Journal of Cellular Biochemistry 68:457–471 (1998)

A Novel AP180-Related Protein in Vesicles That Concentrate at Acetylcholine Receptor Clusters

S. Bursztajn,[1]* S. Vincent,[1] F.M. Brodsky,[2] F. Benes,[1] and S.A. Morris[2]

[1]Psychiatry Department (Neuroscience), Harvard Medical School, Belmont, Massachusetts 02178
[2]Hooper Foundation, University California, California 94143

Abstract Monoclonal antibodies were generated to vesicular membranes of clathrin coated vesicles enriched for acetylcholinesterase (AChE). One of these, C172, recognizes vesicles which accumulate in muscle cells around nuclei associated with acetylcholine receptor AChR clusters. Immunoblots of muscle extracts and brain purified clathrin coated vesicles show that C172 recognizes a 100 kd band in muscle, but a 180 kd band in brain. Western blots of purified AP180 protein stained with the two antibodies AP180.1 and C172 displayed the same staining pattern. Tryptic digests probed with peptide antibodies (PS26 and PS27) generated to known sequences of AP180 were used to map the epitope for C172 within the brain AP180 sequence. On immunoblots of digested AP180, all AP180 antibodies and C172 recognized a 100 kd tryptic fragment, however only C172 recognized a smaller 60 kd. Our results suggest that the C172 epitope is located within amino acids 305–598 of the AP180 sequence. Confocal fluorescence microscopy of myoblasts and myotubes stained with the C172 antibody gives a punctate immunofluorescence pattern. Myoblasts stained with C172 revealed a polarized distribution of vesicles distinct from that observed when cells are stained with γ adaptin antibody which is known to localize to trans Golgi network. Myotubes stained with C172 antibody reveal a linear array of vesicular staining. Quantitative analysis of C172 reactive vesicles revealed a significant increase in number of vesicles present around the nuclei associated with the acetylcholine receptor clusters. These vesicles did not colocalize with the Golgi cisternae. These results indicate that a protein with homology to the neuron-specific coated vesicle protein AP180, is present in muscle cells associated with vesicles showing significant concentration around postsynaptic nuclei present in close proximity to AChR clusters. J. Cell. Biochem. 68:457–471, 1998.

Key words: coated vesicles; acetylcholine receptors; AP180; myotube

INTRODUCTION

Clathrin coated vesicles have been shown to participate in intracellular membrane transport, selective receptor mediated endocytosis, as well as membrane retrieval and recycling at the synapse [Goldstein et al., 1985; Brodsky, 1988; Morris et al., 1989; Pley and Parham, 1993; Heuser, 1989; Maycox et al., 1992; Heuser and Reese, 1973]. These vesicles have been purified and characterized from numerous tissues including mammalian brain [Pearse and Robinson, 1990].

We and others have shown that some clathrin coated vesicles contain newly synthesized acetylcholine receptors (AChRs) and the enzyme acetylcholinesterase (AChE) [Bursztajn and Fischbach, 1984; Porter-Jordon et al., 1986; Bursztajn et al., 1987; Park and Bursztajn, 1990]. These vesicles, which appear to participate in postsynaptic membrane remodeling and accumulate beneath the nerve muscle contacts, are characterized by reactivity with the epitope for the C172 antibody [Bursztajn et al., 1993].

Biochemical and functional studies have allowed for characterization of the coat components and other factors involved in coat assembly and disassembly during the vesicle transport process [Pearse, 1987, 1989; Murphy et al., 1991; Morris et al., 1993; Ye and Lafer, 1995]. The major protein component of the coated vesicle is clathrin, composed of triskelions that

Contract grant sponsor: NIH; Contact grant numbers: NS24377, GM38093, MH00423; Contract grant sponsor: a senior postdoctoral fellowship from the California Affiliate of the American Cancer Society.

S. Bursztajn's current address is LSU Medical Center, Biomedical Res. Institute, Shreveport, LA 71104.

S.A. Morris' current address is Department of Pharmacology, University of Cambridge, Cambridge, CB2 10J, England.

*Correspondence to: Dr. Sherry Bursztajn, LSU Medical Center, Biomedical Res. Institute, Shreveport, LA 71104.

Received 5 August 1997; Accepted 10 October 1997

consists of three clathrin light chains and three identical heavy chains [Ungewickell and Branton, 1981; Kirchhausen et al., 1987; Jackson et al., 1987]. The clathrin coated vesicles also contain associated protein complexes or adaptors (APs) [Vigers et al., 1986; Pearse and Robinson, 1990; Keen, 1990]. The AP1 adaptor is localized to clathrin coated membranes within the trans-Golgi network (TGN) and is composed of two distinct 100 kd subunits γ_1 β1 a 47 kd (μ1) and a 19 kd (δ) subunit. The AP2 adaptor localized to clathrin coated vesicles derived from the plasma membrane has 100 kd subunits α, and β2, a 50 kd (μ2), and a 17 kd subunit [Robinson, 1989, 1990; Kirchhausen et al., 1989]. The β1 and β2 subunits are highly homologous while the α and γ have much lower homology [Kirchhausen et al., 1991; Robinson, 1990]. These adaptors are thought to provide a linkage between the clathrin coat and the cytoplasmic domains of the transmembrane receptors responsible for the selection and concentration of the specific cargo molecules within the vesicles.

In coated vesicles isolated from neuronal tissue, two other assembly proteins, AP180 and auxilin, are also part of the clathrin coat complex [Morris et al., 1989, 1993; Keen, 1990]. AP180 was discovered by a number of different laboratories and named AP180 [Ahle and Ungewickell, 1986], AP-3 [Keen and Black, 1986; Keen, 1987], NP 185 [Kohtz and Puszkin, 1988], pp155 [Keen and Black, 1986], and F1-20 [Sousa et al., 1990]. It has been shown that AP-3, pp155, AP180, and NP185 are the same proteins [Murphy et al., 1991]. Later it was found that AP-3 and F1-20 are identical [Morris et al., 1993; Zhou et al., 1993]. AP180 is a phosphoprotein [Keen and Black, 1986; Morris et al., 1990; Murphy et al., 1994] which is acidic with unusual molecular properties [Ahle and Ungewickell, 1986; Keen and Black, 1986; Zhou et al., 1992]. In SDS-PAGE, AP180 has a tendency to migrate at 155,000–185,000, although its molecular weight was determined to be about 112,000–123,000 daltons [Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988; Murphy et al., 1991] using hydrodynamic measurements and 92,000 daltons from its primary sequence [Morris et al., 1993]. In contrast to the adaptor proteins AP180 is a monomer, which like the adaptor proteins interacts with clathrin and promotes its assembly [Ahle and Ungewickell, 1986, 1990]. However, AP180 appears to be more active in inducing clathrin assembly than the other adaptor proteins [Lindner and Ungewickell, 1992]. The protein is sensitive to proteolysis, and can be cleaved by trypsin and elastase into fragments of 30,000 and 100,000 daltons [Prasad and Lippoldt, 1988; Murphy et al., 1991]. The 30 kd fragment is derived from the N-terminus of the protein and contains a clathrin binding site. However, it is not sufficient to promote clathrin assembly and a second site located towards the C-terminus has been suggested to be essential for this function. In PC12 cells, nerve growth factor appears to induce AP180 protein expression and enhances its appearance at nerve endings [Kohtz and Puszkin, 1988; Su et al., 1991]. A number of isoforms of AP180 may exist due to alternate splicing [Zhou et al., 1992, Morris et al., 1993]. However, all of these are thought to be specific to neuronal tissue and experimental evidence has been presented that this protein is synapse specific [Perry et al., 1991, 1992; Sousa et al., 1992]. Recently a protein related, but not identical, to AP180 called NAP185 has been identified in glial cells from chick brain [Solca et al., 1994].

