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14. ABSTRACT

To date this project has demonstrated that calcium-dependent, membrane-binding proteins of the annexin and copine classes have the potential to stabilize lipid bilayers. These proteins may therefore be promising agents for promoting the repair of damaged cell membranes in the case of spinal cord injury.

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INTRODUCTION

In acute spinal cord injury the plasma membranes of spinal neurons are torn allowing high concentrations of calcium to enter the cytoplasm, activating proteolytic cascades and leading to neuronal cell death. Membrane repair mechanisms have evolved that protect cells from this type of damage by repairing the cell membrane as soon as the increase in intracellular calcium is sensed by calcium-binding proteins (1-3). If these repair mechanisms can be strengthened either before or after spinal cord injury it may be possible to reduce cell damage resulting from the injury. In this project we are testing the hypothesis that the action of copine, a human calcium-dependent-membrane-binding protein, in model systems can promote a stable repair of broken membranes that could preserve cell viability. Preliminary data obtained using a novel imaging technology, atomic force microscopy, suggested that calcium-dependent, membrane-binding proteins of the copine class can repair membranes through direct binding to the edges of torn membranes and promoting sealing of the edges.

BODY OF REPORT

Research accomplishments associated with tasks (Aims) described in the statement of work.

Aim 1. Express human copine as a recombinant protein in yeast and purify.

Human copine I (a close homolog of copine VI expressed in neural tissue) was expressed from a galactose 10 (*GAL10)* -regulated yeast expression plasmid (4) and purified by calcium-dependent binding to phospholipids followed by ion exchange chromatography (Figure 1). Yield was approximately 2 mg per four liter yeast culture. Human annexins I and VI, also calcium-dependent, membrane-binding proteins, were similarly expressed and purified from yeast for comparison with copine in the experiments described below.

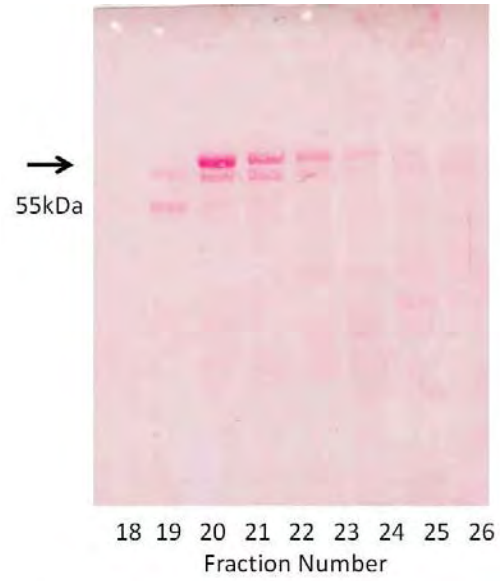

Figure 1: Electrophoretic analysis of purified human copine. Fractions 18 through 26 from the final purification step on a Fast Protein Liquid Chromatography (FPLC) system using the anion exchanger Poros Q were applied to an Sodium Dodecyl Sulfate (SDS) gel. After electrophoretic speparation the gel was blotted to nitrocellulose and the proteins stained with Ponceau S. The prominent band at 55 kilodalton (kDa) is copine; the shadow band slightly lower is believed to be a proteolytic breakdown product of copine.

Aim 2. Examine the association of copine with supported lipid bilayers containing defects by atomic force microscopy (AFM) to assess the ability of the protein to repair the bilayer defects.

A number of methods were tested to reliably prepare damaged membranes as a substrate for these experiments, including mechanical scoring of a supported bilayer with the AFM probe. The most reliable method was found to be limiting the amount of lipid applied to the substrate and reducing the time of incubation after application of the lipid to the substrate.

