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Characterization of a Nitrogen-Regulated Protein Identified by Cell Surface Biotinylation of a Marine Phytoplankton

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The biotinylating reagent succinimidyl 6-(biotinamido)hexanoate was used to label the cell surfaces of the cosmopolitan, marine, eukaryotic microorganism *Emiliania huxleyi* under different growth conditions. Proteins characteristic of different nutrient conditions could be identified. In particular, a nitrogen-regulated protein, nrp1, has an 82-kDa subunit that is present under nitrogen limitation and during growth on urea. It is absent under phosphate limitation or during exponential growth on nitrate or ammonia. nrp1 is the major membrane or wall protein in nitrogen-limited cells and is found in several strains of *E. huxleyi*. It may be a useful biomarker for examining the physiological state of *E. huxleyi* cells in their environment.

The activities of a number of cell surface enzymes are known to be induced or increased by factors limiting the growth of such eukaryotic microorganisms as phytoplankton. Alkaline phosphatases and 5′ nucleotidases can be induced under phosphorus-limited conditions in order to obtain phosphate from organic phosphorus compounds in the environment (7, 12). Nitrogen limitation has been shown to induce or increase cell surface L-amino acid and amine oxidase activities in a number of phytoplankton (26–28), while in others it may induce amino acid and urea transport systems presumably located in the plasma membrane (reviewed in reference 2). Trace metal limitation can induce high-affinity metal transport systems that must have access to the cell surface (32). Cell surface redox enzymes probably involved in nutrient transport may be regulated by growth conditions (21). Silica transport in diatoms appears to be driven by a plasma membrane Na^+-K^+ ATPase (5) whose activity could be governed by growth rate or by silica availability. In general, although we know these many enzyme activities must exist, we are only beginning to understand the diversity of enzymes present at the cell surface and outer membrane of phytoplankton and how that diversity is regulated by environmental factors.

Studies of cell surface enzymes have often relied on methods for derivatizing the cell surface of an organism in order to demonstrate enzyme location. These reagents are often large, hydrophilic molecules that react rapidly with cell surface proteins but do not cross the plasma membrane to react with intracellular proteins. A large number of these reagents are available (18), and some are clearly effective on phytoplankton in seawater media (29). A subset of these, the biotin-containing derivatizing reagents, have been shown to be especially useful for studying cell-surface proteins, their response to environmental stresses, and their turnover rates in a number of experimental systems including both prokaryotes and eukaryotes (6, 13, 14, 16, 17, 23, 33, 35). At least four different classes of biotin-containing reagents are readily available (18). (i) Biotin succinimidyl esters react with primary amines such as lysines in proteins. (ii) *N*-biotinoyl-*N*′-(maleimidohexanoyl)hydrazine reacts with sulfhydryl groups such as cysteines. (iii) Biotin hydrazide reacts with glycoproteins oxidized with periodate. (iv) *p*-Diazobenzoyl biocytin reacts with tyrosine and histidine residues. The variety of reagents available allows some specificity for probing cell surfaces. Different reagents have been shown to label different cell surface proteins in cultured mammalian cells (16).

The biotin moiety in the various reagents allows the labeled proteins to be detected on Western blots (immunoblots) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) because of the high affinity of the protein avidin for the biotin moiety. In this study, that approach was used to look for environmental stress-induced cell surface proteins in a very abundant, cosmopolitan marine phytoplankton, *Emiliania huxleyi*. The goal was to identify cell surface proteins that could act as biomarkers of the physiological state of these cells in the environment.

MATERIALS AND METHODS

Strains. Two *E. huxleyi* strains were obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton, Bigelow Laboratories: CCMP374 was isolated from the Gulf of Maine, and CCMP1516 was isolated from the Galapagos. A third strain, L, was obtained from T. Ietswaart and was originally isolated from waters near Norway.

