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Nanoscale 3D contour map of protein assembly within the astrocyte porosome complex

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Abstract

The astrocyte porosome complex, the secretory machinery at the plasma membrane of astrocytes, is a 10–15 nm cup-shaped lipoprotein structure possessing a central plug. Since the porosome is a membrane-associated, multi-protein complex, it has precluded the generation of 3D crystals for X-ray diffraction studies, nor structural analysis at the atomic level using the solution NMR. These limitations were partially overcome in the current studies, furthering our understanding of the porosome structure in astrocytes. Using atomic force microscopy, electron microscopy, and electron density and 3D contour mapping, finally provides at the nanoscale, the structure and assembly of proteins within the astrocyte porosome complex. Results from this study demonstrate a set of protein units lining the porosome cup, each connected via spoke-like elements to a central plug region within the structure. In contrast to the neuronal porosome, which possess eight globular proteins at the outer rim of the complex, the porosome complex appear to possess 12 such globular structures. Nature has designed the porosome as the universal secretory machinery, but has fine-tuned its use to suite secretion from different cell types. The isolation of intact astrocyte porosomes for near-atomic resolution using cryo-electron diffraction measurements, is finally possible.
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Keywords: Astrocyte porosome complex; Nanoscale protein assembly; 3D contour maps

1. Introduction

In the past 12 years, permanent supramolecular structures called porosomes or fusion pores have been identified at the cell plasma membrane in neurons, astrocytes, exocrine, endocrine, and neuroendocrine cells, where membrane-bound secretory vesicles transiently dock and fuse to expel their contents to the outside during cell secretion (Cho et al., 2002a,b, 2004, 2007; Lee et al., 2008; Jena et al., 2003; Jeremic et al., 2003; Schneider et al., 1997). These findings demonstrate the porosome to be the universal secretory machinery in cells (Jena, 2005, 2007). The overall morphology, composition, and reconstitution of this secretory machinery in exocrine pancreas and in neurons are well documented (Cho et al., 2002a, 2004, 2007; Jena et al., 2003; Jeremic et al., 2003; Schneider et al., 1997), however, much remains to be understood regarding the fine structure and molecular details of the assembly of proteins within the porosome complex, especially in astrocytes. Porosomes are supramolecular lipoprotein structures, composed of several proteins (Cho et al., 2004, 2007; Jena et al., 2003; Jeremic et al., 2003) such as SNAP-23/25, syntaxin, synaptotagmin, the ATPase *NSF*, cytoskeletal proteins (actin, α-fodrin, and vimentin), calcium channels β3 and α1c, chloride ion channels ClC2 and ClC3, and in some cases their isoforms. Recent studies further demonstrate cholesterol to be an integral component of the porosome complex, required for retaining its integrity and intramolecular interactions (Cho et al., 2007). Besides the fact that t-SNAREs and calcium channels are

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present at the base of porosomes (Jena et al., 2003), and the likely association of actin at the opening of the structure to the cell exterior (Jena et al., 2003; Schneider et al., 1997), localization of various proteins within the complex remains to be determined. Ideally, atomic coordinates of the complex using either X-ray crystallography or solution nuclear magnetic resonance spectroscopy (NMR) would provide structural details at the atomic level. However, the size and complexity of the membrane-associated porosome, has precluded determination of its atomic structure, which ultimately would provide a molecular understanding of its function. Solution NMR has not been possible primarily due to the large molecular size of the porosome complex, which is beyond the resolution limits of current NMR's. Similarly, X-ray crystallography of the porosome complex has not been realized, due in part to the solubility problems of this membrane-associated structure. In the present study however, these limitations have been partially overcome in furthering our understanding of the fine structure and nano-arrangement of proteins within the native astrocyte porosome complex, using high resolution atomic force microscopy (AFM) and electron microscopy (EM).

