Supplemental Data

Membrane folds are caused by cortical actomyosin, but do not reflect focal adhesions

The observed patches conceivably reflect sites of interaction with the substratum. In confocal reflection images (Verschueren, 1985), sites of cell-substratum proximity such as focal adhesions are apparent as marked dark (reflection-free) areas. Indeed, a large fraction of the patches colocalizes with sites of membrane-substratum proximity as observed in reflection images (Supplemental Figure 1), independently corroborating the results obtained with the reconstructed PSF imaging technique. However, patches do not reflect focal adhesions, since N1E-115 cells do not possess focal adhesions and the staining pattern of antibodies against vinculin and paxillin does not colocalize with the patches (data not shown). Furthermore, in a significant subset of cells, basal membrane patches were absent, while adhesion to the cover slip was normal. Finally, serum-starved N1E-115 cells are quite immobile in cultures on glass or plastic; the highly dynamic nature of the patches therefore suggests a role different from cell adhesion in these cells. Perhaps these structures serve a role in probing the substratum; however, this hypothesis requires further investigation that is beyond the scope of this paper.

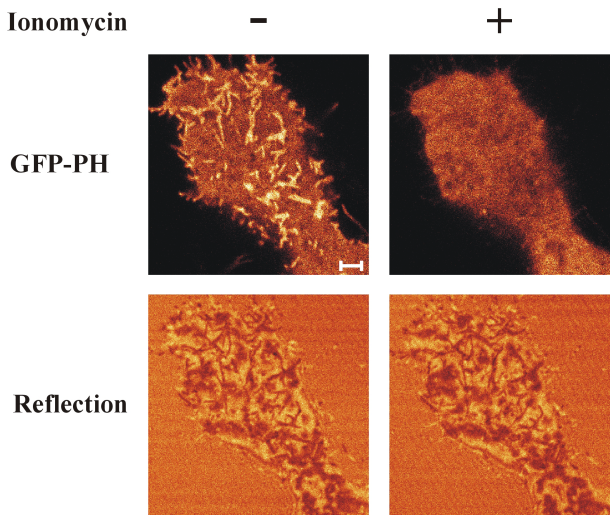
How do motile patches arise? Simultaneous imaging of GFP-actin and DiI confirmed the reported colocalization between patches and F-actin (Supplemental Figure 2) (Tall *et al.*, 2000). Note that while DiI-stained patches strictly overlap with GFP-actin filaments, the reverse is not always true, since not all actin filaments reach the plasma membrane. Other cytoskeletal components, including tubulin, paxillin and vinculin, do not colocalize with DiI (data not shown). We therefore hypothesized that folds are due to actomyosin contractility. To test this, GFP-actin and DiI images were collected simultaneously and analyzed for colocalization. In time-lapse series, both signals showed strikingly similar dynamics. When cells were treated with cytochalasin D (0.5 μg/ml) to disrupt F-actin (Urbanik and Ware, 1989), GFP-actin structures rapidly shrank with a concomitant drop in motility (Supplemental Figure 2B), while the DiI patches stopped moving (not shown). Cytochalasin D (Sigma Chemical Co., St. Louis, MO) also blocked motility of GFP-PH patches completely. Similarly, myosin light chain kinase blockers (1 μM KT5926, supplemental Figure 2C, and 100 nM K252a, not shown; inhibitors were from Calbiochem-Novabiochem Corp., La Jolla, CA) impeded patch motility. We conclude that actomyosin contractility underlies membrane folding and patch formation.

Reference

Tall, E.G., Spector, I., Pentyala, S.N., Bitter, I., and Rebecchi, M.J. (2000). Dynamics of phosphatidylinositol 4,5-bisphosphate in actin-rich structures. Curr.Biol. *10*, 743-746.

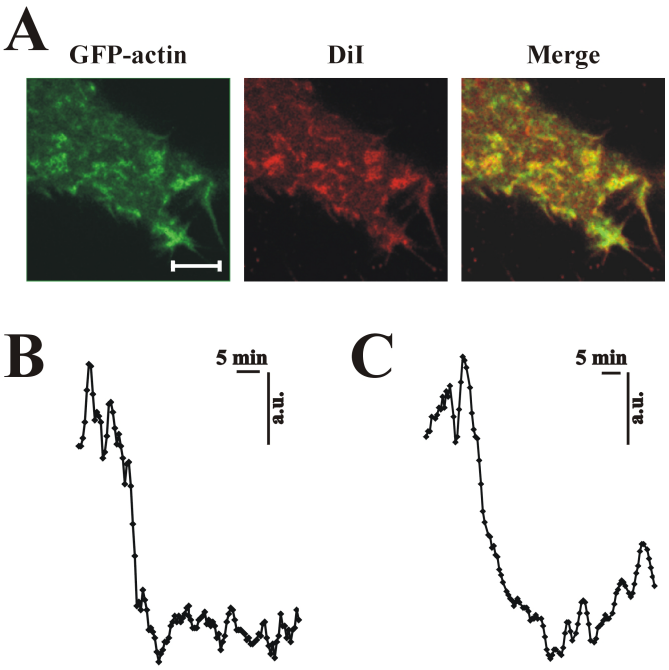
Urbanik, E., Ware, B.R. (1989). Actin filament capping and cleaving activity of cytochalasins B, D, E, and H. Arch.Biochem.Biophys. *269*, 181-187.

Verschueren, H. (1985). Interference reflection microscopy in cell biology: methodology and applications. J.Cell Sci. *75*, 279-301.



Supplemental Figure 1. Colocalization of membrane patches with sites of membrane-substratum proximity

Fluorescence and reflection images were taken from the basal membrane of a N1E-115 cell expressing GFP-PH, using the 488 nm Argon ion laser line. To induce complete translocation of GFP-PH to the cytosol, ionomycin (5 µM) was added. Scale bar: 2 µm.



Supplemental Figure 2. Actomyosin-based forces drive motility of the patches

(A) N1E-115 cells expressing GFP-Actin were stained with DiI. Time lapse series of confocal images were collected from the basal membrane of these cells, simultaneously registering GFP-Actin and DiI. Actin motility was analyzed after **(B)** cytochalasin D (CCD, 0.5 µg/ml) and **(C)** KT5926 (1 µM) treatment using the actin motility assay. Scale bar: 2.5 µm.