LOCALIZATION OF CYTOPLASMIC SPECTRIN FILAMENTS IN SEA URCHIN (STRONGYLOCENTROTUS DROEBACHIENSIS) COELOMOCYTES.

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Sea urchin coelomocytes provide an excellent model experimental system for the study of actin-mediated shape changes. We have recently demonstrated that coelomocytes consist of two distinct cell types which differ in their relative degree of motility, overall morphology, and organization of their actin and tubulin cytoskeletons (Henson et al., 1992. J. Cell Sci. 103: 309-320; Henson et al., 1993. Bulletin MDIBL 32: 4-6; Henson et al., 1993. Mol. Biol. Cell 4: 54a). The polygonally shaped motile cell type possesses a well developed array of microtubules and has actin filaments arranged in parallel bundles similar to stress fibers. The disc shaped stationary cells contain a sparse array of perinuclear microtubules and have actin filaments organized into an extensive cortical network. In order to further elucidate the differences between these cell types we have initiated immunocytochemical localization studies involving several actin-binding and microtubule associated proteins. Here we report results on the immunolocalization of spectrin in coelomocytes and argue that this localization may suggest a novel structural organization of spectrin in these cells.

A mixed population of coelomocytes was collected from coelomic fluid in a calcium-chelating anticoagulant (0.5 M NaCl, 2.5 mM $MgCl_2$, 100 mM EGTA and 40 mM HEPES pH 7.4), isolated by means of centrifugation at 2,000 x g onto a O.8 M sucrose cushion, and maintained in an isotonic coelomocyte culture medium (0.5 M NaCl, 2.5 mM $MgCl_2$, 1 mM EGTA and 20 mM HEPES pH 7.4). Alternatively, the motile and stationary cell subsets were separated by centrifuging the cells into a two step (O.75 M and 1 M) sucrose cushion. The stationary cells were collected at the interface between the coelomic fluid and the 0.75 M layer, while the motile cells were collected at the interface of the 0.75 and 1 M layers. For western blotting with affinity purified anti-sea urchin egg spectrin antibody (from Dr. David Begg, University of Alberta), lysates from each cell type were loaded onto SDS gels at equivalent protein concentrations as determined by a modified Bradford assay. For immunolocalization, cells were allowed to settle onto poly-L-lysine coated coverslips, fixed in buffer A (300 mM sucrose, 75 mM KCl, 2.5 mM $MgCl_2$, 20 mM PIPES pH 7.0) plus 1% formaldehyde, 0.1% glutaraldehyde, and 0.5% Triton X-100, and then blocked in PBS plus 1% BSA and 2% goat serum. Cells were then labeled with the affinity purified spectrin antibody followed by a fluorophore conjungated secondary antibody. In experiments involving the double labeling of actin and spectrin in cells, monomeric and filamentous actin was localized using a monoclonal anti-actin antibody (clone C4, ICN Biomedicals Inc., Irvine, CA), while filamentous actin was localized using fluorescent phalloidin (Molecular Probes Inc., Eugene, OR). Labeled cells were viewed on a epifluorescent microscope using a Nikon 60 X (N.A. 1.4) planapochromatic objective lens.

Western blotting of motile and stationary cell samples with anti-spectrin indicated that both cells appeared to contain the same isoform of spectrin found in sea urchin eggs. It consists of a 240 kDa alpha subunit and a 235 kDa beta subunit.

Immunofluorescent staining with anti-sea urchin egg spectrin resulted in an array of filamentous-like structures within motile cells (figure 1b). Stationary cells exhibited cytoplasmic perinuclear staining, but lacked spectrin-labeled filaments. The motile cell filaments were periodically stained and appeared to radiate away from the perinuclear region of the cell. The discontinuous nature of the staining suggests that spectrin is associated with some other filamentous protein. Since spectrin is known to be associated with actin filaments in a variety of

cell types, motile coelomocytes were double labeled for spectrin and actin. In experiments involving either actin antibody or fluorescent phalloidin labeling, there appeared to be no clear codistribution between the actin (figure 1a) and spectrin (figure 1b) patterns. Using both actin antibodies and fluorescent phalloidin for double labeling with anti-spectrin was important in terms of interpreting the results. Using phalloidin helps counter the argument that stearic hindrance prevents the simultaneous binding of anti-spectrin and anti-actin molecules, since phalloidin is a relatively small molecule compared to a protein antibody (for example a single actin filament can be double labeled with both fluorescent phalloidin and anti-actin). Second, using the anti-actin helps counter the argument that the spectrin-associated actin may be in a filamentous/oligomeric form that is simply not recognized by phalloidin. Naturally, the possibility remains that spectrin is associated with a non-phalloidin binding form of actin which also cannot bind actin and spectrin antibodies at the same time. However, this interpretation is not supported by labeling cells with actin antibodies alone or sequentially labeling first with anti-actin followed by anti-spectrin. In neither of these cases do the actin antibodies label structures which resemble the spectrin stained filaments.