To better understand vesicular traffic and postsynaptic membrane remodeling at the neuromuscular synapse, we have generated monoclonal antibodies to the membranes of coated vesicles [Bursztajn et al., 1993]. These vesicles appeared enriched at nerve-muscle contacts, but we did not know whether these regions contained AChR clusters [Bursztajn et al., 1993]. It is well established that in innervated cultures there are nerve-muscle contacts that lack AChR clusters. In this report, we show that one of our antibodies, C172, cross-reacts with purified AP180 from bovine brain and concentrates over two fold in muscle cells around nuclei in close proximity to AChR clusters. Furthermore, AChR clusters that are not associated with nuclei do not show an increase in the number of C172 stained vesicles. This is the first report of an AP180 homologue in nonneuronal tissue that has a concentrated distribution at the postsynaptic nuclei.

MATERIALS AND METHODS

Coated Vesicle Preparation Enriched for Acetylcholinesterase

Clathrin coated vesicles were isolated and purified from bovine brains as described previously [Bursztajn et al., 1993]. Briefly, brains were homogenized in a Waring blender with three high speed 10 s bursts in two volumes of

Buffer A [(0.1 M 2-N-morpholino acid (MES), 0.5 mM $MgC1_2$, 1 mM ethylene glycol bis (β-aminoethyl ether)-N,N 1-tetra acetic acid (EGTA), 0.02% NaN3, pH 6.5]. The pellets containing the coated vesicles were centrifuged, the supernatant collected and centrifuged at 34,000 rpm in a Beckman type 35 motor. The pellets were resuspended in Buffer A, homogenized, mixed with an equal volume of Ficoll-sucrose solution (12.5% W/V Ficoll-400 and 12.5% sucrose in Buffer A) and centrifuged for 40 min at 19,000 rpm. The supernatant was diluted with three volumes of Buffer A centrifuged for 5 min at 10,000, to remove aggregates, and loaded on a sephacryl S- 1000 column (2.6 x 35 cm) pre-equilibrated with Buffer A. The 280 nm absorbance profile of the eluate showed two peaks. Because previous studies have shown that coated vesicles contain AChRs and AChE [Bursztajn and Fischbach, 1984; Porter-Jordon et al., 1986; Bursztajn et al., 1991], we collected 3 ml fractions, tested for acetylcholinesterase [Ellman et al., 1961], hexosaminidase [Hall et al., 1978], and NADH cytochrome C reductase [Mahler, 1955] activities, and pooled the fractions enriched in protein and AChE activity but low in hexosaminidase (a marker for lysosomes) and NADH cytochrome C reductase (a marker for mitochondria) activities. Column chromatography revealed two peaks, one of which was enriched in protein and AChE activity [Bursztajn et al., 1993]. After obtaining a single peak from the second chromatography, we centrifuged it for 1 h at 34,000 rpm using a Beckman Type 35 rotor, and assessed the purity of the pellet of coated vesicles so obtained by SDS-polyacrylamide gel electrophoresis [Laemmli, 1970] and electron microscopy [Bursztajn et al., 1993]. Vesicular membranes were sedimented in a Beckman airfuge at 130,000 g, the pellet was treated with 0.5% saponin and, after washing, the proteins were subjected to 7.5% SDS polyacrylamide gel electrophoresis. Specific bands of 180 kd, 100 kd, and 50 kd were identified by prestained molecular weight standards. The bands were cut out, the protein was extracted separately from each band, concentrated with Amicon mini concentrators and used for antibody production.

Preparation of Monoclonal Antibodies to 100 kd Vesicular Components

Female BALB/c mice were subcutaneously immunized with 50 mg of the 100 Kd protein band emulsified in MPL and TDM (monophosphoryl lipid A + dimycolate, RIBI; Immunochemical Research, Hamilton, MT). The mice were boosted subcutaneously in incomplete Freud's adjuvant and 2 weeks and after each boost antisera were collected from mice and screened by Elisa [Maecker at al., 1987]. Mice producing the strongest antisera were used in fusion of their spleen cells with SP2 myeloma cells in HAT medium as described previously [Bursztajn et al., 1993]. After twice subcloning colonies of interest using limiting dilution, we tested their supernatants by immunoblotting. For large scale production of monoclonal antibodies, we cultured hybridomas in large flasks and collected the supernatants or injected cells into mice for ascites production employing affinity chromatography on protein-A-sepharose according to manufacturer's instructions to purify the antibodies from the ascites fluid. Antibody subclasses were typed with a kit obtained from Boehringer Mannheim Biochemicals (Minneapolis, MN).

Electrophoretic Methods and Western Blot Analysis

0.5 M Tris extract of braincoated vesicles, chick muscle extract and purified AP180 from bovine brain coated vesicles [Ahle and Ungewickell, 1986; Morris et al., 1993] was run on 7.5% SDS polyacrylamide gels (SDS-PAGE) using buffers and stacking gel as described by Laemmli [1970]. Gels were either stained for proteins with Coomassie brilliant blue or electrophoretically transferred to nitrocellulose using a transfer apparatus (Bio-Rad, Hercules, CA). After incubation in PBS containing 0.05% Tween-20 (TPBS) and 5% powdered nonfat milk, immunoblots were washed in TPBS and incubated with monoclonal antibodies to C172, the α subunit of the AP2 adaptor (100/2), the γ subunit of the AP1 adaptor (100/3) and AP180, (AP180.1), or rabbit antisera PS26 and PS27 against known sequences in AP180. After further washings, the blots were incubated with biotinylated anti IgG (mouse or rabbit), washed, and incubated for 1 h in vectastain ABC reagent (Vector Laboratories, Burlingame, CA). Blots were washed in PBS and developed using 4-chloro-1-napthol as a substrate.

Tryptic Digests

AP 180 purified from bovine brain by the method of Ahle and Ungewickell [1986] at a concentration of 0.1–0.2 mg/ml was digested in 50 mM Tris-HCl, pH 8.0 with trypsin at a weight

ratio of 1:375 on ice for the time indicated. The reaction was stopped by the addition of a 10-fold molar excess of soybean trypsin inhibitor.

