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SHORT COMMUNICATION

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Novel glycoproteins of the halophilic archaeon *Haloferax volcanii*

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Abstract Archaea possess many eukaryote-like properties, including the ability to glycosylate proteins. Using oligosaccharide staining and lectin binding, this study revealed the existence of several glycosylated *Haloferax volcanii* membrane proteins, besides the previously reported surface layer (S-layer) glycoprotein. While the presence of glycoproteins in archaeal S-layers and flagella is well-documented, few archaeal glycoproteins that are not part of these structures have been reported. The glycosylated 150, 98, 58 and 54 kDa protein species detected were neither precursors nor breakdown products of the 190 kDa S-layer glycoprotein. Furthermore, these novel glycoproteins were outwardly oriented and intimately associated with the membrane.

Key words Archaea · Halophiles · *Haloferax volcanii* · Glycoproteins · Membranes · S-layer

Introduction

Long-thought to be restricted to eukaryotes, it is now established that prokaryotes can also *N*-glycosylate specific extra-cytoplasmic proteins (Messner 1997; Moens and Vanderleyden 1997). The surface layer (S-layer) protein of the halophilic archaeon *Halobacterium halobium* was the first prokaryotic glycoprotein to be described in detail (Lechner and Sumper 1987). It has since become clear that the cell walls of many archaeal species are composed of a single S-layer glycoprotein (Messner and Sleytr 1992).

Archaea are best known for their abilities to survive drastic conditions, including extremes of temperature, pH and salinity (Woese et al. 1990) and may represent part of the ancestry of eukarya (Martin and Muller 1998). Thus, eukaryal *N*-linked protein glycosylation may have originated from a similar process in archaea. As such, further understanding of protein glycosylation will be aided by better characterization of this phenomenon in archaea. Although current understanding of archaeal protein glycosylation is limited, parallels to the eukaryal protein glycosylation system are clear. In both eukarya and archaea, *N*-glycosylation occurs at asparagine residues of the Asn-X-Ser/Thr consensus motif (Spirig et al. 1997). Dolichyl-linked oligosaccharides, similar to those involved in eukaryal protein glycosylation, have been detected in archaea (Lechner et al. 1985; Kuntz et al. 1997). Moreover, many enzymes involved in the eukaryal protein glycosylation process find counterparts in archaea (Meyer and Schafer 1992; Zhu and Laine 1996; Spirig et al. 1997; Burda and Aebi 1999).

In attempting to further characterize protein glycosylation in archaea, the present report reveals the presence of glycoproteins in the plasma membrane of *Haloferax volcanii*, including 150, 98, 58 and 54 kDa species not previously shown.

Materials and methods

Culture and radiolabeling conditions

Haloferax volcanii DS2 (ATCC No. 29605) was grown as previously described (Mevarech and Werczberger 1985). Radiolabeling was performed in minimal medium (Mevarech and Werczberger 1985) at 40 °C with 15 μCi [^{35}S]methionine per ml culture. Cells were harvested (8,000×g, 30 min, room temperature), resuspended in a small volume of 1.75 M NaCl, 50 mM Tris-HCl, pH 7.2, and stored at –20 °C.

Periodic acid-Schiff reagent (PAS) staining

The protein of *H. volcanii* was precipitated with 15% TCA, pelleted in a microfuge (10,000×g, 15 min, 4 °C), washed with acetone, repelleted and resuspended in sample buffer (4% SDS, 20% (v/v) glycerol, 0.02% bromphenol blue, 1.5% β-mercaptoethanol,

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125 mM Tris-HCl, pH 6.8). Gels were PAS-stained as described previously (Dubray and Bezard 1982).

Concanavalin A-Sepharose binding

Radiolabeled *H. volcanii* membranes (10^6 cpm) were added to 100 µl of concanavalin A (ConA)-Sepharose beads (prewashed in 1 ml lectin buffer: 1 mM Ca^{2+}, 1 mM Mn^{2+}, 1 mg/ml BSA, 1% Triton X-100, 50 mM Tris-HCl, pH 7.9) and lectin buffer in a final volume of 1 ml and rocked overnight at 4 °C. The beads were washed four times in lectin buffer without BSA, heated (3 min, 95 °C) in the presence of 35 µl sample buffer and pelleted in a microfuge (5,000×g, 3 min). The supernatant was transferred to clean microtubes and examined by 7.5% SDS-PAGE and fluorography.

Isolation of membranes

Membranes were isolated by osmotic lysis of cells upon transfer into 1 ml water, addition of DNase (6 U) and ultracentrifugation (140,000×g, 10 min, 4 °C). Pelleted membranes were resuspended (100 µl lectin buffer) and probed with ConA as above.

