Vol. 194, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1993 Pages 490-495

DETECTION OF ELECTROPHORETIC VARIANTS OF *Notch*, PS INTEGRIN, AND DROP-1 PROTEINS IN *Drosophila* FOLLOWING EXTRACTION IN GUANIDINE HYDROCHLORIDE

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Received May 24, 1993

SUMMARY: A method is presented for the rapid extraction of proteins from *Drosophila* tissues. This method involves lysis of embryos in high concentrations of guanidine hydrochloride, followed by ultracentrifugation in a guanidine hydrochloride step gradient. Several membrane-associated antigens, including *Notch* and the ß subunit of PS integrin are enriched in this preparation. The quantity of the proteoglycan, DROP-1, obtained from *Drosophila* eggs and testes was also greatly improved by the guanidine hydrochloride extraction method. This method should prove useful in the isolation and characterization of many *Drosophila* antigens, particularly those associated with cell membranes.

Recent genetic and cell biological studies in *Drosophila melanogaster* have led to the identification of a large number of genes and proteins central to processes of embryonic development. While many of these proteins can be followed with antibody probes, biochemical analysis of these proteins is often hindered by proteases prevalent in *Drosophila* embryos and tissues (1). In this paper, we present an extraction procedure for *Drosophila* eggs and tissues which leads to the separation and identification of polypeptides not previously described. This method, adapted from a method for purifying mammalian desmosomal membranes (2), utilizes the powerful denaturant and chaotropic agent guanidine hydrochloride not only for cell lysis, but also as a medium for buoyant density ultracentrifugation. We find that an embryo fraction that reaches buoyant density equilibrium in a guanidine hydrochloride step gradient is also enriched for several proteins, including the membrane markers *Notch* (3) and PS integrin α- and ß-subunits (4). These polypeptides appear in forms not previously described: The *Notch* protein appears to be full length, while the PS integrin ß subunit is primarily in a 49 kD form. Low molecular weight forms

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Abbreviations used: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; PMSF, phenyl methyl sulfonyl fluoride; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; TBST, 30 mM Tris-Cl,pH 7.5, 20 mM NaCl, 0.1% Tween-20; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, Nitro Blue Tetrazolium; Mabs, monoclonal antibodies; anti-DROP-1, monoclonal antibody that recognizes a high molecular weight heparan sulfate proteoglycan in *Drosophila* embryos.

0006-291X/93 $4.00

of the *Drosophila* proteoglycan, DROP-1, are also greatly enriched in guanidine hydrochloride extracts. This method could prove useful in the analysis of many *Drosophila* antigens.

MATERIALS AND METHODS

Fly Stocks and Culture. Wild type *Drosophila melanogaster* (Oregon R strain) were grown under standard conditions in a temperature controlled environment at 25° C in plexiglass population cages (40cm x 40cm x 40cm) containing approximately 20,000 flies per cage. Flies were fed on grape juice/Cream of Wheat (Nabisco) food plates supplemented with yeast.

Preparation of Embryo Extracts. Embryos from overnight collections were harvested from food plates, collected onto nylon mesh screens, blotted dry and weighed. Three grams of embryos were transferred to a pre-chilled glass-teflon homogenizer and homogenized with 4-5 strokes of a motor-driven pestle in buffer 6G (6 M guanidine hydrochloride [Ameresco biotechnology grade, 99% pure], 0.1 M citric acid/sodium citrate, pH 2.6) at a ratio of 6 ml buffer per gram of embryos. This homogenate was centrifuged at 10,000 rpm (12,000 x g) in a SS-34 rotor at 4° C for 10 min. The supernatant (designated "primary supernatant") was filtered through Miracloth (Calbiochem), and was then evenly distributed into 13 ml polyallomer ultracentrifuge tubes (Sarstedt). The tube contents were then overlaid with buffer 3G (3 M guanidine hydrochloride, 0.1 M citric acid/sodium citrate, pH 2.6) to about 0.5 cm from the top of each tube. The tube contents were then overlaid with buffer 0G (0.1 M citric acid/sodium citrate, pH 2.6) to the top of each tube. The samples were then centrifuged at 40,000 rpm (270,000 x g) for two hours or 30,000 rpm (154,000 x g) overnight at 4° C in an SW 41.Ti rotor in a Beckman L5-50 ultracentrifuge.

