Identification of Cytoskeletal, Focal Adhesion, and Cell Adhesion Proteins in Growth Cone Particles Isolated From Developing Chick Brain

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Growth cones are intimately involved in determining the direction and extent of neurite elongation during development. They are able to monitor their environment and respond to it by undergoing directed motility. We have isolated a fraction enriched in growth cone particles from embryonic chick brain. Assayed by immunoblots, this fraction is enriched in GAP-43, and contains the cytoskeletal proteins actin, myosin II, neurofilament protein, tubulin, kinesin, and dynamin. All of the major components of focal adhesions are also present: alpha-actinin, vinculin, talin, and integrin. In addition to integrin, we also identify the cell adhesion molecules A-CAM, L1, fibronectin, and laminin in these particles. This preparation of isolated growth cone particles may be a useful model system for studying growth cone adhesion and motility.

Key words: growth cones, cytoskeleton, cell adhesion

INTRODUCTION

The leading tips of neurites, the growth cones, are versatile and dynamic structures. They are specialized to perform two functions essential to neurite growth and neuronal regeneration. First, they perceive their environment and detect changes in it. Second, they respond appropriately to their environment and use that information to undergo directed motility. While performing these sensory and motile functions, growth cones transduce extracellular signals into changes in cytoskeletal organization and function.

Many studies have examined the behavior of growth cones and the distributions of known cytoskeletal components within them. More recently, attention has turned to the proteins that interact directly with the extracellular substrate; the cell adhesion molecules, and their receptors on and within growth cone membranes. This study reports the first demonstration that proteins found in focal adhesions, and extracellular cell adhesion proteins, are present in isolated growth cone particles (GCPs).

As they move, growth cones extend filopodia and lamellipodia, which make adhesive contacts with various substrates, such as other cells and the extracellular matrix. Differences in the adhesiveness of these contacts with different substrates influence the direction of motility related events, but it is also through these adhesive contacts that growth cones can exert mechanical force on the substrate. Although the transmembrane linkage between substrates and the cytoskeleton has been well studied (reviewed in Burridge et al., 1988), the details of the interactions between substrates and cytoskeletal proteins remain unclear.

The major components of focal contacts are actin filament bundles and the actin-associated proteins alpha-actinin, vinculin, and talin. Alpha-actinin can bind directly to and cross-link actin filaments (Bennett et al., 1984). It has also been shown to bind vinculin (Otto, 1983; Wilkins et al., 1983), which in turn binds with high affinity to talin (Burridge and Mangeat, 1984). The reported binding of talin to the transmembrane, cell adhesion protein integrin, however, occurs with relatively low affinity (Horwitz et al., 1986). It has recently been reported that alpha-actinin can bind directly to integrin (Otey et al., 1990). While these results suggest several viable models for the transmembrane linkage of the cytoskeleton with extracellular substrates, it is possible that additional interactions and proteins are involved.

One suitable preparation in which to study these interactions is a homogenous population of isolated, purified growth cones. While it has not proven feasible

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to obtain populations of growth cone particles from a single type of neuron, there are two published procedures for isolating fractions enriched in GCPs from developing brain (Pfenninger et al., 1983; Gordon-Weeks and Lock-erbie, 1984). Both procedures yielded subcellular fragments that were initially characterized as being enriched in growth cones by morphological criteria. In addition, radiolabeled growth cones microdissected from neuronal explant cultures copurified with GCPs from fetal rats (Pfenninger et al., 1983).

This initial characterization has been reinforced by demonstrations that these fractions are enriched in particular proteins that have been shown to be concentrated in growth cones by immunofluorescence. For example, the 5B4 antigen (Wallis et al., 1985), growth associated protein-43 (GAP-43) (Meiri et al., 1986, 1988), and the protooncogene product pp60c-src (Maness et al., 1988) have all been shown to be concentrated in growth cones by immunofluorescence, and all are enriched in isolated GCPs.

