Salt-induced Changes in the Plasma Membrane Proteome of the Halotolerant Alga *Dunaliella salina* as Revealed by Blue Native Gel Electrophoresis and Nano-LC-MS/MS Analysis*[S]

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The halotolerant alga *Dunaliella salina* is a recognized model photosynthetic organism for studying plant adaptation to high salinity. The adaptation mechanisms involve major changes in the proteome composition associated with energy metabolism and carbon and iron acquisition. To clarify the molecular basis for the remarkable resistance to high salt, we performed a comprehensive proteomics analysis of the plasma membrane. Plasma membrane proteins were recognized by tagging intact cells with a membrane-impermeable biotin derivative. Proteins were resolved by two-dimensional blue native/SDS-PAGE and identified by nano-LC-MS/MS. Of 55 identified proteins, about 60% were integral membrane or membrane-associated proteins. We identified novel surface coat proteins, lipid-metabolizing enzymes, a new family of membrane proteins of unknown function, ion transporters, small GTP-binding proteins, and heat shock proteins. The abundance of 20 protein spots increased and that of two protein spots decreased under high salt. The major salt-regulated proteins were implicated in protein and membrane structure stabilization and within signal transduction pathways. The migration profiles of native protein complexes on blue native gels revealed oligomerization or co-migration of major surface-exposed proteins, which may indicate mechanisms of stabilization at high salinity. *Molecular & Cellular Proteomics 6:1459–1472, 2007.*

The halotolerant alga *Dunaliella salina* uses a unique osmoregulatory mechanism and is able to proliferate in environments with extreme salt content (1). This is achieved through remarkable changes in metabolism and ion transport brought about by up-regulation or down-regulation of multiple enzymes and membrane proteins. Elucidation of the molecular basis for this remarkable adaptation might provide tools to improve the resistance of crop plants to the progressive salinization of soils, which is already a major limitation for agricultural productivity worldwide. By characterizing the changes in the soluble subproteome of *D. salina*, we demonstrated previously that the alga responds to hypersaline conditions by up-regulating key enzymes in photosynthetic CO_2 fixation and in energy metabolism that divert carbon metabolism to massive synthesis of glycerol, the osmotic element in *Dunaliella* (2).

Salinity stress destabilizes biological membranes and affects the solubility of many essential substrates and ions (3). Adaptation to salinity stress might also alter the composition and organization of the membrane proteome associated with the stabilization and enhancement of ion transporters. Early studies revealed the accumulation of two carbonic anhydrases, dCAI[1] and dCAII (4, 5), and a transferrin-like protein (6) that presumably compensated the impaired availability of bicarbonate and iron under high salt. Salt-induced changes in organellar membranes, such as the expression of ER fatty acid elongase, were also reported (7). High salinity also affects sodium transport (8), lipid organization (9, 10), and activation of PM protein kinases (11). Taken together, the accumulated evidence suggested that short and long range reorganization of the structure and composition of the PM is at the core of molecular mechanisms responsible for the salinity response.

In this study we report the effect of salinity stress on the composition and organization of *D. salina* plasma membrane proteome. To circumvent well known limitations in separation and quantification of membrane proteins by conventional 2D gel electrophoresis, we used blue native (BN)/SDS-PAGE to separate hydrophobic membrane proteins and complexes in

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[1] The abbreviations used are: dCA, *Dunaliella* carbonic anhydrase; CA, carbonic anhydrase; PM, plasma membrane; BN, blue native; BN/SDS-PAGE, two-dimensional blue native SDS-PAGE; 1D, one-dimensional; 2D, two-dimensional; HSP, heat shock protein; ER, endoplasmic reticulum; Ttf, transferrin-like protein; DM, *n*-dodecyl β-D-maltoside; MS BLAST, MS-driven Basic Local Alignment Search Tool; sup, supernatant; NHS, *N*-hydroxysuccinimide; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EST, expressed sequence tag.

their native state. The method was previously applied for separating mitochondrial membrane protein complexes (12), chloroplast protein complexes (13), whole cell lysates (14), and in some preliminary reports on plant plasma membrane (15).

We performed a proteomics analysis of the PM-enriched preparation from *D. salina* that was extensively characterized in our laboratory (8, 16, 17). To enhance the analysis specificity, we tagged surface-exposed proteins in intact cells with a membrane-impermeable biotin tag, which specifically labeled plasma membrane proteins.

Proteins and protein complexes separated by BN/SDS-PAGE were identified by nano-LC-MS/MS sequencing followed by conventional and sequence similarity-driven database searches. For the first time, we obtained a comprehensive overview of membrane and membrane-associated proteins in *D. salina* and characterized its adaptation in response to salinity stress. We identified a total of 55 proteins, including novel surface coat proteins, lipid-metabolizing enzymes, a new family of membrane proteins, ion transporters, small GTP-binding proteins, and heat shock proteins. The abundance of 20 protein spots increased and that of two protein spots decreased under high salt, thus elucidating the mechanisms of plasma membrane proteome adaptation via concerted changes in its composition and organization.

EXPERIMENTAL PROCEDURES

Algal Material

D. salina, a green species, was obtained from the culture collection of Dr. W. H. Thomas, La Jolla, CA. Algae were cultured for several weeks in 0.5 M NaCl (low salt) or in 3 M NaCl (high salt) medium as described previously (18). Cells were usually cultured at 10 watts/m^2 on a shaker in a 26 °C temperature-controlled room.

Membrane Preparation

Cells of 6-liter cultures at logarithmic growth phase containing 2–3·10^6 cell/ml were harvested and washed in glycerol buffer containing glycerol osmotically equivalent to the NaCl concentration in the growth medium (for 0.5 M NaCl, 0.8 M glycerol; for 3 M NaCl, 4 M glycerol), 10 mM Na^+-MOPS, pH 7.2, 10 mM KCl, and 2 mM $MgCl_2$. The cells were osmotically lysed (1:4 dilution) by suspension of the pellet at a final concentration of 2·10^8 cells/ml in bursting buffer (10 mM Na^+-MOPS, pH 7.2, 10 mM KCl, 2 mM $MgCl_2$, 5 mM ε-aminocaproic acid, 1 mM benzamidine, and plant protease inhibitor mixture (Sigma, catalog number P-9599) diluted to 1:200. The lysed cells were incubated on ice for 30 min, and 100 mM KCl was added in the last 5 min. The lysed cells were centrifuged at 800 × *g* for 15 min at 4 °C and separated to sup I and pellet. The pellet was washed with washing buffer (0.5 M glycerol, 10 mM Na^+-MOPS, pH 7.2, 2 mM $MgCl_2$, 100 mM KCl, and protease inhibitors as above) and centrifuged as above. The pellet fraction, which contained the chloroplast, was discarded, and the supernatant, sup II, containing the membranes was combined with sup I and incubated for 30 min at 4 °C in the presence of 4 mM EDTA to eliminate ribosomes, ribulose-bisphosphate carboxylase/oxygenase, and other contaminating proteins. The supernatants were centrifuged for 1 h at 17,000 × *g* and 4 °C. The pellet, which contained plasma membranes, was washed by suspension buffer (0.5 M glycerol, 10 mM Na^+-MOPS, pH 7.2, 2 mM $MgCl_2$, and 10 mM KCl) and centrifuged again as above. The sedimented PM was suspended in a small volume of suspension buffer, and aliquots were stored in liquid nitrogen until further use. Total protein was determined by BCA (Sigma, catalog number B-9643) using BSA as standard.

Biotinylation of Surface Proteins

D. salina cells were biotinylated with an impermeable biotin reagent sulfo-NHS-LC-biotin (Pierce, catalog number 21335) as described previously (19). In brief, cell samples were washed with biotinylation buffer containing PBS, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM $NaHCO_3$, and glycerol osmotically equivalent to the NaCl concentration in the growth medium, pH 7.8. Cells were incubated with 0.5 mg/ml NHS-LC-biotin at 10 °C for 30 min. Following the cells were washed twice with biotinylation buffer containing 50 mM glycine, pH 6.5, and then subjected to fractionation as described above. The biotinylated proteins were identified after BN/SDS-PAGE separation and blotting with streptavidin as described below.