2. Materials and methods

2.1. Astrocyte culture

Neuron-glia cultures from neonatal rat cerebral cortex were established as described. Cortex from three newborn pups (1–3 days old) were dissected, and the tissue was enzymatically treated in 2 ml of papain solution (1.54 mg/ml of EBSS; 40 min at 37 °C), followed by a 5 min wash using EBSS-trypsin inhibitor (stock of soybean trypsin inhibitor 7000 U/mg in 3 ml EBSS, final concentration 300 ml of the stock/5 ml EBSS). The tissue in DMEM containing high glucose, 10% FBS, and penicillin/streptomycin (1 ml/100 ml DMEM) was then mechanically dissociated using pipettes. Cells were plated in the same media (DMEM high glucose + 10% FBS, +penicillin/streptomycin) in culture flasks (25 mm flasks) and maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere. The cells were maintained by changing the medium every 2–3 days. After mixed cultures reach confluence in 9–12 days, the flasks were ''pre-shaken'' (260 rpm) for 90 min to remove microglia and the dividing type I astroglia. The cultures were agitated overnight (12–18 h) at 260 rpm at 37 °C. Cultures enriched in type I astroglia were obtained by trypsinizing (0.25%) the attached cells for 3 min. Trypsin was inactivated by adding 3 ml DMEM (the same media as above) supplemented with 10% heat-inactivated FBS (serum contains protease inhibitors). Astrocytes plated for 1–3 days on poly-L-lysine (100 mg/ml; MW 100,000) coated glass coverslips are used for AFM imaging, and isolated for use in EM and biochemistry.

2.2. Atomic force microscopy

Astrocytes cultured on glass coverslips, and isolated astrocyte porosomes reconstituted in lipid membrane, were immersed in PBS, pH 7.5, and imaged using the AFM (BioScope III, Digital Instruments, Santa Barbara, CA). AFM imaging was performed using the ''tapping'' mode, using silicon nitride tips with a spring constant of 0.06 N/m, and an imaging force of <200 pN. Images were obtained at line frequencies of 1.98 Hz, with 512 lines per image, and constant image gains. Tip velocity 11.4 mm/s; tip spring constant 0.06 N/m; sample/line 512; integral gain 2.0; proportional gain 1.0; amplitude set point 0.12–0.28 V; drive frequency 7.76–8.12 KHz; and drive amplitude 150–400 mV, were used. Topographical dimensions of both native and lipid-reconstituted porosomes were analyzed using the NanoScope IIIa version 4.43r8 software, supplied by Digital Instruments.

2.3. Transmission electron microscopy

Isolated rat astrocytes were fixed in 2.5% buffered paraformaldehyde for 30 min, and the pellets were embedded in Unicryl resin and were sectioned at 40–70 nm. Thin sections were transferred to coated specimen TEM grids, dried in the presence of uranyl acetate and methylcellulose, and examined using a transmission electron microscope. For negative staining electron microscopy, purified protein suspensions in PBS, were adsorbed onto hydrophilic carbon support films that were mounted onto formvar-coated, metal specimen grids (EMS, Ft. Washington, PA). The adsorbed protein was washed in double-distilled water and negatively stained using 1% aqueous uranyl acetate. After the grids were dried in the presence of the uranyl acetate solution, they were examined by transmission electron microscopy. To prevent bleaching by the electron beam, micrographs were obtained on portions of the grid not previously examined.

2.4. Astrocyte porosome isolation and reconstitution

The porosome complex from astrocyte was immunoisolated using SNAP-23 specific antibody conjugated to protein A-sepharose beads. Fifty micrograms of astrocyte homogenate, prepared by solubilizing astrocytes in Triton/Lubrol solubilization buffer (0.5% Lubrol; 1 mM benzamidine; 5 mM Mg-ATP; 5 mM EDTA; 0.5% Triton X-100, in PBS) containing a protease inhibitor cocktail (Sigma, St. Louis, MO), was used. SNAP-23 antibody conjugated to the protein A-sepharose was incubated with the solubilized homogenate for 1 h at room temperature followed by washing with wash buffer (500 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.5). The immunoprecipitated sample attached to the immuno-sepharose beads was eluted using low pH buffer to obtain the porosome complex. To prepare lipid membrane on mica for AFM studies, freshly cleaved mica disks were placed in a fluid chamber. Two hundred microliters of the bilayer bath solution containing 140 mM NaCl, 10 mM HEPES, and 1 mM $CaCl_2$, was placed at the center of the cleaved mica disk. Ten microliter of the brain lipid vesicles were added to the above bath solution. The mixture was then allowed to incubate for 60 min at room temperature, before washing (×10), using 100 μl bath solution/wash. The lipid membrane on mica was imaged by the AFM before and after the addition of immunoisolated porosomes