We have slightly modified the procedure of Pfenninger et al. (1983) to obtain a fraction from developing chick brain that is enriched in GCPs. Here we characterize this fraction with respect to its cytoskeletal proteins, proteins associated with focal adhesions and cell adhesion molecules. We report for the first time that isolated GCPs contain myosin II, the high molecular weight neurofilament protein, and two proteins that interact with microtubules, kinesin and dynamin. In addition, we find all of the major components of focal adhesions and several cell adhesion molecules. These results suggest that this preparation may be a useful system with which to study the interactions between these components.

MATERIALS AND METHODS

Materials

Electrophoresis and blotting reagents were obtained from Bio-Rad (Richmond, CA) and ICN (Cleve-land, OH). The BCA protein assay reagent was obtained from Pierce (Rockford, IL). All other chemicals were from Sigma (St. Louis, MO). Controlled-pore glass (CPG03000, 300 nm mean pore size) was obtained from Electro-Nucleonics (Fairfield, NJ).

Antibodies

Antibodies were generously provided from the following sources: monoclonal anti-GAP-43, Dr. P. Skene, Stanford University; rabbit anti-actin, Dr. J. Scholl-meyer, Roman L. Hruska Lab, NE; monoclonal antibodies against chick brain myosin II, Dr. A.H. Conrad, Kansas State University; monoclonal antibodies to the high molecular weight neurofilament protein, Dr. G. Shaw, University of Florida; monoclonal anti-beta-tubulin, Amersham (Arlington Heights, IL); rabbit antikinesin, Dr. J.R. McIntosh, University of Colorado; rabbit anti-dynamin, Dr. R.L. Margolis, Fred Hutchinson Cancer Research Center, WA; rabbit anti-alpha-actinin and anti-talin, Dr. K. Burridge, University of North Carolina; anti-vinculin and anti-A-CAM, Dr. B. Geiger, Weizman Institute, Israel; antibodies against the beta 1 integrin subunit, Dr. S. Johansson, University of Uppsala, Sweden, and Dr. A. Horwitz, University of Illinois; anti-L1, Dr. V. Lemmon, University of Pittsburgh; mouse anti-fibronectin, Dr. L. Furcht, University of Minnesota; anti-laminin, Dr. S. Palm, University of Minnesota. Secondary antibodies were from Promega (Madison, WI).

Growth Cone Particle Isolation

GCPs were isolated after Pfenninger et al. (1983). All procedures were done at 4°C. Whole brains from E13–E14 chick embryos were homogenized in six volumes of buffered sucrose (320 mM sucrose, 2 mM EDTA, 0.02% NaN3 and 5 mM HEPES, pH 7.3) with 1 μg/ml aprotinin, 1 μM pepstatin, and 0.1 mM phenylmethanesulfonyl fluoride. This crude homogenate was spun at 1,300g for 15 min. The supernatant from this low speed spin was layered over buffered sucrose (as above but with 750 mM sucrose) and spun at 150,000g for 60 min in a swinging bucket rotor. The material at the interface of this discontinuous sucrose gradient was removed and made 0.1 mM in phenylmethanesulfonyl fluoride. This interface fraction was then chromatographed on a controlled-pore glass (CPG) column equilibrated with a modified Krebs’ buffer (145 mM NaCl, 5 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 0.02% NaN3, and 5 mM HEPES, pH 7.3) (Gordon-Weeks and Lock-erbie, 1984). Fractions were monitored by absorbance at 280 nm. GCPs are found in the first peak off the column. When required, they were concentrated from pooled fractions by centrifugation at 84,000g for 30 min and resuspended in modified Krebs’ buffer.

Other Methods

Protein concentrations were measured with the Pierce BCA protein assay reagents using BSA as a standard. SDS-PAGE (Laemmli, 1970) was performed on 7 or 7.5% gels. Gels were either stained according to Neu-hoff et al. (1988) or blotted to nitrocellulose after Towbin et al. (1979) with 0.1% SDS in the transfer buffer. Blots were washed with, and antibody incubations performed in, Tris buffered saline (150 mM NaCl, 0.02% NaN3, and 10 mM Tris, pH 8.0) with 0.05% Tween 20. Alka-line phosphatase conjugated secondary antibodies were used according to the provider’s instructions.
RESULTS

We have isolated and begun to characterize a fraction from embryonic chick brain that is enriched in GCPs. Following an existing procedure (Pfenninger et al., 1983; see also Gordon-Weeks and Lockerbie, 1984), with slight modifications, we consistently obtain a uniform population of spherical, membrane-bound particles that are 1.5 to 2.0 μm in diameter. These are found in the first peak off the last step of the isolation procedure, chromatography on a CPG column. This preparation routinely yields approximately 0.5 mg of GCPs per gram (wet weight) of embryonic chick brain. When allowed to settle onto polyornithine-coated coverslips, these particles adhere firmly, flatten, and assume irregular shapes (Fig. 1).

