I. Introduction

A. General Introduction

The cytoskeleton and the plasma membrane are likely to carry out many functions *inter*dependently. These structures work collaboratively, and together are largely responsible for determining the movements, shapes, and shape changes of the cell. The plasma membrane dynamics, such as endocytosis, exocytosis, membrane extension, and membrane resealing after cell wounding, could be regulated by the tension exerted on the membrane by the cytoskeleton (Sheetz and Dai, 1996; Sheetz, 2001). Signal transduction by glycosylphosphatidylinositol (GPI)-anchored proteins is mediated by the binding of stimulation-induced

clusters of GPI-anchored proteins to actin filaments (Suzuki *et al.*, 2007a,b). Tension exerted on integrin clusters in the plasma membrane by the force generated by actomyosins and by binding to the extracellular matrix strengthens the linkages between the integrin clusters and the cytoskeleton (Choquet *et al.*, 1997), possibly by way of the force-induced conformational changes of the Src family kinase substrate p130Cas at the integrin clusters (Sawada *et al.*, 2006).

The cytoskeleton has been a target of comprehensive studies by electron microscopy (EM). The "cortical" cytoskeleton, the cytoskeleton located near the plasma membrane, has also been studied quite extensively (for example, see Hartwig *et al.*, 1989; Svitkina *et al.*, 2003; Yin and Hartwig, 1988), but since many of these studies employed detergents, the structures at the exact interface between the cytoskeleton and the plasma membrane were not clear. One of the best ways to observe the membrane surface is to prepare the specimens using "freeze etching", an EM technique for sample preparation. In this technique, the biological specimen is rapidly frozen, faster than the growth of the ice microcrystals, and then, after the removal of excess ice, the ice on the membrane surface is sublimed (etching) so that the membrane surface is exposed outside the ice. The surface morphology is replicated by coating this surface with platinum, and the platinum coat is observed by EM.

This technique was greatly improved and modified for its application to investigations of the plasma membrane and the cytoskeleton near the plasma membrane by Heuser and his colleagues, as well as others, in the late 1970s and early 1980s (Chandler and Heuser, 1979; Heuser and Salpeter, 1979; Heuser *et al.*, 1979; Landis and Reese, 1981). Among them, two papers published by Heuser, Hirokawa, and their colleagues stand out in the history of the studies of both the plasma membrane and the cytoskeleton (Hirokawa and Heuser, 1981; Hirokawa *et al.*, 1982). By using intestinal epithelial tissue, and by devising various ways of identifying a variety of intracellular structures, they clearly and impressively showed images of the cytoskeleton closely associated with the microvilli and the apical plasma membrane. Actin bundles in microvilli were shown vividly. The barbed ends (fast-growing ends) were attached to the cytoplasmic surface of the tip of the villus, while the pointed ends (slowly-growing ends) at the root of the villus were linked to a structure called the "terminal web", which was further linked to the bulk cytoskeleton consisting of actin, myosin, and other intermediate filament structures. The structures linking the actin bundles as well as those linking the actin filaments and the plasma membrane were clearly shown in their electron micrographs.

Here, we list only several of the membrane-related, representative investigations using the freeze-etching technique during the last 20 years: Fujita *et al.* (2007), Hartwig *et al.* (1989), Hanson *et al.* (1997, 2008), Heuser and Anderson (1989), Heuser (2005), Italiano *et al.* (1999), Katayama *et al.* (1996), Kanaseki *et al.* (1997, 1998), Kajimura *et al.* (2000), Ohno and Takasu (1989), and Rothberg *et al.* (1992), and Nakata and Hirokawa (1992).

We have recently adopted this technique to observe the undercoat structures of the plasma membrane. In addition, we showed that the platinum replica of the rapidly-frozen, deep-etched, plasma membrane is suitable for the three-dimensional (3D) reconstruction of the cytoplasmic surface of the plasma membrane with its membrane-associated part of the cytoskeleton, using electron tomography. Hence, the objectives of this review are:

1. To show the high potential of freeze-etch EM for studying the interface between the plasma membrane and the cytoskeleton;
2. To briefly review the structure of the plasma-membrane-associated part of the cytoskeleton;
3. To briefly summarize the protocols for preparing the plasma membrane specimen with its undercoat structures, and for the rapid-freezing, deep-etching, and platinum replication of the plasma membrane specimen, with several recent improvements; and
4. To present the 3D reconstruction data and the meshwork of the actin filaments associated with the cytoplasmic surface of the plasma membrane (within 0.85 nm from the surface), and to compare the mesh size with the size of the compartments for the diffusion of plasma-membrane molecules, detected by single-molecule tracking of phospholipids and proteins.

B. Introduction to Terminology: Membrane-Associated Part of the Cytoskeleton (Membrane Skeleton[MSK])

Distinguishing the plasma membrane-associated part of the cytoskeleton from the bulk skeleton is difficult because the membrane-associated part of the cytoskeleton is continuous with the bulk cytoskeleton. In this review, *we define the membrane-associated part of the cytoskeleton as the part of the cytoskeleton that is located within several tens of nanometers from the plasma membrane, and we call this structure the "membrane skeleton (MSK)" for convenience.* The vague distance stated here is due to the large variations in the association of the cytoskeleton with the plasma membrane among different cell types.

The reasons why the term MSK, separate from the bulk cytoskeleton, is used are as follows:

1. Since the MSK-part of the cytoskeleton often functions in close cooperation with the plasma membrane, it is often more logical and easy to consider the MSK as a part of the plasma membrane, rather than as a part of the cytoskeleton, in terms of cellular functions. As mentioned in the first paragraph of this review, the proteins in the plasma membrane constantly interact with the cytoskeleton adjacent to the plasma membrane.

2. For the interaction with the plasma membrane, the MSK is likely to involve protein species and to have structures different from those of the bulk cytoskeleton. The proteins that are more often found in the MSK, rather than the bulk cytoskeleton, include spectrin, ankyrin, band 4.1, adducin, villin, gelsolin, supervillin, filamin, dystrophin, and utrophin.

3. Since the term membrane skeleton or MSK is often used as an adjective, such as the "MSK fence model", the following term, the membrane-associated part of the cytoskeleton, which might sound more proper, is inconvenient.

C. Introduction to the MSK: The MSK of the Human Erythrocyte Ghost

EM investigations of the MSK structure have long been conducted using human erythrocytes. Since the human erythrocyte can easily be obtained, and since its plasma membrane can be readily isolated due to its lack of intracellular membrane compartments, the biochemical and structural analyses of the erythrocyte plasma membrane were much easier than those of other cell types. Therefore, it has been used as an important paradigm for studying the interaction between the MSK and the plasma membrane.