Western blotting

SDS-PAGE-separated proteins were transferred to nitrocellulose and probed with antibodies (1:10,000 in 5% milk powder, 0.5% Tween-20, PBS) raised against either a peptide corresponding to the S-layer glycoprotein N-terminal 13-amino-acid residues (Sumper et al. 1990) or the purified protein, or normal rabbit serum. Antibody binding was revealed by horseradish-peroxidase-conjugated goat anti-rabbit antibodies and ECL.

Materials

Bovine serum albumin, DNase, periodic acid, Triton X-100, Tween 20 were from Sigma (St. Louis, Mo.). Proteinase K was purchased from Boehringer-Mannheim (Mannheim, Germany). Schiff reagent and horseradish-peroxidase-conjugated goat anti-rabbit antibodies were from BioRad (Hercules, Calif.). SDS-PAGE molecular weight markers were from Novex (San Diego, Calif.). Tryptone and yeast extract were from Biolife (Milan, Italy). ConA- and CnBr-Sepharose were from Pharmacia (Uppsula, Sweden). Nitrocellulose (0.45 µM) came from Schleicher and Schuell (Dassel, Germany); Centricon-30 concentrating units were from Amicon (Beverly, Mass.). [^{35}S]methionine (>1000 Ci/mmol) and an ECL kit were from Amersham (Buckingham, UK).

Results

H. volcanii membranes contain five glycoproteins

Examination of the *H. volcanii* proteins by SDS-PAGE and PAS staining revealed numerous putative glycoproteins (Fig. 1A). An 190 kDa protein was most intensely stained, although 98 kDa and 70 kDa protein bands were also clearly visible (lane 1). In addition, 150, 58 and 54 kDa bands were also stained as was a far weaker 80 kDa band. When water replaced periodic acid in the staining protocol, no labeling was detected, confirming the specific nature of the labeling pattern (lane 2).

The existence of multiple *H. volcanii* glycoproteins was also confirmed using lectins. Since ConA strongly labels intact *H. volcanii* cells (Gilboa-Garber et al. 1998), membranes from radiolabeled cells were isolated and the extracted proteins were incubated with ConA-Sepharose beads. SDS-PAGE and fluorography revealed the capture of 190, 150, 98, 58 and 54 kDa bands (Fig. 1B, lane 2). The addition of excess glucose during incubation of membranes with the lectin beads prevented glycoprotein capture, apart from a low level of the 58 kDa glycoprotein (lane 3). Similarly, when the membranes were incubated with underivatized beads, only a small amount of the 58 kDa species was precipitated (lane 4).

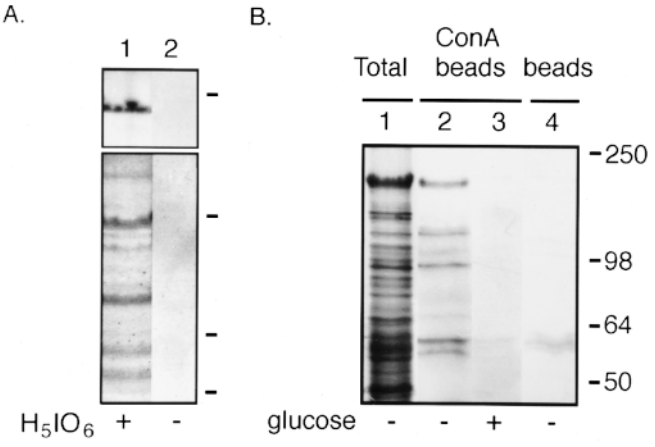

Fig. 1A,B *Haloferax volcanii* contains 190, 150, 98, 58 and 54 kDa glycoproteins. **A** The proteins of *H. volcanii* were PAS-stained (*lane 1*). To compensate for intense staining of the 190 kDa band, 25- and 150-µl aliquots were probed, corresponding to higher and lower molecular mass gel regions, respectively. As control (*lane 2*), H_5IO_6 was omitted from the reaction. **B** Radiolabeled membranes (*lane 1*) were incubated with concanavalin A (ConA) beads (*lanes 2, 3*) or underivatized beads (*lane 4*). In *lane 3*, 0.5 M glucose was included in the reaction. Molecular mass markers are shown on the *right* of each figure, with kDa values given in **B**

The 150, 98, 58 and 54 kDa glycoproteins are not derived from the 190 kDa S-layer glycoprotein