Methods. Axenic cultures were grown in continuous fluorescent cool white light (0.3×10^{16} to 0.5×10^{16} quanta s^{-1} cm^{-2}) at 20°C. Stock cultures were maintained on a light-dark cycle (in f/20 nutrients) to foster calcifying strains. The three strains used here produced calcite scales (coccoliths). Medium was made with filtered (pore size, 0.2 μm) coastal seawater autoclaved with nutrients except for ammonia and urea, which were added from filter-sterilized stocks. To obtain nitrogen-limited cells, cultures were typically grown in 25 ml of medium in 50-ml glass tubes in f/2 (15) with only 50 μM nitrate until growth (as determined by increasing chlorophyll fluorescence on a Turner-Designs fluorometer or cell numbers counted microscopically) had stopped for 24 h. For phosphate-limited cells, only 1 μM phosphate was added with the other f/2 nutrients, and cells were labeled after growth slowed or stopped relative to that of phosphate-replete cultures, depending on the experiment. High-nitrogen cultures included 500 μM nitrate, 250 μM urea (and 100 nM nickel), or 100 μM ammonia with the other f/2 nutrients and were assayed during the exponential-growth phase and were found to have a growth rate of approximately one division per day. Only 100 μM ammonia was used, as higher levels have toxic effects on the cultures. Growth of cells with alanine or putrescine as the sole nitrogen source was tested with f/2 nutrients without nitrate and various amounts of L-alanine (0 to 500 μM) or 100 μM putrescine. For nrp1 purification, 8-liter cultures of strain CCMP374 were grown with stirring, with 25 μM nitrate being used as the nitrogen source.

Labeling protocol. The following stocks were prepared: (i) 0.1 M L-lysine in 1 M Tris, pH 8, stored frozen; (ii) 1 M $NaHCO_3$, freshly made; and (iii) 2 mg of succinimidyl 6-(biotinamido)hexanoate (SBH) (Molecular Probes, Inc.) in 100 μl of dimethyl sulfoxide (freshly made). To a 25-ml culture (about 1.5×10^6 cells

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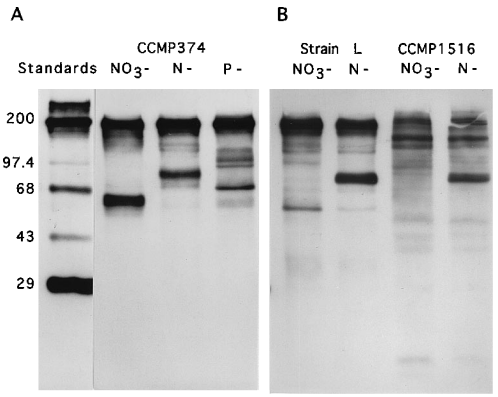

FIG. 1. (A) The patterns of biotin-containing proteins run on SDS-PAGE gels following SBH treatment of intact preconditioned *E. huxleyi* (CCMP374). Lanes: high nitrate (NO_3), nitrogen (N) limited, and phosphate (P) limited. Biotin-containing protein standards are also shown. The numbers at the left are sizes in kilodaltons. (B) The nitrogen-regulated protein nrp1 is present in different strains of *E. huxleyi* (L and CCMP1516) under nitrogen-limited but not nitrate-replete growth conditions.

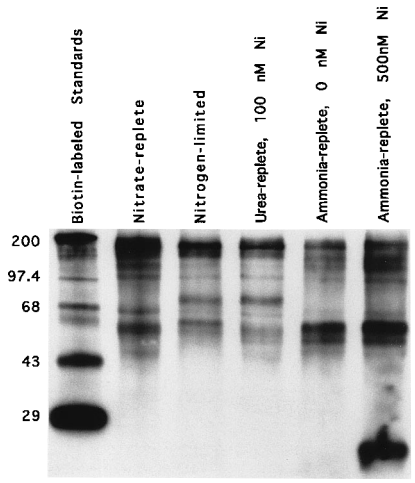

FIG. 2. The patterns of biotin-containing proteins on SDS-PAGE gels following SBH treatment of intact preconditioned *E. huxleyi* (CCMP374). The numbers at the left are sizes in kilodaltons.

ml^{-1}), 0.25 ml of 1 M $NaHCO_3$ was added to buffer the seawater (final concentration, 10 mM). SBH stock (50 μl) was added, mixed, and incubated for 20 min. Lysine stock (0.25 ml) was added to stop the reaction. Cells were harvested by centrifugation. Electrophoresis loading buffer was added to the cell pellet, and the sample was boiled for 2 min and centrifuged to clear debris just before it was loaded on SDS-PAGE gels. Longer or repeated boiling significantly affects band sharpness in our hands. Biotinylated protein standards were made according to the method of Della-Penna et al. (8) with high-molecular-weight protein standards from GIBCO BRL. Biotin standards in the figures are given at their unlabeled molecular weights, although the migration on the SDS-PAGE gels shifted in some cases after biotin labeling.