Due to the clarity of the results as well as the historical importance, first, we will describe the MSK structure, as revealed by EM (+AFM) as well as biochemical investigations. The MSK of the human erythrocyte even now provides a basic paradigm for studies of MSK structure and function. The schematic structure of the human erythrocyte MSK, obtained as a result of pioneering studies, is shown in Fig. 1 (Bennet, 1990; Byers and Branton, 1985). This amazing structure is completely different from the MSKs of other cell types, which will be described in the latter part of this review. Short actin filaments of approximately 40 nm in length, each consisting of 12–18 G-actin molecules, are bound by a tropomysin

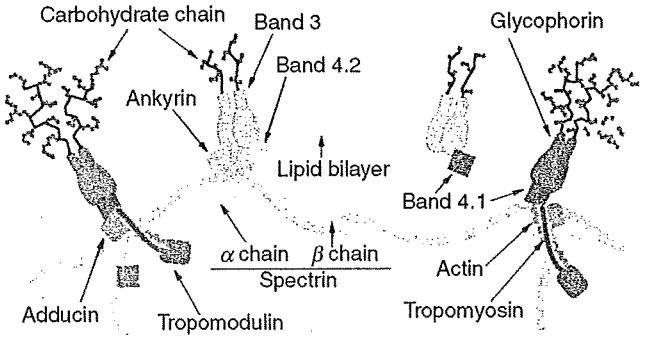

Fig. 1 Schematic model of the MSK of the human erythrocyte. Reproduced from Morone *et al.* (2006). © 2003 The Rockfeller University Press.

molecule on its side (this molecule is considered to determine the length of these short actin filaments), and are linked to the plasma membrane on its barbed end (fast-polymerizing end) and to the tropomodulin at its pointed end (slow-polymerizing end). These filaments are densely distributed throughout the cytoplasmic surface of the plasma membrane. The short actin filaments are linked to the plasma membrane by way of protein complexes, called junctional complexes, consisting of a single-pass transmembrane protein, glycophorin C, band 4.1, and adducin, occurring most frequently every 78 nm (Byers and Branton, 1985). These junctional complexes are linked to each other sideways by flexible spectrin tetramers with lengths between 50–80 nm, which can be extended up to ≈200 nm. Schematically, this structure can be envisaged as a picket-fence structure, like a garden fence. In this analogy, the short actin filaments can be considered as the pickets, each of which is fixed to the ground by a glycophorin C-band 4.1-adducin complex, and these junctional complexes are linked by spectrin fences. The difference from the picket fences found in the agricultural farm is that the spectrin fences are not attached to the short actin pickets, but to the ground protein complex structures (junctional complexes + spectrin). The flexibility of individual spectrin molecules is considered to provide both the elasticity and resilience of erythrocytes in circulation (Evans, 1989; Mohandas and Chasis, 1993; Vertessy and Steck, 1989).

Observations of the erythrocyte's MSK without using detergent solubilization and negative staining of the erythrocyte membrane, which might modify the MSK structure greatly, have been carried out by several methods, including scanning EM (Hainfeld and Steck, 1977), thin-section EM (Tsukita *et al.*, 1980), and EM after freeze-etching and platinum replication (Nermut, 1981; Ursitti *et al.*, 1991; also see Fig. 2A in the review by Coleman *et al.* (1989), which was provided by Dr. J. Heuser of Washington University). These studies showed that the membrane skeleton is a dense, complex, three-dimensional network of filaments. Atomic force microscopy of the cytoplasmic surface of the erythrocyte plasma membrane, taking advantage of its high sensitivity to small height variations on the surface, were conducted using freeze-dried human erythrocyte ghosts (Takeuchi *et al.*, 1998). The average mesh size of the spectrin network was 3000 nm^2, which is basically consistent with the number density of junctional complexes detected by the negative-staining EM.

II. Protocol for Visualization of the Three-Dimensional Structure of the MSK of the Cytoplasmic Surface of the Plasma Membrane

A. Methods for Exposing the Cytoplasmic Surface of the Plasma Membrane

Generally, two methods have been used for exposing the cytoplasmic surface of the plasma membrane of cultured cells, for immunolabeling and visualization of the cytoplasmic surface (Fig. 2). One is used to observe the upper plasma membrane (top surface in the cell culture on the coverslip) from inside the cell; and the

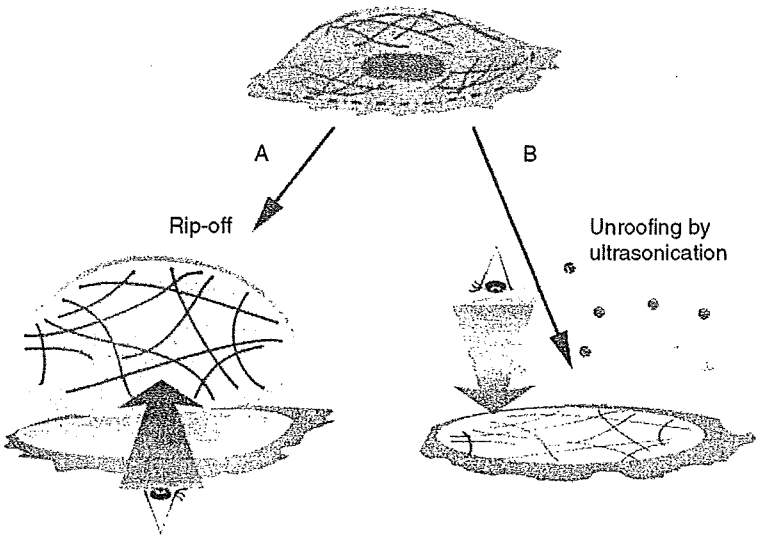

Fig. 2 The "rip-off" method (A) and the "unroofing" method (B) for exposing and observing the cytoplasmic surface of the plasma membrane of cultured cells. Reproduced from Morone *et al.* (2006). © 2003 The Rockfeller University Press.

other is employed for the observation of the bottom cell membrane (the cell membrane facing the cover slip), again from inside the cell. The former method is often called the "rip-off" protocol, as it involves the placement and attachment of a coverslip from the top, and the subsequent removal of the top coverslip to rip the top membrane off from the rest of the cell. The latter method is referred to as "unroofing", because the upper plasma membrane and the majority of the cytoplasmic structures and molecules are removed by very brief, weak sonication, leaving the bottom membrane attached to the coverslip on which the cell was originally cultured. Both methods can work reasonably well, but they both have their own potential problems and limitations. Further details are given in the following subsections.

Another method frequently used to visualize the actin filament meshwork and its binding proteins near the plasma membrane is to solubilize the plasma membrane using detergents. Triton X-100 solutions, containing other stabilizing reagents such as polyethylene glycol, glycerol, and sucrose, are the most popular ones. However, these detergent solutions tend to disintegrate caveolae and clathrin-coated pits. Using this detergent-solubilization method, the development of cortical actin filaments was clearly observed (Svitkina *et al.*, 1995, 2003).

a. The "rip-off" method for the observation of the cytoplasmic surface of the upper plasma membrane (Fig. 2A)

No.1 coverslips are cut into 5-mm square pieces. These small coverslips are cationized (coated) with alcian blue, a small-molecule reagent. The cationized coverslips are placed on cells cultured on the cell sheet at 4 °C for 10 min in order to allow the coverslips to attach to the plasma membrane (Fig. 3A). A buffer solution is then gently introduced into the gap between the coverslips and the cell sheet (Fig. 3B). The surface tension of the buffer forces the coverslips to float up, which could rip off the upper cell membrane from the rest of the cell, sometimes with very small amounts of the membrane skeleton or with contamination by whole cells (Fig. 3B). This buffer usually contains chemical fixatives such as 2% formaldehyde, so that the plasma membrane ripped off from the rest of the cell is immediately fixed, before its component proteins dissolve away into the rip-off buffer.