Cell Culture

Pectoral cells were obtained from 11-day-old chick embryos and plated in collagenized Falcon tissue culture dishes, as previously described [Bursztajn, 1984; Bursztajn and Fischbach, 1984]. Cells were maintained in Eagle's balanced salt solution supplemental with horse serum (10% vol/vol) and chick-embryo extract (2% vol/vol). The medium was replaced every 2 days [Bursztajn and Fischbach, 1984].

Immunofluorescence Labeling

At various periods after plating cells were fixed for 30 min in 2% paraformaldehyde in a buffer containing 37.5 mM sodium phosphate, 0.75 M Lysine, and 10 mM sodium periodate, permeabilized them with 0.1% Triton X-100 in PBS for 5 min, washed, and incubated overnight at 4°C with the following vesicular monoclonal antibodies: C172, AP180.1, anti-AP2 clone 100/2, or anti-AP1 clone 100/3 (1:100 dilution of culture supernatin). Cells were washed and incubated with biotinylated anti-mouse IgG followed by streptavidin Texas red or streptavidin FITC as previously described. For double labeling of AChR clusters and the vesicular proteins, cells were first incubated with α-bungarotoxin conjugated to tetramethylrhodaminine (αβTX-TMR) fixed, and then exposed to the vesicular antibodies. In some double label studies wheat germ agglutinin (WGA; Molecular Probes) conjugated to fluorescein was used to localize the Golgi complex, and establish a relationship between the Golgi cisternal and the vesicle distribution. The cytoskeletal elements were observed with an antibody to β-tubulin (Sigma, St. Louis, MO) which binds to microtubules, and actin filaments were visualized with BODIPY FL phallacidin (Molecular Probes, Eugene, OR) which bind to F-actin. Permeabilized cells were stained with one unit of phallacidin for 10 min at 37°C in accordance with the manufacturer's protocol. The coverslips containing cells were mounted on slides in a 1:9 solution of PBS:glycerol p-phenylenediamine (0.1%) as a quenching agent. The slides were viewed using a digital confocal microscope (ONCOR Imaging Systems, Oncor, Inc., Gaithersburg, MD) mounted on a Leitz microscope.

Digital Confocal Imaging

To obtain high resolution images of fluorescently-labeled vesicles, AChR clusters, microtubules, and actin filaments, labeled cell cultures were viewed at high magnification using digital confocal microscopy. Digital images were obtained from representative labeled cells using the epifluorescence optics of a Leitz Diaplan light microscope equipped with specific dichroic filters for FITC and rhodamine. A Dage-MTI SIT-68 video camera with a DSP-100 signal processor (DAGE-MTI Inc., Michigan City, IN) was used to amplify the intensity of the fluorescent image. The microscope and video camera system were interfaced with a Z-axis stage controller, an Apple Macintosh Quadra-950 computer, and ONCOR-IMAGE processing software. The DSP-100 signal processor was operated in the manual mode in order to calibrate the black level, kV, and gain settings of the camera to optimize the visualization of the respective image fields. The specific fluorescent emissions were standardized relative to each other to obtain a video image with comparable signal in situ concentration in paired images acquired with the FITC and rhodamine filters, respectively. High magnification digitized images were made with a 100X oil immersion lens (1.32 NA) coupled to a 2X magnification converter. A Z-series stack of images through the depth of a selected cell profile was acquired using 1.5-μm-step intervals for 10 consecutive focal planes. For double-labeled cells, two Z-series image stacks were produced with the FITC and rhodamine filters, respectively. Single-labeled and double-labeled parallel pairs of image stacks for each selected cell were initially processed with a deconvoluting subroutine of the ONCOR-IMAGE software that mathematically reduces image distortion effects produced by fluorescent background flare. From each set of deconvoluted stacks, a single focal plane that best represented the fluorescent probe localization was selected. The brightness and contrast levels of these later images were adjusted in order to optimize the inherent localization of probe with the respective cytoplasmic element. Using a registration subroutine of ONCOR-IMAGE software, matched pairs of selected images were assigned colors and superimposed in register to form a single composite image.

Image Analysis

Semi-quantitative measurements were obtained from laser printer copies of gray scale composite images using BIOQUANT OS/2 Advanced Image Analysis software (R and M Biometrics, Nashville, TN) interfaced with a DTK 486pc computer via a Hipad digitizing board. The magnification of the gray scale images was calibrated by using a digitally recorded micrometer scale. The outline of each 1) selected cell profile in an image field, 2) nuclear profile and, 3) AChR cluster profile was traced with the digitizing board and the area determined. The cell area was adjusted by subtracting the total area of measured nuclei. The numerical density of labeled vesicle profiles was determined for: 1) regions overlying ACh- receptor clusters that were adjacent to nuclei and, 2) regions overlying ACh-receptor clusters that were not adjacent to nuclei. In addition, the numerical density of labeled vesicle profiles for an equivalent ribbon thickness around nuclei was determined for: 1) nuclei adjacent to AChR clusters and, 2) nuclei not adjacent to AChR clusters. A mean and standard error of the mean were obtained for each variable to assess the consistency of vesicle densities between these different intracellular relationships. A two-tailed Student's *t*-test was used to evaluate the significance of differences for these variables.

RESULTS

Characterization of C172 Antibody Recognizing a Homologue of AP180 in Skeletal Muscle

We have produced monoclonal antibodies to the membranes of clathrin coated vesicles as well as clathrin in order to examine their role in membrane transport and remodeling in the postsynaptic cell. We have shown in our previous report that the C172 antibody on Western blots of chick skeletal muscle recognizes a 100 kd protein. We therefore evaluated the relationship of the 100 kd protein recognized by the C172 antibody and three clathrin associated proteins. Immunoblots of muscle extracts and brain purified coated vesicles (Tris extract) show that C172 recognizes proteins of different molecular weights (Fig. 1A). In the brain extract, C172 recognizes a 180 kd band and smaller breakdown products, but in muscle it recognizes a 100 kd band and two other bands probably representing breakdown products. The C172 antibody does not appear to recognize the same molecular weight band as an antibody to the γ subunit of the AP1 adaptin. Immunoblots of brain coated vesicles show that C172 stains a 180 kd band whereas the anti γ antibody (100/3) and an antibody to the α subunit of the AP2 adaptin (100/2) both recognize 100 kd bands in the same extract (Fig. 1D,E). Interestingly, when pure AP180 protein was electrophoretically transferred to nylon membrane, both antibodies AP180.1 and C172 displayed essentially the same molecular weight labeling patterns (Fig. 1B,C). However, the intensity of the band staining is not similar (Fig. 1B,C). A variety of factors may account for these differences. These may include the following: the immunoblots were not processed at the same time, the C172 epitope may be more accessible than AP180.1, and/or there is more C172 than Ap180 accessible protein. Furthermore, the lower molecular weight band which is probably the breakdown product of AP180 is also more intense when the blot is stained with the C172 than with AP180.1 antibodies (Fig. 1B,C). The Pure AP180 appears to be less sensitive to proteolytic degradation than the protein extracts obtained from coated vesicles.