from astrocytes. All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Preparation of lipid vesicles and their reconstitution with proteins were performed using previously published procedures (Cho et al., 2007). Briefly, a 10 mM lipid stock solution was prepared by mixing lipid solution in chloroform-DOPC (1,2-dioleoyl phosphatidylcholine): DOPS (1,2-dioleoyl phosphatidylserine) in 70:30 mol/mol ratios, or DOPC:DOPS:cholesterol in 56:24:20 mol/mol/mol ratios in glass test tubes. The lipid mixture was dried under gentle stream of nitrogen and resuspended in decane. The lipids were then suspended in buffer containing 10 mM Hepes-NaOH [pH = 7.5] and 140 mM NaCl by vortexing for 5 min at room temperature. Large unilamellar vesicles were formed following sonication for 2 min. Liposomes ranging in size from 0.2 to 2 μm in diameter were obtained, as assessed by zeta sizer and AFM (data not shown). As previously demonstrated in published studies on immunoisolated neuronal porosomes (Cho et al., 2004), these immunoisolated astrocyte porosomes reconstitute into artificial lipid (PC:PS or PE:PC) membrane.

2.5. Electron microscopy of the isolated astrocyte porosome complex

Aliquots (2.5 μl) of immunoisolated porosomes from astrocyte culture were adhered to carbon-coated 400-mesh copper grids (Cu-400CN, Pacific Grid-Tech, San Francisco, CA) previously rendered hydrophilic by glow discharge for 10 s. The grids were washed with drops of deionized water and then exposed to drops of 2% (w/v) uranyl nitrate as previously reported (Pettersen et al., 2004). Images at 80,000 × magnification were recorded at low defocus on 4K × 4K Gatan UltraScan CCD under low electron dose conditions using a Tacnai 20 electron microscope (Philips Electron Optics/FEI, Eindhoven, The Netherlands) operating at 200 kV. Each pixel of the micrographs corresponds to 1.4 Å at the level of the specimen. Particles in micrographs were selected and windowed using EMAN software (Ludtke et al., 1999). Each window size is 160 pixels, corresponding to 225 Å in specimen.

2.5.1. Contour mapping

To display the protein structure in the astrocyte porosome complex at greater detail, contour maps of proteins was created by Spider software (Frank et al., 1996). All particles are first normalized using a mean density of 0 and standard deviation of 10, then, contoured by the lowest ring at 0.5. Outside the protein complex, the background is flattened and smoothed. The protein boundary is defined by the darkest shadow around the particles, which results from the contrast transfer function (CTF) of electrons.

2.5.2. Porosome topography from electron density maps

Heavy metal staining of the astrocyte porosome, enables an estimation of the relative size and arrangement of proteins in electron micrographs of the complex. Thus, density of negative stained proteins in electron micrographs, reflect the dimension, concentration, and arrangement of protein at the various locations. Higher density in the micrograph corresponds to a greater amount of protein at that location. Using Chimera software developed at UCSF (Pettersen et al., 2004; Goddard et al., 2005), protein density distributions were determined and revealed in 3D. Here, the colors from red, yellow, green to blue, correspond to the protein image density from lowest to the highest. The highest peak in each image is presented at 27 Å.