b. Unroofing by low-power ultrasonication for the observation of the cytoplasmic surface of the bottom plasma membrane (Fig. 2B)

A probe-type, low-power ultrasonic generator is used to remove the top membrane and the bulk cytoplasmic materials (Heuser, 2000). This method has often been plagued by the limited observation areas, the broken MSK meshwork

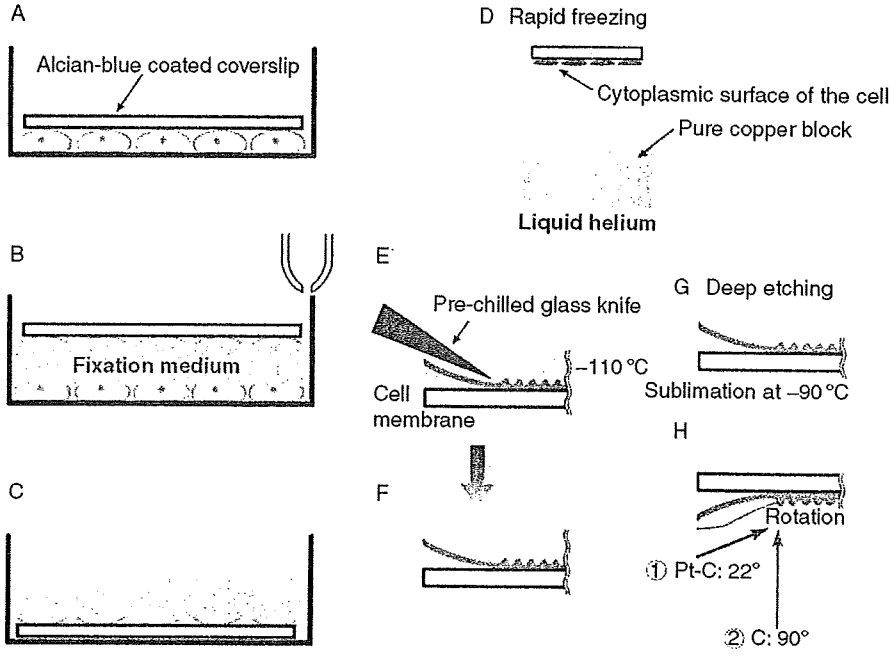

Fig. 3 Specimen preparation protocol for ripping-off the upper plasma membrane, followed by rapid-freezing, deep-etching, and platinum replication.

structures, and the loss of small membrane invaginations, including caveolae and clathrin-coated pits. Careful adjustments of the various conditions for the sonication step are required, such as the use of smaller probes (e.g., 1 mm in diameter) and lower output powers (e.g., up to several milliwatts), for the observations of the MSK, caveolae, and clathrin-coated pits spread throughout the cytoplasmic surface of the bottom plasma membrane, just like those of the upper cell membrane.

In both cases, the plasma membrane is (further) fixed by an incubation in fresh, ice-cold 1% paraformaldehyde/0.25% glutaraldehyde for 10 min (Fig. 3C).

B. Immunolabeling of the Proteins on the Cytoplasmic Surface of the Plasma Membrane

Immunogold labeling is an excellent way to reveal the molecular identifications in the observed structures in the electron micrograph. Immunolabeling of the isolated plasma membrane on its cytoplasmic surface can be conducted in the normal manner for fixed samples. As we intend to use platinum replication of the plasma membrane specimens, colloidal gold particles are generally recommended as probes. Since each gold particle exhibits a clear dot surrounded by a halo of platinum-carbon coating, these gold probes can be easily identified in the platinum replicated specimens. Transmembrane proteins can be labeled from the extracellular surface, and these proteins with gold probes can be retained after chemical fixation and platinum replication on the replica (Fujimoto, 1995; Fujimoto *et al.*, 1996).

C. Rapid-Freezing

The plasma membrane with the exposed cytoplasmic surface is next frozen quickly from the cytoplasmic surface, with its external surface still attached to the coverslip. One of the surfaces of a pure-copper block is polished with diamond paste to make a mirror surface, and the block is placed in liquid helium, with its mirror surface slightly exposed over the helium surface. Each coverslip is placed on the plunger tip of the rapid-freezing device, with the cytoplasmic surface of the membrane facing down (Fig. 3D). The plunger is slammed down onto the polished, pure-copper block (metal-contact method). This method takes advantage of the feature of pure copper, for which its thermal conductivity is maximal around the temperature of liquid-helium. Usually, within 20 microns from the frozen surface, the growth of ice crystals is sufficiently suppressed, due to the rapid freezing rate (10^5 °C/s, Heuser *et al.*, 1979) so that they do not alter the cellular structures (Fig. 3D).

D. Deep-Etching and Platinum Replication

The frozen specimen is placed in liquid nitrogen, and then it is transferred into the freeze-etching-shadowing chamber, where the pressure can be lowered to approximately 10^{-6} Pa. The excess ice covering the cytoplasmic surface of the

membrane is shaved off, with a prechilled glass or diamond knife, using a microtome placed in the chamber at −110 °C or below (Fig. 3E). Under optimal conditions, this shaving process leaves the structures (in ice) approximately 0.2 to 1 micron from the plasma membrane (Fig. 3F). The surface of the ice layer is then sublimed by slightly raising the temperature of the specimen to approximately −100 to −70 °C, so that the structures hidden in the ice layer are exposed (Fig. 3G). This process is called "etching (or deep-etching as, in this protocol, the etching is more extensive than other methods)", and hence this whole specimen preparation protocol is named "freeze-etching." The etched specimen surfaces are then rotary shadowed with platinum at an angle of approximately 20° from the surface, with a thickness of 1–2 nm, and then with carbon from the top (Fig. 3H). By lowering the specimen temperature and the pressure during shadowing, the platinum grains become smaller, giving images with higher resolutions. The molecules as well as the gold probes localized on the cytoplasmic surface of the cell membrane are immobilized by the deposited platinum (Fujimoto, 1995; Fujimoto *et al.*, 1996).

E. Recovering the Platinum Replicas

The following is the procedure we learned from Drs. T. Baba and S. Ohno of Yamanashi University Medical School. Collodion is applied immediately after the platinum-carbon replicas are removed from the cold chamber, to fortify them. The platinum-carbon replica is removed from the glass coverslip by an incubation in 1% hydrofluoric acid in distilled water. After the replicas are successfully removed from the glass surface and mounted on the grid, the collodion coat is dissolved away in *n*-pentyl acetate. In this protocol, the sodium hypochlorite solution, which is generally used to remove the replicas from the coverslip and also to clear the membrane and the undercoat structure of the replicas, is replaced with 1% hydrofluoric acid, in order to keep the cell membrane, the undercoat structure, and the immunogold probes attached to these structures on the platinum replicas (Fujimoto, 1995; Fujimoto *et al.*, 1996; 1% hydrofluoric acid is likely to only dissolve the glass, leaving the cell membrane molecules bound to the platinum replica). An additional advantage of using 1% hydrofluoric acid is that the platinum replicas break less often, probably because the membrane components are not removed from the replicas, leaving them rather intact.