BN/SDS-PAGE

First dimension BN-PAGE was performed essentially as described previously (12) with some modifications. For each lane of BN-PAGE 100 μg of membrane proteins were pelleted and resuspended in 30 μl of solubilization buffer (50 mM bis-Tris, pH 7, 500 mM ε-aminocaproic acid, 2 mM EDTA, 10% glycerol, 0.5 mM PMSF, and plant protease mixture diluted to 1:100). For solubilization, *n*-dodecyl β-D-maltoside (DM) was added to a final concentration of 2%. After incubation on ice for 20 min, insoluble material was pelleted at 100,000 × *g* for 30 min at 4 °C. Three microliters of sample buffer (2.5% Coomassie Brilliant Blue G-250 in 0.5 M ε-aminocaproic acid) were added, and after a short spin the entire sample was loaded for 5–14% gradient BN-PAGE (100 × 100 × 1.5-mm gel). Electrophoresis was carried out at 4 °C at a constant current of 4 mA. After the tracking dye reached the second third of the gel, the cathode buffer was replaced by cathode buffer containing 10% Coomassie Brilliant Blue G-250 (final concentration, 0.002%). Molecular mass markers were purchased from GE Healthcare (product number 17-0445-01). After electrophoresis, the gel was rinsed with water for 10 min, and the lanes were cut out.

Second dimension SDS-PAGE gradient gels (6–18% acrylamide, 150 × 170 × 1.5 mm) were prepared according to conventional Laemmli (20) conditions. Each of the one-dimensional (1D) BN lanes were denatured by incubation in 5 ml of equilibration buffer (50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and traces of bromphenol blue) including 10 mg/ml DTT for 1 h followed by incubation in equilibration buffer containing 25 mg/ml iodoacetamide for 30 min and rinsing with running buffer. The strips were loaded horizontally on top of the second dimension stacking gel and covered with sealing buffer (0.5% agarose in running buffer). Two lanes were loaded side by side on the same gel, 0.5 M NaCl cell membranes and 3 M NaCl cell membranes, to enable optimal comparison of protein patterns. Electrophoresis was performed at 70 V until the front passed into the stacking gel. The electrophoresis was stopped, and the 1D strip was removed from the top of the gel, and the run was continued at 120 V. The gels were either stained with Coomassie Brilliant Blue R-250 or blotted onto a nitrocellulose membrane (where specified), and proteins were detected with antibodies as described below. To visualize biotin-labeled membrane proteins, only 10 μg of protein samples were loaded on each lane of the BN/SDS-PAGE gel due to the extreme high sensitivity of the avidin-biotin detection.

1D Band Separation on Second Dimension SDS-PAGE—Bands 1–14 (see Fig. 1) were excised from several lanes of the 1D BN-PAGE gel. Four identical bands were loaded in one lane of a 6–18% gradient SDS-PAGE gel and run as described above.

Low salt and high salt plasma membrane preparations and separation by BN/SDS-PAGE were repeated at least four times. Spot detection and quantitative analysis were performed using the Z3 software version 3.0 (Compugen, Tel Aviv, Israel), which determines spot areas at the pixel level. Differential expression represented the ratio of spot abundance in the 3 M NaCl gel image to its abundance in the 0.5 M NaCl gel image.

Immunoanalysis

After electroblotting of the 2D gels, the membranes were blocked with 10% skimmed milk in PBS, probed with different first antibodies (as described in the legend of Fig. 4) for 1 h, and incubated with horseradish peroxidase-coupled second antibody using ECL analysis. For the detection of the biotinylated proteins, the nitrocellulose membranes were blocked with 5% albumin in TBS and probed with 20 ng/ml horseradish peroxidase-conjugated streptavidin (Pierce catalog number 21126) for 1 h followed by six washes in TBS and ECL analysis.

Identification of Protein Spots by LC-MS/MS

In-gel Digestion—Spots visualized by Coomassie Brilliant Blue R-250 staining were excised and in-gel digested with trypsin as described previously (21, 22). Trypsin was applied at the concentration of 12.5 ng/μl in 50 mM ammonium bicarbonate buffer containing 10% (v/v) acetonitrile with 0.01% (w/v) 5-cyclohexyl-1-pentyl-β-D-maltoside added to enhance the recovery of hydrophobic peptides (23).

Analysis by LC-MS/MS—Samples were redissolved in 15–20 μl of 0.05% TFA and analyzed on a nano-LC-MS/MS Ultimate system (Dionex, Amsterdam, The Netherlands) interfaced on line to a linear ion trap LTQ (Thermo Fisher Scientific, San Jose, CA) as described previously (24). From raw files, MS/MS spectra were exported as individual files in dta format using the extract_msn.exe program (a part of BioWorks 3.2 software from Thermo Fisher Scientific) under the following settings: peptide mass range, 500–3500 Da; minimal total ion intensity threshold, 1000; minimal number of fragment ions, 15; precursor mass tolerance, 1.4 amu; group scan, 1; minimum group count, 1.

Database Search—Extracted MS/MS spectra were merged into a single Mascot generic format file (mgf) and searched against MSDB (Mass Spectrometry Protein Sequence Database, updated May 15, 2005, containing 2,011,572 entries) or a *D. salina* EST database (from the National Center for Biotechnology Information (NCBI), updated March 16, 2006, containing 3998 entries) by Mascot version 2.1 software (Matrix Science Ltd., London, UK) installed on a local server with two central processing units. Tolerance for precursor and fragment masses was 2.0 and 0.5 Da, respectively; the instrument profile was as follows: ESI-trap; fixed modification, carbamidomethyl (cysteine); variable modification, oxidation (methionine). Biotinylation of lysine residues was not listed among variable modifications because under the reaction conditions used it occurred at a negligible extent.

De Novo Sequencing and Sequence Similarity Searches—Tandem mass spectra were interpreted *de novo* by PepNovo software (kindly provided by Prof. P. Pevzner, University of California San Diego) that was modified to generate several redundant, degenerate, and possibly partially inaccurate sequence candidates per each interpreted spectrum (25). PepNovo estimated the expected confidence in the produced sequence candidates by assigning a score, which corresponds to the expected number of correct amino acids in the best sequence candidate. All sequences whose score exceeded a threshold of 2.0 were merged into a single query string and submitted to MS-driven BLAST (MS BLAST) (26) search against a non-redundant database (nrdb95) at the dedicated server. Prior to *de novo* sequencing, the entire pool of acquired MS/MS spectra was filtered to remove spectra originating from common background proteins (trypsin and keratins) and from non-peptide precursors (detergents, plasticizers, etc.) as described previously (27).

Criteria of Statistical Confidence of Database Searching Hits—The threshold ions scores suggested by Mascot for confident single peptide identifications in MSDB was 53 (all species) ($p < 0.05$). Searches were performed without restricting species of origin; therefore we considered as confident hits those with at least two matched peptides with combined peptide ion score exceeding 95. Hits produced by matching of at least one peptide with a minimum score of 40 were considered borderline and subjected to subsequent validation by *de novo* sequencing and MS BLAST database searching as described previously (23). Because of the small size of the *D. salina* EST database, we then accepted as confident hits those identified by matching one peptide whose ion score was above 40. The threshold ion score suggested by Mascot for confident single peptide identifications in the EST database was 31 ($p < 0.05$). Annotated spectra of single peptide hits are provided in the supplemental materials. In sequence similarity searches, statistical significance of hits was evaluated according to the MS BLAST scoring scheme (28). Because of the high redundancy of the queries, only hits identified with a total BLAST score above 100 or with at least one high scoring segment pair above 72 were considered in the scoring scheme. When MS BLAST was used as a validation tool (23), the high scoring segment pair overlapping with the Mascot hit sequence should have a score of 62 or higher. When proteins were solely identified with MS BLAST, at least one high scoring segment pair with the score above 72 and the total score of the hit above 200 were required.