3. Results and discussion

Neuronal communication depends on both the fusion of 40–50 nm in diameter membrane-bound synaptic vesicles containing neurotransmitters at the presynaptic membrane, as well as similar size vesicles fusing at the plasma membrane of astrocytes. In earlier studies (Cho et al., 2004, 2007; Figs. 1 and 2)–17 nm in diameter cup-shaped neuronal porosomes at the presynaptic membrane have been demonstrated. The porosomes are permanent structures at the presynaptic membrane, where synaptic vesicles transiently dock and fuse to release neurotransmitters. The morphology, isolation, composition, and functional reconstitution of porosomes present at the nerve terminal, have also been described (Cho et al., 2004, 2007). Similarly, 12–15 nm in diameter porosomes have been identified at the astrocyte plasma membrane, where 40–50 nm in diameter membrane-bound secretory vesicles dock and fuse to release neurotransmitters (Lee et al., 2008). However, in contrast to the neuronal porosome, which possess eight globular proteins at the outer rim of the complex, the astrocyte porosome complex, appear to possess 12 such globular structures. Nature has designed the porosome as the universal secretory machinery, but has fine-tuned its use to suite secretion from different cell types. Hence, the size of porosomes in different cells may represent a form of such fine-tuning. In the current study, AFM, EM, and electron density measurements followed by contour mapping, and 3D topography, provide for the first time, the arrangement of proteins at nm resolution within the astrocyte porosome complex. Results from this study demonstrate that proteins at the central plug of the porosome, interact with proteins at the periphery of the complex, conforming to its 12-fold symmetry. Furthermore, at the center of the porosome complex representing the porosome base, where secretory vesicles dock and transiently fuse, proteins, possibly comprised of t-SNAREs, were found assembled in a ring conformation.

Electron micrographs of astrocyte membrane, demonstrate presence of approximately 12 nm cup-shaped porosomes (Lee et al., 2008; Figs. 1A,B), where 30–40 nm secretory vesicles (SV) dock and fuse to enable the release of neurotransmitters. Studies (Lee et al., 2008; Fig. 1) using AFM on live astrocytes (Fig. 1C) and isolated astrocyte porosomes reconstituted into PC:PS (Fig. 1D), confirm the presence of 12–15 nm in diameter cup-shaped porosomes in astrocytes. These studies show that porosomes are permanent structures at the astrocyte membrane, where synaptic vesicles transiently dock and fuse to release neurotransmitters. Although, the general morphology, isolation, composition, and functional reconstitution of neuronal and astrocyte porosomes, have previously been described (Cho et al., 2004, 2007; Lee et al., 2008), high-resolution images using AFM on isolated astrocytes in buffered solution, and of reconstituted