An electrophoretic analysis of the polypeptide composition of all the steps in the isolation procedure is shown in Figure 2. In the gel shown here, GCPs from the first peak off the CPG column were concentrated by centrifugation for gel analysis. Under the conditions used, 94% of the protein in this peak is pelleted, indicating that it is indeed found in a particulate fraction.

The Coomassie stained gel in Figure 2 reveals many polypeptides in all fractions, including the pelleted GCPs, where more than twenty bands are seen. Silver stained gels of GCP proteins reveal more than three times this number of bands (not shown). While some polypeptides appear in all fractions, most notably the tubulins and actin (see below), it is clear that some polypeptides are enriched or depleted in various fractions. For example, compared to the crude brain homogenate the GCPs are enriched in polypeptides of approximately 187, 145, 89, 87, 75, and 28 kD. Compared to the interface fraction from the discontinuous sucrose gradient or the second peak off the CPG column, however, the GCPs are depleted in at least five polypeptides running above the myosin molecular weight marker, three bands of about 167 kD and bands of approximately 133, 124, and 92 kD. None of these polypeptides has been identified, although these differences in protein composition are consistently observed. Indeed the compositions of GCPs from separate isolations are always remarkably similar.

While there are no known unique protein markers for growth cones, there are proteins known to be enriched in growth cones. For example, GAP-43 has been shown to be enriched in growth cones by immunoblots (Meiri et al., 1986) and immunofluorescence (Meiri et
Fig. 3. GAP-43 immunoreactivity in the various purification steps. A gel of all fractions in the procedure, similar to Figure 2, was transferred to nitrocellulose that was probed with a monoclonal antibody to GAP-43. The lanes are labeled as in Figure 2. The following lanes are enriched in GAP-43: the sucrose interface fraction (lane E), the second pellet (lane F), and especially the GCPs (the pooled first CPG peak) (lane G). Each lane was loaded with the same amount of protein. The molecular weight markers to the right are: 200, 116, 97, 66, and 43 kD.

al., 1988). In order to determine if our preparation of GCPs was enriched in GAP-43, an unstained gel, containing all the fractions of the purification procedure, like that shown in Figure 2, was blotted onto nitrocellulose that was probed with a monoclonal antibody to GAP-43 (Fig. 3). While there is apparently proteolysis of GAP-43 at several steps in the procedure, it is clear that the isolated GCPs are greatly enriched in GAP-43.

We have further characterized these GCPs by qualitatively assaying for three groups of proteins: cytoskeletal proteins, proteins associated with focal adhesions, and cell adhesion molecules. Proteins within each of these groups were directly detected on immunoblots of GCP proteins that were probed with specific antibodies to the protein. In all cases the antibodies reacted with an antigen of the appropriate molecular weight.

The immunoblots for cytoskeletal proteins are shown in Figure 4. In agreement with many studies using immunofluorescence or electron microscopy (e.g., Letourneau, 1981; Bridgman and Dailey, 1989; Letourneau and Shattuck, 1989), actin is a prominent component of GCPs, as is myosin II. In addition, there is some neurofilament protein present, as reflected by the presence of the high molecular weight neurofilament subunit. Tubulin is also present and densitometer scans of Coomassie stained gels show that the tubulins are the major component of this fraction (not shown). Kinesin (Vale, 1987) and dynamin (Shpeter and Vallee, 1989), two proteins that interact with microtubules, are also found in the isolated GCPs.

Because substrate adhesion is a major growth cone function, the GCPs were examined for several components of focal adhesions. In our preparations we can identify alpha-actinin, vinculin, talin, and the beta 1 subunit of integrin (Fig. 5). These are all of the major components currently thought to be in focal adhesions (Burridge et al., 1988).