In addition, to keep as many colloidal gold particles attached to the platinum replicas as possible, all of the solutions included 0.5–1% Kodak Photo-Flo 200, a detergent used to prevent water-drop stains on the photographic film (advice from John Heuser). After the replicas are washed with distilled water, they are mounted on 100–200 mesh copper grids coated with polyvinyl formvar, and then observed at magnifications of 10,000 ~ 70,000 with a transmission electron microscope (~80 kV).

F. Summary of the Methods for Producing Large Plasma Membrane Fragments and Avoiding Excessive Fragmentation of Replicas

The following methodological precautions and improvements will help to reproducibly produce large plasma membrane fragments and replicas without excessive fragmentation. Although individually these are minor modifications, but collectively they exert a substantial impact.

1. Employ an alcian-blue coat, rather than a poly-L-lysine coat (Rutter *et al.*, 1988; Sanan and Anderson, 1991).

2. In the protocol to observe the top membrane, before overlaying the alcian-blue-coated coverslips, remove the excess water from the specimen, leaving just enough buffer to cover the cell.

3. To cleave off the upper membrane attached to the overlaid coverslip, float the coverslip off very gently by adding cleavage medium (using the surface tension of the buffer to float the coverslip). If this is not done gently enough, the membrane will be fragmented.

4. Shave off the frozen sample with a glass or diamond knife, with the angle between the knife and the cover glass adjusted to a shallow angle (less than 6°), so that most of the excess water and the cytoplasm are removed and the cytoplasmic surface of the cell membrane could be exposed after light etching. Since replicas with too many variations in height tend to break when they are removed from the coverslip and placed on the water surface, removal of the excess cytoplasm helps to avoid replica breakage.

5. Apply collodion immediately after the replicas are removed from the cold chamber (before the replicas are removed from the coverslip on the water surface), to fortify the replica. This step helps to prevent replica breakage when the replicas are removed from the cover slip. Dissolve away the collodion coat in *n*-pentyl acetate, after the large replicas are successfully removed from the glass surface.

6. Use a solution of 1% hydrofluoric acid to slightly dissolve the glass surface, to facilitate the removal of the replicas from the cover slip.

7. To keep as many colloidal gold particles attached on the platinum replicas as possible, include 0.5–1% Kodak Photo-Flo 200 in all of the solutions used to remove the replicas from the coverslips.

G. Creation of Stereo Views (Anaglyphs)

The term "anaglyph" refers to any image that has the appearance of being raised from the surface of the paper. For the stereo views (anaglyphs), image pairs have to be obtained by tilting the stage at ±12.5° from the vertical axis. For details on the production of anaglyphs, see Heuser (2000).

H. 3-D Reconstruction of the MSK by Electron Tomography

In X-ray tomography, an increasingly popular technology for disease diagnosis, X-rays irradiate the body at various angles, and from the X-ray images taken at various rotated or tilted images, sliced images of the body are calculated for reconstructing the 3-D images of the body. In electron tomography, the specimen stage is tilted with respect to the incident electron beam in the transmission electron microscope. The mathematical formulation is basically the same, except that the tilt angle may be limited within $\pm\sim 70°$, limiting the z-resolution. In addition, since the axis of rotation slightly shifts around upon stage tilting, inducing large shifts in the location of the observed detailed structures (large as compared to the subnanometer size of the object we observe under the electron microscope), correction to compensate for this effect is needed for three-dimensional reconstruction. Usually, EM images are taken every 1°, and therefore, for the tilt angles between ±70°, 141 tilt images are taken for a single view field (Fig. 4).

Such quantitative data acquisition and three-dimensional image reconstruction in EM were developed and automated in the 1990s, resulting in the current prevalence of this method. Four key features in the development were: (1) the development of the tilting stage that allows accurate rotation; (2) the development of an electron microscope with z-axis correction and a side-entry specimen holder; (3) the development of a scientific CCD camera with high sensitivity, resolution (1024 × 1024 pixels or more, with a pixel size of 0.85 nm or better on the sample, which should be finer than the platinum grain size), and linearity; (4) the creation of software for the automatic data acquisition of a series of tilted images (Medalia *et al.*, 2002). Recently, this method has increasingly been used to determine molecular forms as well as cellular structures (Lucic *et al.*, 2005).

We generally use the "IMOD" software package, created by Dr. J. R. McIntosh of the University of Colorado at Boulder (Kremer *et al.*, 1996), running on Linux,

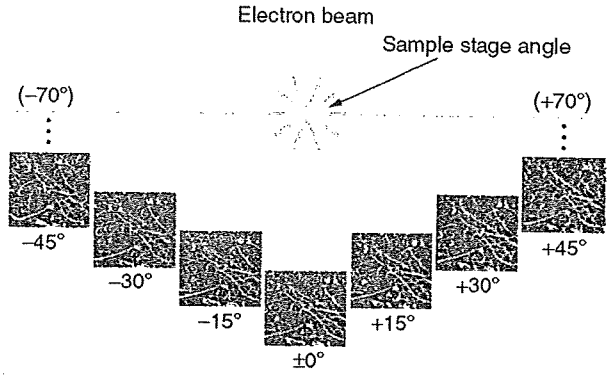

Fig. 4 EM images obtained at different tilt angles of the specimen stage with respect to the incident electron beam. Reproduced from Morone *et al.* (2006). © 2003 The Rockfeller University Press.

for the generation of a series of sliced images (for example, about 100 image sections of every 0.8–1.3 nm) from a series of tilted images. Corrections for the tilt and the long-wavelength undulations of the membrane can also be achieved with the IMOD software. 3D rendering (displaying 3D images in different ways) can be carried out using the Templace Graphics AMIRA software package, operating on a Linux system.

III. 3D Structure of the Cytoskeleton-Plasma Membrane Interface

A. The Cytoplasmic Surface of the Plasma Membrane of Cultured Cells is Entirely Coated with the Meshwork of the Actin-Based MSK

A typical electron micrograph, providing a bird's-eye view of the cytoplasmic surface of a large area of the upper cell membrane of a cultured normal rat kidney (NRK) cell line, is shown in Fig. 5. A number of such EM images showing the cytoplasmic surfaces of large cell membrane fragments were obtained for NRK and fetal rat skin keratinocyte (FRSK) cells, suggesting that the entire (upper) plasma membrane, except for the places where clathrin-coated pits and caveolae exist, is coated with a filamentous, net-like structure.