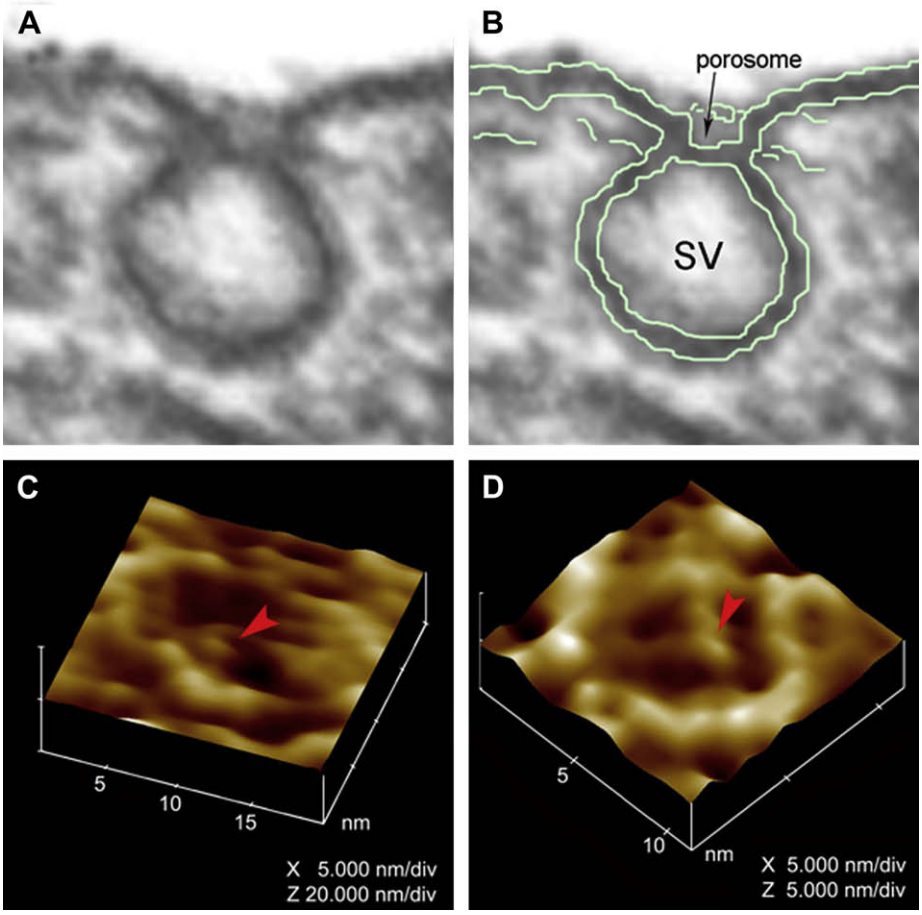

Fig. 1. Electron and atomic force micrographs of the astrocyte porosome complex. (A, B) Electron micrographs of the cup-shaped astrocyte porosome, measuring ~12 nm, present at the cell plasma membrane, with a 40 nm secretory vesicle (SV) docked at its base. (C) Atomic force micrograph of an astrocyte porosome at the plasma membrane of an astrocyte. (D) Atomic force micrograph of an immunoisolated astrocyte porosome, reconstituted in lipid membrane. Note the central plug (red arrowhead) of the porosome complex and the presence of approximately 12 globular units arranged at the lip of the complex.

astrocyte porosomes in lipid membrane are shown in Figs. 1C,D, to orient the reader. Preparation of lipid membrane and their reconstitution with immunoisolated astrocyte porosomes were performed using previously published procedures (Cho et al., 2004, 2007; Jeremic et al., 2003; Lee et al., 2008). Isolated astrocytes, and immunoisolated astrocyte porosomes reconstituted in the lipid membrane in PBS, pH 7.5, were imaged using AFM in the tapping mode in fluid, using silicon nitride tips with a spring constant of 0.06 N/m, and an imaging force of <200 pN. Close examination of porosomes in the atomic force micrographs reveal an array of 12 globular units arranged at the lip of the porosome opening to the outside. The globular elements appear to be tethered to a central plug-like structure (red arrowhead) in the atomic force micrographs (Figs. 1C,D).

High resolution negative staining EM (Fig. 2) of the immunoisolated native astrocyte porosome complexes, followed by contour mapping (Fig. 3) and 3D topology measurements (Fig. 4), provide for the first time, the nano-arrangement of proteins within the structure. Using SNAP-23-specific antibody, immunoisolation of astrocyte porosomes from Triton/Lubrol-solubilized astrocytes is demonstrated, as it readily reconstitutes into artificial PC:PS membrane and exhibits in AFM micrographs, all of the characteristic features exhibited in the native structure (Figs. 1C,D). Information from Fig. 1, has helped to generate a model of the porosome complex, as a cup-shaped structure having a centrally located plug, and an array of 12 globular units arranged at the lip of the complex.

Negative staining EM was performed using low electron dose, in a Tacnai 20 electron microscope operating at 200 kV (Ludtke et al., 1999). Results from this study demonstrate that proteins at the central plug of the porosome complex interact with proteins at the periphery of the structure. Similar to AFM micrographs, approximately 10–12 interconnected protein densities are observed at the lip or periphery of the porosome complex in the electron micrographs (Fig. 2). The 10–12 interconnected protein densities are also connected to the central plug, via spoke-like structures (Figs. 2–4). Electron density and contour maps and resultant 3D topology profiles of the astrocyte porosome complex, provide further details of the circular arrangement of proteins, and their connection to the central plug via distinct spokes (Figs. 3 and 4). The contour map of proteins within the astrocyte porosome complex was obtained using a published procedure (Frank et al., 1996; Goddard et al., 2005; Pettersen et al., 2004). Furthermore, the center of the porosome complex representing the porosome base where vesicles dock and fuse, show ring-like arrangement