Proteins were separated on SDS-Tricine PAGE gels (31) on a Hoefer mini gel apparatus. Following electrophoresis, the gels were equilibrated with 25 mM Tris and 192 mM glycine, pH 8.3 (Towbin buffer without SDS or methanol), and the proteins were electrophoretically transferred at 4°C overnight at 15 V or for 2 h at 100 V to Immobilon-polyvinylidene difluoride membrane (Millipore) by using the same buffer with 0.025% SDS. After the protein transfer, the membrane was blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS)–0.05% Tween for 30 to 60 min at room temperature, which was followed by a 1-h incubation with a 1:10,000 dilution of avidin D-horseradish peroxidase complex (Vector Laboratories) in fresh blocking reagent. After the membranes were washed for 1 h with PBS-Tween, Amersham ECL chemiluminescence detection reagents and X-ray film were used for detection of the protein-biotin-avidin-peroxidase complex following the manufacturer's instructions.

nrp1 was partially purified. To develop the purification, SBH-labeled cells of CCMP374 from 1 liter of culture in 1 ml of buffer (20 mM Tris, pH 8, and 50 mM NaCl) were broken by vortexing for 3 min with a slightly smaller volume of glass beads. Cell breakage was assessed microscopically. The broken-cell extract was resuspended with an additional 1 ml of buffer and centrifuged at 10,000 rpm (12,800 × *g*) for 10 min, and the subsequent supernatant was centrifuged at 28,000 rpm (100,000 × *g*) for 1.5 h. The 10,000-rpm pellet (containing any unbroken cells, coccoliths, and possibly some cell wall material) and the 28,000-rpm pellet (cell membranes and cell wall material) were resuspended in 1 ml (i.e., 2× concentrated) and 200 μl (10× concentrated) of buffer, respectively. All steps, including centrifugations, were carried out at about 20°C. An equal volume of 2× loading buffer (31) was added to the fraction samples, and 15 μl of the different fractions was run on SDS-PAGE gels and probed as described above.

Glycoprotein staining of the 28,000-rpm fraction on SDS-PAGE gels was performed by the periodic acid-Schiff stain protocol (22). Protein was quantitated by the Bio-Rad DC method (Bio-Rad).

RESULTS

Biotin-containing reagents such as SBH were used on a strain of the ubiquitous marine phytoplankton *E. huxleyi* (strain CCMP374) to find a protein under nitrogen-limited but not nitrate-replete growth conditions. This protein can be seen in a representative blot (Fig. 1A). This nitrogen-regulated protein, which will be referred to as nrp1, has a molecular weight by reducing SDS-PAGE of approximately 82,000, as calculated with unbiotinylated standards. This protein was not found under phosphate-limited conditions (Fig. 1A). Cell surface proteins specific to phosphate limitation were also found, and some proteins present in nitrogen-replete cultures disappeared in nutrient-limited cultures (Fig. 1A). When unlabeled cells were probed for biotin for the three types of preconditioned cells, only a small amount of background from endogenous biotin-containing proteins was found at much longer exposures (data not shown).

To further investigate the regulation of nrp1, cells were grown with ammonia (100 μM), high nitrate (500 μM), or high urea (250 μM) and labeled during the exponential-growth phase (except as noted). In each case, cells were grown with or without nickel, as this trace metal is required for urease activity. nrp1 was not detectable in cells growing exponentially on nitrate with (Fig. 2) or without (data not shown) nickel or on ammonia with (Fig. 2) or without (Fig. 2) nickel. nrp1 was detectable in urea-grown cells with nickel and in nitrogen-limited cells without nickel (Fig. 2). As can be seen from the developed blots, these cells were labeled at approximately the same amounts, although nitrogen limitation for longer than 1 day shows slightly higher nrp1 levels (data not shown). Urea-grown cells without added nickel had high levels of nrp1 (data not shown). The latter cells grew slowly, as nickel is required for urea utilization in *E. huxleyi*, and these cells were effectively nickel and nitrogen limited, as was found previously for a marine diatom (30).

The nitrogen-regulated protein nrp1 can be differentially labeled by varying the SBH concentration (data not shown). In addition, cells were labeled to the same extent in the presence of added biotin (data not shown). This suggests that SBH is not being transported into the cells as part of a biotin transport system before it labels nrp1. The addition of lysine to stop further labeling, which is not found in some protocols, was useful for controlling the extent of labeling and for ensuring that intracellular proteins were not labeled during cell harvesting and lysis. Without this addition, the Western blots had significantly higher backgrounds (data not shown).