Clear documentation was obtained that the protein associated with the regions of membrane defect, and, over time tended to repair the defect so that a continuous bilayer was obtained (Figures 2 and 3). However, regions of the disrupted bilayer that had not been extensively imaged with the AFM probe did not exhibit the same extent of repair. This made it apparent that the mechanical action of the probe on the lipid bilayer was contributing to the repair, probably by transporting some lipid into the damaged areas of the membrane. Indeed, very forceful application of the probe was able to repair membranes alone without the addition of copine (Figure 4). Since the AFM could not be used in this fashion as a practical repair tool for damaged spinal cord membranes it became important to establish the assay using lipid vesicles described below (Aim 4) to test the functionality of the protein in membrane repair.

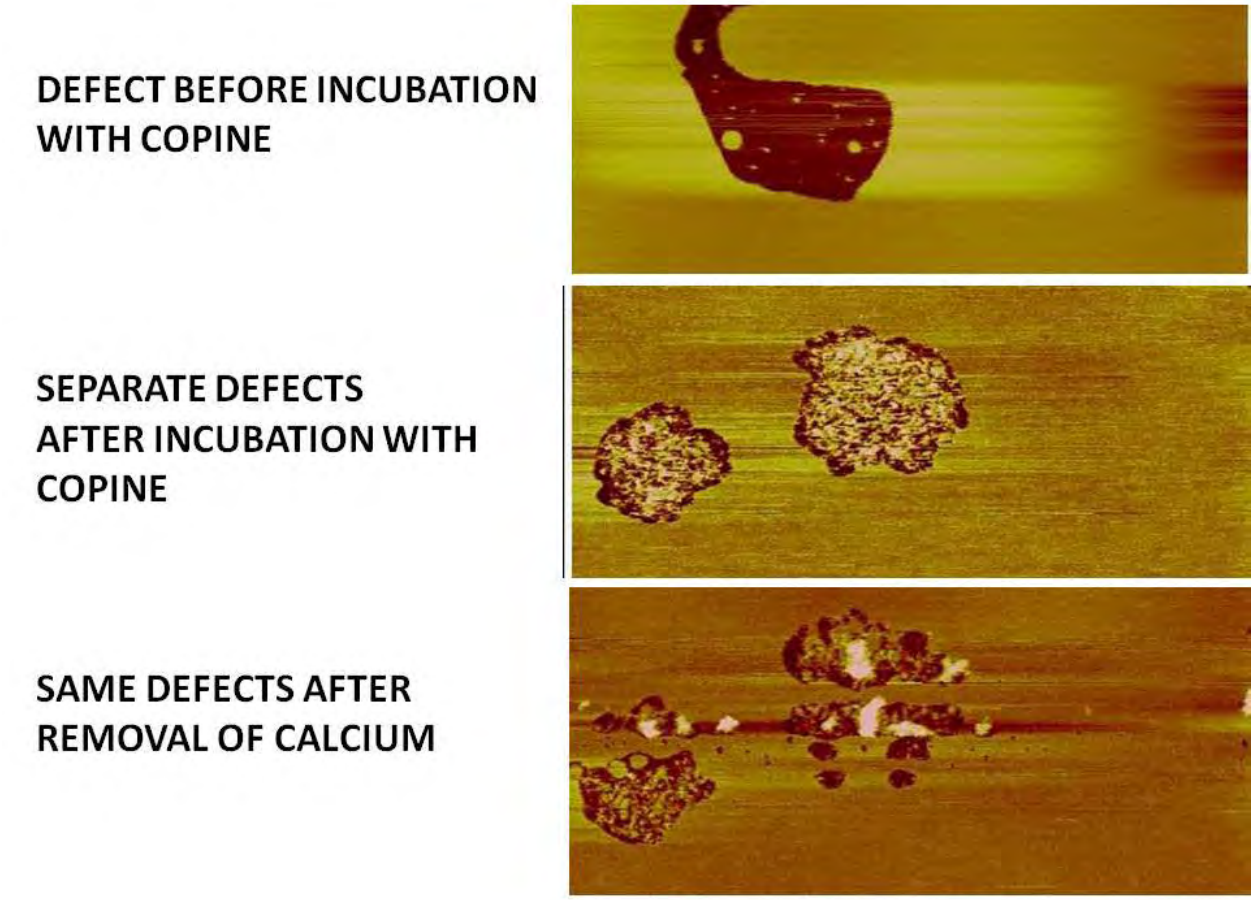

Figure 2: Application of copine to a supported bilayer with defects results in attachment of copine to the defects and formation of a continuous bilayer at the site of the defects.
Top: DEFECT BEFORE INCUBATION WITH COPINE. By limiting the amount of lipid applied to the mica substrate, and the time of incubation, it was found that bilayers with holes (dark areas) in them could be reproducibly formed. Visualization by atomic force microscopy, width of field 3micrometers.

Middle: SEPARATE DEFECTS AFTER INCUBATION WITH COPINE. In a different field of view on the same membrane after incubation with copine for 40 minutes, similar defects are partially filled with material representing both copine and transported lipid.

Bottom: SAME DEFECTS AFTER REMOVAL OF CALCIUM. In order to visualize the membrane underneath the accumulated copine protein, the membrane was incubated in the calcium chelator ethylene glycol tetraacetic acid (EGTA). In the absence of calcium copine is released from the membrane and reveals portions of continuous bilayer partially filling the original holes. The white material is aggregated protein and/or lipid.