It has been shown previously that AP180 can be readily cleaved into smaller fragments by trypsin and elastase [Murphy et al., 1991]. To map the epitope for C172 to the brain AP180 sequence we digested pure AP180 with trypsin as previously described [Murphy et al., 1991; Morris et al., 1993]. Aliquots of the digest were removed at time 0 to 30 min, run on SDS-PAGE gradient gels and immunoblotted with C172, AP180.1, or peptide antibodies (PS26 or PS27) generated against known sequences within AP180 (Fig. 2A–C). Immunoblots of AP180 digest blotted with C172, AP180.1, or peptide antibodies revealed an identical 100 kd band. An additional 60 kd band is recognized only by C172 (Fig. 2B). This short lived 60 kd band is probably derived from the 100 kd band and lacks the epitopes for AP180.1 and the peptide antibodies. These experiments were repeated three times with identical results. Based on peptide sequencing, we mapped the position of the C172 epitope within the brain AP180 sequence (Fig. 2C). Our previous work on peptide sequencing shows that the N-terminus of the 100 kd band is residue 305, and PS27 is at residue 613 [Morris et al., 1993]. This suggests that the 60 kd sequence must end before resi-

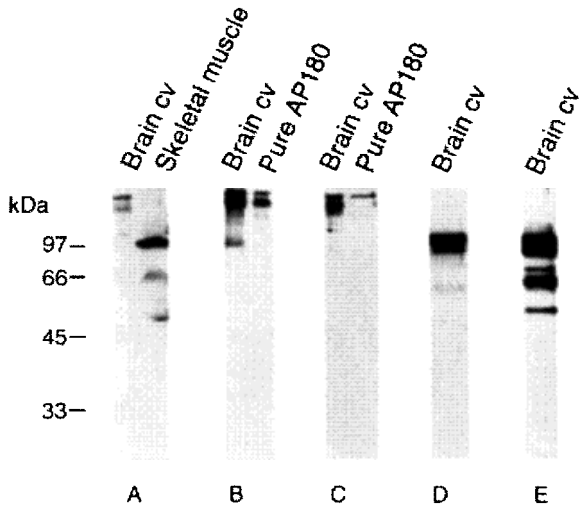

Fig. 1. Western blots of brain coated vesicles (Tris extract) and chick muscle cells extracts labeled with antibodies against different coated proteins. Five µg of brain coated vesicles, muscle extract, and purified AP180 were SDS solubilized and loaded on 7.5% polyacrylamide gradient gels. Molecular weight markers (in kilodaltons) are indicated at left. Nitrocellulose blots were stained with the following antibodies. **A,B**: Incubated with C172, **(C**) with AP180.1, **(D**) with 100/3, and (**E**) with 100/2. A: C172 recognizes a 180 kd band in brain coated vesicles, but a 100 kd band in muscle cells. The lower molecular mass bands labeled with the antibody C172 are probably breakdown products. B,C: Antibodies to C172 and the AP-3 adaptor protein antibody AP180.1 recognize the same molecular weight band on purified AP180 protein. The lower molecular weight bands are probably breakdown products since it is known that the AP180 protein is susceptible to proteolysis. D,E: The C172 antibody does not recognize the γ or α subunits of AP1 and AP2, respectively, as shown by the different molecular weights on brain coated vesicles.

due 613 and that the most likely trypsin site is at residue 598. Therefore, the C172 epitope is probably contained within amino acids 305–598 of the AP180 sequence (Fig. 2C). This region whilst only coding for a 28 kd peptide is known to contain the sequence which appears to be responsible for the anomalous migratory pattern of AP180 on SDS-polyacrylamide gel electrophoresis hence giving rise to a band of 60 kd apparent molecular weight. As C172 clearly recognizes AP180 derived from brain tissue the 100 kd protein detected in Western blots of skeletal muscle must be related to AP180.

Asymmetric and Linear Distribution of Vesicles Labeled With C172 Antibodies

We have reported previously that myotubes stained with the antibodies to C172 and C-33 which recognizes clathrin, give a punctate immunofluorescent pattern [Bursztajn et al., 1993]. Here we have employed digital confocal microscopy and computer assisted image analysis software in order to determine whether vesicles stained with clathrin associated proteins localize to specific intracellular compartments. Myoblasts permeabilized and stained with C172 antibody revealed a punctuate staining pattern that appears to be polarized (Fig. 3). This staining polarization pattern was further confirmed by dissecting the image through the depth of the cell at consecutive intervals of 1.5 µm (Fig. 3A–E). Examination of the myoblasts, which appear spindle-like in all planes of the series, reveals C172 vesicles concentrated toward one side of the cell (Fig. 3B–E), whereas the opposite side of the same cell contains very few C172 labeled vesicles (Fig. 3B–E). We obtained a similar polarized punctate staining pattern when we stained cells with an antibody to the α subunit of the AP2 adaptor (100/2), suggesting that these vesicles are endocytic. This polarity was not observed when cells were stained with an antibody to the γ subunit of the AP1 adaptor (100/3). The AP1 staining was perinuclear, and previously shown to be localized to the trans Golgi network [TGN; Robinson 1987, 1990], and differed from the C172 staining topography. In myoblasts (Fig. 4A–D) and fused myotubes (Fig. 4E), AP1 immunoreactivity was localized in close proximity to nuclei where the Golgi apparatus is known to be present. The antibody staining patterns we have observed are specific because cells processed the same way as in Figs. 3c-3e except for omission of the primary antibody, or incubation with irrelevant antibody show no staining [Bursztajn et al., 1993].

Fused myoblasts stained with C172 antibody revealed a linear array of vesicular staining patterns (Fig. 5A–C). This linear pattern tends to be localized toward the cell periphery and toward the flat surface of the cell suggestive of specialized contact sites which form neuromuscular junctions. Double label studies with BODIPY FL phallacidin which stain the actin filaments showed that some of these vesicles line up along the filaments (Fig. 5C). Cells double labeled with tubulin and C172 antibodies do not show alignment of vesicles along the microtubules (data not shown). These results supports the idea that transport of these vesicles from the cell surface, or to the cell surface may take place preferentially, but not exclusively along the filamentous structures. These filaments may serve as railroad tracks on which these vesicles are guided to the proper destination.