The glycosylated nature of the *H. volcanii* S-layer protein as well as the transfer of activated glucose subunits to a 190 kDa protein identified as the S-layer protein have been previously shown (Sumper et al. 1990; Zhu et al. 1995). To confirm that the 190 kDa band labeled by PAS and captured by lectin beads corresponds to the S-layer glycoprotein, antibodies were raised against the N-terminal 13 residues of the protein (Sumper et al. 1990). Immunoblotting revealed intense staining of a 190 kDa band (Fig. 2, lane 1) that was not recognized by normal rabbit serum (lane 2). Identification of the 190 kDa band as the S-layer glycoprotein was confirmed by the absence of labeling upon addition of the peptide used for antibody generation to the immunoblot incubations (lane 3). Antibodies to purified S-layer glycoprotein also recognized the 190 kDa protein (lane 4). Finally, the ConA-bound 190 kDa protein was also labeled by the antibodies

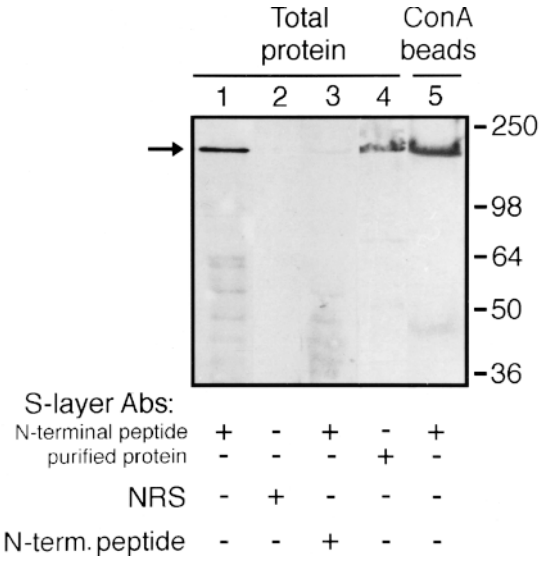

Fig. 2 The 150, 98, 58 and 54 kDa glycoproteins are not part of the S-layer glycoprotein. Cells (*lanes 1–4*) or ConA-bound glycoproteins (*lane 5*) were probed with antibodies raised against the N-terminal 13 amino acids of the S-layer glycoprotein (*lanes 1, 3, 5*) or the purified protein (*lane 4*), or with normal rabbit serum (*NRS, lane 2*). In *lane 3*, the peptide to which antibodies were raised was included during antibody incubation. The *arrow* shows the position of the S-layer glycoprotein. Molecular mass markers are shown on the *right*

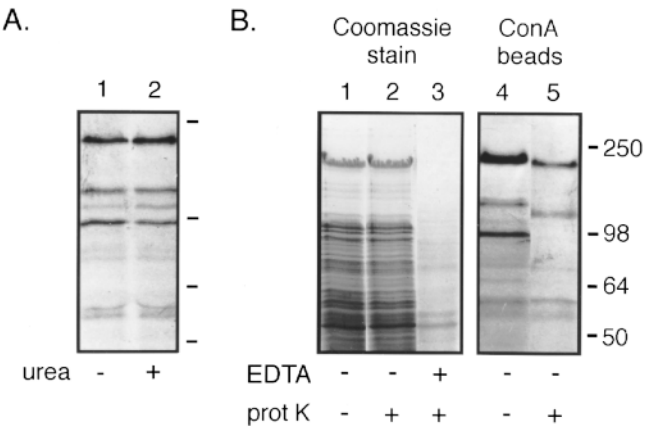

Fig. 3A,B *H. volcanii* glycoproteins are externally oriented and intimately associated with the membrane. **A** Control (*lane 1*) and urea-treated (*lane 2*) radiolabeled membranes were incubated with ConA-Sepharose beads. **B** Radiolabeled cells were incubated in the absence (*lanes 1, 4*) or presence (*lanes 2, 3, 5*) of proteinase K (2 mg/ml, 30 min, room temperature). In *lane* 3, 150 mM EDTA was also included. Samples were examined by SDS-PAGE and Coomassie stain (*lanes 1–3*) or incubated with lectin beads and examined by SDS-PAGE and fluorography (*lanes 4, 5*). Molecular mass markers are shown on the *right*

(lane 5). Thus, the lectin-captured 190 kDa species is the S-layer glycoprotein. The failure of anti-S-layer glycoprotein N-terminus antibodies to recognize the 150 and 98 kDa glycoproteins (lane 1) suggests that these are not precursors of the S-layer glycoprotein. Indeed, neither antibody preparation labeled the 150, 98, 58 or 54 kDa glycoproteins. Had these species originated from degradation of the 190 kDa S-layer glycoprotein, then these fragments should have contained at least some of the epitopes recognized by the antisera. The absence of labeling suggests that these protein bands do not arise from S-layer glycoprotein degradation.

