Oligomeric Nature of the Integral Membrane Protein Stomatin*

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The 31-kDa integral membrane protein stomatin (protein 7.2b) is not only an important component of the red cell membrane but can also be found in abundance in different tissues and cell lines. The protein is thought to be anchored to the membrane by a hydrophobic domain while both N and C termini are exposed to the cytoplasm. We have previously shown in the human cell line UAC that stomatin concentrates preferentially in plasma membrane folds and protrusions. There is also evidence that stomatin is linked to the cortical actin cytoskeleton, suggesting a role in cortical morphogenesis of the cell. In this study, we demonstrate that the fundamental structure of stomatin is oligomeric. Whereas interaction of stomatin with itself was suggested by cross-linking experiments, we show by density gradient centrifugation analysis that soluble homo-oligomeric complexes of this protein are present in Triton X-100 extracts of UAC cells. We also show the existence of these oligomers by co-immunoprecipitation of the endogenous stomatin and a recombinantly expressed myc-tagged stomatin, using an anti-myc antibody. The data indicate that these complexes comprise between 9 and 12 monomers of stomatin. Two C-terminally truncated forms of stomatin do not incorporate into these oligomers, suggesting an involvement of the C terminus in the homo-oligomeric interaction.

The 31-kDa protein stomatin was first identified and purified from the erythrocyte membrane as a major component of band 7 (band 7.2b) (1, 2). cDNA cloning and amino acid sequence analysis (3, 4) revealed the presence of a putative 29-amino acid membrane-associated domain preceded by a hydrophilic 24-amino acid N-terminal region and followed by the C-terminal part containing most of the 287 residues. Both N- and C-terminal regions have been shown to be exposed at the cytoplasmic side of the membrane (5). Analysis of stomatin expression allowed its presence to be detected in abundance in most tissues (although significantly lower in brain tissue) as well as in various cell lines (2, 4, 6, and 7).

Although the wide distribution of stomatin and its constitutive expression suggest an important role for this protein in cell biology, perhaps as a “house-keeping” component, its function remains undetermined. However, some clues to a possible function are provided by features related directly to stomatin or to homologous proteins. First, stomatin is apparently absent in hereditary stomatocytosis, an autosomal dominant hemolytic anemia (8, 9). As this disease is associated with an increase in erythrocyte membrane permeability, the protein may play an important role in the maintenance of membrane integrity. Notably, stomatin was also found to interact specifically with phospholipids in the erythrocyte membrane (10).

Second, at least two proteins related to human stomatin exist in the worm Caenorhabditis elegans. MEC-2 appears to be one of the essential components of mechano-sensory neurons and is specifically expressed in these cells (11). UNC-24 is required for normal locomotion of the worm but was not assigned to any type of cell or tissue (12). Although the amino acid sequences of these proteins display some homology with stomatin, they also show important differences that might imply modified or different functions.

We have described the 5–6-fold up-regulation of stomatin in the human amniotic cell line UAC after treatment with interleukin-6 (IL-6)1 and dexamethasone (13). The same treatment induces expression of metallothionein in these cells and a correlated increase of resistance against oxidative stress. While the significance of stomatin induction in this system remains hypothetical and may be related to a more complex tissue response, in a previous study we took advantage of the high expression to analyze the cellular localization of stomatin by immunofluorescence and electron microscopy (14). These data showed that the protein concentrates preferentially in plasma membrane protrusions and also in a juxta-nuclear region that might represent vesicles derived from the Golgi apparatus. In addition, stomatin appears to co-localize to some extent with cortical actin microfilaments, even after disruption of actin filaments by cytochalasin D.

The massive presence of stomatin in membrane-protruding folds and extensions (14) suggests a possible structural role for this protein in the formation of these structures and/or the anchorage to the actin cytoskeleton. Other proteins involved in cortical morphogenesis typically form oligomers (15). We also noticed that gold particles often appeared as doublets or triplets on immunoelectron microscopy with an anti-stomatin antibody (14). In this study, we present evidence, using two different approaches, that stomatin actually forms oligomers. One is the analysis of detergent extracts of UAC cell membranes by density gradient centrifugation and the other is co-immunoprecipitation of the endogenous stomatin when a constitutively expressed epitope-tagged stomatin is immunoprecipitated with an epitope-specific antibody. The results of both studies led to the conclusion that stomatin is present in the membrane in an oligomeric form.