The extensive filamentous, net-like structures shown in the magnified images of the cytoplasmic surface of the plasma membrane in Figs. 6A and B, which were

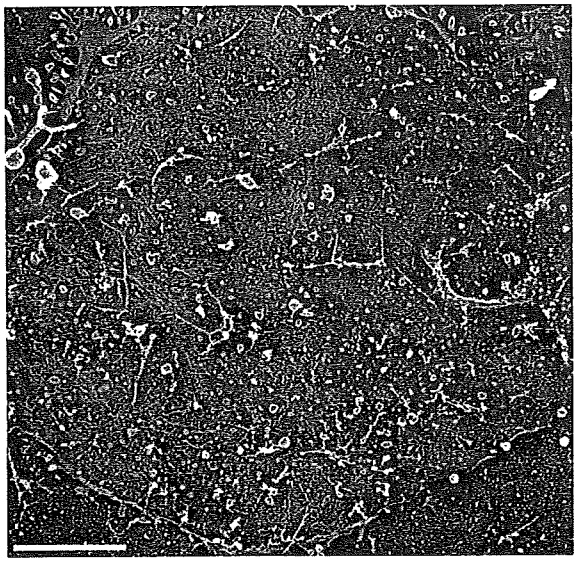

Fig. 5 EM image of the cytoplasmic surface of the upper plasma membrane (bird's-eye view). The plasma membrane fragment shown here represents about a quarter of the upper plasma membrane. Reproduced from Morone *et al.* (2006). © 2003 The Rockfeller University Press.

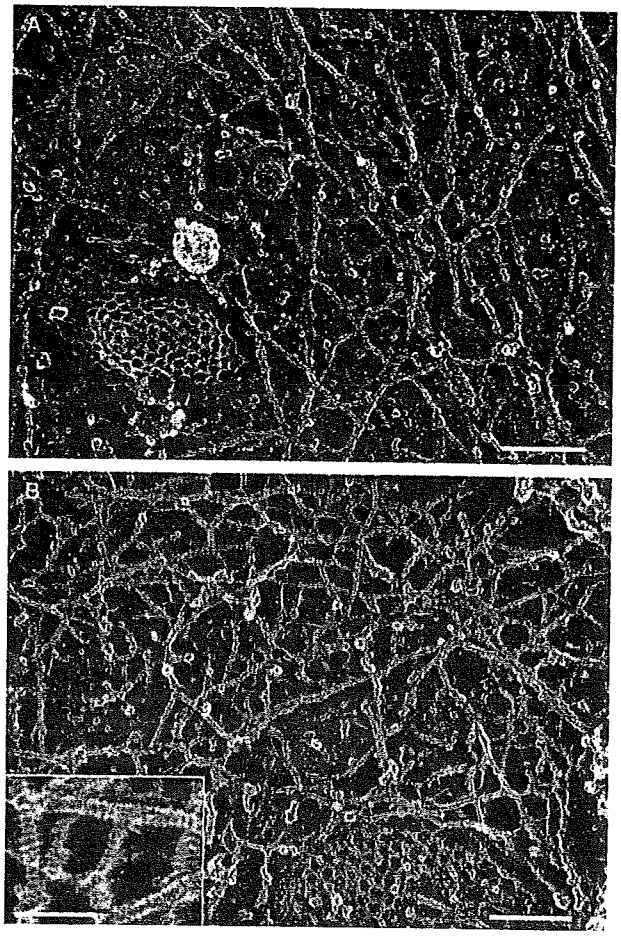

Fig. 6 EM images of the MSK of the upper plasma membrane. (A) NRK cell. (B) FRSK cell. Clathrin-coated structures (A and B) and a caveola (A) show the cytoplasmic surface. The striped banding patterns with the 5.5-nm periodicity on individual filaments are characteristic of actin filaments. These images reveal the close links of the MSK actin filaments with the clathrin-coated structures and caveolae. Bars = 100 nm. The bar in the inset in B = 50 nm. Reproduced from Morone *et al.* (2006).

obtained for an NRK cell (A) and an FRSK cell (B), respectively, are the MSK. The presence of clathrin-coated structures shows that this is indeed the cytoplasmic surface. The striped banding patterns with a 5.5-nm periodicity on individual filaments are characteristic of actin filaments, and thus indicate that these are actin filaments (Heuser and Kirschner, 1980; Katayama, 1998; Schoenenberger *et al.*, 1999). Since almost all of these filaments contain this striped pattern, it was thus concluded that *the MSK is predominantly composed of actin filaments*. This was also confirmed by immunogold staining.

The inset in Fig. 6B indicates the spatial resolution: since each band in the striped pattern with a 5.5-nm periodicity is visibly separated, the effective resolution is thought to be ≈ 2 nm (both the thickness of the platinum coating and the platinum granule size are ≤ 2 nm: Heuser and Kirschner, 1980; Heuser, 1983).

The MSK structure observed here on the upper cell membrane is similar to that on the bottom cell membrane (the part of the cell membrane facing the coverslip) observed previously (Heuser and Anderson, 1989). Based on these observations, it was concluded that the entire cytoplasmic surface of the plasma membrane is coated with the filamentous actin network (MSK), except for the places where clathrin-coated pits and caveolae are present. The notion of the complete coverage of the cytoplasmic surface of the plasma membrane by actin filaments might have existed for over 30 years in part of the EM community (Byers and Porter, 1977; see Sheetz *et al.*, (2006) for a review), but the data specifically indicating that the actin filaments of the MSK may cover the entire plasma membrane had neither been presented in the literature, as done by Morone *et al.* (2006), nor shared in the cell biology community. The EM observations described here are consistent with the MSK-fence and anchored transmembrane protein picket models, in which the entire plasma membrane, except for specific membrane domains, is parceled up into apposed domains, with regard to the lateral diffusion of the molecules incorporated in the plasma membrane (Fujiwara *et al.*, 2002; Kusumi *et al.*, 2005a,b).

B. View of the MSK Using Anaglyphs

A representative anaglyph produced from images taken at $\pm 12°$ is shown in Fig. 7. In these images, because of their 3D representation, it is especially clear that the MSK, which is mostly composed of actin filaments, generally spreads along the plasma membrane, covering almost the entire cytoplasmic surface of the upper plasma membrane. In addition, clathrin-coated pits and caveolae are very closely associated with the actin filaments in the MSK, as seen in this image as well as in Figs. 6A and B. These results are consistent with those reported by Fujimoto *et al.* (2000), Rothberg *et al.* (1992), and Parton, (2003), but in the NRK cells studied here, 92 and 93% of clathrin-coated pits and caveolae ($n = 200$) are bound by the actin filaments. Furthermore, in these images, many actin filaments are associated with each clathrin-coated pit or caveola. These results are consistent with the requirement of f-actin for clathrin-coated pit internalization (cf. Merrifield *et al.*, 2002; Qualmann *et al.*, 2000).