H. volcanii glycoproteins are found on the outer surface of the cell

To examine the mode of membrane association of the glycoproteins, membranes from radiolabeled cells were incubated with 6 M urea to remove peripherally bound proteins. Subsequent removal of urea followed by extraction of remaining integral membrane proteins and incubation with ConA beads revealed the failure of urea to remove any of the glycoproteins (Fig. 3A). Thus, *H. volcanii* glycoproteins are intimately membrane-associated.

To determine the topology of the membrane-associated glycoproteins, proteinase K was added to radiolabeled cells. Coomassie staining demonstrated the ineffectiveness of protease treatment unless cell integrity was first compromised by EDTA (Fig. 3B, compare lanes 2 and 3). To determine whether the membrane-associated glycoproteins were accessible for proteolytic attack, radiolabeled membranes were isolated and transferred to lectin-binding conditions following protease treatment (and inhibition of the protease). As a result of proteolysis, capture of the 190 kDa glycoprotein was reduced 55% (as determined densitometrically), with the bulk of the surviving S-layer glycoprotein migrating slightly faster in SDS-PAGE (Fig. 3B, lane 5). Protease treatment also led to the appearance of a novel 120 kDa band. Whether this band originates from the S-layer or from the 150 kDa glycoprotein is unclear. Proteolysis also led to total and partial losses in capture of the 98 and 54 kDa glycoproteins, respectively. A 58 kDa band survived proteolysis, but it is unclear whether this is the 58 kDa glycoprotein or a breakdown product of the heavier glycoproteins. Thus, the results suggest outward orientation of at least the 150, 98 and 54 glycoproteins.

Discussion

PAS staining and lectin binding revealed the existence of novel 150, 98, 58 and 54 kDa *H. volcanii* glycoproteins. The inability of antisera to the S-layer glycoprotein to label these glycoproteins and the failure to detect increased appearance of these species upon proteolysis suggests that they are unrelated to the S-layer glycoprotein. Furthermore, unlike the S-layer glycoprotein, membrane association of the novel glycoproteins is Mg^{2+}-independent (not shown). Only a few examples of archaeal glycoproteins that are not part of the S-layer or flagella have been reported (Yang and Haug 1979; Grogan 1996; Messner 1997; Moens and Vanderleyden 1997). *H. volcanii* does not contain flagella (Mullakanbhai and Larsen 1975).

Earlier studies mentioned binding of radionucleotide-activated sugars to several *H. volcanii* proteins besides the S-layer glycoprotein (Zhu et al. 1995). The 98, 58 and 54 kDa glycoproteins identified in the present study may correspond to the 105, 56 and 52 kDa species suggested in the earlier work. The 150 kDa glycoprotein revealed in the current report has not been previously described. Interestingly, PAS also weakly labeled a 150 kDa protein in *H. halobium* (Mescher et al. 1974). As the 150 kDa species did not bind activated glucose (Zhu et al. 1995), it may contain little if any of this sugar. ConA, which bound the 150 kDa protein, also binds mannose and *N*-acetylglucosamine, although the latter is not found in *H. volcanii* (Zhu et al. 1995; Gilboa-Garber et al. 1998). PAS staining also labeled 70 and 80 kDa bands that were not precipitated by the lectin beads. While false-positive PAS staining has been reported (Kimura and Stadtman 1995), these species may alternatively contain sugars not recognized by ConA or may not incorporate ^{35}S radiolabel, thereby being undetectable.

The *H. volcanii* glycoproteins are intimately membrane-associated, as suggested by their urea non-extractability. Protease susceptibility of the 150, 98 and 54 kDa glycoproteins suggests that, like the S-layer glycoprotein, these species are outwardly oriented. Protease sensitivity of pulse-labeled S-layer glycoprotein was unexpected given the resistance of the existing pool of S-layer glycoprotein to proteinase K as well as the reported protease resistance of other S-layer proteins (Engelhardt and Peters 1998). The different protease susceptibilities of newly synthesized and existing S-layer glycoproteins could reflect conformational differences in the two pools or differences in the integration of the nascent molecules into the S-layer relative to the pre-existing pool. Although *H. volcanii* has a generation time of several hours, de novo S-layer glycoprotein synthesis occurs in under 2 min, suggesting substantial turnover of *H. volcanii* S-layer glycoprotein. In *H. halobium*, the S-layer glycoprotein is degraded in vivo by an endogenous protease (Mescher et al. 1974).

What function might protein glycosylation serve in archaea? In *H. halobium*, glycosylation of the S-layer protein is responsible for maintaining the cell's rod-like shape (Mescher and Strominger 1976). The glycan moieties of the *H. volcanii* S-layer glycoprotein may create a "periplasmic" space between the plasma membrane and the S-layer (Kessel et al. 1990). The additional outward-facing *H. volcanii* glycoproteins reported here could exist in such a compartment, with their glycan structures participating in the architecture of this space.

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