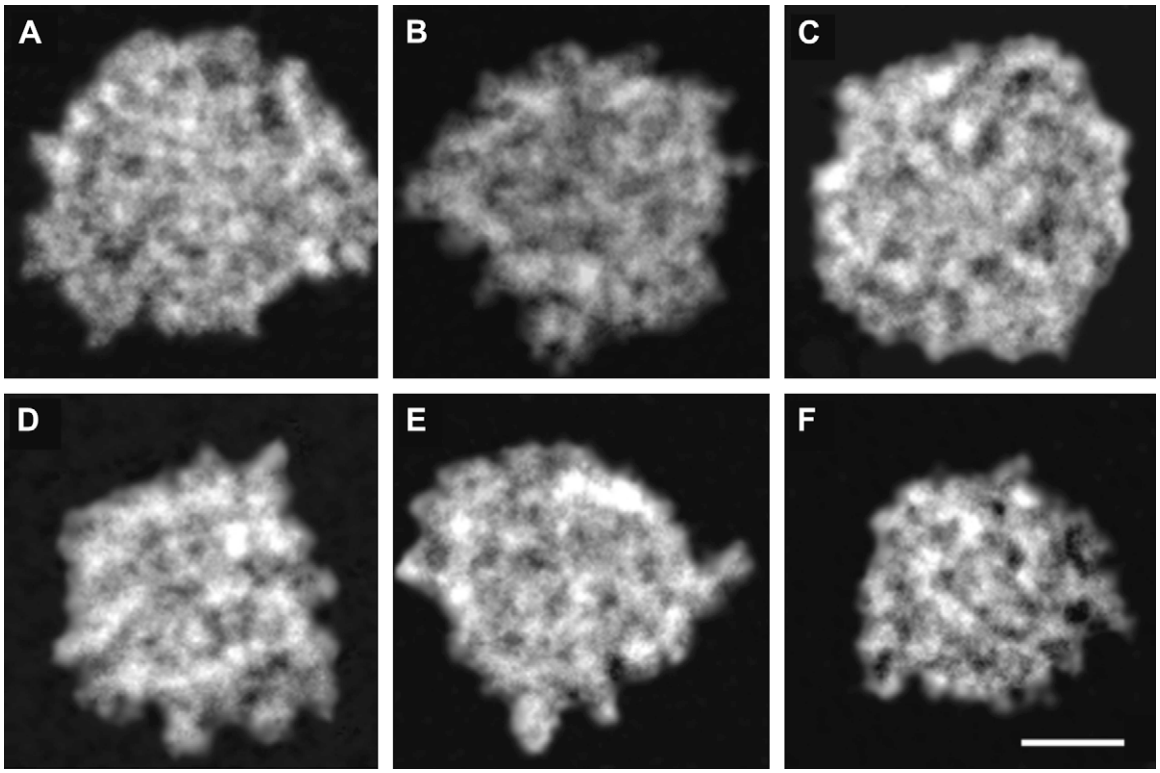

Fig. 2. Negatively stained electron micrographs of isolated astrocyte porosome protein complexes (A–F). Note the 10–15 nm complexes exhibiting a circular profile and having a central plug. Approximately 10–12 interconnected protein densities are observed at the rim of the structure, which are connected to a central element via spoke-like structures. At the center of the structure, which correspond to the porosome base, there are proteins arranged in rings. Bar = 5 nm.