To examine the strain distribution of nrp1, two other *E. huxleyi* strains, L and CCMP1516, were biotin labeled with SBH. nrp1 (or a protein of identical size and regulation) was also present in these two strains under nitrogen-limited but not nitrate-replete conditions, suggesting that its presence is a gen-

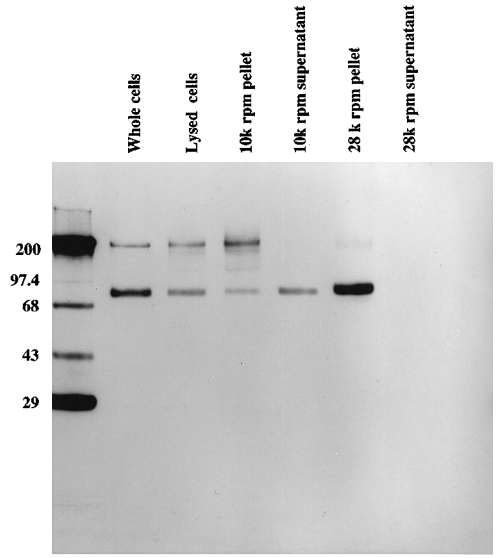

FIG. 3. Fractionation of biotin-labeled cells shows nrp1 in the high-speed pellet (28,000-rpm) fraction but not the supernatant, indicating that it is a membrane or cell wall protein. From the left, the lanes show biotin standards, nitrogen-limited control cells (whole cells), broken-cell extract (lysed cells), the 10,000-rpm pellet at a 2× concentration, the 10,000-rpm supernatant, the 28,000-rpm pellet at a 5× concentration, and the 28,000-rpm supernatant. The numbers at the left are sizes in kilodaltons.

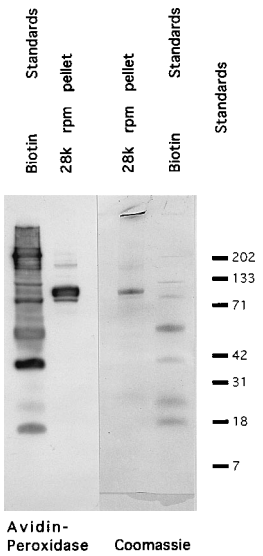

FIG. 4. Comparison of a biotin detection blot and a Coomassie-stained SDS-PAGE gel for the 28,000-rpm pellet shows that nrp1 is the major protein in this fraction in nitrogen-limited cells. The numbers at the right are sizes in kilodaltons.

eral characteristic of *E. huxleyi* strains (Fig. 1B). The two other strains had some differences in biotin-labeling patterns and did not grow as well as CCMP374 on urea as a nitrogen source, possibly because of different nickel requirements. The three strains, isolated from different geographical locations, thus clearly were not identical. Strain L is a type A strain. We do not know the immunological types of the other strains (34).

nrp1 was partially purified in order to investigate its characteristics. Most of this protein was present in the 28,000-rpm pellet which would have contained cell membranes and possibly some cell wall-type material (Fig. 3). None was detected in the 28,000-rpm supernatant, which is consistent with nrp1 being a cell surface protein. nrp1 is the major protein in the 28,000-rpm pellet of nitrogen-limited cells, as can be seen by Coomassie blue-stained gels (Fig. 4). This preparation, used on cells starved for nitrogen for several days (which further depletes cellular proteins), is sufficient to obtain material for an N-terminal protein sequence (data not shown).

It was also found that the presence of SDS and β-mercaptoethanol, not boiling, was required to run nrp1 into an SDS-PAGE gel (data not shown). This suggests that the protein exists in vivo as a larger-molecular-weight protein, either with a disulfide-bonded oligomer or with a modification (for example, lipid) removable by β-mercaptoethanol reduction. In addition, the 82-kDa subunit of nrp1 had a positive reaction under periodic acid-Schiff staining, suggesting that it is probably a glycoprotein (data not shown).

DISCUSSION

Assessing the role of nutrient limitation in controlling the growth rate and cell yield of phytoplankton in the environment is a long-standing issue in the ecology of marine and freshwater environments (24). Some arguments for nutrient limitation in specific cases are based on the low or undetectable nutrient concentrations themselves. Biological markers for nutrient stress (such as glutamate/glutamine ratios for nitrogen limitation) have been proposed (11). Less effort has gone into identifying particular proteins that might indicate nutrient stress and assaying for these with specific antibody probes. This approach potentially has the advantage of being applicable at the single-cell and species levels but the disadvantage of not necessarily being applicable to more than one species of phytoplankton.