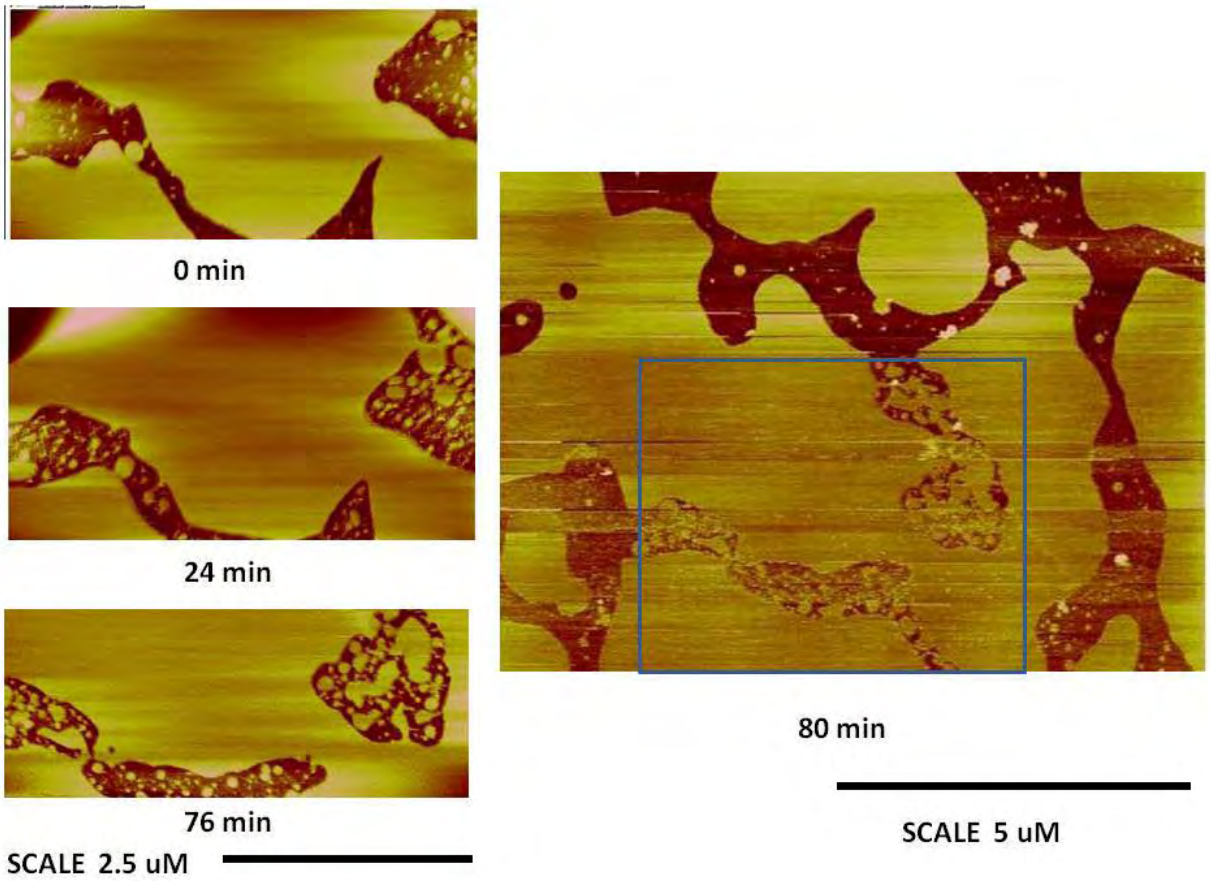

Figure 3: Bilayer repair is accelerated by the addition of lipid vesicles and is dependent on action of the AFM probe. ***0 min****: A bilayer is initially formed with significant defects as in Figure 2.* ***24 min, 76 min:*** *After addition of copine and brain lipid vesicles (Folch fraction I) and incubation for 24 or 76 minutes, new patches of bilayer appear in the membrane holes and defects.* ***80 min:****At 80 minutes after addition of copine and lipid vesicles the field of view is expanded to 10 by 10 micrometers revealing that membrane deposition and repair was dependent on the action of the AFM probe during the imaging (area inside the blue box corresponding to the areas imaged in the left panels).*