Following centrifugation, off-white material banding just above the interface between the 3G and 6G layers (designated "primary buoyant material") was collected and combined, and diluted with approximately 5 volumes of buffer 8G (8 M guanidine hydrochloride, 0.1 M citric acid/sodium citrate, pH 2.6). This was distributed between 4 ultracentrifuge tubes. Each tube was layered with buffers 3G and 0G as above, and the centrifugation repeated. This step resulted in material (designated "membrane fraction") banding near the interface of the buffer 3G and buffer 8G layers.

Samples from all three fractions (primary supernatant, primary buoyant material, and membrane fraction) were dialyzed against a large volume of dialysis buffer (1 mM EDTA , 1 mM EGTA, 10 mM CAPS pH 10.0) overnight at 4°C. The buffer was replaced twice over the course of the dialysis. In some preparations, the protease inhibitor PMSF was added to the dialysis bag, but this practice was discontinued in later experiments.

Dialyzed samples were pelleted by centrifugation at 30,000 rpm (approx. 60,000 x g) in a TLS-55 rotor in a Beckman TL-100 tabletop ultracentrifuge for 30 min. at 4°C. The supernatants were discarded, and pellets were resuspended in membrane buffer (1 mM EDTA, 1 mM EGTA, 10 mM HEPES pH 7.4).

"Standard" embryo extractions: Embryos from overnight collections were harvested as described above. Three grams of embryos were transferred to a pre-chilled glass-teflon homogenizer and homogenized in 3 ml PBS buffer with ten strokes of a motor-driven pestle. The homogenate was clarified by centrifugation in a Beckman TL-100 ultracentrifuge at 70,000 rpm (200,000 x g) for 10 min. at 2° using a TLA-100.2 rotor. The resulting supernatant was transferred to new tubes and centrifuged in the same rotor at 100,000 rpm (approx, 400,000 x g) for 20 min. at 2°. This supernatant was made to 50% in ammonium sulfate and the precipitated proteins pelleted by centrifugation and dialyzed exhaustively against PBS.

Preparation of Testis Extracts. Testes from *Drosophila* males were hand-dissected into buffer 6G, and homogenized with a loose-fitting pestle in a 1.5 ml microfuge tube. Samples were pelleted in a microfuge for 15 min. The pellets were discarded, and the supernatants were dialyzed and pelleted in the microfuge. The pellets were discarded, and the supernatants were used in subsequent experiments.

"Standard method" testis extraction consisted of hand dissection of testes from young *Drosophila* males into *Drosophila* Ringers (5) and homogenization with a loose-fitting pestle in a 1.5 ml microfuge tube. Samples were pelleted in a microfuge spun at full speed for 15 min. The pellets were discarded, and the supernatants were used in subsequent experiments.

SDS-PAGE and Western Blot Analysis. Samples were analyzed by SDS-PAGE as described by Laemmli (6) utilizing 5%-15% linear gradient gels. Gels containing up to 10 μg of protein per lane, as determined by the BCA method, (Pierce Chemical Co.) were either stained with Coomassie Brilliant Blue or electrotransferred to nitrocellulose membranes by the method of

Towbin et al. (7). Total protein on blots was visualized by reversibly staining with Ponceau Red. Prestained molecular weight markers (BioRad) were used as molecular size standards.

Blots were probed with mouse monoclonal antibodies (MAbs) from either hybridoma culture supernatants or ascites fluids at dilutions described in figure legends. MAb HC1 to the *Drosophila* PS integrin α2 subunit and MAb Dx4C8.67-4 to the *Drosophila* PS integrin ß subunit (4) were generous gifts from Dr. Danny Brower, University of Arizona. MAb C17.9C6 to the intracellular domain of *Notch* protein (8) was a generous gift from Dr. Richard Fehon, Yale University. Anti-DROP-1, a monoclonal antibody which recognizes the *Drosophila* proteoglycan DROP-1, was produced in the Karr laboratory. Antibody dilutions were made in TBST.