The results of these experiments suggest that spectrin in coelomocytes may be organized in a novel way. It is generally accepted that spectrin in many cell types is a major constituent of the membrane cytoskeleton, and immunofluorescent localization results in a pattern of peripheral, sub-plasma membrane staining. Spectrin is also known to associate with actin filaments in a number cell types, including in the cortex of early sea urchin embryos (Fishkind et al., 1990. Dev. Biol. 142: 453-464). However, results presented here indicate that spectrin in motile coelomocytes does not associate with the membrane cytoskeleton or with actin filaments. Instead it is associated with cytoplasmic filaments whose identity and function are unknown. Currently we are investigating the possibility that these filaments may correspond to microtubules or intermediate filaments. Microtubules are abundant in motile coelomocytes (Henson et al., 1992. loc cit.), however no evidence exists for the presence of intermediate filaments in these cells. Cytokeratin containing intermediate filaments have been localized in sea urchin oocytes (Boyle and Ernst, 1989. Dev. Biol. 134: 72-84). Our future line of experimentation will include the ultrastructural localization of spectrin in coelomocytes.

(Supported by NIH grant GM47693-01, NSF Young Investigator Award MCB-9257856, and NSF ILI grant USE-9050842.)

Figure 1: Immunofluorescent labeling of actin (panel a) and spectrin (panel b) in a motile sea urchin coelomocyte. Note that the splayed arrangement of spectrin labeled filaments does not codistribute with the parallel arrays of actin filament bundles. Magnification = 1,500X

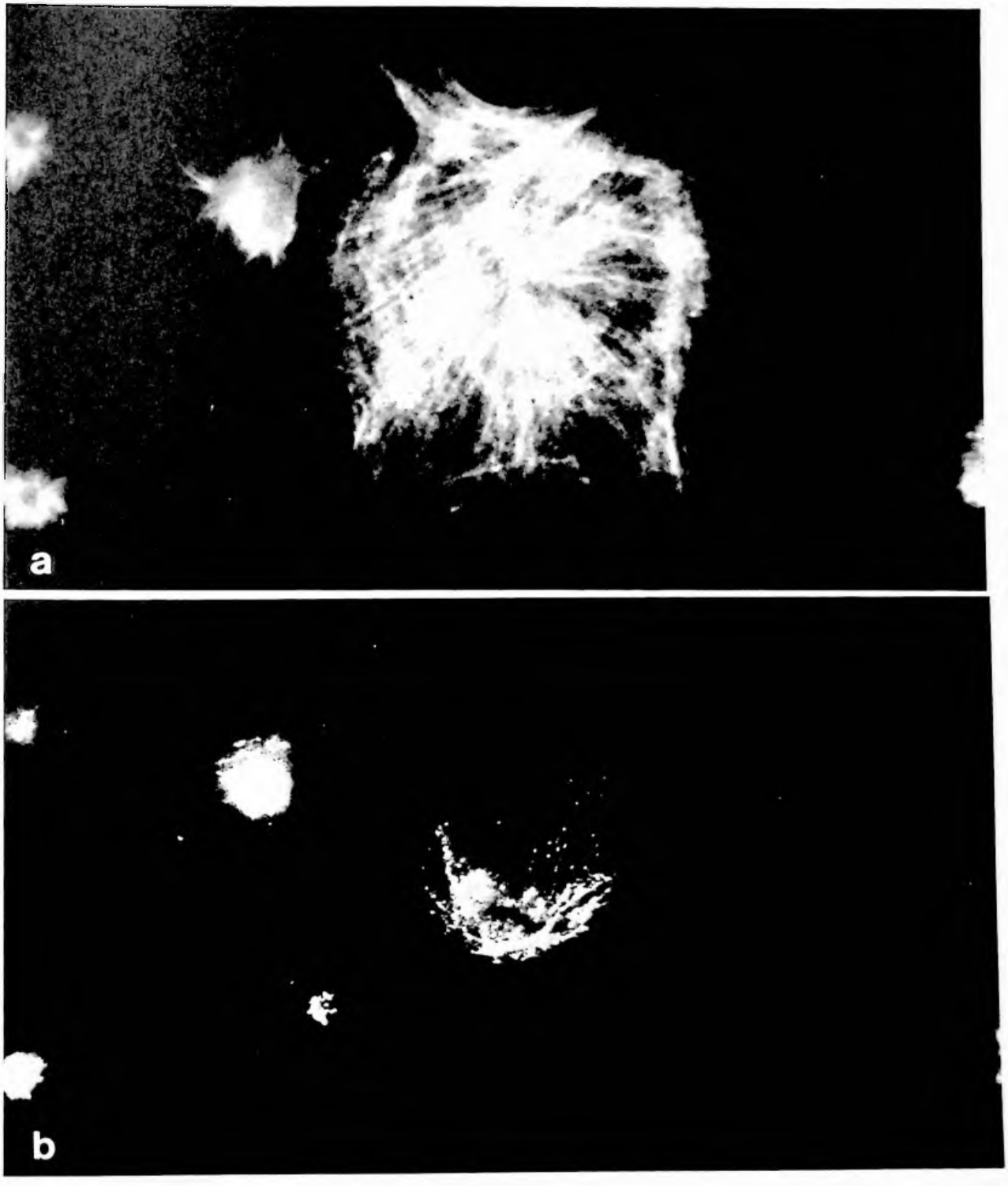

FIGURE 1