RESULTS

Membrane Preparation

A proteomics analysis was carried out on a plasma membrane-enriched preparation from *D. salina* that was described previously (2, 8, 17). To reduce the amount of contaminating soluble proteins, we modified the isolation procedure. First we increased the salt concentration during cell lysis, and second we optimized the differential centrifugation conditions to eliminate ribosomal proteins and ribulose-bisphosphate carboxylase/oxygenase. An alkaline carbonate wash, which is often applied to remove peripheral proteins, dissociated membrane protein complexes and was not applied (Fig. 1*A*). Instead we used an impermeable biotin reagent to tag the surface-exposed plasma membrane proteins and hence enable their identification. To identify changes in the membrane protein composition and organization at high salinity, we compared membranes obtained from cells cultured either at 0.5 M (low salt) or at 3.0 M (high salt) NaCl.

BN Analysis

To determine optimal conditions for the solubilization of membrane proteins within intact protein complexes, we tested various detergents, Triton X-100, zwittergent, DM, and digitonin, within the concentration range of 0.3–4% (data not shown). Best results were achieved with 2% DM, which was used in all subsequent experiments. Fig. 1*B* shows a representative 1D BN-PAGE separation of membrane proteins from low salt and high salt preparations. Gradient gels of 5–14%

acrylamide resolved 14 distinct bands whose molecular masses were in the range of 60–800 kDa. The relative abundances of several bands, as judged by the intensity of their Coomassie staining, differed substantially in low and in high salt membrane preparations, suggesting that the expression or oligomerization state of the corresponding proteins was affected.

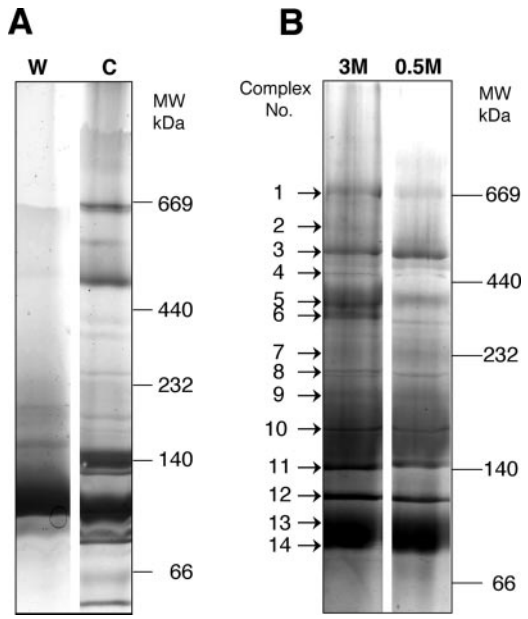

FIG. 1. **First dimension BN-PAGE separation of membrane preparations isolated from *D. salina* cells.** Membrane proteins were solubilized in 2% DM, as described under "Experimental Procedures," and 100 μg were applied on each lane. *A*, alkaline wash: membrane preparations were either treated (*w*) or not treated (*c*) with Na_2CO_3. *B*, separation of membranes from low salt cells grown in 0.5 M NaCl and high salt cells grown in 3 M NaCl. For better visualization of bands the gel was stained with Coomassie Blue R-250 after the separation. The *numbers* and *arrows* on the *left* indicate the protein complexes.

For separation in the second dimension, BN gel strips carrying membrane proteins, isolated from cells grown at low and high salinity, were loaded horizontally on the same SDS-PAGE gel to minimize the differences in running conditions (Fig. 2). Alternatively bands were excised from 1D BN gels and loaded as individual lanes for SDS-PAGE (Fig. 3). Fig. 2 shows a representative BN/SDS-PAGE map of membrane proteins from 0.5 M NaCl and 3 M NaCl. More than 50 spots were recognizable on each BN/SDS-PAGE map. The comparison of the low salt and high salt protein maps revealed that 20 spots were reproducibly increased by high salt by more than 2-fold, whereas two were almost completely suppressed.

Interestingly a number of major proteins appeared in several BN bands having different apparent molecular weights. To test whether this represented different oligomeric states of the same protein, we performed an immunoblot analysis with specific antibodies against several previously characterized *Dunaliella* plasma membrane proteins. The Western blotting (Fig. 4) demonstrated that the transferrin-like protein Ttf (4), the previously characterized plasma membrane P-type ATPase,[2] and two carbonic anhydrases, dCAI and dCAII (4, 5), migrated in different spots and therefore most likely represented their different oligomeric states.

To identify *bona fide* plasma membrane proteins, we tagged them with an impermeable biotin derivative. This reagent was expected to react primarily with surface-associated proteins and integral plasma membrane proteins having extracellular hydrophilic domains. Proteins resolved by BN/SDS-PAGE were blotted and incubated with avidin. As shown in Fig. 5, the protein profiles are quite similar to the total protein maps (Fig. 2) confirming that the preparation contained mostly plasma membrane proteins. The major biotiny-

[2] M. Weiss and U. Pick, unpublished data

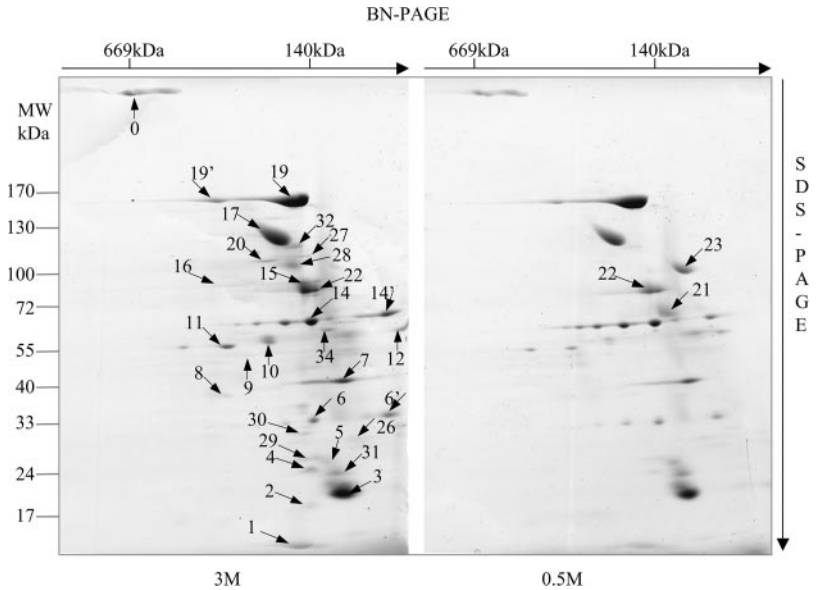

FIG. 2. **BN/SDS-PAGE proteomic maps of membrane proteins from 0. 5 and 3 M NaCl cells.** 1D BN strips were loaded and separated by 6–18% acrylamide SDS-PAGE. The 1D BN strips were oriented with top to the left and bottom to the right. Two samples were run in parallel in the same SDS gel. The presented proteomic maps are typical representatives of Coomassie Brilliant Blue-stained membrane protein gels obtained from 0.5 M (*right*) and 3 M NaCl (*left*) cells. At least four independent gel separations were performed for each sample, and they showed a high degree of reproducibility; one typical gel separation is shown. *Arrows* represent spots that were excised and identified by MS and correspond to the numbers shown in Table I.