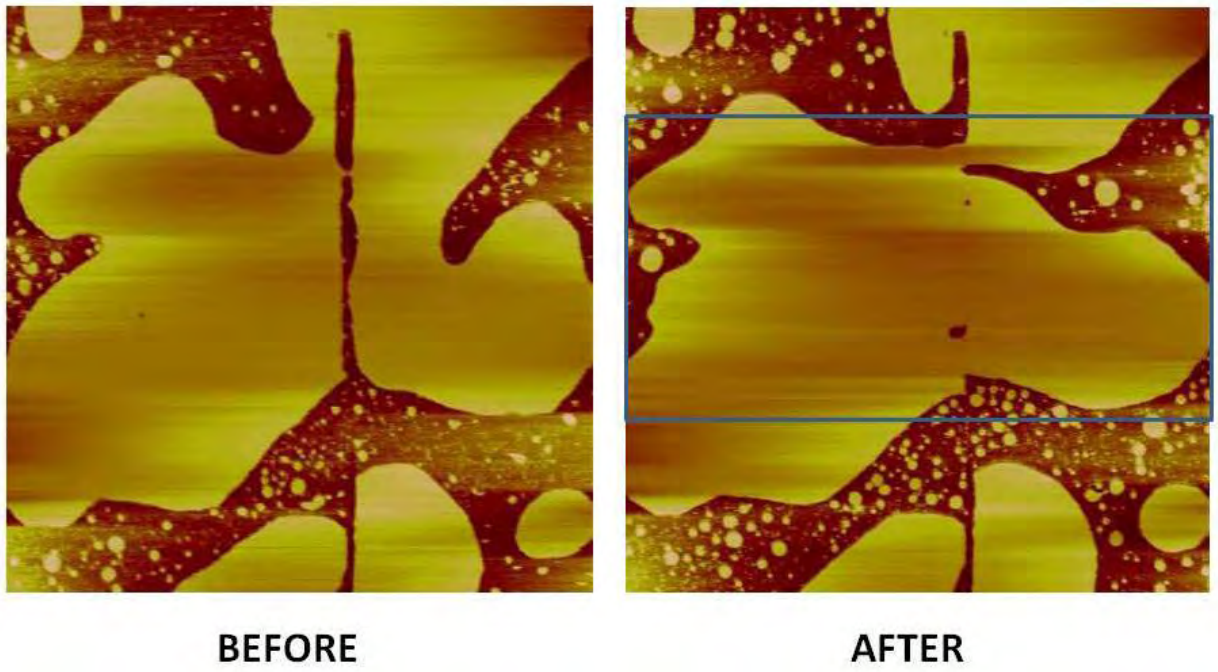

Figure 4:The action of the AFM probe alone can repair broken membranes. ***BEFORE:*** *An incomplete supported bilayer was further damaged by scoring it with the AFM probe in contact mode with high force. This resulted in the formation of the dark vertical wound crossing the bilayer patches. This wound is stable to imaging when the AFM is operated in tapping mode with normal imaging force (i.e., the region on the left was continuously imaged with several passes of the probe and the membrane topography did not change).* ***AFTER:*** *The same area was then subjected to increased force of the tapping probe (too great to permit imaging) in the area outlined by the blue box. In subsequent examination with a normal imaging force, as shown on the right, it is apparent that the vertical wound has been repaired by the action of the probe.*

Aim 3. Determine whether the addition of lipid vesicles would enhance the repair process mediated by copine in supported bilayers.

Evidence was obtained that the addition of vesicles to the medium over the bilayer resulted in greater transport of lipid into the regions of broken membrane. This is also shown in Figure 3 above. However, as in the case described above without vesicles, it was apparent that the AFM probe was contributing significantly to the repair process.

Aim4. In order to verify that true membrane sealing has been promoted by copine, a complementary assay will be developed based on a measure of the ability of the protein to reseal large unilamellar vesicles after rupture of the vesicles by osmotic shock.

A model system was developed using Large Unilamellar Vesicles (liposomes, or LUV's) prepared by extrusion through polycarbonate filters of defined pore size (5). Using a pore size of 100 nm, vesicles with an average diameter of 100 nm were obtained. Such vesicles are stable, have a modest curvature typical of cell organelles, and a large capacity for capture of carboxyfluorescein at self-quenching concentrations. In order to approximate the lipid composition of biological membranes we used mixtures characteristic of the cytoplasmic face of cell membranes – containing phosphatidylcholine (PC),

phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and cholesterol. To perform the permeability studies, carboxyfluorescein (CF; molecular weight 376 dalton) was encapsulated in the liposomes during their formation at a self-quenching concentration (100 mM) (6). Leakage of carboxyfluorescein was detected as an increase in the (unquenched) fluorescence over time (excitation at 495nm, emission at 525nm). Leakage experiments were performed at 37 degrees in 150mM KCl, 25 mM HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) -NaOH, pH 7.4, 5 mM $MgCl_2$, and 1mM $CaCl_2$.

In the experiment illustrated here osmotic shock of lipid vesicles led to transient breakage of the membrane and this was accompanied by a large burst of release of carboxyfluorescein. The presence of annexin A6 was found to significantly reduce leakage due to this stress.