Nitrocellulose membranes were first treated with blocking solution (5% non-fat dry milk [Carnation] in TBST) for 20 min., after which the membranes were rinsed for 3 X 5 min washes in TBST. The membranes were then incubated in primary antibody solutions for 30-60 min, followed by 3 X 5 min washes in TBST. The membranes were then incubated with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Chemicon International) at 1:2500 dilution in blocking solution for 30-60 min., followed by the TBST rinses. Alkaline phosphatase activity was detected utilizing the chromogenic substrates BCIP and NBT (Research Organics Inc.) made in alkaline phosphatase substrate buffer (0.45 M NaCl, 5 mM $MgCl_2$, 0.1 M Tris-Cl pH 9.5).

Electron Microscopy. For transmission electron microscopy, small samples were pelleted in a microfuge, fixed for 1 hr. at ambient temperature in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate pH 7.2. After transfer to BEEM capsules, samples were post-fixed in buffered 1% OsO_4 for 1 hr, and were then stained *en bloc* with 2% uranyl acetate for 1 hr. Samples were dehydrated in an alcohol series and embedded in an Epon/Araldite mixture. Silver sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined in a Phillips 400 transmission electron microscope.

RESULTS AND DISCUSSION

Comparison of total proteins in the primary supernatant (Fig 1, lane a) with those in the final membrane fraction (Fig 1, lane b) reveals that numerous proteins are enriched in the final membrane fraction. Arrows in lane b indicate examples of prominent bands in the final membrane fraction that are present at reduced amounts in the primary supernatant. The most prominent band, migrating at an apparent mol. wt. of approximately 45 kD, could be the yolk proteins. Protein quantification of fractions indicate the amounts of protein obtained starting from 3 grams of embryos were, in one typical experiment: 19.3 mg in primary supernatant; 3.2 mg in the primary buoyant material; and 1.2 mg in the final membrane fraction.

Several monoclonal antibodies were used to compare the three stages of separation in the guanidine extraction procedure using Western blot analyses as shown in Figure 2. A MAb directed against the *Drosophila* PS α2-integrin reveals the presence of two high molecular weight (157 kD and 143 kD) forms of the protein in the primary supernatant (Fig 2, lane a). The primary buoyant fraction contains only the 143 kD high mol. wt. form, but also a band migrating at approximately 57 kD (Fig 2, lane b). In the final membrane fraction, the 142 kD band is clearly visible, and minor amounts of the 57 kD form are detected (Fig 2, lane c).

The three fractions probed using a MAb directed against *Drosophila* PS ß-integrin gave surprisingly different results. A very prominent, broad band is detected in the primary supernatant at 118 kD (Fig 2, lane d), along with several minor bands of lower molecular weight. In the primary buoyant material (Fig 2, lane e), the 118 kD band is greatly diminished, and a minor band at 49 kD is observed. The final membrane fraction contains only trace levels of the 118 kD form which is replaced by a strong immunoreactive band at 49 kD (Fig 2, lane f).

