A Structurally Novel Transferrin-like Protein Accumulates in the Plasma Membrane of the Unicellular Green Alga Dunaliella salina Grown in High Salinities*

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The alga Dunaliella salina is outstanding is its ability to withstand extremely high salinities. To uncover mechanisms underlying salt tolerance, a search was carried out for salt-induced proteins. The level of a plasma membrane 150-kDa protein, p150, was found to increase with rising external salinity (Sadka, A., Himmelhoch, S., and Zamir, A. (1991) Plant Physiol. 95, 822–831). Based on its cDNA-deduced sequence, p150 belongs to the transferrin family of proteins so far identified only in animals. This, to our best knowledge, is the first demonstration of a transferrin-like protein in a photosynthetic organism. Unlike animal transferrins, p150 contains three, rather than two, internal repeats and a COOH-terminal extension including an acidic amino acid cluster. In intact cells p150 is degraded by Pronase, indicating that the protein is extracellularly exposed. The relationship of p150 to iron uptake is supported by the induction of the protein in iron-deficient media and by its radioactive labeling in cells grown with 59Fe. Accumulation of p150 is transcriptionally regulated. It is proposed that p150 acts in iron uptake other than by receptor-mediated endocytosis and that its induction permits the cells to overcome a possible limitation in iron availability under high salinities.

An exceptionally interesting model to uncover mechanisms conferring salt tolerance to photosynthetic organisms is provided by the unicellular green algae Dunaliella. Algae belonging to this genus are remarkable in their ability to proliferate in salinities as high as saturating NaCl concentrations. Within a broad range of external salinities, the algae maintain a relatively low internal salt concentration (1) and balance the external osmotic pressure by accumulating iso-osmotic levels of glycerol (2).

Although glycerol-mediated osmotic adjustment is a necessary element in the salt tolerance of Dunaliella, it is reasonably insufficient to permit growth in high salinities. To unravel additional mechanisms contributing to salt tolerance it was postulated that participating components might be specifically induced under high salt. Accordingly, screens were performed for proteins preferentially accumulating in high salt-grown cells (3, 4). Following this approach, a protein of 150 kDa, p150, was observed to rise markedly with an increase in external salinity. Furthermore, in cells transferred from low to high salt, an increase in p150 roughly coincided with resumption of cell division following the hyperosmotic shock (3). The protein was characterized biochemically and by immunoelectronic microscopy as a major plasma membrane component and was accordingly postulated to be involved in the control of salt or nutrient fluxes across the cell membrane (3).

Evidently, structural and functional characterization of p150 could provide important new insights concerning the nature of factors limiting growth in high salinities as well as the mechanisms that evolved to counteract these limitations.

We now show that p150 is a unique new member of the transferrin family of proteins which, to the best of our knowledge, has been identified so far only in animals (5–7). The transferrin family includes mainly iron-binding proteins found in serum and other body fluids that perform essential roles in iron binding and transport. All members of the transferrin family characterized to date are typically composed of two homologous halves, and most are not membrane-associated. The membrane-associated p150 is distinctly different in encompassing three, rather than two, internal repeats. The involvement of p150 in iron uptake is suggested by its induction under iron limitation and supported by 59Fe labeling of the protein. Thus, transferrin-homologous proteins have probably evolved far earlier than thought so far to function in the uptake of iron, or other nutrients, by unicellular eukaryotes.

MATERIALS AND METHODS

Algae and Growth Conditions—The source of Dunaliella salina, the method used to obtain axenic cultures, the medium composition, and growth conditions were essentially as described (4, 8). The standard growth medium contained 0.5 M NaCl. Deviations from the standard medium are indicated.

Preparation and Screening of a D. salina cDNA Library—D. salina cells grown in 0.5 M NaCl were transferred in two steps to 3.5 M NaCl (hyperosmotic shock), essentially as described (4). Total RNA was prepared from cells harvested 9 h after the shock by extraction with 1 ml of Tri Reagent/107 cells (9) (Molecular Research Center, Inc.). Poly(A)+ mRNA was isolated using the Poly(A)+ tract mRNA Isolation System (Promega). Synthesis of cDNA, using 3 μg of poly(A)+ mRNA, was by the ZAP-cDNA Synthesis Kit (Stratagene). Cloning in the λ Uni-ZAP XR vector used the Stratagene cloning kit. The original library contained 7 × 105 plaque-forming units. An amplified library was screened with anti-p150 antibodies (3) using the Stratagene picoBlue Immuno-screening Kit. Positive clones were processed further to rescue the corresponding recombinant plasmids.