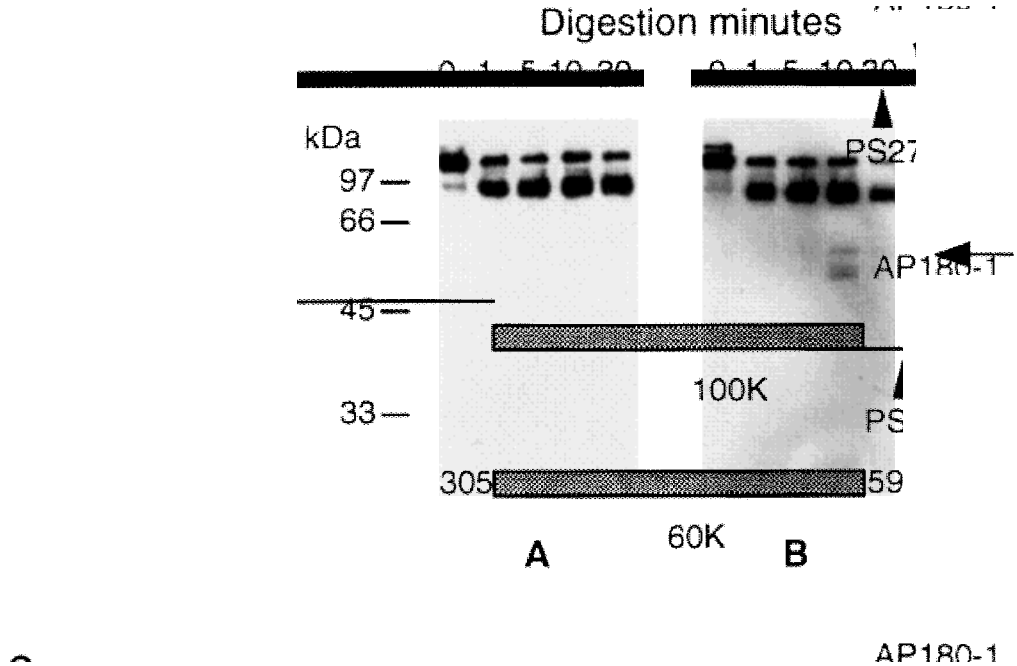

Fig. 2. Immunoblots of AP180 protein digested with trypsin. At left of the blot are the position of the molecular weight markers. At specific times indicated on the top of the blot, aliquots of digested AP180 protein were fractionated on 7.5–19% polyacrylamide gradient gels, transferred to nitrocellulose and immunoblots stained with (**A**) PS27 antibody whose position in the AP180 protein is known (see below). Incubation with AP180.1 or PS26 gave identical staining. **B**: C172 antibody. **C**: Diagrammatic representation of antibody epitope mapping. Antibodies PS26 and PS27 were raised to specific defined peptides from the predicted sequence of rat AP180. PS26 is to the peptide CFDPSVFDGLGDLL centered at amino acid residue 730. PS27 is to the peptide CAEDDRHVPLFFTA centered at amino acid residue 620 (Morris et al., manuscript submitted). Digestion of intact AP180 gives initially fragments of 30 kd, 100 kd, and 14 kd. The 100 kd fragment contains the epitopes for C172, AP180.1, PS26 and PS27. Further digestion gives a 60 kd fragment derived from the 100 kd. This contains only the epitope for C172. Thus the epitope for C172 appears to be contained within the region of amino acid residues 305-598 shown at the bottom of the diagram.

Localization of Vesicles Around Nuclei Associated With AChR Clusters

One of the striking characteristics of C172 labeling was its staining pattern around nuclei of certain myoblasts. This concentrated perinuclear staining pattern has been observed previously in our innervated muscle cells [Bursztajn et al., 1993]. It was found that acetylcholine receptors (AChR) cluster around nuclei which are beneath nerve terminals. We therefore in-

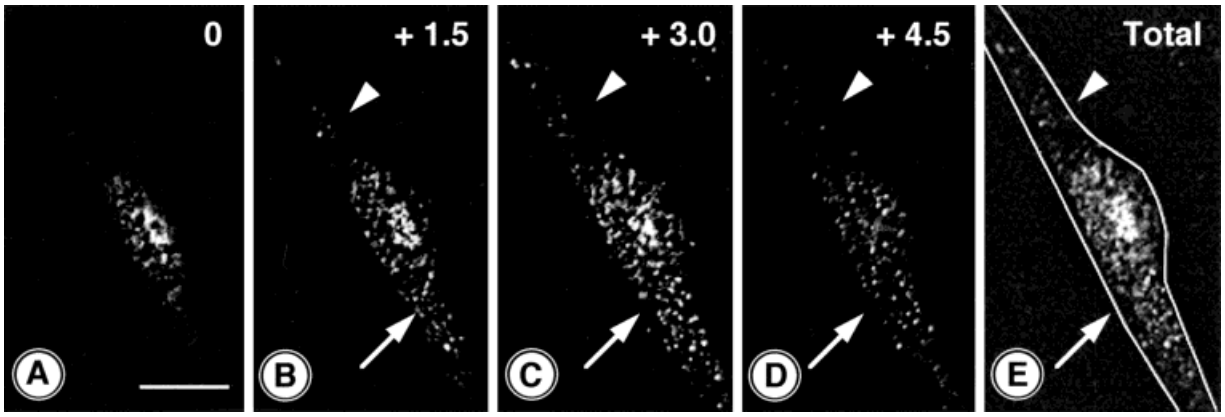

Fig. 3. Immunofluorescence confocal localization of C172 staining of myoblasts. A series of stack images taken through the depth of cell was acquired using 1.5 μm step intervals for eight consecutive focal planes. Four of these images are shown (**A–D**) and the compiled image is shown in **(E)**. Dissecting the cell through any of the focal planes shows concentration of C172 vesicular epitopes toward one side of the cell (arrow), and at the other side of the cell the C172 vesicular epitopes are sparse (arrowhead). Scale bar = 10 μm.

vestigated whether the C172-positive vesicles accumulate around the nuclei associated with AChR clusters or whether they accumulate around nuclei, even in the absence of AChR clusters. In our previous studies we have used innervated muscle cells, but did not carried out double labeled studies, or quantitative analysis. Because it is well established that not all nerve muscle contacts contain AChR clusters, it became important to carry out these studies. Myotubes were double stained with α-bungarotoxin (αBTX), which labels AChR clusters, and with C172 vesicular antibody. Our results show that C172 vesicles are concentrated around nuclei which are associated with AChR clusters (Fig. 6A–B). Quantitative analysis of C172 vesicles localized around nuclei which are AChR cluster associated, and compared to nuclei which are not AChR cluster associated, revealed a 2.3-fold increase in number of vesicles present around the nuclei associated with the receptor clusters (Table I). The number of vesicles/μm^2 of the surface of nuclear membrane showed an increase in vesicles around nuclei of AChR clustered regions. Furthermore, the distribution and number of C172 vesicles present in areas of myotube that did not have nuclei, did not differ when we compared AChR clusters and nonclustered regions per unit area (μm^2; Fig. 6C,D and Table I).