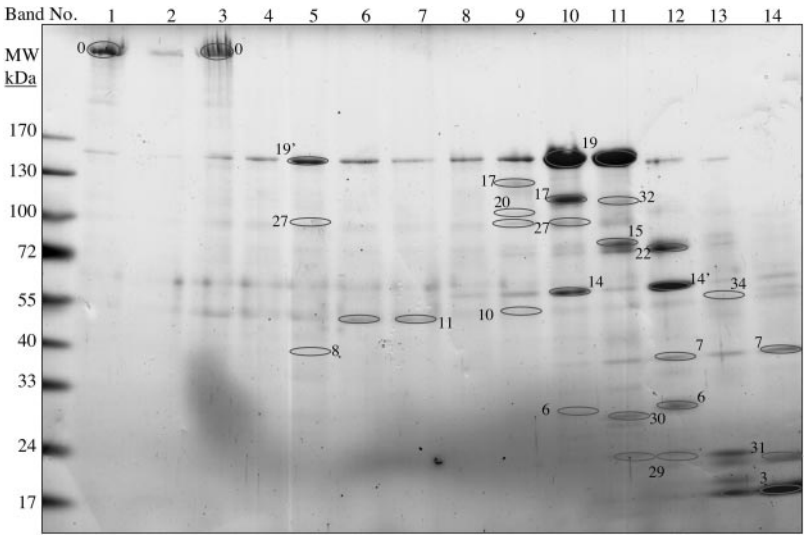

FIG. 3. **Analysis of BN bands by SDS-PAGE.** Bands numbered as shown in Fig. 1 were cut out from BN 1D gels. Four identical bands were loaded separately in each lane of a 6–18% acrylamide SDS gel and separated as described under "Experimental Procedures." The *numbers* of bands in the gel correspond to spot numbers in Fig. 2 and in Table I.

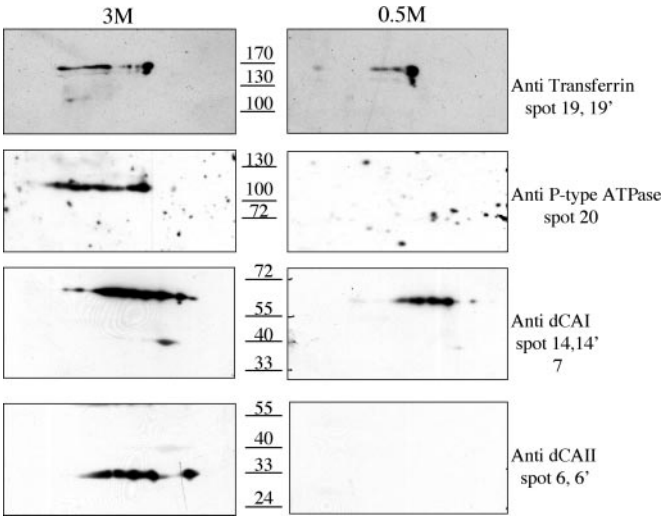

FIG. 4. **Differential expressions of proteins at 0. 5 and 3 M NaCl as revealed by immunoblot analysis.** Proteins were separated by BN/SDS-PAGE as in Fig. 2. Gels were electroeluted to nitrocellulose, and each blot was analyzed with a different antibody: anti-transferrin, anti-P-type ATPase, and anti-carbonic anhydrases dCAI 64 kDa and dCAII 32 kDa. The spot *numbers* indicate the matching spots in Fig. 2 and in Table I.

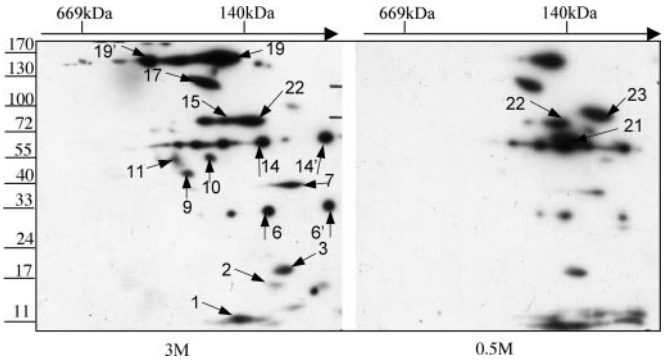

FIG. 5. **Cell surface biotinylated proteins in membrane preparations from 0. 5 and 3 M cells.** *D. salina* cells grown in 0.5 M or in 3 M NaCl were biotinylated, plasma membranes were prepared, and their proteins were separated on 2D BN/SDS gels (as in Fig. 2) and analyzed by Western blotting using horseradish peroxidase-conjugated streptavidin. One representative Western analysis of four separate repetitions is shown.

lated spots corresponded to Ttf, dCAI, and dCAII (spots 19, 14, and 6) located at the surface of *D. salina* plasma membrane (4, 6). The biotinylation maps also showed many minor spots, presumably representing uncharacterized plasma membrane proteins.

Spot Identification

Thirty-five individual protein spots, which were consistently detected by Coomassie staining (designated by *arrows* in Figs. 2 and 3), were excised and digested with trypsin, and recovered peptides were sequenced by nano-LC-MS/MS (Table I). The table also indicates whether the particular spot was up- or down-regulated by high salt and whether it was biotin-labeled.

We found that 20 spots contained more than one protein, and in total, 55 unique proteins were identified. Co-migration of several proteins within a single spot is commonly observed when the analytical methods with enhanced dynamic range (such as, for example, nano-LC-MS/MS) are routinely applied for the identification of spots from 2D gels (29). The identified proteins include some previously characterized plasma membrane *Dunaliella* proteins, several integral membrane proteins and a novel family of putative integral membrane proteins (discussed below), membrane-associated proteins, and a few soluble enzymes. A few membrane proteins from several internal compartments were also identified, including chloroplasts, mitochondria, and endoplasmic reticulum.

A sizable fraction of proteins were identified by searching against a *Dunaliella* EST database. The translated protein sequences of some of these ESTs were not homologous to

TABLE I

Proteins identified in 2D BN/SDS-PAGE maps

Spot numbers correspond to the 2D BN/SDS map in Fig. 2. Salt, differential expression of spot under low and high salt: 2< or 2, up regulation; 0.5>, down regulation; 0.5 < 2, not affected; Biotin, labeling by biotin; Species, origin of the corresponding retrieved database sequence; CMW, calculated molecular weight for the database entry; Ma, peptide ion scores combined for all matched peptides; PP, number of peptides matched by Mascot to the database entry; Spec., number of spectra matched; Cov., protein sequence coverage; MB, combined scores of matched high scoring segment pairs reported by MS BLAST. Parentheses indicate the number of the peptides matched by MS BLAST to database entry. Chl., chlorophyll; DH, dehydrogenase; hm., homolog; pp., polypeptide; PS, photosystem; pc., precursor; comp., complex; prot., protein; transp., transporting.

Spot	Apparent molecular mass	Salt	Biotin	Protein name	Species	Accession no.	CMW	Ma	PP	Spec.	Cov.	MB
	kDa										%	
0	>200	0.5 < 2	+	Transferrin-like prot. Ttf-1	*D. salina*	P93125	139.55	70	2	5	1	3401 (70)
1	15	2	+	Triplicated transferrin Ttf-1 (ttf1)	*D. salina*	P93125	139.55	153	2	61	1	
				Light-harvesting comp. prot. LHCG12	*Bigelowiella natans*	AF268321	36.08					233 (5)
2	20	2<	+	Nucleoside-diphosphate kinase	*Dunaliella tertiolecta*	AAK38732	24.07	172	4	5	22	
				Peptidoglycan-associated lipoprot.	*Pseudomonas syringae*	AAO57430	17.78	91	2	3	11	77 (1)
3	22	0.5 < 2	+	EST, CX160759	*D. salina*	CX160759		294	4	10		
				EST, CX120094	*D. salina*	CX120094		276	6	72		
				EST, CX120245	*D. salina*	CX120245		101	2	7		
4	25	2<	+	EST, hm. to ATP synthase δ chain	*D. salina*	BM448537	24.06	296	6	13		
				EST, CX120245	*D. salina*	CX120245		101	2	5		
				EST, CX160725[a]	*D. salina*	CX160725		93	1	2		
				EST, CX160887[a]	*D. salina*	CX160887		61	1	3		
				EST, hm. to light-harvesting comp. I[a]	*D. salina*	BM447790	23.9	54	1	4		73 (1)
31	25	0.5 < 2		EST, CX120245	*D. salina*	CX120245		123	2	7		
				Fe-superoxide dismutase (EC 1.15.1.1)	*Pseudomonas putida*	Q88PD5	21.97	95	3	3	18	
				EST, CX160725[a]	*D. salina*	CX160725		94	1	4		
29	26	2<	+	EST, hm. to glutathione *S*-transferase	*D. salina*	BM447040	22.63	427	9	34		
				GTP-binding prot. YptC4	*Chlamydomonas reinhardtii*	AAA82726	23.81	314	7	10	41	204 (3)
				Chl. *a*/*b*-binding prot. 28.5-kDa pc.[a]	*D. tertiolecta*	AAA62772	27.3	45	1	2	7	259 (4)
				Lecithinase	*Vibrio mimicus*	AAC63951	53.19					224 (5)
5	28	0.5 < 2		EST, hm. to glutathione *S*-transferase	*D. salina*	BM447040	22.63	334	7	9		
				EST, CX120245	*D. salina*	CX120245		130	2	3		
				Ethylene-responsive small GTP-binding prot.	*Oryza sativa*	AAS88430	23.97	104	3	4	15	
				Murein lipoprotein lpp pc.	*Escherichia coli*	BAA16044	8.38	101	2	2	33	
30	30	2<		EST, hm. to Ras-related prot.Yptc6	*D. salina*	BM449137	24.73	642	10	20		414 (7)
				EST, BM448806	*D. salina*	BM448806		589	10	34		
				Chl. *a*/*b*-binding protein pc.	*D. salina*	AAA33278	29.31	211	4	40	15	592 (9)
				EST, hm. to nascent pp.-associated comp.	*D. salina*	BM447417	22.09	172	3	3		
				Succinyl-CoA synthetase, α subunit	*P. putida*	AAN69766	30.49	122	2	6	10	202 (4)
26	30	0.5 < 2		EST, hm. to Ras-related prot.Yptc6	*D. salina*	BM449137	24.19	409	7	7		314 (5)
				EST, hm. to glutathione *S*-transferase	*D. salina*	BM447040	22.63	369	8	18		
				Chl. *a*/*b*-binding prot. 28.5-kDa pc.	*D. tertiolecta*	AAA62772	27.30	195	4	20	15	633 (10)