DNA Sequencing Procedures—Phage-rescued plasmids were subjected to DNA sequencing analysis by the dye deoxy sequencing method in the Applied Biosystems 373A DNA sequencer.

Northern Blot Analysis—Fifteen μg of total RNA extracted as described above was analyzed on each lane of a formaldehyde agarose gel (10). The probe used contained the full-length cDNA for p150.

*(Received for publication, August 6, 1996, and in revised form, October 12, 1996)
Partial Protein Sequencing—Purification of p150 was as described (3). The band containing p150 was excised from the preparative gel and transferred to an SDS-containing 10–20% polyacrylamide gradient gel in the presence of 2 µg of Staphylococcus aureus V8 protease/lane (11). The digestion was continued for 30 min, and the oligopeptide products were separated by electrophoresis and blotted on a polyvinylidene difluoride membrane (12). The NH2-terminal amino acid sequence of the two major proteolytic fragments was determined using the Applied Biosystems model 475A protein microsequencer, equipped with a model 120A on-line high performance liquid chromatography phenylthiodantoin derivative analyzer and a model 900A data acquisition and processing unit.

Pronase Digestion—Algae grown in 0.5 M NaCl to a density of 10^6 cells/ml were harvested and resuspended in fresh medium to a density of 10^7 cells/ml. Pronase, at the indicated final concentrations, was added to 1 ml cell suspensions that were incubated for 1 h at 30°C. The cells were collected by centrifugation, washed twice with 0.5 M NaCl, resuspended in SDS-sample buffer, and incubated at 100°C for 3 min. Aliquots corresponding to 5 x 10^5 cells were analyzed on SDS-PAGE followed by immunoblot analysis with anti-p150 antibodies.

59Fe Labeling—To 200 ml of a 3 x 10^6 cells/ml culture in a medium containing 3.5 M NaCl, 0.1 µM FeCl3, and 4 µM EDTA, 100 µCi of 59Fe (as FeCl3, carrier-free, DuPont NEN) was added. After 2 days growth under standard conditions, the cells were harvested, plasma membranes were isolated as described (4) and solubilized with 0.1% Triton X-100. Aliquots containing 25 µg of protein were resolved by nondenaturing electrophoresis on a 4–10% polyacrylamide gradient gel with a 3% polyacrylamide stacking gel. After blotting onto nitrocellulose, radioactivity was monitored by a PhosphorImager. A parallel lane was cut out from the nondenaturing gel and subjected to a second dimension electrophoresis under denaturing conditions (13). Proteins were localized by staining with Coomassie Brilliant Blue.

1 The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

FIG. 1. Nucleotide and deduced amino acid sequence of the cDNA for p150. Underlined, amino acid sequences matching sequences determined for undigested p150 and two V8 proteolytic products. Position +1, proposed NH2-terminal residue of the mature protein.
RESULTS

Cloning of the cDNA for p150 and Sequence Determination—A cDNA expression library was constructed in the λZap vector using D. salina poly(A)1mRNA isolated 9 h after the cells had been subjected to a hyperosmotic shock. The cDNA library was screened with anti-p150 polyclonal antibodies. Several cross-reacting phages were found to contain cDNA inserts of 4.4 kilobases. Sequencedetermination of the cloned cDNA indicated that it included a 3.822-kilobase open reading frame encoding a 1,274-amino acid polypeptide, flanked on its 5' end by 36 base pairs and on its 3' end by 485 base pairs attached to a poly(A) tail (Fig. 1).