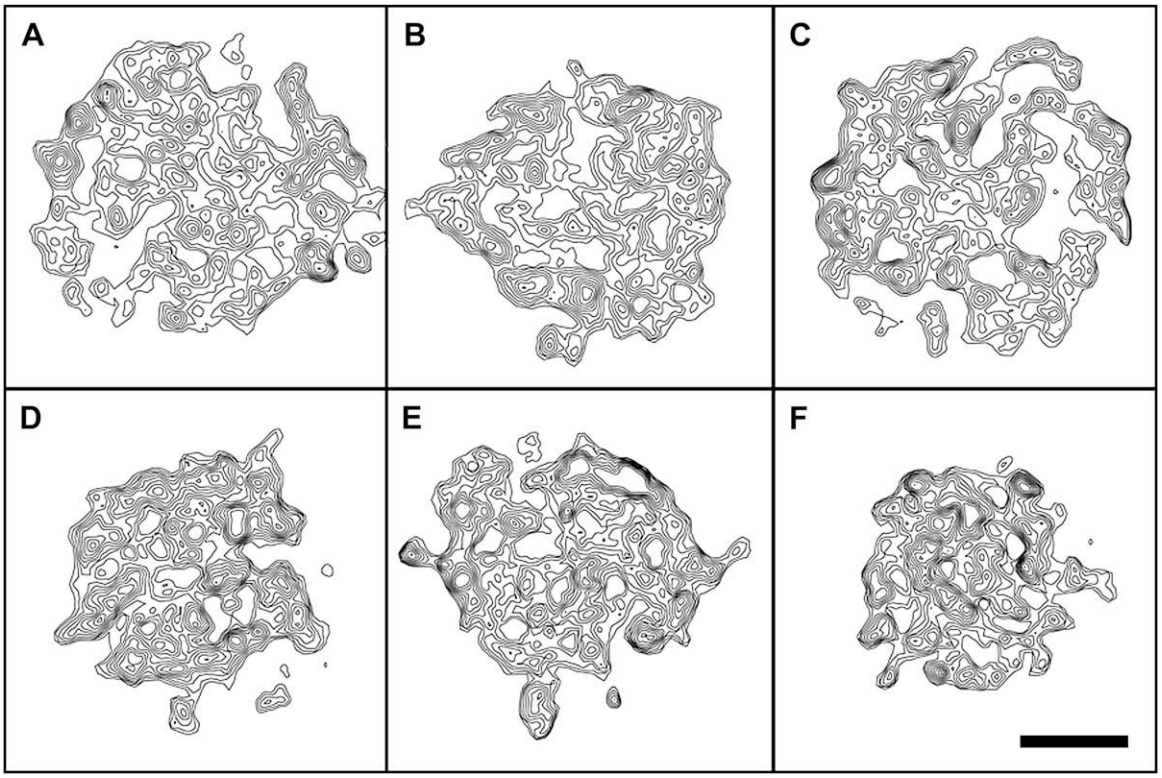

Fig. 3. Electron density maps of negatively stained electron micrographs of isolated astrocyte porosome protein complexes (A–F). Note as in Fig. 2, the 10–15 nm complexes exhibiting a circular profile and having a central plug, with 10–12 interconnected protein densities at the rim of the complex, and connect to a central element. Bar = 5 nm.

