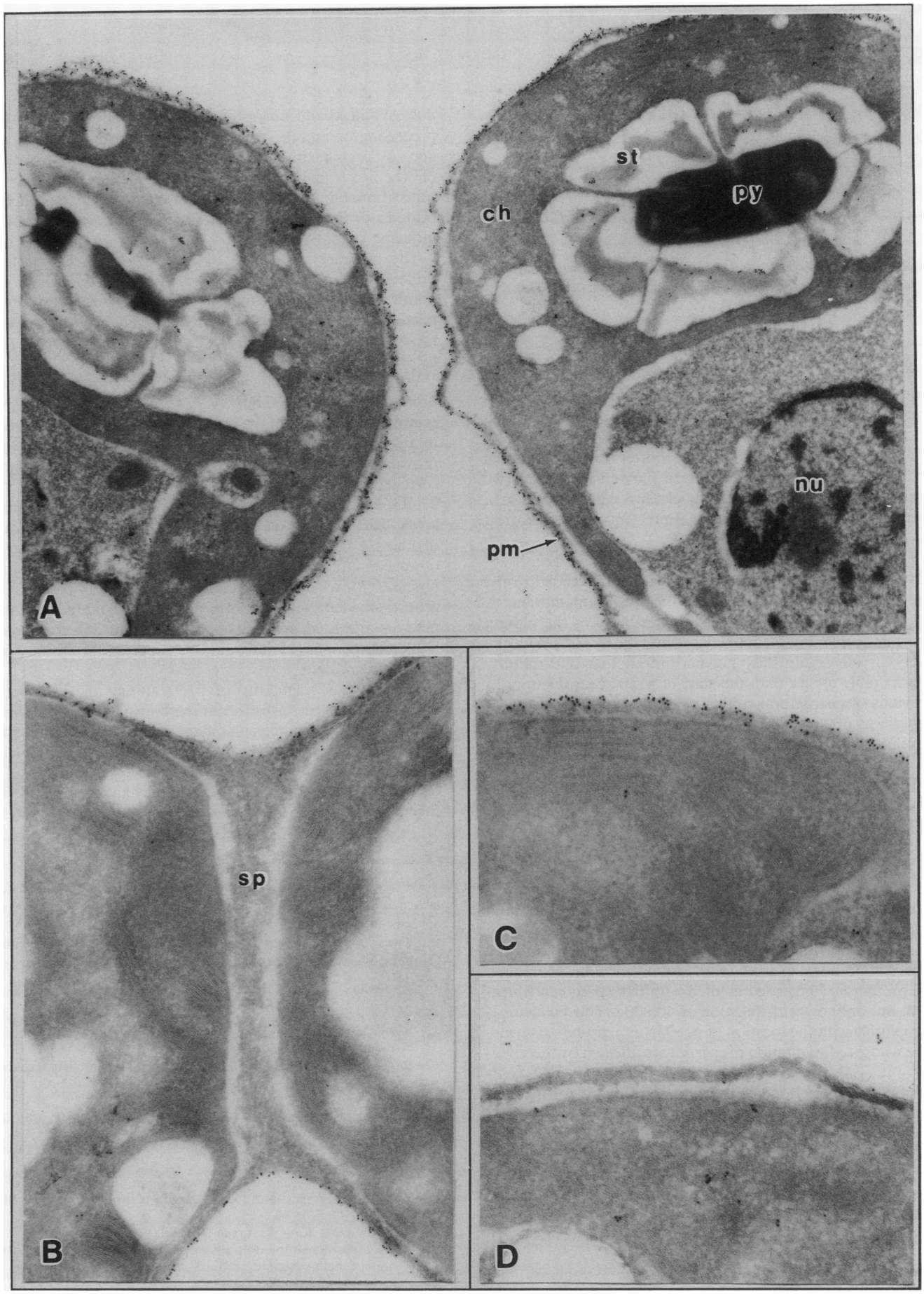

Figure 8. Subcellular localization of p150. Preparation of the cells and immunoelectron microscopy procedures were as described in "Materials and Methods." Abbreviations: nu, nucleus; py, pyrenoid; st, starch, ch, chloroplast; pm, plasma membrane; sp, septum. A, Large cell sections showing protein at cell surface; B, p150 seen in outer membrane but not in septum; C, larger magnification of panel A; D, control for nonspecific labeling.

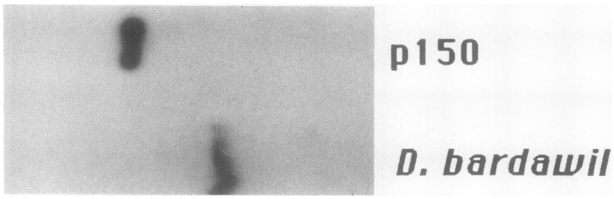

Figure 9. Anti-p150 cross-reacting protein in *D. bardawil*. *D. bardawil* cells grown in 3.0 M NaCl were extracted as described for *D. salina* and analyzed along with purified p150.

one of these requirements: the intracellular level of p150 is strictly dependent on the external salinity; a significant increase in p150 just precedes the restart of cell proliferation after hyperosmotic shock; the growth lag period following hyperosmotic shock is considerably shorter in cells containing an initial higher level of p150. In a previous study (27) we postulated that hyperosmotic shocks arrested the cells at a specific stage of their growth cycle. Emergence from this arrest may then require the action of p150.