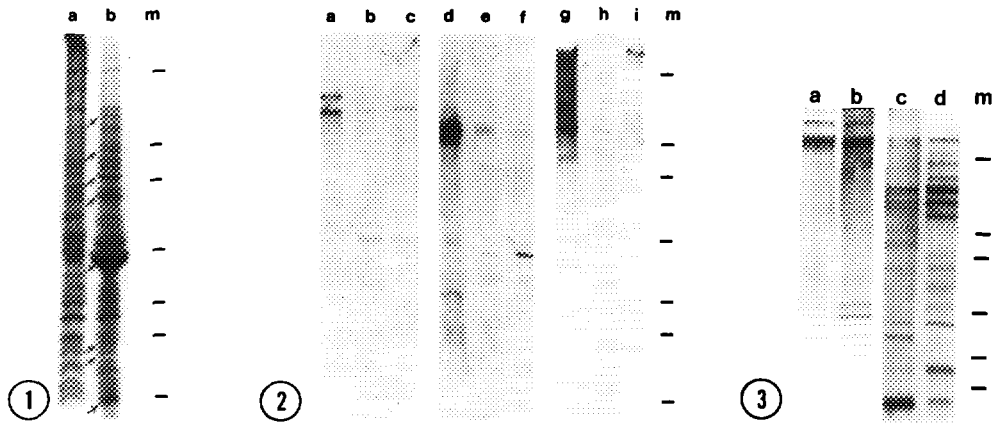

Figure 1. Coomassie Brilliant Blue staining of a) primary supernatant and b) final membrane fraction of *Drosophila* embryos extracted by the guanidine hydrochloride method, 10 µg /lane. Arrows indicate examples of polypeptides enriched in the final membrane fraction. Molecular weight standards are indicated from top to bottom in lane m: myosin, 205 kD; phosphorylase B, 106 kD; bovine serum albumin, 80.0 kD; ovalbumin, 49.0 kD; carbonic anhydrase, 32.5 kD; soybean trypsin inhibitor, 27.5 kD; lysozyme, 18.5 kD.

Figure 2. Western blot analysis of *Drosophila* embryo fractions with monoclonal antibodies. a, d, g: primary supernatant, 10 µg/lane; b,e,h: primary buoyant material, approx. 7 µg/lane; c,f,i: final membrane fraction, 10 µg/lane. Antibody probes: a, b, c: anti-*Drosophila* PS integrin α subunit hybridoma supernatant diluted 1:50; d, e, f: anti-*Drosophila* PS integrin ß subunit ascites fluid diluted 1:2000; g, h, i: anti *Notch* protein hybridoma supernatant diluted 1:10. m: molecular weight standards, as in Figure 1.

Figure 3. Western blot analysis of embryos and tissues probed with the anti-DROP-1 Mab. a: embryos extracted by "standard method" (12 µg loaded). b: primary supernatant fraction of embryos extracted by the guanidine hydrochloride method (4 µg loaded). c: testes extracted by "standard method" (2 µg loaded). d: testes extracted by guanidine hydrochloride method (2 µg loaded). m: molecular weight standards, as in Figure 1.

A MAb directed against the intracellular domain of *Drosophila Notch* protein (8) reveals the separation of apparent full-length *Notch* protein (9) in the final membrane fraction. In the primary supernatant (Fig 2, lane g), a ladder of proteins similar to that seen with other extraction procedures (10) is observed. Only a faint band at ≈120 kD, is found in the primary buoyant material (Fig 2, lane h). The final membrane fraction (Fig 2, lane i) contains one prominent band at ≈300 kD, corresponding to the top band in the protein ladder. Thus, enriched fractions that apparently represent full length *Notch* protein can be obtained using this extraction and separation procedure.

A MAb directed against a widely distributed *Drosophila* proteoglycan, DROP-1, (M.G. and T.L.K, manuscript submitted) was used to compare guanidine extracts to extracts generated by the standard method. Shown in Figure 3, the primary supernatant fraction from embryos (Fig 3, lane b) contains several immunoreactive bands: a triplet around 350-250 kD; and single bands at approximately 53 kD and 47 kD. In marked contrast, standard embryo extracts do not contain the lower molecular weight doublet (Fig 3, lane a), and shows comparatively less of the higher molecular weight forms (note, guanidine extracted sample is loaded at approximately one-third the amount of the standard extract). Likewise, primary supernatant fractions of *Drosophila* testes extracted in guanidine (Fig 3, lane d) contain a variety of different immunoreactive bands when compared to the standard extraction (Fig 3, lane c).