**EXPERIMENTAL PROCEDURES**

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1 The abbreviations used are: IL-6, interleukin-6; PBS, phosphate-buffered saline; DSS, dissuccinimidyl suberate; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum.
clonal anti-stomatin antibody GAR50 has been described (2, 14) and was used from hybridoma supernatant. Polyclonal anti-calscinitin antibody was a kind gift of Dr. I. Ivecova (University of Vienna, Vienna, Austria), and monoclonal anti-epidermal surface antigen was purchased from Zymed Laboratories (San Francisco, CA). Eukaryotic expression plasmid for PLO-2 (plasmid for PLO-2) was rescued from pPL-2, which was a kind gift of Dr. J.-C. Renaud (Ludwig Institute for Cancer Research, Brussels, Belgium). Disuccinimidyl suberate (DSS) cross-linking agent was from Pierce. Pfu DNA polymerase was purchased from Stratagene. [35S]methionine (1368 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Cells (human, embryonic cells of amniotic origin) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS). For maximum expression of stomatin, they were grown to confluence and treated with IL-6 (200 units/ml) and dexamethasone (0.1 μM) 1 day before experiments as described (13, 14).

Vector Construction and Transfections—To express an epitope-tagged stomatin in UAC cells, we used the eukaryotic expression vector pEF-Puro.PL3. This vector contains a BsrXI site downstream of the promoter of elongation factor 1α and a puromycin resistance gene. A double-stranded oligonucleotide having the sequence coding for the myc epitope EKLISEEDL followed by a STOP codon and preceded by a KpnI site was inserted into the BsrXI site. The resulting plasmid contains a unique KpnI site directly upstream of the oligonucleotide was created with the appropriate sequencing code and two KpnI compatible ends, of which only the 5’ end was used as a cleavable site. This was inserted into BsrXI site in frame by five copies of the myc epitope (pEFKmyc5). A full-length DNA polymerase, the coding region of KpnI site followed BstXI site at both ends. The resulting fragment was cloned into pEFKmyc5 leading to the C-terminal tagged stomatin (pEFSTmyc5). The construct was checked by dideoxy sequencing which showed that no mutations were introduced through the cloning process (3, 6). UAC cells were stably transfected using LipofectAMINE (Life Technologies, Inc.) as transfection reagent. After selection in medium supplemented with 2 μg/ml puromycin, individual clones were picked by trypsinization using cloning rings. Expression of the recombinant protein was screened by Western blot analysis using the anti-stomatin antibody GAR50, and correct membrane localization was monitored by immunofluorescence using the anti-myc antibody. C-terminally truncated forms of stomatin fused to an myc tag at the C terminus were prepared as described above. Amino acids from 1 to 198 were fused to three copies of the myc epitope for better visualization on Western blot.

Metabolic Labeling and Immunoprecipitation—Clones expressing epitope-tagged stomatin were grown to confluence in a 10-cm dish and treated with IL-6 and dexamethasone or left untreated. They were rinsed in methionine-free medium supplemented with dialyzed serum and incubated for 5 h in 5 ml of the same medium containing 50–100 μCi/ml [35S]methionine. After rinsing with PBS, cells were treated with IL-6 and dexamethasone and harvested from the dish using 1 ml of homogenization medium (50 mM Tris/Hepes, pH 7.4, 30 mM mannotol, and 5 mM CaCl2) containing protease inhibitors. Homogenization was performed on ice in a tight fitting Dounce homogenizer. Unbroken cells and nuclei were pelleted at 5700 g for 15 min, and the supernatant was centrifuged at 65,000 × g for 30 min. The resulting pellet was used directly for lysis and subsequent gradient centrifugation as described above.

Western Blotting—Fractions collected from the gradients were separated on 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose. Rainbow molecular weight marker was used (Amershams Pharmacia Biotech Inc.). Blots were blocked for 60 min in Tris-buffered saline, pH 8, 0.1% bovine serum albumin, and 5% non-fat dry milk. After incubation with anti-stomatin antibody GAR50 (diluted 1/15 in Tris-buffered saline, 0.1% bovine serum albumin) for 60 min, they were incubated with anti-mouse alkaline phosphatase conjugate (Promega, Madison, WI) before visualization with the substrate (NBT, BCIP). In Fig. 2, a secondary antibody conjugated to horseradish peroxidase was used and visualized with SuperSignal chemiluminescent substrate (Pierce).

Immunofluorescence—Immunofluorescence of IL-6 and dexamethasone-treated UAC cells with anti-stomatin antibody was performed as described (14). The same procedure was used to visualize myc epitope-tagged stomatin in stably transfected cells except that anti-myc monoclonal antibody served as a primary antibody at 1 μg/ml in PBS containing 1% FCS. Preparations were examined using a Zeiss Axioskop microscope and photographed with a Quantix camera (Photometrics).

Cross-linking Experiments—IL-6 and dexamethasone-treated UAC cells were washed twice with PBS. DSS first dissolved in Me2SO was applied onto the cells at a final concentration of 2 μM in PBS. After incubation at room temperature for 30 min, cells were washed and directly dissolved in SDS-PAGE sample buffer containing 0.1 units/ml Benzon Nuclease (Merck) to digest genomic DNA. The resulting extract was analyzed by immunoblotting using anti-stomatin antibody.