TABLE I—*continued*

Spot	Apparent molecular mass	Salt	Biotin	Protein name	Species	Accession no.	CMW	Ma	PP	Spec.	Cov.	MB
	kDa										%	
76	33	2<	+	Carbonic anhydrase	*D. salina*	1Y7WB	31.68	549	10	51	47	571 (10)
	38	0.5 < 2	+	EST, BM447054	*D. salina*	BM447054	336		5	54		
				Carbonic anhydrase (EC 4.2.1.1)[a]	*D. salina*	AAC49378	64.45	38	1	5	2	429 (8)
				Carbonic anhydrase[a]	*D. salina*	AAO83593	53.52	64	1	13	2	
8	38	2<		PS II reaction centers CP47 apoprot.	*C. reinhardtii*	AAA84154	56.24	534	11	25	19	755 (10)
				PS II Chl. *a*-binding prot. psbC	*Chlamydomonas eugametos*	AAA84148	50.87	511	11	34	26	819 (13)
				NADP-glyceraldehyde-3-phosphate DH	*Chlamydomonas* sp.	BAA94304	39.98	249	6	7	14	245 (3)
				H^+-transp. two-sector ATPase γ chain pc.	*C. reinhardtii*	AAA33079	39.08	99	2	3	5	306 (5)
9	55	2<	+	High affinity nitrate transporter	*D. salina*	AAU87579	58.66	450	10	13	25	
				PS II reaction centers CP47 apoprot.	*C. reinhardtii*	AAA84154	56.24	297	6	9	10	255 (4)
				EST, hm. to ammonium transporter	*D. salina*	BM446979	57.54	207	5	6		
				Low CO_2-inducible prot. LCIC	*C. reinhardtii*	BAD16683	47.65					656 (13)
10	60	2<	+	Tubulin β chain	*C. reinhardtii*	AAA33101	50.15	126	3	3	8	121 (2)
				Dihydrolipoamide DH (EC 1.8.1.4)[a]	*Pseudomonas fluorescens*	1LPFA	49.53	60	1	2	3	248 (5)
11	60	2<	+	ATP synthase β subunit (fragment)	*D. parva*	BAC20416	40.87	1183	17	68	51	2031 (32)
				ATP synthase α chain (EC 3.6.3.14)	*C. reinhardtii*	P26526	54.7	454	9	19	19	
12	60	2<		Calreticulin[a]	*C. reinhardtii*	CAB54526	47.41	66	1	7	2	1053 (22)
34	60	2<		Enolase	*D. salina*	AAO86694	52.36	1526	23	85	55	1820 (29)
14′	65	2<	+	Carbonic anhydrase (EC 4.2.1.1)	*D. salina*	AAC49378	64.45	1265	23	70	51	1885 (21)
14	65	0.5 < 2	+	Carbonic anhydrase (EC 4.2.1.1)	*D. salina*	AAC49378	64.45	1173	23	216	47	2433 (30)
21	66	0.5>	+	EST, CX120245[a]	*D. salina*	CX120245		86	1	3		
				Lysophospholipase A	*Legionella pneumophila*	Q8GNM7	34.45					205 (4)
22	78	0.5 < 2	+	Acyl-CoA synthetase-like prot.	*Arabidopsis thaliana*	AY049239	74.51					295 (6)
15	80	2<		DnaK protein PA4761 (imported) HSP-70	*Pseudomonas aeruginosa*	B83052	68.47	404	6	7	11	883 (21)
				Flagellar adenylate kinase	*C. reinhardtii*	Q6JIY9	70.57					476 (10)
16	80	2<	+	Polyubiquitin	*Pinus sylvestris*	CAA66667	85.22	120	3	3	4	205 (4)
				70-kDa heat shock prot.	*C. reinhardtii*	AAB00730	71.3	93	3	3	5	307 (7)
				Acyl-CoA synthetase-like prot.	*A. thaliana*	CAB43885	74.51					244 (5)
23	90	0.5>	+	Hypothetical prot.[a,b]	*Rhodopirellula baltica*	CAD78080	55.25	43	1	10	1	62 (1)
28	108	2<	+	P-type ATPase	*D. salina*	ABB88698	99.86	3004	56	181	39	607 (11)
				ClpC protease	*Spinacia oleracea*	AAD02267	99.59	467	11	15	15	417 (7)
				Tetraubiquitin	*Saccharum* hybrid cv.	AAC67551	34.2	151	3	3	13	
27	110	2<	+	Heat shock protein 90	*O. sativa*	AAL79732	93.04	461	9	13	8	731 (16)
				Ubiquitin	*Neurospora crassa*	P13117	8.6	130	2	2	32	331 (7)
				2-Oxoglutarate DH, lipoamide DH	*P. syringae*	AAO55717	50.22	122	3	3	10	
20	110	2<		P-type ATPase	*D. salina*	ABB88698	99.86	2024	39	150	32	783 (15)
32	120	2<	+	Transferrin-like prot.Ttf-1	*D. salina*	P93125	139.55	905	21	21	23	837 (17)
				EST, hm. to Golgi apparatus prot. 1	*D. salina*	BM448994	122.02	404	8	8		
				H^+-exporting ATPase (EC 3.6.3.6)	*Dunaliella bioculata*	CAA52107	124.28	268	6	6	7	566 (13)
17	125	0.5 < 2	+	H^+-exporting ATPase (EC 3.6.3.6)	*D. bioculata*	CAA52107	124.28	1349	23	43	27	1078 (20)

TABLE I—*continued*

Spot	Apparent molecular mass	Salt	Biotin	Protein name	Species	Accession no.	CMW	Ma	PP	Spec.	Cov.	MB
	kDa										%	
				Transferrin-likeprot.Ttf-1	*D.salina*	P93125	139.55	1420	29	53	32	2166 (41)
				EST, CX161084[a]	*D. salina*	CX161084		42	1	13		
19	150	2	+	Transferrin-like prot. Ttf-1	*D. salina*	P93125	139.55	3020	57	245	49	4774 (69)
19′	150	2	+	Transferrin-like prot. Ttf-1	*D. salina*	P93125	139.55	2833	52	218	49	4595 (70)

[a] Annotated MS/MS spectra in supplemental materials.
[b] Validated by *de novo* and MS BLAST, high scoring segment pair alignment in supplemental materials.

any known proteins and therefore have not been functionally annotated, yet they likely represented a new family of integral plasma membrane proteins. They were identified in spots 3, 4, 5, and 31 (molecular mass, 22–26 kDa) and corresponded to six different *Dunaliella* ESTs (maximal length, 837 base pairs). Alignment of their predicted amino acid sequences revealed substantial sequence identity (Fig. 6), suggesting that they might originate from the same gene family. Their sequences contained three to four transmembrane-spanning domains, suggesting integral membrane localization, and indeed spots 3 and 4 were tagged by biotin (Fig. 5). High salt had a minor effect on most of the spots except for spot 4 that was up-regulated. Although the EST sequences cover most of the gene coding sequences (as it could be concluded from the apparent molecular weight of corresponding protein spots), there is no significant sequence similarity of the predicted amino acid sequences to any known protein.