The use of biotinylating reagents makes it possible to identify cell surface proteins that vary in response to cell growth conditions (6, 13, 14, 16, 17, 23, 33, 35). In applying this approach to a eukaryotic phytoplankton, *E. huxleyi*, we found cell surface proteins that were regulated by phosphorus and nitrogen availability (Fig. 1 and 2). This approach also works on other phytoplankton groups, such as diatoms (25) and dinoflagellates (9), and could be a potentially important tool for investigating phytoplankton responses to any environmental stress. In addition, it may be useful, as was seen with the different labeling of *E. huxleyi* strains, for looking at strain variability in phytoplankton.

In *E. huxleyi*, a protein, nrp1, that was greatly induced by nitrogen-limited conditions and was virtually absent under any other growth conditions tested, except for growth on urea, was found. It is thus clearly a protein regulated by the amount and/or availability of nitrogen; whether or not it is specific to nitrogen-limited conditions is still equivocal (see below). This protein has an 82-kDa subunit that is detectable by SDS-PAGE only under reducing (β-mercaptoethanol) conditions. This suggests that the protein exists in vivo as a larger-molecular-weight protein. It may have other subunits that were not detected by biotinylation because of the protection of lysines by their location in the protein or by the absence of lysines altogether.

nrp1 is the major membrane or wall protein in nitrogen-limited cells and is found in the three strains of *E. huxleyi* that were tested. It is not yet known whether the protein is found in other coccolithophorids or prymnesiophytes, including members of such closely related genera as *Gephyrocapsa*. The exact nitrate or urea levels or degree of nitrogen limitation causing nrp1 production has not been defined, though these will be

necessary for the interpretation of any field experiments using antibodies to nrp1.

The function of nrp1 is unknown at this time. nrp1 may be a kind of protective protein that somehow improves the cell's ability to survive nitrogen starvation.

Alternatively, nrp1 may be a low-nitrogen-induced transporter of a nitrogen source, such as amino acids, amines, purines, pyrimidines, urea, etc. Some strains of *E. huxleyi* can grow on a few amino acids, such as alanine, as their sole nitrogen source (20). nrp1 does not appear to be an alanine or a general amino acid transporter, however, as it was not possible in our hands to grow cells of any of the three strains on alanine (data not shown).

nrp1 is not a cell surface amino acid oxidase that allows some phytoplankton to grow on these extracellular nitrogen sources via oxidation and ammonia transport (26, 27). This was demonstrated by the lack of amino acid oxidase activity on whole cells and the lack of growth on alanine (data not shown). It was not possible to grow cells on putrescine (data not shown), a substrate of some amine oxidases, so it is unlikely that nrp1 is a cell surface amine oxidase (28) (or a putrescine transporter). It is possible that nrp1 is a another kind of degradative enzyme, such as a DNase, RNase, or proteinase, that makes larger-molecular-weight forms of nitrogen more available.

The possible role of nrp1 as a urea transporter or urease is equivocal in that the protein is present in CCMP374 under urea growth conditions, possibly enough to account for growth on urea. This protein would then be further induced under nitrogen-limited conditions in order to increase the maximum uptake or hydrolysis rate of urea. A component of the urea transporter in *Saccharomyces cerevisiae* is predicted to have a similar molecular weight of 80,000 (10). Unfortunately, as seen by ourselves and others, growth of *E. huxleyi* on urea is slightly slower than that on nitrate by approximately 5% (this paper) or 25% (1). Cells may thus be effectively slightly nitrogen limited, even in the presence of excess urea nitrogen. Further clues to the function of nrp1 may come from urea transport and urease assays, but these alone would not be definitive. Also, the function of nrp1 may be predicted from the amino acid sequence of the protein when it becomes available.

E. huxleyi is a cosmopolitan marine phytoplankton. Blooms of this organism under certain conditions can be detected by satellite because of the light scattering by their calcite scales (19). These scales are a major source of calcite in marine sediments. In addition, this organism is a thought to be an important source of the sulfur gas dimethyl sulfide for the atmosphere. Final cell yields of blooms of *E. huxleyi* in the Gulf of Maine may be limited by nitrogen availability, as suggested by the effects of nutrient additions (3, 4). Potential protein biomarkers for nitrogen limitation, such as nrp1, and markers for phosphorus limitation could help in understanding the relationships among nutrients, bloom dynamics, and dimethyl sulfide and calcite production in the marine environment.

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