RESULTS

Cross-linking of Stomatin in UAC Cells—The first evidence of an oligomeric structure of stomatin in UAC cells came from cross-linking experiments. Cells were treated with IL-6 and dexamethasone and total proteins were subjected to chemical cross-linking using DSS. Western blots of whole cell extracts were performed using the stomatin-specific antibody GAR50 (Fig. 1). In addition to the 31-kDa stomatin monomer, we observed bands that the mobility of which suggested the presence of cross-linked dimers, trimers, and hexamers as well as higher order oligomers. Other minor bands might correspond to a covalent linking of stomatin to different cytoplasmic or membrane proteins or result from intramolecular bonds. Various other chemical or light-activated cross-linkers were used and gave identical results (not shown).

Sucrose Gradient Centrifugation Analysis of Stomatin Oligomers—Because of the efficiency inherent in the cross-linking reaction, we thought that higher order oligomers might be the major stomatin species found in the cell. Triton X-100 readily solubilizes the majority of UAC cell stomatin, although a significant proportion of the protein (20 to 30%) remains in the pellet, which might represent the part of stomatin linked to the cytoskeleton (Fig. 2). Oligomers would be present in the soluble part of these extracts if interaction between monomers would be resistant to disruption by Triton X-100. Purified membranes of induced UAC cells were solubilized in 1% Triton X-100 and subjected to 5–25% sucrose density gradient centrifugation. Proteins of the different fractions were separated by SDS-PAGE and analyzed by immunoblotting with the anti-stomatin antibody or by silver staining (Fig. 3A). The results show that the major stomatin species has a high molecular weight (fractions 10, 11, and 12) and migrates away from the bulk of other proteins in the extract. Comparison with molec-
ular weight standards separated under the same conditions indicates that stomatin oligomers have an apparent mass of 250–300 kDa. As stomatin oligomers are likely to exist in a micellar form, fractions were also probed with antibodies against two other membrane proteins (Fig. 3B). Calnexin and epidermal surface antigen (ESA) separate at an apparent molecular mass slightly lower than that expected from the globular markers, demonstrating that the size of the oligomers is not overestimated.

The stomatin immunoblot shows that very little stomatin is present between fraction 10 and fractions 2 and 3, where a small amount of monomer can be detected. This indicates that the oligomers are very stable under these conditions. They are also stable in 0.5M KCl (not shown). Analysis of total protein in the fractions does not show any other protein co-migrating with stomatin in the gradient. In conclusion, if no other protein of the same molecular weight is masked by stomatin, this implies the presence of homo-oligomers. To rule out the presence of unspecific aggregates of stomatin, samples were routinely centrifuged at 20,000 × g after lysis in Triton X-100. Also, ultracentrifugation at 100,000 × g for 20 min resulted in only partial loss of the complexes (not shown).

**Analysis of Stomatin Oligomers by Co-immunoprecipitation**—We further investigated the homo-oligomeric interaction of stomatin by co-immunoprecipitation of the endogenous protein with a C-terminal tagged stomatin expressed in UAC cells. Cells were transfected with a eukaryotic expression vector containing the whole coding region of stomatin fused at the C terminus to five copies of the myc epitope (STmyc5) to increase affinity for the specific antibody 9E10. Individual clones were isolated and screened for the presence of the expected 38–40-kDa product, using immunoblotting with anti-stomatin and anti-myc antibodies (Fig. 4A). They were also checked by immunofluorescence for the correct cellular localization of ST-my5. Fig. 4B shows that the C-terminal tagged stomatin displays a staining pattern identical to endogenous stomatin in UAC cells. Fluorescence is concentrated in fine plasma membrane folds and extensions and also in the intra-cytoplasmic pool within the Golgi region. Interestingly, stomatin fused to the myc-epitope at the N terminus did not reach the plasma membrane but was blocked in the Golgi apparatus and/or the endoplasmic reticulum (not shown). One clone expressing ST-my5 was metabolically labeled, after having been induced by...
IL-6 and dexamethasone or left untreated, and Triton X-100 extracts of the cells were immunoprecipitated with anti-myc monoclonal antibody. Fig. 5, left panel, shows that two proteins were specifically immunoprecipitated. One has the expected size of STmyc5 (38–40 kDa) and the other has the molecular weight of stomatin. This latter appears more abundant in in extracts of cells expressing these two truncated stomatin forms were immunoprecipitated with anti-myc antibody, no co-immunoprecipitation of endogenous stomatin was observed, in contrast to full-length STmyc3 and STmyc5. However, the use of the more sensitive chemiluminescent detection system in similar experiments allowed us to see marginal amounts of stomatin co-immunoprecipitated with ST198myc3 but to a level far below what is observed for STmyc3 and STmyc5 (not shown).