Biotinylated spot 23, which was abundant in low salt and down-regulated by high salt (Figs. 2 and 5), remained unidentified. Several high molecular mass protein bands observed in 1D BN-PAGE (bands 1–4 in Fig. 1*B*; apparent molecular mass of 500–660 kDa) were poorly resolved by SDS-PAGE (Figs. 2 and 3). One major spot (spot 0) was analyzed by LC-MS/MS and identified as Ttf; however, many peptide peaks were left unassigned.

Functional Classification of Identified Proteins

According to their functional annotation, the identified proteins were divided into eight major classes.

Ion Transporters—This group included four integral membrane ion transporters and a surface-associated iron transporter: the previously characterized H^+-ATPase (17), a second P-type ATPase whose function remains unknown, and two newly discovered transporters involved in nitrogen acquisition, a high affinity nitrate transporter and an ammonium transporter. The iron-binding transferrin-like protein Ttf is a surface-associated protein that has been well characterized in our laboratory as a mediator of iron uptake (30, 31).

Bicarbonate Acquisition—At high salinity, the solubility of carbon dioxide is reduced and it becomes a rate-limiting step in photosynthesis (3). We found that two surface-associated carbonic anhydrases were up-regulated in *D. salina* hence enhancing carbon acquisition. Previously characterized dCAI and dCAII (4, 5) were identified in spots 6 and 14. A new putative plasma membrane carbonic anhydrase was identified in spot 7 Expressed Sequence Tag (EST) database for Dunaliella accession number BM447054), as this spot reacted positively with anti-CA antibodies (dCAI) (Fig. 4), but it had only partial sequence similarity to two previously identified CAs from *D. salina* (Genepept accession numbers AA083593 and AAC49378). Another protein that may be associated with bicarbonate acquisition is an ortholog of a low CO_2-inducible soluble protein in *Chlamydomonas* whose exact function remains unknown (spot 9).

Surface Coat and Flagellar Proteins—This group includes mainly structural proteins associated with the extracellular integrity of the membrane and with flagellar motion. We detected two extracellular lipoprotein homologs, a peptidoglycan-associated lipoprotein (32) and murein lipoprotein (33), which stabilize cell envelope structure by bridging the outer membrane and the peptidoglycan layer in bacteria. These bacterial proteins have no known orthologs in eukaryotes. Two proteins that are associated with flagellar motion were identified: tubulin β chain, known to compose the flagellum and also to associate with proteins in the plasma membrane (34), and a flagellar adenylate kinase involved in assembly of the outer dynein arm of the flagellum in *Chlamydomonas* (35).

Signal Transduction—Small GTP-binding proteins were identified in four different spots (spots 5, 26, 29, and 30), suggesting that they are important components in *Dunaliella* plasma membrane. All peptides identifying small GTP-binding proteins were observed in two *Dunaliella* ESTs (EST database for Dunaliella accession numbers CX120183 and BM449137). These EST are homologous to yptV2/Rab8 and yptC6/Rab11 subgroups of the Ras superfamily, respectively. Small GTP-binding proteins mediate membrane vesicle trafficking between ER and Golgi and between Golgi and the plasma membrane and were shown to act as pivotal elements in signal transduction pathways that stimulate cell response to external signals (36). Recently Ras-related small GTP-binding proteins, which act as molecular switches in signal transduction cascades, have also been identified in plants (37, 38).

Protein Synthesis, Stability, and Degradation—This group includes several ubiquitous proteins: a nascent polypeptide-

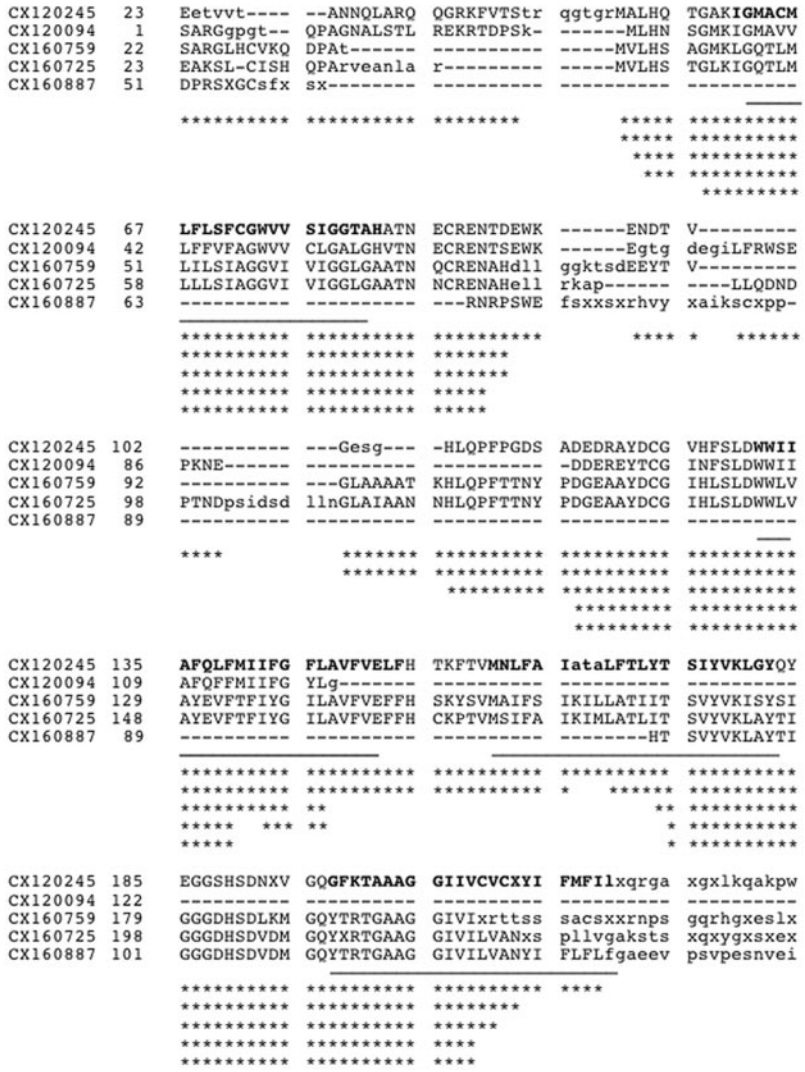

FIG. 6. **Multiple sequence alignment of ESTs representing a novel membrane protein family.** ESTs identified in different spots were aligned by using the DIALIGN program (83) (University of Bielefeld, Bielefeld, Germany), which is based on segment to segment comparison. Five ESTs were identified in the following spots: CX120245 in spots 3, 4, 31, 21; CX120094 in spot 3; CX160759 in spot 3; CX160725 in spots 4 and 31; and CX160887 in spot 4. Predicted transmembrane-spanning domains, according to TMHMM program, are *underlined*. The *asterisks* indicate the degree of local similarity among sequences.

associated protein, which is involved in protein sorting and translocation by preventing mistargeting of nascent polypeptide chains to the ER; the heat shock proteins HSP-70 and HSP-90, which are highly conserved stress-related proteins that act as chaperones involved in protein stabilization; and ubiquitin, tetraubiquitin, polyubiquitin, and ClpC protease, proteins associated with protein degradation. Ubiquitin-mediated degradation of proteins plays an important role in regulation of several processes such as signal transduction and transcription regulation.