In myotubes, the Golgi apparatus is usually close to the nuclei. To determine whether the vesicular accumulation around nuclei as seen by the C172 antibody colocalizes with the Golgi cisterna and vesicles, we double labeled cells with wheat germ agglutinin lectin which marks predominantly the Golgi apparatus [Tartakoff and Vassalli, 1983]. To improve image resolution, we analyzed individual cells by optical sectioning and image reconstruction using digital confocal microscopy and silicon graphic software. Figure 7A,B and C,D show two different myotubes double labeled with vesicular antibody C172 and wheat germ agglutinin, optically sectioned through the Z axis of the cell and aligned in register with the same plane of section for each label. Intracellular lectin staining was readily visualized by the green fluorescence (Fig. 7B,D) but the staining patterns were distinctly different from the C172 vesicular staining. Although wheat germ agglutinin stained perinuclear regions, its pattern of staining did not coincide with the C172 vesicular labeling, and the antibody staining was not concentrated in this region. The C172 vesicular labeling is also not consistent with the staining for the AP1 adaptor, and thus the C172 antibody staining of vesicles around nuclei must reflect intracellular vesicles which are not associated with the TGN.

DISCUSSION

The various membrane transport functions that have been assigned to coated vesicles together with our studies showing that these vesicles participate in AChR and AChE transport [Bursztajn and Fischbach, 1984; Bursztajn et al., 1987; Park and Bursztajn, 1990], prompted us to further characterize the proteins involved. Previous studies showed that

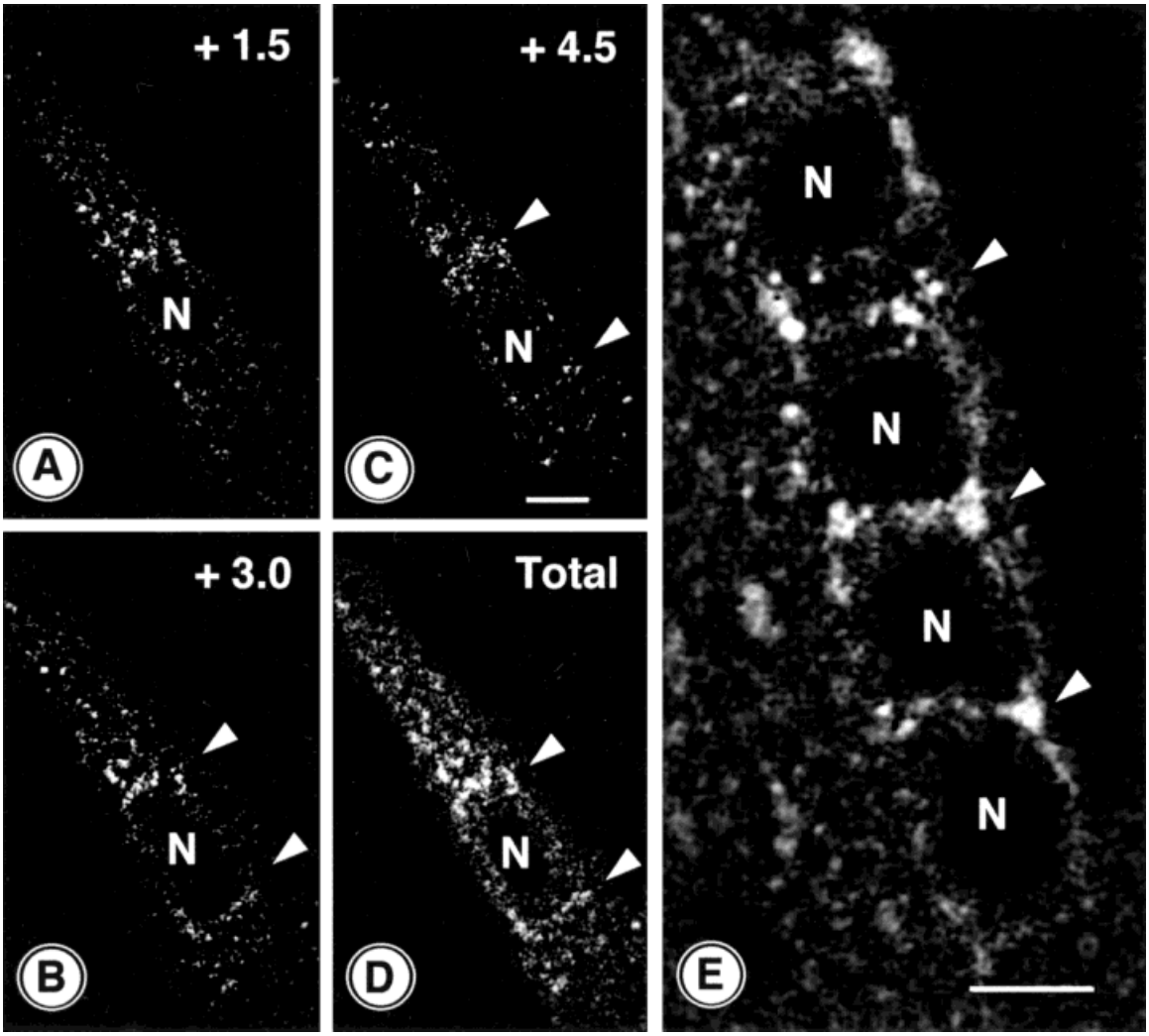

Fig. 4. Confocal immunofluorescent images of myoblasts (**A–D**) and myotube (**E**) stained with anti-AP1 antibody (clone 100/3). Images were taken through the dept of the cell at 1.5 μm intervals. Arrows point to discrete punctate staining of AP1 immunoreactivity which previously was shown to identify Golgi associated vesicles. In fused muscle cells the immunoreactivity of AP1 appears to be highly concentrated in close proximity to nuclei (arrows). N, Nuclei. Scale bar = 10 μm.

monoclonal antibody C172, generated to vesicles stripped of clathrin coats, bound epitopes concentrated around nuclei beneath nerve-muscle contacts [Bursztajn et al., 1993]. It is beneath these nerve terminals that AChRs cluster. On Western blots of skeletal muscle C172 recognizes a 100 kd band and two smaller bands. Immunofluorescent studies showed a punctate surface distribution. It was therefore thought that the C172 antibody recognized a protein homologous to the α subunit of the AP2 adaptor complex. To test this hypothesis, we compared C172 immunochemical properties in purified coated vesicles and muscle extract. We compared immunoblots of brain coated vesicles stained with C172 and anti-AP2 antibody 100/2 with immunoblots of purified AP180 stained with AP180.1 and C172 antibodies. These results show that C172 does not recognize the AP2 adaptor, but it does recognize AP180. This result was rather surprising, because AP180 is thought to be confined only to neurons. We therefore tested our C172 antibody properties further by carrying out tryptic digests to map the epitope for C172 in the brain AP180 sequence. These tryptic digests generated a 100 kd band recognized by both C172 and AP180.1 as well as antipeptide antibodies PS26/PS27 (Fig. 2), but only C172 recognized a short lived triplet of bands 60–66 kd. Thus, the 60 kd band must be derived from the 100 kd, but lacks the epitopes for AP180.1, PS26, and PS27. From