From its subcellular localization at the cell surface, it appears likely that p150 might fulfill a function related to the permeability or flux properties of the plasma membrane. A role in the mechanical strength or flexibility of the plasma membrane does not seem likely because the hyperosmotically shocked cells regain their normal morphology and motility considerably earlier than the start of p150 accumulation. In previous ultrastructural studies of *Dunaliella tertiolecta* evidence was obtained for the presence of glycocalyx-type cell envelope, probably made up of the carbohydrate and protein moieties of glycoproteins embedded in the plasma membrane (25). It is likely that p150 might constitute one of these glycoproteins.

The membrane localization of p150 is in accord with additional observations. In our initial fractionation attempts, cell lysis was performed under mild conditions as used in the release of plasma-membrane fractions containing a vanadate-sensitive H^+-ATPase (32). The p150 protein was not released under these conditions, and remained in the low speed pellet (data not shown). When the cells were burst under more drastic conditions, as in the standard purification procedure, the protein no longer sedimented with low speed centrifugation, but only by centrifugation at 40,000*g*. This behavior is compatible with the location of p150 in a membranous structure whose sedimentation properties are affected by the severity of the lysis step. The evidence for p150 being a detergent-soluble glycoprotein constitutes further support for a membrane localization of the protein.

Glycoproteins in the range of 140 to 170 kD were previously identified as surface components in a variety of eukaryotic cells and also have prokaryotic counterparts. Members of this group of structurally conserved proteins have been proposed to act as energy-dependent efflux pumps (for a review see ref. 10). The possibility that p150 might be related to this group of proteins is currently being examined.

Because immunoblot analysis of crude extracts did not reveal potential precursor forms of p150, we initially concluded that its accumulation was most probably due to *de novo* synthesis and not to regulation on the level of protein processing or modification. The inhibition of p150 appearance by cycloheximide supports this conclusion. Regardless of whether regulation acts on the level of transcription or translation, our results indicate that the elevated salt concentrations, or perhaps osmotic stress in general, is the primary inducing factor of p150.

The presence of a p150 immunologically cross-reacting protein in *D. bardawil* is not surprising in view of the taxonomic relatedness and similar halotolerance of the two organisms. The apparent size difference between the proteins from the two strains could be partly accounted for by variation in the degree of glycosylation.

While salt-induced changes in the protein profiles have not been previously studied in *Dunaliella*, numerous studies have examined the effects of the interrelated salt and water stresses in higher plants. Analyses on both protein and mRNA levels provided evidence for a variety of salt-related changes, some correlated with hormonal regulation (*e.g.* refs. 9, 11, 14, 18, 22, 23, 26, 28, 30). Of the salt-induced proteins, several play a well-defined metabolic adaptive role (22), whereas the role of others, *e.g.* the extensively studied 26 kD osmotin originally observed in tobacco suspension cells (11, 18, 28), still remains obscure. More recently, additional osmotically regulated genes were identified in graminea (9, 12, 23), but none of the newly synthesized proteins appears to be an integral membrane component (15) as would be expected for an ion flux controlling protein.

The obvious singularity of p150 among the salt stress induced proteins described so far in photosynthetic organisms is in its being a high molecular mass, cell surface glycoprotein. Furthermore, the function of p150, or a coinduced protein with a similar stability, appears to be necessary for the cells to proliferate in high salt and thus to play an essential role in the halotolerance of *Dunaliella*.

ACKNOWLEDGMENTS

We are indebted to M. Avron, A. Ben Amotz, and U. Pick for advice and useful discussions, to M. Wilchek for help in glycoprotein analysis, and to A. Segal and U. Faber for technical assistance. This study was supported in part by the Forchheimer Center for Molecular Genetics, Weizmann Institute of Science.

LITERATURE CITED

1. **Avron M** (1986) The osmotic components of halotolerant algae. Trends Biochem Sci **11:** 5–6
2. **Bayer EA, Ben Hur H, Wilchek M** (1987) Enzyme-based detection of glycoproteins on blot transfers using avidin-biotin technology. Anal Biochem **161:** 123–131
3. **Ben Amotz A, Avron M** (1978) On the mechanism of osmoregulation in *Dunaliella*. *In* SR Caplan, M Ginzburg, eds, Energetics and Structure of Halophilic Microorganisms. Elsevier, Amsterdam, pp 529–541
4. **Ben Amotz A, Avron M** (1983) Accumulation of metabolites by halotolerant algae and its industrial potential. Annu Rev Microbiol **37:** 95–119
5. **Ben Amotz A, Katz A, Avron M** (1982) Accumulation of beta-carotene in halotolerant algae: purification and characterization of beta-carotene globules from *Dunaliella bardawil*. J Phycol **18:** 529–537
6. **Berman J, Gershoni JM, Zamir A** (1985) Expression of nitrogen fixation genes in foreign hosts. J Biol Chem **260:** 5240–5243