Antioxidative Stress Enzymes—Several proteins that are associated with oxidative stress, which is a common secondary response to salt stress in plants, were identified. They include Fe-superoxide dismutase, involved in neutralization of superoxide radicals; glutathione *S*-transferase (the major component in spots 29, 5, and 26), which is associated with oxidative stress and induced in plants under salt stress (39); and nucleoside-diphosphate kinase, which in general regulates cellular protein functions probably through its phosphotransferase activity. In *Arabidopsis* nucleoside-diphosphate kinase interacts with mitogen-activated protein kinase (MAPK)-mediated H_2O_2 signaling, and its overexpression down-regulates the accumulation of reactive oxygen species, and this, in turn, enhances the tolerance of *Arabidopsis* to abiotic stresses (40).

Lipid-metabolizing Enzymes—Three lipid catabolism enzymes were identified: lecithinase, involved in the degradation of phosphatidylcholine (spot 29); lysophospholipase A, an enzyme involved in phospholipid degradation and interconversions (spot 21); and long-chain acyl-CoA synthetase (spots 16 and 22), an enzyme that is known to have a role in lipid biosynthesis, fatty acid catabolism, and transport of fatty acids to subcellular compartments including translocation across the plasma membrane (41).

Energy Metabolism in Mitochondria and Chloroplasts—This group mainly includes contaminating proteins from membranes of chloroplasts and mitochondria. It includes three mitochondrial citric acid cycle enzymes, 2-oxyglutarate dehy-

drogenase (spot 27), succinyl-CoA synthetase (spot 30), and dihydrolipoamide dehydrogenase, the E3 subunit of pyruvate dehydrogenase (spot 10); mitochondrial nucleoside-diphosphate kinase (spot 2); and enolase (spot 34), NADP-glyceraldehyde-3-phosphate dehydrogenase (spot 8), and ATP synthase (spot 11), three chloroplast enzymes associated with energy metabolism and metabolic flexibility. The abundance of energy metabolism enzymes is consistent with our previous proteomics analysis that revealed up-regulation of energy metabolism in *D. salina* at high salinity (2).

Plasma Membrane Complexes at High and Low Salinity

To dissect the protein complexes separated by BN gels, corresponding bands were excised from the 1D BN-PAGE gels and resolved, each in a lane, by second dimension SDS-PAGE. A careful examination of BN/SDS-PAGE profiles in Figs. 2 and 3 revealed that salinity stress induced compositional changes in membrane protein complexes.

The two previously characterized carbonic anhydrases from *Dunaliella* plasma membrane termed dCAII and dCAI, with molecular masses of 32 and 64 kDa, respectively, comigrated in a series of bands (spots 6 and 14) thus suggesting that they might be present as heterodimers that further oligomerize within the membrane. Such interactions might be physiologically significant. It was reported previously that interactions between the two internal domains of dCA contributed to the catalytic activity and stability of the protein in saline solutions (42). Furthermore the putative carbonic anhydrase identified in spot 7, which was also biotin-positive and co-migrated with dCAI or dCAII in BN band 12 (Fig. 3), might represent interactions between different plasma membrane carbonic anhydrases in *Dunaliella*.

High salinity appears to promote the oligomerization of Ttf and of carbonic anhydrases within the membrane: BN band 5, probably representing oligomerized Ttf, appears only in 3.0 M and not in 0.5 M NaCl (Fig. 1*B*). Also for carbonic anhydrases, the higher molecular weight states appear more pronounced at 3.0 M NaCl (see the Western immunoanalysis in Fig. 4).

Several pairs of proteins were identified exclusively in the same BN bands. For example, the transferrin-like protein, Ttf (spot 19), peaked in BN *lanes 5* and *10* (Fig. 3) in parallel with spot 27, which contained HSP-90. The P-type ATPase and tubulin β chain (molecular masses, 100 and 50 kDa, respectively) are the major components in spots 20 and 10, respectively, which co-migrate in BN band 9. The possibility for protein interactions within the membrane will be discussed below.

DISCUSSION

It is generally accepted that proteomes of living cells are organized into a complex interaction network (43), which enables the cell to sense the environment and adapt the internal protein content to enhance the cell viability. To understand the molecular mechanism of *Dunaliella* adaptation to high salt, we analyzed the composition and organization of the plasma membrane proteome.

Although membrane protein complexes are labile and insoluble in common buffers used in protein electrophoresis, they were well resolved by BN/SDS-PAGE. When combined with biotin surface tagging and nano-LC-MS/MS sequencing, several new membrane-associated proteins were identified. Considering their sequence similarity to known proteins (Table I), we estimated that more than 60% of the identified proteins are either directly membrane-associated or have a functional relationship to membrane activities as discussed below. This study also demonstrated that the abundance of 22 protein spots was altered upon salinity stress: 20 protein spots were up-regulated, and two protein spots were down-regulated. Only a few of these proteins were previously known to be involved in salinity stress response in plants.

We also found several major components that were identified in *Dunaliella* ESTs that had no apparent homology to any database proteins, and many of the acquired high quality tandem mass spectra were not assigned. The corresponding peptide might originate from unique plasma membrane proteins and will likely be identified in the future once *Dunaliella* EST or genomic databases mature.

Identification of Plasma Membrane Proteins

Identification of membrane proteins in general and of plasma membrane proteins in particular is hampered by a typically low purity of membrane protein preparations and difficulties in resolving and identifying membrane proteins by conventional proteomics methodologies (44). Therefore, in this work we decided against a multistage procedure of protein purification. Instead we used differential centrifugation to enrich for membrane protein complexes in their native form and biotin tagging of the surface-exposed plasma membrane proteins. Although it is difficult to completely avoid cytosolic protein contaminations, biotin surface labeling demonstrated that major proteins in the preparation were indeed associated with the plasma membrane. Conversely spots whose intensities strongly varied in different preparations or contained mostly chloroplast proteins were either weakly tagged or not tagged by biotin at all.

Integral Membrane Proteins

Plasma membrane localization of studied proteins was primarily supported by the biotin tagging. However, this should be considered with caution because the relative labeling efficiency is influenced by the protein orientation within the membrane. Therefore, if a biotinylated spot contained more than one protein, we also considered the predicted localization of protein orthologs in related organisms.

Proteins identified previously in the PM and clearly labeled by biotin are Ttf, the two carbonic anhydrases, and two P-type

ATPases, which served as positive controls. Integral membrane proteins that were tagged by biotin and predicted to be plasma membrane proteins are the nitrate and ammonium transporters and the new putative family of integral membrane proteins described above.

The P-type ATPase, which accumulates at high salinity (spots 20 and 28), is a plasma membrane ion transporter previously cloned in our laboratory whose function is currently unknown. The identification of a high affinity nitrate transporter and an ammonium transporter is consistent with previous physiological studies, suggesting that *Dunaliella* can grow either on nitrate or on ammonium as a nitrogen source. The nitrate transporter of *Dunaliella* is of special interest with respect to salinity tolerance because, in contrast to most other algae, nitrate uptake in *D. salina* is coupled to sodium rather than proton transport, which is a secondary adaptation mechanism utilizing sodium electrochemical gradients to drive nitrate uptake (8, 45).

A Functional Network Involved in Salinity Tolerance

Our previous proteomics analysis of the soluble proteome in *D. salina* revealed that, under high salinity, the alga diverts its metabolic pathways to massive synthesis of glycerol, its major osmotic defense element. Our present analysis enabled identification of several novel plasma membrane components and revealed unexpected interactions between proteins that provide clues to the exceptional salinity tolerance of *Dunaliella.*

Sensing and Signaling under Salt Stress—*Dunaliella* cells respond to osmotic (or salt) shocks by rapid changes in cell volume associated with structural changes in the plasma membrane, which in turn activate massive synthesis or elimination of glycerol (46, 47). Recent studies suggest that the mitogen-activated protein kinase cascade may mediate the osmotic response in *Dunaliella* (48). However, it is not clear which proteins are involved in sensing and signaling at the early stages in the osmotic response. Our work identified two proteins as plausible osmotic response mediators: GTP-binding proteins and lysophospholipase A.