To confirm that the cloned cDNA encoded for p150, the predicted amino acid sequence was compared with several sequences determined for undigested p150 and two protease V8 digestion products. All of the directly determined amino acid sequences were identified in the sequence predicted from the isolated cDNA. However, the sequence reported previously as corresponding to the NH2 terminus of the intact p150 (3) was not found at the expected site, but rather close to the carboxyl terminus of the protein. The most plausible explanation for this discrepancy is that the NH2 terminus of the intact protein is blocked and that the sequence determined previously is of a small COOH-terminal peptide. This peptide is generated by proteolytic cleavage occurring after the intact protein had been isolated by SDS-PAGE. The likelyhood of such a cleavage is supported by the occasional appearance of a polypeptide somewhat shorter than p150 in purified p150 preparations after storage (3). The small COOH-terminal peptide remains unde-tected in this analysis.

The ATG assigned as the initiation codon is the most up-stream in the cDNA, is in frame with the following open reading frame, and together with flanking nucleotides conforms to the core consensus sequence for translation initiation in plants (14, 15). For reasons explained below, the Gly numbered +1 (Fig. 1) was designated as the NH2-terminal residue of the mature protein. According to this assignment, the 17 mainly hydrophobic amino acid residues at the NH2 terminus probably constitute the leader peptide responsible for the ultimate direction of the protein to the plasma membrane.

Internal Repeats in p150 and Relationship to the Transferrin Family—The predicted amino acid sequence of p150 (Fig. 2) includes three internally homologous segments ranging in length from approximately 350 to 400 amino acid residues. The NH2-terminal (a1) and central (a2) repeats are 64% identical, whereas the COOH-terminal repeat (b) shows 28% identity to either the a1 or a2 repeats. Although it is not possible to establish definitively the boundaries of the three repeats, it is clear that they are separated by relatively short connecting sequences. Particularly striking is the peptide connecting a2 with b which includes four consecutive Asn residues. The unique -100-amino acid COOH-terminal sequence is outstanding in containing a dense cluster of acidic residues which is followed by a relatively hydrophobic sequence.

Data base searches revealed a clear homology between the repeated units in p150 and transferrins, a family of proteins so far identified only in animals (Fig. 3). The functionally best characterized members of this family are the serum transferrins, major iron-binding proteins of ~80 kDa, which are essential for iron delivery to different types of cells (16). The serum ferritin transferrin is internalized by target cells via receptor-mediated endocytosis and releases its bound iron after the internal pH of the vesicles turns acidic (7). Other transferrins include lactoferrin, ovotransferrin, and melanotransferrin, p97. The last is a membrane-anchored transferrin of 97 kDa, first identified on the surface of human melanoma cells (17). Transferrins and related proteins are composed of two homologous halves, each similar in length to the repeat unit in p150 (16). The internally duplicated sequence is reflected in the three-dimensional structure, which is composed of two similar lobes as determined for human lactoferrin (18) and rabbit se-
rum transferrin (19). In these proteins each lobe binds a single Fe$^{3+}$ cation synergistically with a single HCO$_3^-$ (or CO$_3^{2-}$) anion.

The assignment of the NH$_2$-terminal residue of the mature p150 (Figs. 1 and 2) is based on sequence resemblance to the human lactoferrin. In lactoferrin, a Gly residue, immediately preceded by an Ala (in the nascent protein) located 9 residues NH$_2$-terminal to the first conserved Cys, was identified as the NH$_2$-terminal residue of the mature protein (16). In other instances, NH$_2$ termini of mature transferrins were found 7–8 residues NH$_2$-terminal to the first conserved Cys residue.

To permit alignment with animal transferrins (Fig. 3), the $\alpha_1$ repeat of p150 was omitted and the alignment was started with the $\alpha_2$ repeat. The juxtaposition of the $\alpha_2$ and $\beta$ repeats with the NH$_2$ and COOH halves of the transferrins shows that the $\alpha$ repeats include several insertions with respect to the NH$_2$ halves of the transferrins. The $\beta$ repeat is closer in size to the COOH halves of animal transferrins but still contains a 12-residue insertion as well as several shorter insertions.