This experiment confirms that oligomers of stomatin do not result from artifactual aggregation of the protein during lysis, through the hydrophobic domain or the membrane proximal part of the molecule, because the truncated polypeptides (which contain these regions) do not incorporate into the complexes. It also suggests that the C terminus of stomatin is involved in the homomeric interaction of this protein.
endogenous stomatin with a recombinant form of this protein. Both approaches led to the conclusion that stomatin oligomers detected in Triton X-100 extracts of UAC cells contain at least 9 monomers and might comprise up to 12 monomers per complex. Oligomers of stomatin are not a peculiarity of UAC cells because we also observed complexes of the same size in Madin-Darby canine kidney (MDCK) cells by sucrose gradient centrifugation.2

Throughout this study we have examined stomatin from total cellular membranes or whole cellular extracts. It is likely that stomatin exists in an oligomeric form in both the plasma membrane and the juxta-nuclear region detected by immunofluorescence (14) because almost the totality of the protein is in the high molecular weight form in the sucrose gradient. This would be consistent with a model in which oligomers of stomatin are built very early in the biosynthetic pathway and processed to the plasma membrane via Golgi-derived vesicles. In addition, a specific role of stomatin in these vesicles is not excluded.

Various proteins playing a structural role in the plasma membrane have been shown to oligomerize. Ezrin, in particular, is a peripheral membrane protein believed to function as a membrane cytoskeletal linker. It is abundant underneath the dorsal plasma membrane in all actin-containing structures and is partially detergent-insoluble (17, 18). Ezrin oligomer formation has been proposed to drive the assembly of cell surface structures, a process regulated by phosphorylation in certain types of cells (15). While some aspects of stomatin staining (14) are reminiscent of ezrin, there is no indication of a post-translational control of stomatin. Moreover, stomatin is an integral membrane protein, which would fit to a more constitutive role. Another integral membrane protein, caveolin, has drawn our attention as it has topological features similar to stomatin, with a ‘hairpin-like’ membrane domain and cytoplasmic N and C termini (19) and, like stomatin, contains three palmitoylated cysteine residues (20). Preliminary data indicate that stomatin is also palmitoylated on cysteine residues in UAC cells as has been described for red cells (1). Caveolin is thought to have a scaffolding function in caveolae membranes by forming homo-oligomers (21, 22). These oligomers interact side-by-side to form more complex particles (23). It is tempting to speculate that stomatin oligomers could also serve as a basis for building up a widespread structure. Such a possibility is also suggested by staining of stomatin and actin by double immunofluorescence in cytochalasin D-treated cells. In these studies, stomatin staining appears as a reticular structure centered around actin patches, suggestive of a meshwork-like organization (14). Triton X-100 action could be sufficient to disaggregate this meshwork, whereas the oligomeric complex would remain intact in this detergent.

In which part of stomatin is the homo-oligomerization domain localized? In caveolin, such a domain has been mapped to the membrane proximal region of the more important cytoplasmic N terminus (21). It is reasonable to think that part of or the whole cytoplasmic C terminus of stomatin could act as a domain responsible for interaction of the protein with itself, as this is part of the region conserved in the two homologous proteins described in C. elegans. MEC-2, a membrane component of mechano-sensory neurons, is thought to act as a linker between the cytoskeleton and an ion channel MEC-4/MEC-10 (24). In addition to the stomatin-like region, MEC-2 possesses two extended regions: one at the N terminus, possibly linked to microtubules (11), and the other at the C terminus, possibly regulating the ion channel. Genetic studies indicate a probable

**DISCUSSION**

The data presented in this study demonstrate the homo-oligomeric organization of the integral membrane protein stomatin in an epithelial cell line. This was suggested by chemical cross-linking and confirmed and characterized by density gradient centrifugation analysis and co-immunoprecipitation of

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2 L. Snyers, unpublished results.
interaction of MEC-2 with itself (24). A likely candidate region for this homomeric interaction could be the stomatin-like part of the protein. UNC-24 homology extends only from amino acid 25 to 198 of stomatin (12). The remaining C-terminal portion of UNC-24 resembles a nonspecific lipid transfer protein, which suggests a biochemical function. Interestingly, truncated stomatin containing residues 1–198 had a reduced plasma membrane localization when stably expressed in UAC cells. When further reduced to amino acids 1–174, the protein access to the plasma membrane was blocked almost completely and staining was confined to intracytoplasmic compartments. Furthermore, these truncated forms of stomatin do not associate into oligomeric complexes when stably expressed in cells. Because protein-protein interaction and correct assembly into complexes are thought to be essential for efficient processing of membrane and secreted proteins (25), these data represent an indication that the C terminus is involved in stomatin interaction with itself.

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