Many short, thin filaments protrude toward the cytoplasm, mostly perpendicularly, from the membrane surface (arrows in Fig. 7; they were short probably because they were broken when the membrane was ripped off). Note that these perpendicular filaments are almost always connected to the MSK network lying on the cytoplasmic surface (see the tips of the arrows). Thus, the part of the MSK that is located on the cytoplasmic surface is connected three-dimensionally to

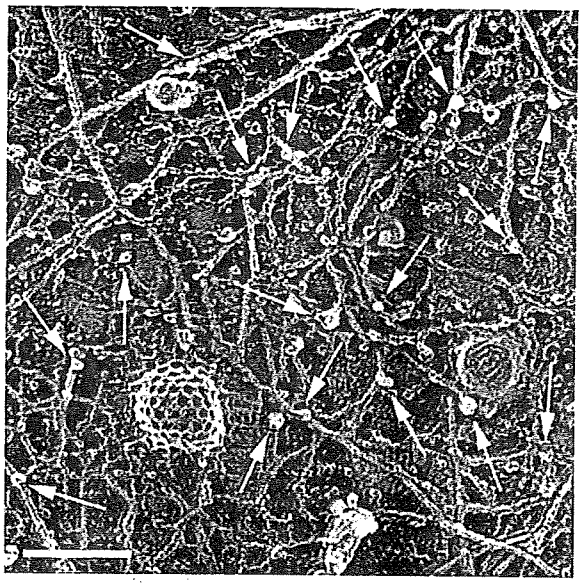

Fig. 7 Stereo electron micrograph (anaglyphs; left = red, right = green) of the plasma membrane undercoat structure generated at ±12° of the tilt angle among the 131 tilt images (acquired in the range of ±65° with 1° steps). For the 3D view, one will need red-blue viewing glasses. These glasses are widely available online or via novelty sources, such as comic book shops and toy stores, and are often attached to Journals [for example, J. Cell Biol. 2006 vol.174, No.6)] or confocal microscopes. We will send a pair upon request at singlemolecules111<at>frontier.kyoto-u.ac.jp. Arrows: actin filaments protruding from the membrane cytoplasmic surface toward the cytoplasm. The arrows point to the places where the protruding actin filaments intersect with the actin filaments lying horizontally on the plasma membrane. Bar = 100 nm. Reproduced from Morone *et al.* (2006). © 2003 The Rockfeller University Press. (See Color Plate no. 5 in the Color Plate Section.)

the cytoskeleton. Together, they will provide the mechanical support for the membrane and the force for deforming the membrane.

C. Quantitative 3D Reconstruction of the Undercoat Structure on the Cytoplasmic Surface of the Plasma Membrane Using Electron Tomography

The 3D structure of the undercoat within 100–134 nm from the cytoplasmic surface of the plasma membrane, which includes clathrin-coated pits, caveolae, and the actin-based MSK, was reconstructed using electron tomography for the platinum replicated samples. Based on the 97–141 tilt images acquired in the range of ±48–70° every 1° step for a single EM view field, 100–121 sliced images of every 0.85–1.34 nm perpendicular to the z-axis (parallel to the image obtained at 0° of the tilt angle) were calculated by a computer (long-wavelength [≥~500 nm] undulations of the cell membrane were corrected by the 3D-reconstruction software, IMOD). The 3D-image was reconstructed based on these serial thin slices.

In Fig. 8A, a typical MSK structure quantitatively analyzed in the present work is shown in an anaglyph, and its 8.5-nm thick sections (created by superimposing ten 0.85-nm sections) of the MSK of an NRK cell, starting from the cytoplasmic side toward the membrane, are shown (Fig. 8B). The actin-based MSK is visible on image sections 81 through 110. Individual actin filaments, forming a network as well as bundles, can be identified. Given the high density of the actin filament meshwork, which is much smaller than the optical resolution, conventional fluorescence microscopy cannot be used to observe the individual actin filaments, and can visualize only the bundles of actin filaments.

D. Interface Structure of the MSK on the Cytoplasmic Surface of the Plasma Membrane

The part of the actin-based MSK that contacts the cytoplasmic surface of the plasma membrane has been proposed to partition the cell membrane into 30–230 nm compartments, by the "fence and picket" effect (Edidin *et al.*, 1991; Kusumi and Sako, 1996; Kusumi *et al.*, 2005a,b), for the diffusion of membrane molecules. If these fence and picket models are correct, then the distribution of the mesh size of the MSK on the cytoplasmic surface of the plasma membrane would be practically the same as that of the compartment size determined by diffusion measurements of membrane molecules. To carry out this examination, the 3D reconstruction of the MSK by electron tomography provides a unique opportunity, because the obtained images provide quantitative data on the distance between the individual filaments and the membrane surface.

The actin filaments of the MSK that are directly associated with the cytoplasmic surface of the plasma membrane and may be involved in partitioning the plasma membrane were systematically determined. Out of the stack of 121 image slices taken every 0.85 nm from the cytoplasmic surface ($\approx$ 100-nm thick altogether), 16 consecutive image slices from the membrane surface ($\approx$ 13.6-nm thick altogether) were used for this analysis (Figs. 9A and B).

In Figs. 9A (four images on the right) and 9B (from the second to the fourth images), the square boxes in the left-most images were expanded, and the sections of every 1.7 nm (superposition of two 0.85-nm-thick slices, 330 $\times$ 330 nm) are displayed, between 0 and 11.9 nm. Using these sections, the filaments that are closely associated with the cytoplasmic surface of the cell membrane were determined. Since the width of the actin filament after platinum shadowing is between 9–11 nm (consistent with Heuser, 1983) and the thickness of the platinum replica is $\leq$ 2 nm (consistent with Heuser, 1983 and Moritz *et al.*, 2000), the height of the actin filament that is associated with the membrane will be 7–9 nm (because the height is given by the actin thickness and one replica thickness, whereas the width of the actin filament in the image is determined by the actin thickness plus two replica thicknesses), with 8 nm being a reasonable estimate.

The electron tomography sections shown in Figs. 9A and B revealed three major classes of filaments, with regard to the distance from the membrane surface.