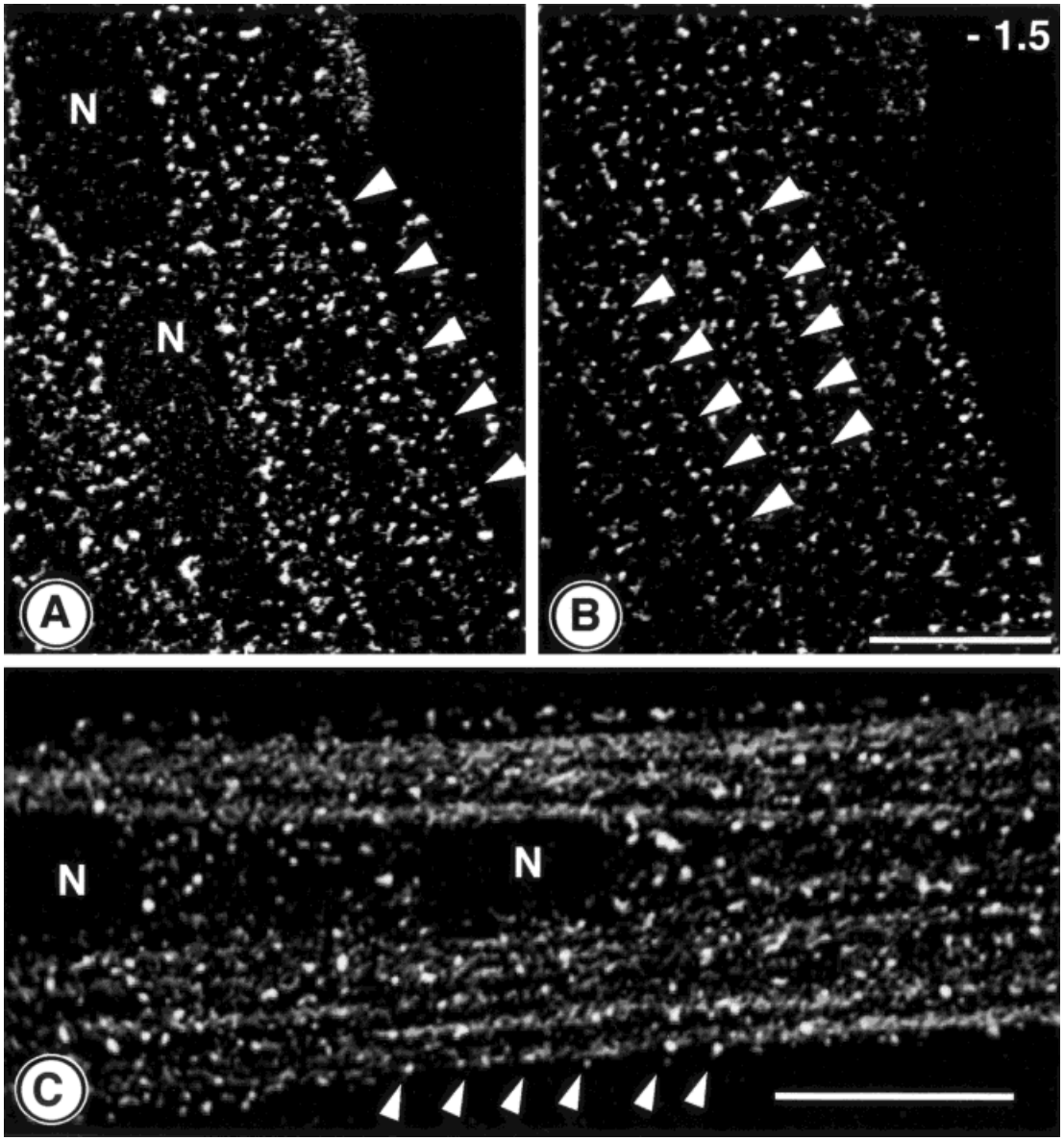

Fig. 5. Immunofluorescence confocal images of myotubes stained with C172 antibody. Images were captured using a video camera interfaced with an A axis drive stage controller and BDS image processing software (Oncor). Images were taken through the depth of the cell at 1.5 μm intervals. **A**: A linear arrangement of vesicles along the periphery of the cell is readily seen (arrows). N, Nuclei. **B**: 1.5 μm below the image seen in (A) rows of vesicles displaying linear arrays (arrows) are numerous. **C**: Myotube double labeled with C172 antibodies and phallacidin which binds to F actin show punctate immunostaining characteristic of vesicles which appear to track along the actin filaments. Scale bar = 10 μm. (Color image available upon request to author.)

peptide sequencing we know that the N-terminus of the 100 kd tryptic fragment is at residue 305 and PS27 is raised to a defined peptide centered at residue 613. Thus, the 60 kd peptide region must end before residue 613 with the most likely trypsin site from the predicted sequence occurring at residue 598. This region codes for a predicted peptide of 28 kd, which is seen as a band of apparent molecular weight 60 kd and must contain the region responsible for the strange behavior of AP180 on gels. A clathrin binding site has been identified in the N-terminal region of the AP180 protein. However a second site located within the C-terminal region is necessary for its assembly promotion function. Thus both of these proteins may contain a clathrin binding site at the N-terminus, but the C-terminal portion of AP180 needed for assembly promotion of clathrin by AP180 is either different or lacking in C172.