The amino acid residues identified as iron ligands in rabbit serum transferrin and human lactoferrin (numbering as in the human lactoferrin, Fig. 3) are Asp-60, Tyr-92, Tyr-192, and His-253 in the NH$_2$ lobe and Asp-395, Tyr-435, Tyr-528, and His-597 in the COOH lobe. In p150, all three repeats contain a Gly instead of Asp-60, the two Tyr residues are conserved, and the His is replaced by Asn in $\alpha_1$ and $\alpha_2$ and by Gln in $\beta$.

Residues involved in HCO$_3^-$ (CO$_3^{2-}$) binding in the NH$_2$ and COOH halves of animal transferrins are Arg-121 and Arg-465, respectively. In all three repeats of p150 these Arg residues are replaced by Lys, which, interestingly, is invariably preceded by an Arg residue.

In addition to iron and anion liganding residues, transferrins characteristically contain conserved Cys residues involved in disulfide bond formation (16). Of the 11 Cys residues conserved between the NH$_2$ halves of the transferrins shown (Fig. 3) 10 are conserved in the $\alpha$ repeats of p150. Of the 14 Cys residues conserved in the COOH halves, 12 are conserved in the $\beta$ repeat of p150. Altogether, p150 retains many of the structural features characteristic of animal transferrins.

Transcriptional Analysis of p150—Northern blot hybridization was used to assess whether p150 induction following a hyperosmotic shock involved gene activation. The analysis for p150 transcripts was conducted in parallel to immunoblot analysis for p150 antigens. The results (Fig. 4) indicate that the p150 transcript level rises coordinately with the level of the protein. Hence, p150 accumulation appears to be transcriptionally regulated.

Regulation of p150 by Iron Availability—Because p150 was originally found to be induced in high salinities, the possibility was considered that such conditions imposed an iron limitation on the cells which could be compensated by p150 overproduction. One could therefore expect that p150 may be induced by iron limitation per se, even in relatively low NaCl concentrations.

To examine this possibility, cells growing in complete medium containing 0.5 M NaCl were resuspended in a medium without added iron (concentration of iron introduced as a contaminant of the salts used to prepare the medium was less than 0.5 µM). In a medium buffered at pH 7.4, a considerable increase in p150 was already noted 8 h after transfer to the new medium, and by 24 h the level of p150 increased severalfold over its initial level. No induction of p150 was observed in cells incubated in the same medium but with added iron (Fig. 5).

Pronase Sensitivity and Iron Binding to p150—To examine the extracellular exposure of p150, Pronase digestion was conducted with cells grown in 0.5 M NaCl (Fig. 6A). Although the level of the protein is relatively low in this salt concentration...
(3), it was still easily detectable in the immunoblot analysis. The results indicate that p150 is degraded in intact cells treated with Pronase, the extent of digestion depending on the enzyme concentration. Microscopic examination indicated that Pronase digestion did not cause cell lysis.

To explore the possibility that the function of p150 involved iron binding, algal cells were grown for several generations in a standard medium with 0.5 M NaCl and analyzed as described under “Materials and Methods.” Pronase digestion did not cause cell lysis.

FIG. 4. Analysis of p150 and its mRNA at different times after a hyperosmotic shock. D. salina cells were subjected to a two-step hyperosmotic shock from 0.5 to 3.5 M NaCl as described previously (4). Culture aliquots were removed at different times after the cells were transferred to a medium with 3.5 M NaCl and analyzed for p150 by immunoblotting (panel A) or for the p150 corresponding transcript (panel B, arrow) by Northern blot hybridization. Std, blots hybridized with a standard probe as described previously (4).

FIG. 5. Effect of iron limitation on p150 induction. Panel A, D. salina cells grown in a standard medium with 0.5 M NaCl were collected by centrifugation and resuspended to 2 × 10^5 cells/ml in a 0.5 M NaCl medium containing 25 mM NaHCO₃ and 50 mM HEPES, pH 7.4, with (+) or without (−) added iron (added iron: 2 mM FeCl₃, 5 mM EDTA, as in the standard medium). The cells were incubated under standard growth conditions for 45 h, and aliquots containing 5 × 10⁵ cells were fractionated on SDS-PAGE and analyzed by immunoblotting with anti-p150 antibodies. Panel B, cells grown in 0.5 M NaCl were collected and resuspended to 6.5 × 10⁵ cells/ml in the medium without added iron as described above. At the times indicated, cell samples were withdrawn, and aliquots containing 5 × 10⁵ cells were analyzed as in panel A.