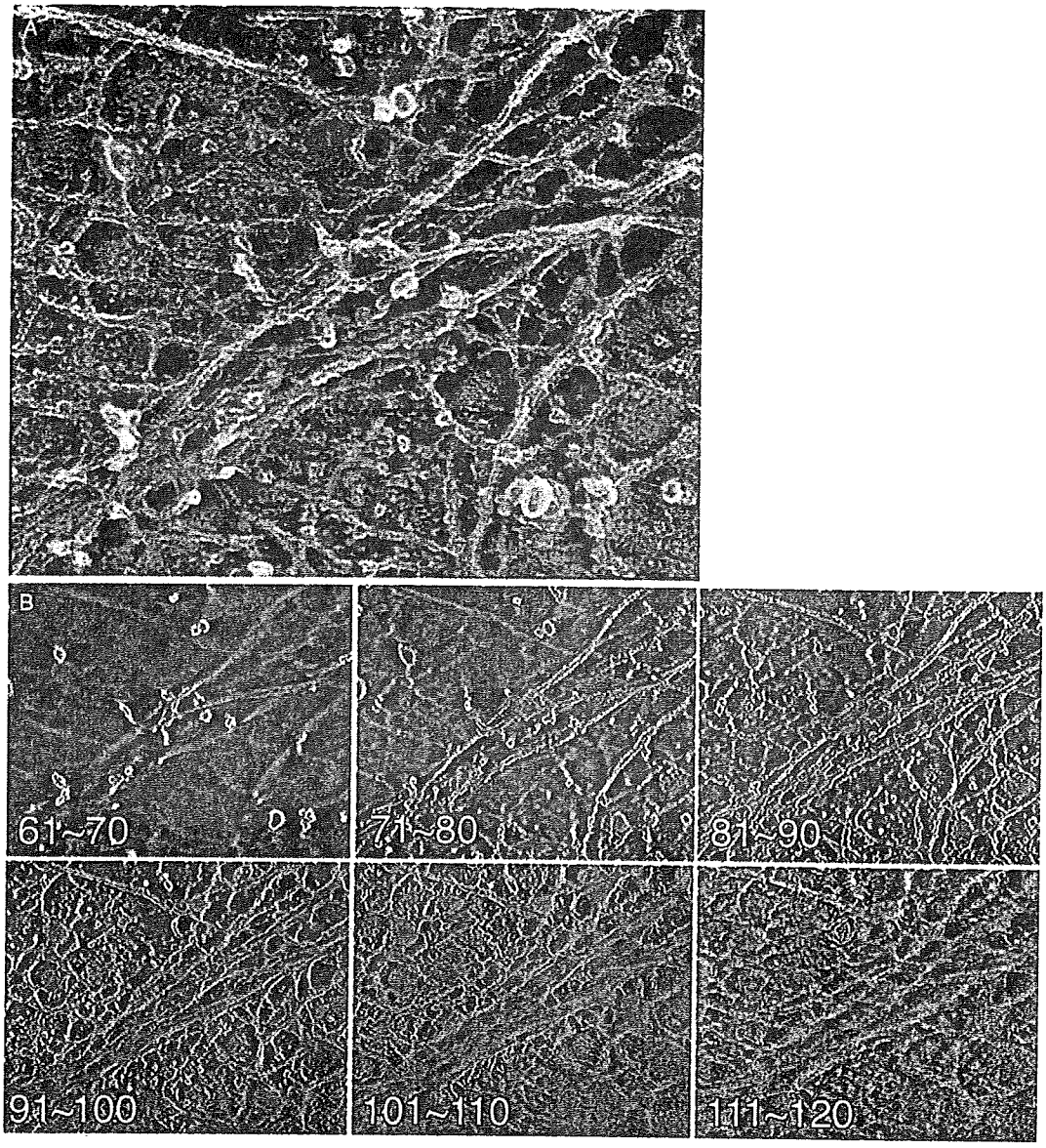

Fig. 8 A typical actin MSK structure on the cytoplasmic surface of the plasma membrane of an NRK cell. (A) An anaglyph of a typical actin MSK structure generated at ±12° of the tilt angle among the 97 tilt images (acquired in the range of ±48° with 1° steps). (B) A typical series of sliced images of the actin MSK. Ten consecutive sections, each 0.85-nm thick, are superimposed, and six of these superimposed images, representing 60 image sections out of 121 image sections, are shown from the cytoplasmic side toward the plasma membrane side. The numbers here indicate the number of slices counted from the cytoplasmic side. The actin-based MSK near the cytoplasmic surface of the plasma membrane is visible on images 81 through 110. Reproduced from Morone *et al.* (2006). (See Color Plate no. 6 in the Color Plate Section.)

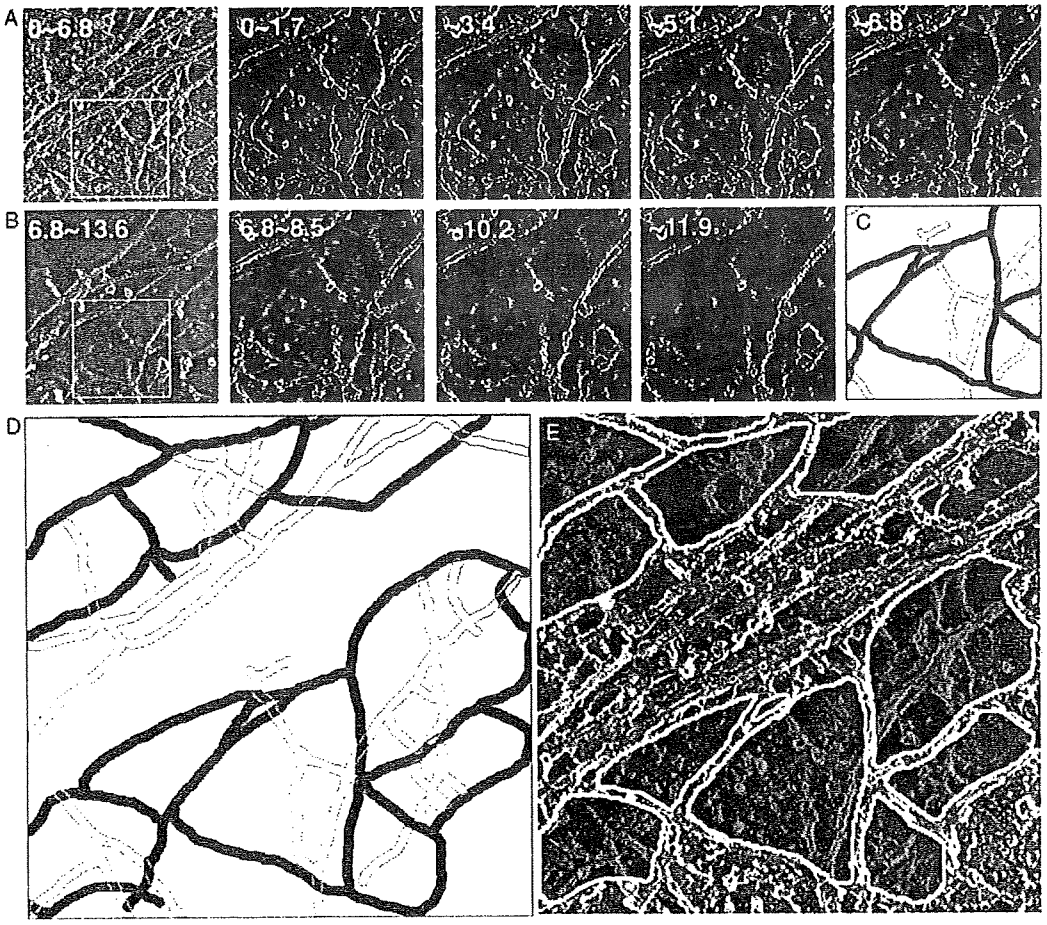

Fig. 9 The method for determining the MSK mesh on the cytoplasmic surface of the plasma membrane, which possibly delimits the compartments of the plasma membrane, using the 3D-reconstructed images of the MSK (an NRK cell). (A, B) The images on the far left are the 0 ~ 6.8 nm or 6.8 ~ 13.6 nm sections, each of which is a stack of eight 0.85-nm sections of 670 × 670 nm. These are from a series of 121 image sections (0.85-nm thick) from the cytoplasmic surface, after the tilt and the long-wavelength undulation of the cell surface were corrected. The images in the white squares in (A) and (B) (330 × 330 nm) are expanded on the right of these image stacks, with a section thickness of 1.7 nm (two 0.85-nm sections are superimposed)(330 × 330 nm for each image). (C) The outline of each actin filament adjacent to the membrane surface (green, which could not be observed above 10.2 nm) and that of each actin filament that could be observed above 10.2 nm (red). The view field and magnification are the same as those for the thinner sections shown in (A) and (B) (330 × 330 nm). See the Methods section for details. (D) The outline of actin filaments in a greater view field, which is the same as those in the thick sections (0 ~ 6.8 nm and 6.8 ~ 13.6 nm) in (A) and (B) (670 × 670 nm, expanded here). (E) The image of the 0 ~ 6.8 nm sections (670 × 670 nm), superimposed on the image of the areas surrounded by the filaments outlined in green in (D) (green areas with yellow outlines). According to the "fence" and "picket" models, these areas are likely to be the compartments where the membrane molecules are temporarily confined. Reproduced from Morone *et al.* (2006). (See Color Plate no. 7 in the Color Plate Section.)