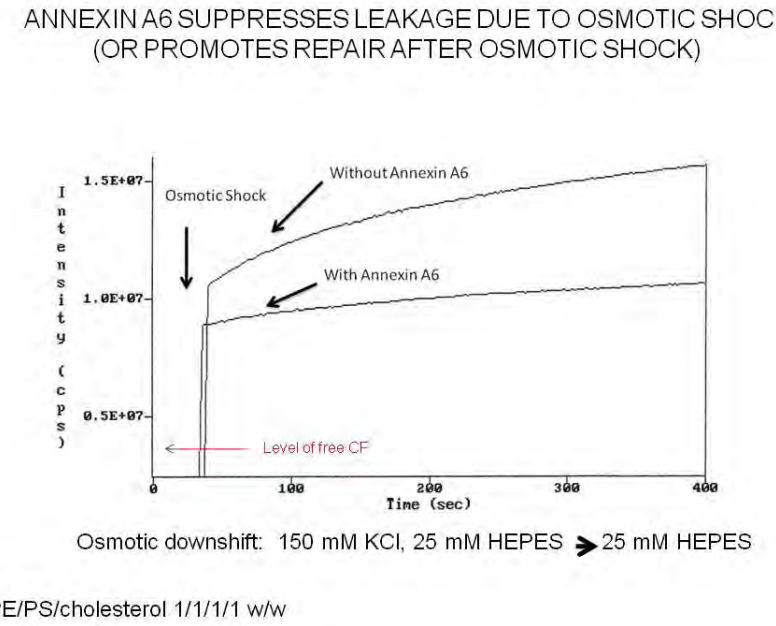

Figure 5: Carboxyfluorescein-loaded vesicles were diluted into a medium without osmotic support (containing only 25 mM HEPES-NaOH, 5mM $MgCl_2$, 1 mM $CaCl_2$, but no KCl). This caused a burst of release of carboxyfluorescein (at 40 sec). The initial baseline fluorescence due to unencapsulated carboxyfluorescein (determined in a separate experiment) was 0.3 X 10^7 CPS (photomultiplier Counts per Second). In the absence of annexin A6 the fluorescence intensity increased to 1.1 X 10^7 CPS due to partial osmotic lysis and subsequent resealing of the vesicles. If annexin A6 (11.7 ug/ml) is present the fluorescence increases only to 0.8 X 10^7 CPS. The annexin A6 apparently bound to the liposome membrane and inhibited lysis, or accelerated repair after lysis so that 37.5% less carboxyfluorescein escaped. Subsequent to the lysis the rate of leakage, indicated by the slopes of the curves at 300 to 400 sec, is reduced by 65.6%.

Although it is possible the annexin made the vesicles more resistant to osmotic lysis, we think it is more likely the annexin prevented membrane tears from becoming more extensive or promoted their resealing. This is because the osmotic shock would be expected to occur on a faster time scale than the diffusion and binding of the annexin to the vesicles.

Using this liposome assay, the annexin was also found to be able to prevent or repair disruption of membranes due to lysophosphatidic acid or lipid peroxidation by hydrogen peroxide (see Figures 3 and 4 of the appended Preliminary Patent Application).

Similar studies are now being conducted with human copine to determine the ability of this protein to similarly promote membrane repair after osmotic lysis.

Aim 5: In order to test whether copine can repair cell membranes in a living cell system the protein will be expressed in cultured neuronal cells. The cell membranes will be damaged by electroporation or mechanical injury and cell viability after injury compared to control cells not expressing copine.

As a prelude to these planned experiments we have taken advantage of the expression of copine and annexins in our yeast expression system and have designed protocols for mechanical (sonication) or electroporation disruption of the yeast cell membranes. These preliminary studies will then be extended to neuronal cell cultures after the construction of appropriate expression vectors.

KEY RESEARCH ACCOMPLISHMENTS

1. Establishment of yeast expression systems for human copine and annexins.
2. Documentation of the ability of copine to repair supported lipid bilayers by atomic force microscopy.
3. Determination that the repair was influenced by the interaction with the atomic force microscope probe.
3. Establishment of an unsupported bilayer system consisting of liposomes for study of membrane repair.
4. Demonstration that annexin A6 promotes repair of liposome membranes subjected to osmotic shock.

REPORTABLE OUTCOMES

U.S. Provisional Patent Application Serial No. 61/467,140
Filed on March 24, 2011
Title: Compositions and Methods for Maintaining and Repairing Membranes
Reported to Edison on 3/8/11 under invention report #1526401-11-0016

An abstract is in preparation for presentation of the Key Research Accomplishments at the Annual Meeting of the Biophysical Society in February, 2012.

CONCLUSION

To date this project has demonstrated that calcium-dependent, membrane-binding proteins of the annexin and copine classes have the potential to directly repair or stabilize lipid bilayers. They may therefore be promising agents for repairing damaged cell membranes in the case of spinal cord injury.

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