A protein accumulating in the unicellular alga D. salina in response to high salt is shown here to be closely related to the animal transferrin family. Most known representatives have been implicated in functions of iron binding and delivery as well as the control of free iron levels in biological fluids (5–7).

A considerable number of transferrins, e.g., melanotransferrin, salmon transferrin, and insect transferrins, do not retain the entire set of the five amino acid ligands for Fe³⁺ and HCO₃⁻ (or CO₃²⁻), as determined for serum transferrin and lactoferrin. In some cases different residues occupy these positions in each of the two lobes, e.g., in the salmon transferrin (Fig. 3) the NH₂ lobe Arg-121 is replaced by Lys. In the human melanotransferrin COOH lobe, Ser replaces Asp-395 as well as Arg-465. In insect transferrins (21) and the bullfrog saxiphilin (22), replacements of the canonical liganding residues occur in both lobes.

The presence of identical sets of putative liganding residues in the three repeats of p150 suggests the existence of three similar iron binding sites. Of the three replacements of canonical ligands (Asp to Gly, His to Asn or Gln, and Arg to Lys) the potentially most significant is the Asp to Gly substitution as Gly cannot serve as an iron ligand. In this respect it is noteworthy that in all three p150 repeats, an Asn residue, not conserved among animal transferrins, is found two residues COOH-terminal to the position of the Asp to Gly replacement. This Asn might potentially contribute to iron coordination.

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DISCUSSION

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In its localization on the cell surface p150 most resembles melanotransferrin (17, 23), a 97-kDa protein first characterized
as a cell surface marker for human melanoma cells, as well as an 80-kDa transferrin-like protein from fetal intestinal epithelial cells (24). Both of these proteins are attached to the cell membrane not through membrane-spanning helices but via a glucosylphosphatidylinositol anchor added to the COOH-terminal residue exposed by proteolytic removal of a short COOH-terminal peptide. The algal p150 is a largely hydrophilic protein in which the transferrin-resembling repeats are followed by a ~100-residue COOH-terminal extension ending with a mildly hydrophobic sequence. Whether p150 is also attached to the plasma membrane by a post-translationally added anchor and what is the functional significance of its COOH-terminal extension in membrane attachment are intriguing questions yet to be answered.

The involvement of p150 in iron uptake is suggested by its induction under conditions of iron limitation, even in relatively low salinities. In view of these results, the induction of p150 in high salinities presumably reflects a decline in effective iron availability under these conditions. This decline may result, for example, from the effect of salt on Fe$^{3+}$ solubility or the interference by salt in the iron uptake machinery. Moreover, in cells grown with $^{59}$Fe$^{3+}$, p150 becomes radioactively labeled, demonstrating the ability of this protein to bind iron.

The mode of action of p150 might resemble that of the membrane-anchored p97, which has been shown recently to act in iron uptake via a mechanism other than receptor-mediated endocytosis of Fe-transferrin (23). The details of this mechanism are still largely unknown except for the distinction between an initial iron-binding step and a subsequent energy-dependent iron internalization. Similar to p97, p150 in intact cells is Pronase-sensitive, implying that p150 is extracellularly exposed. Thus, p150 is a transferrin-like protein that retains activity in the high salinities characteristic of the natural habitats of the algae. The significance of the potentially triple-lobe structure or other structural features in the function and presumed salt stability of p150 remains to be clarified.

Transferrin-like proteins were so far identified only in animals. In bacteria, several periplasmic proteins involved in active transport of ions, amino acids, and sugars show similarity in three-dimensional structure, but not in amino acid sequence, to single lobes of transferrins (6). Of special interest is a 34-kDa periplasmic Fe$^{3+}$-binding protein from pathogenic Neisseria which resembles human transferrin spectroscopically and functionally but not in primary sequence (25). The identification of a transferrin variant in a green alga points to an earlier evolution of the eukaryotic-type transferrins than hitherto thought. The original function of such proteins could have been of the type exhibited by Dunaliella, i.e. in the uptake of iron or possibly other nutrients from the medium.

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