In addition to integrin, four other cell surface molecules involved in cell adhesion are also present in the GCPs. They are A-CAM (equivalent or closely related to N-cadherin), L1 (equivalent to Ng-CAM), fibronectin, and laminin (Fig. 6). All of the antibodies used recognize antigens of the appropriate molecular weight. In the case of L1, the polyclonal antibody used recognizes both the 200 and 80 kD subunits (Rathjen and Schachner, 1984). In the case of laminin, the antibody recognized only the 205–215 kD beta subunits (Martin, 1987).

**DISCUSSION**

We have modified slightly the procedure used by Pfenninger et al. (1983) to isolate a fraction enriched in growth cone particles from developing chick brain. We have taken some additional care to limit proteolysis and have used a modified Krebs’ solution (Fried and Blaustein, 1978, cited in Gordon-Weeks and Lockerbie, 1984) for chromatography on the CPG column. This is the last step in the isolation procedure and separates the
Fig. 5. Components of focal contacts in isolated growth cone particles. Blotted proteins from GCPs were probed with antibodies to alpha-actinin (lane A), vinculin (lane B), talin (lane C), and the beta 1 subunit of integrin (lane D). The molecular weight markers to the right are: 200, 116, 97, and 66 kD.

GCPs from soluble proteins as well as smaller particles. The protein compositions of the interface fraction from the sucrose gradient that was applied to the column, and the two peaks off the CPG column, are distinctly different (Fig. 2). This suggests that this is an important purification step in the procedure. We use this column with the modified Krebs’ solution of Gordon-Weeks and Lockerbie (1984), rather than sucrose, to facilitate pelleting the GCPs after chromatography.

We have used whole brains from chick embryos (E13–E14) rather than fetal or neonatal rat brain. Embryos of this age were chosen because by day 12 of chick neural development, cell proliferation has subsided in most areas of the brain while cell differentiation continues (Romanoff, 1960). During this period, days 11–13, there is an approximately 50% increase in the amount of actin present in chick brain expressed as percent of total protein (Santerre and Rich, 1976). Microtubule protein, also expressed as percentage of total protein, reaches its maximum concentration between days 9 and 17, when it is about double the percentage found in adult brain (Bamberg et al., 1973). While nerve fiber tracts start to form very early in chick development (Romanoff, 1960), these increases in the amounts of cytoskeletal proteins, after most cell division has been completed, probably reflect extensive neurite outgrowth during this period.

The GCPs obtained from chick brain appear, by phase microscopy, similar in size to those isolated from fetal rat brain (Pfenninger et al., 1983) or neonatal rat forebrain (Gordon-Weeks and Lockerbie, 1984) (Fig. 1). In addition, they adhere to and flatten out on coated coverslips as do those from rat forebrain (Gordon-Weeks and Lockerbie, 1984).

Protein profiles of the entire isolation procedure as presented here (Fig. 2), we believe, have not been reported. The major cytoskeletal proteins tubulin and actin appear to be present throughout the procedure, as one would expect. There are, however, distinct differences between some of the fractions, which will need to be examined in the future. It is difficult to compare the GCP protein profile obtained here with those from the other isolation procedures without examining them on the same gel. They do appear to be similar. However, as the three preparations are derived from different sources and at different developmental stages, there may well be differences in protein composition.

Compared to the crude brain homogenate, the chick GCPs are greatly enriched in GAP-43 (Fig. 3). The antigen appears in Figure 3 to be a 57-kD polypeptide. GAP-43 has been assigned molecular weights of 43 to 57 kD, depending upon the electrophoretic conditions used (Skene, 1989). GAP-43 is a growth-associated protein whose expression is correlated with neurite growth during development and neuronal regeneration (Skene, 1989). It has been shown to be enriched in GCPs from neonatal rats by immunoblots and immunofluorescence (Meiri et al., 1986, 1988). Although the function of GAP-43 is not known, expression of GAP-43 in several cell types causes the cells to extend long filopodial-like processes (Zuber et al., 1989). While GAP-43 is apparently absent from dendritic growth cones (Goslin et al., 1990), it may still be important for more rapid axonal growth.