7. **Borowitzka MA, Borowitzka LJ** (1988) *In* MA Borowitzka, LJ Borowitzka, eds, Micro-algal Biotechnology, Cambridge University Press, Cambridge, pp 27–58
8. **Borowitzka LJ, Kessly DS, Brown AD** (1977) The salt relations of *Dunaliella*: further observations on glycerol production and its regulation. Arch Microbiol **113:** 131–138
9. **Claes B, Dekeyser R, Villarroel R, Van den Bulcke M, Bauw G, Van Montagu M, Caplan A** (1990) Characterization of a rice gene showing organ-specific expression in response to salt stress and draught. Plant Cell **2:** 19–27
10. **Endicott JA, Ling V** (1989) The biochemistry of *p*-glycoprotein-mediated multidrug resistance. Annu Rev Biochem **58:** 137–171
11. **Ericson MC, Alfinto SH** (1984) Proteins produced during salt stress in tobacco cell culture. Plant Physiol **74:** 506–509
12. **Gomez J, Sanchez-Martinez D, Stiefel V, Rigau J, Puigdomenech P, Pages M** (1988) A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. Nature **334:** 262–264
13. **Gromoff ED V, Treier U, Beck CF** (1989) Three light-inducible heat-shock genes of *Chlamydomonas reinhardtii*. Mol Cell Biol **9:** 3911–3918
14. **Hurkman WJ, Tanaka C** (1987) The effects of salt on the pattern of protein synthesis in barley roots. Plant Physiol **83:** 517–524
15. **Hurkman WJ, Tanaka CK, DuPont FM** (1988) The effects of salt stress on polypeptides in membrane fractions from barley roots. Plant Physiol **88:** 1263–1273
16. **Jorgensen PL, Collins JH** (1990) Tryptic and chemotryptic cleavage sites in the sequence of the α-chain of Na,K-ATPase from the outer medulla of mammalian kidney. Biochim Biophys Acta **860:** 570–576
17. **Karni L, Avron M** (1988) Ion content of the halotolerant alga *Dunaliella salina*. Plant Cell Physiol **29:** 1131–1314
18. **King GJ, Hussey CE, Turner VA** (1988) A protein induced by NaCl in suspension cultures of *Nicotiana tabacum* accumulates in whole plant roots. Plant Mol Biol **10:** 401–412
19. **Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
20. **Lazard D, Tal N, Rubinstein M, Khen M, Lancet D, Zupko K** (1990) Identification and biochemical analysis of novel olfactory-specific cytochrome P-450IIA and UDP-glucouronyl transferase. Biochemistry **29:** 7433–7440
21. **Lers A, Biener Y, Zamir A** (1990) Photoinduction of massive beta-carotene accumulation by the alga *Dunaliella bardawil*. Plant Physiol **93:** 389–395
22. **Michalowski CB, Olson SW, Piepenbrock M, Schmitt JM, Bohnert HJ** (1989) Time course of mRNA induction elicited by salt stress in the common ice plant (*Mesembryanthemum crystallinum*). Plant Physiol **89:** 811–816
23. **Mundy J, Chua NH** (1988) Abscisic acid and water stress induce the expression of a novel rice gene. EMBO J **7:** 2279–2286
24. **O'Farrell PH** (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021
25. **Oliveira L, Bisalputra T, Antia NJ** (1980) Ultrastructural observation of the surface coat of *Dunaliella tertiolecta* from staining with cationic dyes and enzyme treatments. New Phytol **85:** 385–392
26. **Ramgopal S** (1987) Differential mRNA transcription during salinity stress in barley. Proc Natl Acad Sci USA **84:** 94–98
27. **Sadka A, Lers A, Zamir A, Avron M** (1989) A critical examination of the role of de novo protein synthesis in the osmotic adaptation of the halotolerant alga *Dunaliella*. FEBS Lett **244:** 93–98
28. **Singh NK, LaRosa PC, Handa AK, Hasegawa PM, Bressan RA** (1987) Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. Proc Natl Acad Sci USA **84:** 739–743
29. **Tokuyaso KT** (1973) A technique for ultracryotomy of cell suspensions and tissues. J Cell Biol **57:** 551–565
30. **Tokuyaso KT** (1978) A study of positive staining of ultrathin frozen sections. J Ultrastruct Res **63:** 287–307
31. **Vreeland RH** (1987) Mechanisms of halotolerance in microorganisms. CRC Crit Rev Microbiol **14:** 311–356
32. **Weiss M, Sekler I, Pick U** (1989) Characterization of soluble and membrane-bound forms of a vanadate-sensitive ATPase from plasma membranes of the halotolerant alga *Dunaliella salina*. Biochim Biophys Acta **974:** 254–260