The actin filaments of the first class are distinct even in the first 0 ~ 1.7 nm section (since the contrast is reversed in these micrographs, they look more lucent or white), but they fade out of the reconstructions 8–10 nm away from the membrane surface. These filaments are drawn in green in Fig. 9C. We interpreted this to mean that these filaments are in close contact with the plasma membrane, with the gap between the filament and the inner membrane surface being less than 0.85 nm, because they can be seen clearly even in the first 0.85-nm section. These filaments are likely to be the significant ones for generating membrane corrals (for more quantitative analyses and descriptions, see Morone *et al.*, (2006)).

The filaments of the second class are also clearly visible in the sections very close to the membrane surface, but they do not fade out until about 14 nm away from the surface. These are probably the actin filaments that caught the platinum coating all around their surfaces because they resided slightly off the surface. The extra coating slightly exaggerated their thickness and made them look as though they were in contact with the plasma membrane, when in fact they probably were not quite in direct contact. We did *not* consider these filaments to be close enough to generate membrane corrals.

The actin filaments of the third class are not apparent in the sections closest to the plasma membrane, but they become clear some distance away from it (greater than 2–4 nm), and they also do not fade out until ~14 nm. We interpreted this to mean that these filaments are those that definitely do not contact the plasma membrane directly, and hence should not contribute to forming corrals. The second and third classes of filaments are drawn in red in Fig. 9C.

Therefore, we considered that only the first class of filaments (those drawn in green in Figs. 9C and D) forms the MSK fences and pickets, and the area surrounded by these filaments is colored green in the 0–6.8 nm section shown in Fig. 9E. Note that there are regions that were not amenable to such an analysis. They were the areas where the bundles of actin filaments are present (e.g., the structure crossing diagonally from the lower left to the upper right in Fig. 8), the actin filaments are too crowded to be individually discerned, the actin filament is terminated in the middle of a domain (domains that contain a loose end of an actin filament) or the clathrin-coated pits, caveolae, and the smooth-surface membrane invaginations are present. They were excluded from this analysis (the white regions in Fig. 10C).

E. Distribution of the MSK Mesh Size on the Plasma Membrane Determined by Electron Tomography

A similar determination of the MSK meshwork was also made for FRSK cells. Representative meshes of the MSK are shown in Fig. 10 (for an FRSK cell, colored to aid visualization). We carried out such analyses for 10 representative stacks of image sections (1290 nm × 1290 nm plane) each for NRK cells and FRSK cells (eight different cell membrane sheets for each cell type), and identified 76 and 1300 areas bounded by the MSK meshwork, respectively. The two-dimensional area size

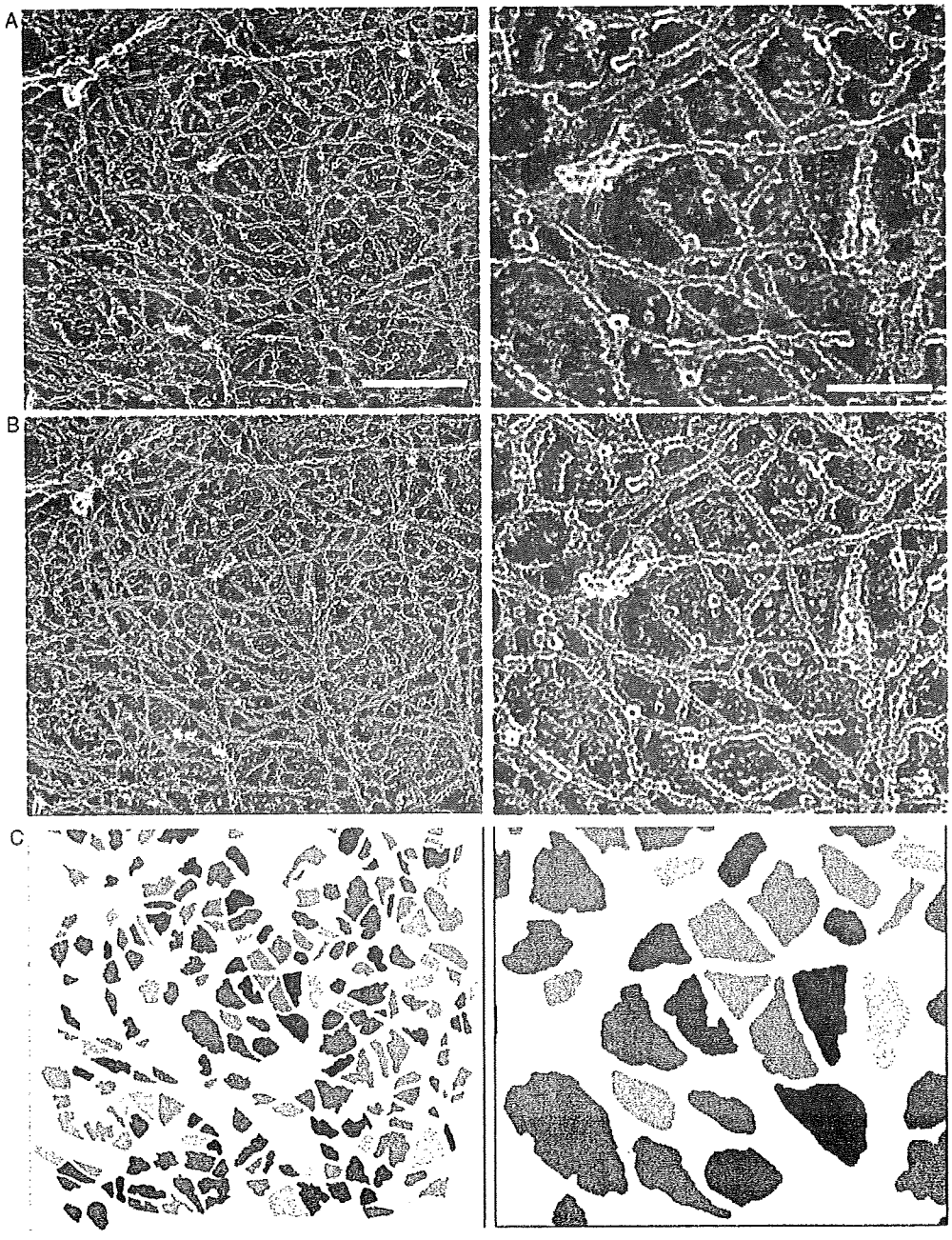

Fig. 10 The MSK meshwork directly located on the cytoplasmic surface of the plasma membrane of an FRSK cell. The central parts of the figures in the left column (bar = 300 nm) are magnified by a factor of 3, and are shown in the right column (bar = 100 nm). (Row A) Typical stereo views of the plasma membrane specimen (anaglyph; left = red, right = green). (Row B) Normal electron