The chick GCPs possessed all of the cytoskeletal proteins examined (Fig. 4). In agreement with many other studies on intact and isolated growth cones, actin, myosin II, and tubulin are all present (e.g., Letourneau, 1981; Gordon-Weeks, 1987; Bridgman and Dailey, 1989; Simkowitz et al., 1989). In intact growth cones, actin filament bundles are found in the filopodia and as a dense network in the lamellipodia (Bridgman and Dailey, 1989). Anti-myosin II immunofluorescence appears punctate and is found at the bases of filopodia and the
proximal edge of the thinly spread peripheral region (Bridgman and Dailey, 1989). The exact roles of actin and myosin II in growth cone motility are still not clear. In this regard, it is noteworthy that we do have preliminary immunoblot data, using a monoclonal antibody elicited by chicken brush border myosin I, that myosin I is greatly enriched in the GCPs compared to the crude homogenate (Cypher and Moosiker, unpublished observation). The role of myosin I in growth cone motility also needs to be elucidated.

We report here for the first time the presence of neurofilament protein in GCPs (Fig. 4). Neurofilaments are not usually thought to be components of the growth cone cytoskeleton. They are not seen as formed filaments in growth cones and are thought to form proximal to the more dynamic actin and microtubule cytoskeleton, adding stability to the forming neurite (Shaw, 1987). The high molecular weight neurofilament protein identified here may be a contaminant in the GCP preparation from the distal regions of neurites.

The presence of kinesin and dynamin in GCPs is reported here for the first time (Fig. 4). The role of kinesin in microtubule-associated motility and the transport of membrane-bound vesicles has been well studied (reviewed in Vale, 1987). In intact growth cones microtubules are found centrally, splaying out of the neurite (e.g., Bridgman and Dailey, 1989). Some individual microtubules, extending from the central region, reach as far forward as the bases of individual filopodia (Letourneau and Ressler, 1983). Kinesin may have an important role in the transport of materials to and within the growth cone during neurite elongation. Less is known about dynamin (Shipetner and Vallee, 1989), which mediates the ATP-sensitive bundling of microtubules and may associate with membranes as well. Dynamin has been localized to the cell bodies and growth cones of PC12 cells by immunofluorescence (Scaife and Margolis, 1990). We have made similar observations in primary cultures of dorsal root ganglion neurons (Cypher and Letourneau, unpublished observations).

The adhesive properties of isolated GCPs have not been extensively studied. The chick GCPs will adhere to and flatten out on polyornithine-coated coverslips. Gordon-Weeks and Lockerbie (1984) reported similar flattening of rat GCPs on poly-D-lysine-coated coverslips. Lockerbie et al. (1989) have reported that rat GCPs adhere poorly to untreated plastic, and only somewhat better to poly-L-ornithine-treated plastic. However, up to 18% of labeled GCPs were observed to adhere to a transformed, mouse glial cell line. This adhesion was inhibited by pretreatment of the GCPs with trypsin and conditions that would elevate cAMP levels, suggesting that the adhesion was dependent on particle surface proteins and under regulation.

We have found that isolated chick GCPs contain all of the known major components of focal adhesions; alpha-actinin, vinculin, talin, and integrin (Fig. 5). In addition to integrin, they contain the cell adhesion molecules A-CAM, L1, fibronectin, and laminin (Fig. 6). A current model of focal contacts (Burridge et al., 1988) proposes that cytoskeletal actin filaments, cross-linked by alpha-actinin, are linked via vinculin and talin to transmembrane integrin receptors that bind to extracellular matrix molecules such as fibronectin and laminin. The results reported here identify all of these components in isolated GCPs.

The several cell adhesion molecules we have identified in this preparation suggest that there may be others. It must be remembered that these GCPs were derived from whole brain at a particular period in chick neural development. The cell adhesion molecules present in GCP preparations may vary with the age of the embryos used and the regions of brain sampled. Taken together, the results presented here suggest that this preparation of chick GCPs may be a useful model system with which to study growth cone adhesion.

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