The identification of small GTP-binding proteins yptV2/Rab8 and yptC6/Rab11, subgroups of the Ras superfamily, in four spots (spots 5, 26, 29, and 30) up-regulated (except for spot 29) at high salinity suggests that they are abundant in *Dunaliella* plasma membrane. The Ras-encoded small GTP-binding proteins function in animal and plant cells as molecular switches in signal transduction cascades in response to external signals (38, 49). The subfamily of Ypt/Rab proteins, which were identified in other green algae as well (50, 51), participates in the regulation of intracellular vesicle transport to the plasma membrane (52). Previous studies in *Dunaliella* demonstrated dynamic interactions between the plasma membrane and internal small vesicles in response to osmotic/salinity changes (53, 54). It is possible that these early observations actually represented part of an osmotic signal transduction pathway mediated by small GTP-binding proteins. Such a mechanism might be expected to be up-regulated at high salinity. Furthermore small GTP-binding proteins were localized in plasma membrane lipid rafts, structures that were suggested to be involved in different signaling events (55). Proteins found to be associated with lipid rafts in mammalian and plant cell membrane have also been identified in *D. salina* plasma membranes: for example, the heat shock protein HSP-90, involved in targeting G-proteins to lipid rafts (56), and tubulin β chain as stabilizers of microdomains (58).

Another protein that might control the osmotic response in *Dunaliella* is lysophospholipase A (spot 21), which is largely suppressed at high salinity. Interestingly lysophospholipases have been directly implicated in triggering the stress response in bacteria and in fission yeast presumably by mediating changes in membrane fluidity (59, 60). Early studies in *Dunaliella* reported global changes in plasma membrane fluidity in response to osmotic shock. Specifically hyperosmotic shocks resulted in rigidification, whereas hypoosmotic shocks led to transient fluidization of the membrane core (9, 10, 14). Moreover we previously showed that sterols have a crucial role in osmotic sensing in *D. salina* (61). Because sterols largely decrease membrane fluidity, these results also suggested that osmotic signaling is mediated by dynamic changes in plasma membrane fluidity. The suppression of lysophospholipase A in plasma membranes is indicative of a slower hydrolysis of phospholipids to lysophospholipids and to free fatty acids, which is expected to decrease the plasma membrane fluidity at high salinity. These results are consistent with the suggested role of phospholipases in osmotic signaling in yeast and in bacteria and with the above mentioned studies in *Dunaliella*.

Membrane Structure and Surface Stabilization—An unusual structural feature of *Dunaliella* is that, unlike most unicellular algae, it does not have a rigid cell wall. Therefore, *Dunaliella* cells shrink or expand in response to osmotic changes, like natural protoplasts, and, in principle, might burst even upon mild changes in osmolarity. Paradoxically *Dunaliella* withstands exceptionally large osmotic shocks (up to 3–4-fold) without cell bursting (46). This indicates that *Dunaliella* cells may have some kind of protective outer layer to prevent lysis upon osmotic changes. The present study provides for the first time clues to understand this special quality. We identified two surface coat proteins that are involved in stabilization of cell envelope structure in bacterial cells, peptidoglycan-associated protein and murein lipoprotein, which were not previously reported in plants or algae. In bacteria these proteins are localized at the extracellular cell envelope and help to maintain the cell integrity (32, 33). Notably it has been reported that the surface coat of *Dunaliella parva* is sensitive to lysozyme treatment, suggesting that peptidoglycans at the cell surface are essential for maintaining cell integrity in saline solutions (62). Our results clearly show that *Dunaliella* indeed

contains peptidoglycans at their cell surface, possibly acquired by gene transfer from bacteria, that may stabilize the cell plasma membranes at high salinity.

Another protein that seems to maintain the plasma membrane integrity in *Dunaliella* is tubulin. Tubulin β chain was identified in spot 10, which was biotin-positive, and its abundance was strongly enhanced at high salinity, indicating its association with the plasma membrane. Furthermore tubulin seems to co-migrate with the P-type ATPase. Interestingly it was recently reported that acetylated tubulin interacts with and regulates the activity of the orthologous H^+-ATPase in yeast plasma membranes (63), and it is conceivable that it does the same also with the P-type ATPase in *D. salina*. Other reports demonstrated that tubulin interacts with plasma membranes in plants and algae and functions as a nucleation site for the association of microtubules and hence controls cell expansion and morphology (34, 64, 65). Tubulin is also one of the major flagellar proteins and, together with the flagellar adenylate kinase, might associate with PM domains at the base of the flagella.

The lipid composition of plasma membrane is crucial for maintaining its integrity. As mentioned above, *Dunaliella* plasma membrane contains a relatively high amount of sterols, which can contribute to membrane stabilization (66). A second feature, which may help to prevent cell lysis upon osmotic shocks, is the dynamic incorporation of lipids from small cytoplasmic vesicles (53, 54). The identification of three lipid-metabolizing enzymes in the plasma membrane proteome adds another dimension to the dynamic plasticity of *Dunaliella* plasma membranes. Acyl-CoA synthetase is a major enzyme in phospholipid biosynthesis and has been demonstrated to transport long-chain fatty acids across plasma membranes (41, 67). Lysophospholipase A and lecithinase function in phospholipid hydrolysis. Together these enzymes can catalyze a dynamic turnover in phospholipid composition in response to changes in salinity. Notably the finding that these enzymes were identified in biotin-positive spots 22, 16, 21, and 29 confirms that they are plasma membrane proteins and further suggests that they may act also at the outer leaflet of plasma membrane phospholipids. Taken together, these results suggest that dynamic changes in the plasma membrane lipid composition in *Dunaliella* may be involved either in short term signaling or in long term adaptation to changes in salinity.

Protein Turnover and Stabilization at High Salinity—The remarkable changes in protein composition and abundance at high salinity depend on active synthesis and degradation of proteins and on chaperones that stabilize and mediate the transportation and insertion of newly synthesized proteins. Indeed we observed up-regulation of several classes of proteins that function in protein stabilization, mobilization, and degradation. The heat shock proteins and chaperones HSP-70 and HSP-90 accumulated at high salinity. This is not surprising because orthologous proteins in plants were induced under various stress conditions, including salinity stress. Interestingly these cytoplasmic proteins were identified as major components in biotin-tagged spots indicating that they are surface-exposed. Indeed orthologs of these heat shock proteins were reported to associate with proteins in PM and thus might localize at the extracellular surface both in animals and plants (68–75). It is conceivable that heat shock proteins associate with newly synthesized surface proteins, such as CA or Ttf, and are co-translocated together to the extracellular surface, consistent with their migration pattern on BN gels, possibly conferring protection against high salt concentrations. Another protein that might function as an HSP and that was induced at high salinity was enolase. This abundant glycolytic enzyme is in fact a multifunctional protein and might act as a surface receptor or as a heat shock protein (76). It is therefore likely that in *Dunaliella* enolase is involved in response to the salinity stress.

Ubiquitin, tetraubiquitin, and polyubiquitin are involved in the mono-, oligo- or polyubiquitination of membrane proteins. Ubiquitination regulates membrane protein stability and turnover within the plasma membrane (77–79) and therefore might play a role in the response to salinity stress.

Calreticulin, generally recognized as an ER chaperone, has been reported to be associated with the extracellular membrane surface in mammalian cells and to serve as a mediator of adhesion (80). Calreticulin is also known to be involved in the stress response in plants (81, 82).

Taken together, the characterization of changes in soluble (2) and plasma membrane (this work) proteomes induced by salinity stress demonstrated that *D. salina* responds to high salinity by combined up-regulation of central metabolic and signal transduction pathways altering the plasma membrane lipid and protein composition, transport activities, and carbon and energy metabolism. Thus, concerted changes in multiple pathways could be the important factor contributing to the unique ability of *D. salina* to withstand high salinity.

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