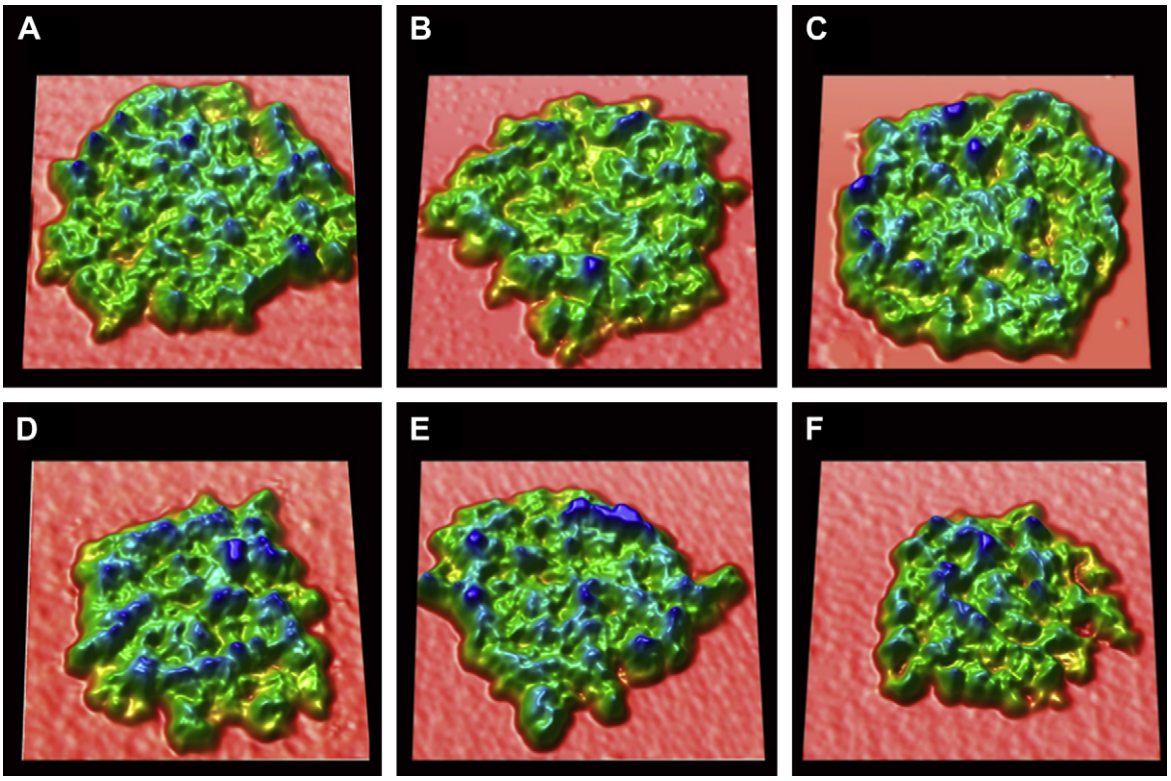

Fig. 4. 3D topography of porosomes obtained from their corresponding electron density maps (A–F). Note the 10–15 nm astrocyte porosome complexes, clearly exhibiting a circular profile having a central plug, with distinct radiating spokes from the central element connecting each of the approximately 10–12 interconnected protein densities at the periphery of the structures. The colors from yellow, through green to blue, correspond to the protein image density from lowest to the highest. The highest peak in each image represents 27 Å.

of proteins, possibly composed of t-SNAREs. Results from these studies provide for the first time the arrangement of proteins at the nm scale within the astrocyte porosome complex. The next level of understanding of this supramolecular structure requires isolation of the intact complex in bulk, for cryo-electron diffraction studies, which are currently under way.

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Appendix A. Supplemental material

Supplementary information for this manuscript can be downloaded at doi: 10.1016/j.cellbi.2008.11.008.

References

Cho S-J, Quinn AS, Stromer MH, Dash S, Cho J, Taatjes DJ, et al. Structure and dynamics of the fusion pore in live cells. Cell Biol Int 2002a;26:35–42.

Cho S-J, Jeftinija K, Glavaski A, Jeftinija S, Jena BP, Anderson LL. Structure and dynamics of the fusion pores in live GH-secreting cells revealed using atomic force microscopy. Endocrinology 2002b;143:1144–8.

Cho WJ, Jeremic A, Rognlien KT, Zhvania MG, Lazrishvili I, Tamar B, et al. Structure, isolation, composition and reconstitution of the neuronal fusion pore. Cell Biol Int 2004;28:699–708.

Cho WJ, Jeremic A, Jin H, Ren G, Jena BP. Neuronal fusion pore assembly requires membrane cholesterol. Cell Biol Int 2007;31:1301–8.

Frank J, Radermacher M, Penczek P, Zhu J, Li Y, Lasjadj M, et al. SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. J Struct Biol 1996;116:190–9.

Goddard TD, Huang CC, Ferrin TE. Software extensions to UCSF Chimera for interactive visualization of large molecular assemblies. Structure 2005;13:473–82.

Jena BP. Molecular machinery and mechanism of cell secretion. Exp Biol Med 2005;230:307–19.

Jena BP. Secretion machinery at the cell plasma membrane. Curr Opin Struct Biol 2007;17:437–43.

Jena BP, Cho SJ, Jeremic A, Stromer MH, Abu-Hamdah R. Structure and composition of the fusion pore. Biophys J 2003;84:1–7.

Jeremic A, Kelly M, Cho SJ, Stromer MH, Jena BP. Reconstituted fusion pore. Biophys J 2003;85:2035–43.

Lee J-S, Cho W-J, Jeftinija K, Jeftinija S, Jena BP. Porosome in astrocytes. J Cell Mol Med 2008 (published on-line ahead of print, "Postprint"; 10.1111/j.1582–4934.2008.00334.x).

Ludtke SJ, Baldwin PR, Chiu WEMAN. semiautomated software for high-resolution single-particle reconstructions. J Struct Biol 1999;128:82–97.

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera – a visualization system for exploratory research and analysis. J Comput Chem 2004;25:1605–12.

Schneider SW, Sritharan KC, Geibel JP, Oberleithner H, Jena BP. Surface dynamics in living acinar cells imaged by atomic force microscopy: identification of plasma membrane structures involved in exocytosis. Proc Natl Acad Sci USA 1997;94:316–21.