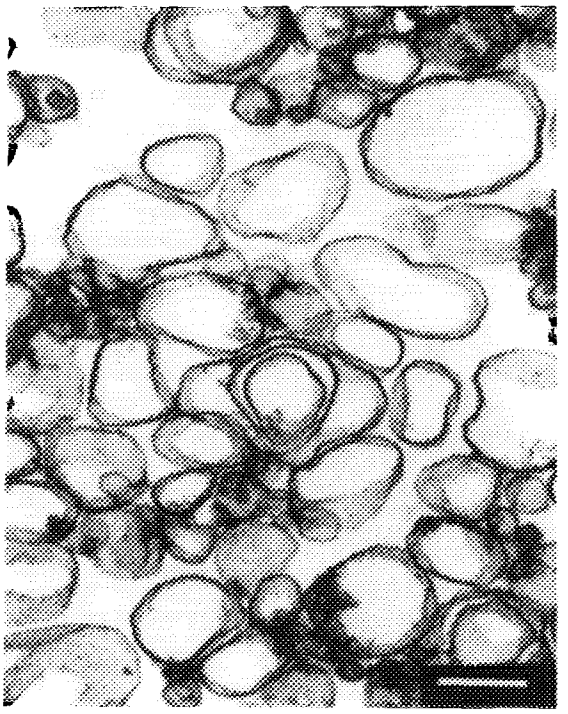

Figure 4. Electron micrograph of final membrane fraction, prepared as described in text. Membrane appears primarily as vesicles, with little evidence of other cell components. Scale bar, 0.2 microns.

Guanidine extraction has previously been used successfully to purify desmosomal membranes from mammalian sources (2). To determine whether guanidine extraction of *Drosophila* embryos yielded a similar enrichment in membranes, we examined the final membrane fraction by electron microscopy. As shown in Figure 4, the final membrane fraction is highly enriched in vesicular material, containing almost exclusively classical lipid bilayer structures. We therefore conclude that the guanidine extraction method is an efficient means for the rapid separation of membranes and membrane associated proteins in *Drosophila*.

Guanidine extraction seems to eliminate the need to use protease inhibitors. Extractions performed with or without the protease inhibitor PMSF present give identical results with any of the antibodies used (data not shown). A simple, plausible explanation for the presence of the 47 kD and 53 kD forms of the DROP-1 antigen in guanidine hydrochloride extracts of embryos, but not in "standard" extracts, is that embryonic proteases are irreversibly denatured by guanidine hydrochloride, but remain active in the "standard" extracts. This latter observation raises the possibility that these smaller forms of the DROP-1 antigen represent either core proteins of the proteoglycan that are not protected from proteolysis by oligosaccharides, or minimally glycosylated forms, or both.

The data presented here demonstrate the potential usefulness of the guanidine extraction procedure for generating greater amounts, and, in some cases novel separations, of antigens found in *Drosophila* embryos. Extraction in guanidine has led to the identification of new forms of integral membrane proteins including apparent full-length *Notch* protein and lower mol. wt. forms

of Drosophila PS-antigens in the final membrane fraction (Figure 2). Previously undetected lower mol. wt. forms of a recently identified proteoglycan in Drosophila, DROP-1, is also observed only upon extraction in guanidine.

The "final membrane" fraction appears, by electron microscopy, to consist of membranes without other cell structures apparent. Guanidine hydrochloride, being both a strong denaturant and salt would be expected to strip away non-integral proteins. Also, due to the high concentrations of guanidine used, the majority of soluble proteins, including most proteases, are either "salted out" or denatured which perhaps explains the lack of significant proteolysis.

The guanidine hydrochloride extraction method presented in this paper should prove useful for immunoblot analysis of other embryo and adult tissue antigens. Although many proteins (including enzymes) are likely to be irreversibly denatured by this method, antigenicity will probably be maintained, facilitating the identification and isolation of other membrane associated proteins in the *Drosophila* embryo.

Acknowledgments: We are indebted to Steven Miklasz at the Hybridoma Center for his expert technical assistance in the production and maintenance of hybridoma lines used in this work. This work was supported by the generous support of the Beckman Institute for Advanced Science and Technology and the Medical Research Council of Canada (to SLZ). SLZ is a Scholar of the Alberta Heritage Foundation for Medical Research.

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