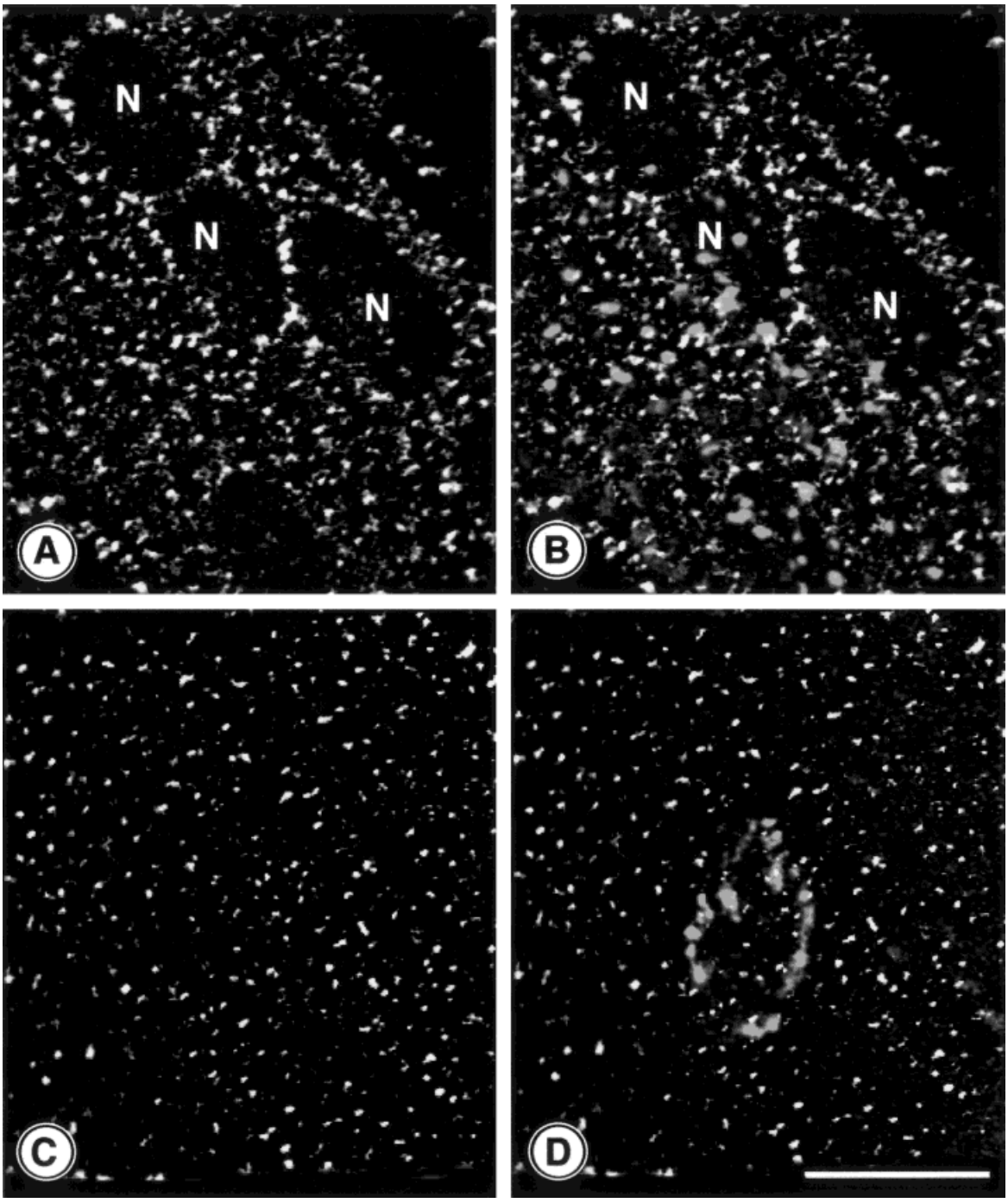

Fig. 6. The C172 vesicular epitopes concentrate around nuclei associated with AChR clusters. Cells were stained with rhodamine-conjugated α-bungarotoxin (αBTX-TMR) followed by C172 antibody and biotinylated anti-mouse IgG and streptavidin FITC. Confocal images of two parallel Z series of stacks were generated with FITC and rhodamine filters, respectively. From each pair of deconvoluted Z series stacks, the focal plane that best represented the labeled vesicles and AChRs were selected. The images were then merged together in register to form a single image. The large punctate stainings represent AChR clusters (**A**) and (**B**). The vesicles tend to line up around nuclei which are present beneath AChR clusters. Spaces between the aggregates may represent internalized receptors. N, Nuclei. **C,D**: Vesicles around AChR clusters not associated with nuclei show a random distribution. Scale bar = 10 μm. (Color image available upon request to author.)

TABLE I. Vesicle Density for Nuclei Associated and Nonassociated With Receptor Cluster‡

	Nucleus area	Vesicles/μm²
Cluster associated	41.51 ± 2.6 (N = 20)	0.63 ± 0.045*
Noncluster associated	64.77 ± 9.8 (N = 16)	0.27 ± 0.039

‡Quantitative analysis of C172 vesicular immunoreactivity was obtained from laser printer copies of gray scale composite images using BIOQUANT OS/2 Advanced Image Analysis software (R and M Biometrics, Nashville, TN) interfaced with a DTK 486pc computer via a Hipad digitizing board. The magnification of the gray scale images was calibrated by using a digitally recorded micrometer scale. The outline of each 1) selected cell profile in an image field, 2) nuclear profile and, 3) AChR cluster profile was traced with the digitizing board and the area determined. The cell area was adjusted by subtracting the total area of measured nuclei. The numerical density of labeled vesicle profiles was determined for: 1) regions overlying ACh-receptor clusters that were adjacent to nuclei and, 2) regions of nuclei that were not associated with ACh-receptor clusters. A mean and standard error of the mean were obtained for each variable to assess the consistency of vesicle densities between these different intracellular relationships. A two-tailed Student's *t*-test was used to evaluate the significance of differences for these variables.
* = (P = 0.0121 two-tailed Student's *t*-test).

These differences between AP180 and C172 suggest a distinct function for the muscle protein.

Morphological distribution of the C172 protein was revealed by immunofluorescent digital confocal microscopy that utilizes computer assisted deconvolution software. C172 appears to have a punctate distribution similar to the described staining pattern for the AP1 adaptor, but in contrast to AP1, C172 does not appear to localize to the TGN [Robinson, 1987; Robinson and Kreis, 1992], and does not colocalize with a WGA staining pattern. This is consistent with our conclusion that C172 does not recognize the AP1 adaptor. In myoblasts, which are spindle shaped cells, C172 exhibits polarity. A similar asymmetry was described in the distribution of the Golgi complex [Jasmin et al., 1989] and in the distribution of microtubules in association with clusters of noncoated vesicles at the postsynaptic membrane folds of Torpedo electric organ [Jasmin et al., 1991]. The C172 antibody did not stain every coated pit or vesicle suggesting that the protein recognized by this antibody functions in a specific subset of clathrin mediated trafficking events. In some cells, linear arrays are observed rather than discrete dots suggestive of cytoskeletal elements with which these vesicles may associate.

If AChR clusters are markers for the embryonic postsynaptic membrane, then organized membrane traffic involving these specialized sites may require vesicles with unique epitopes for targeting, stabilization, or removal. In our previous studies, as well as those of others, electron microscopy of newly formed junctions and sections taken through regions of muscle cells where AChR clustered, showed postsynaptic membrane thickening and an increase in protein density at these sites. Beneath these sites numerous coated vesicles and other vesicular structures are frequently seen [Bursztajn and Fischbach, 1984; Bursztajn et al., 1987; Park and Bursztajn, 1990]. If these microregions where new synapses are formed require vesicular membrane activity, insertion and retrieval of membrane resulting in vesicular accumulation with specific components may require that these targets be removed from this microregion and perhaps recycled. If the increase in membrane traffic is needed for the maintenance of specialized membrane, then this may account for the increase in the number of C172 reactive vesicles around nuclei located beneath AChR clusters. It is of interest that the C172-positive vesicles are not concentrated at AChR clusters that lack associated nuclei. This further suggests an active role for these vesicles in synapse-related membrane transport.

At the neuromuscular junction as well as in newly formed nerve muscle synapse the extracellular matrix contains numerous identified and not yet identified proteins that line the gap formed between nerve and muscle. An association between AChR, extracellular matrix protein, and the cytoskeleton has been proposed. The protein vinculin which is present at adhesion sites in cells as well as in concentrated amounts at AChR clusters [Bloch and Pumplin, 1990] may also provide a link between microfilaments and the plasma membrane [Burridge et al., 1988]. An interaction between vinculin and clathrin heavy chain and an association between microfilaments and coated vesicles has been reported [Faussner et al., 1993; Nishikowa and Kitamura, 1987]. Results have been

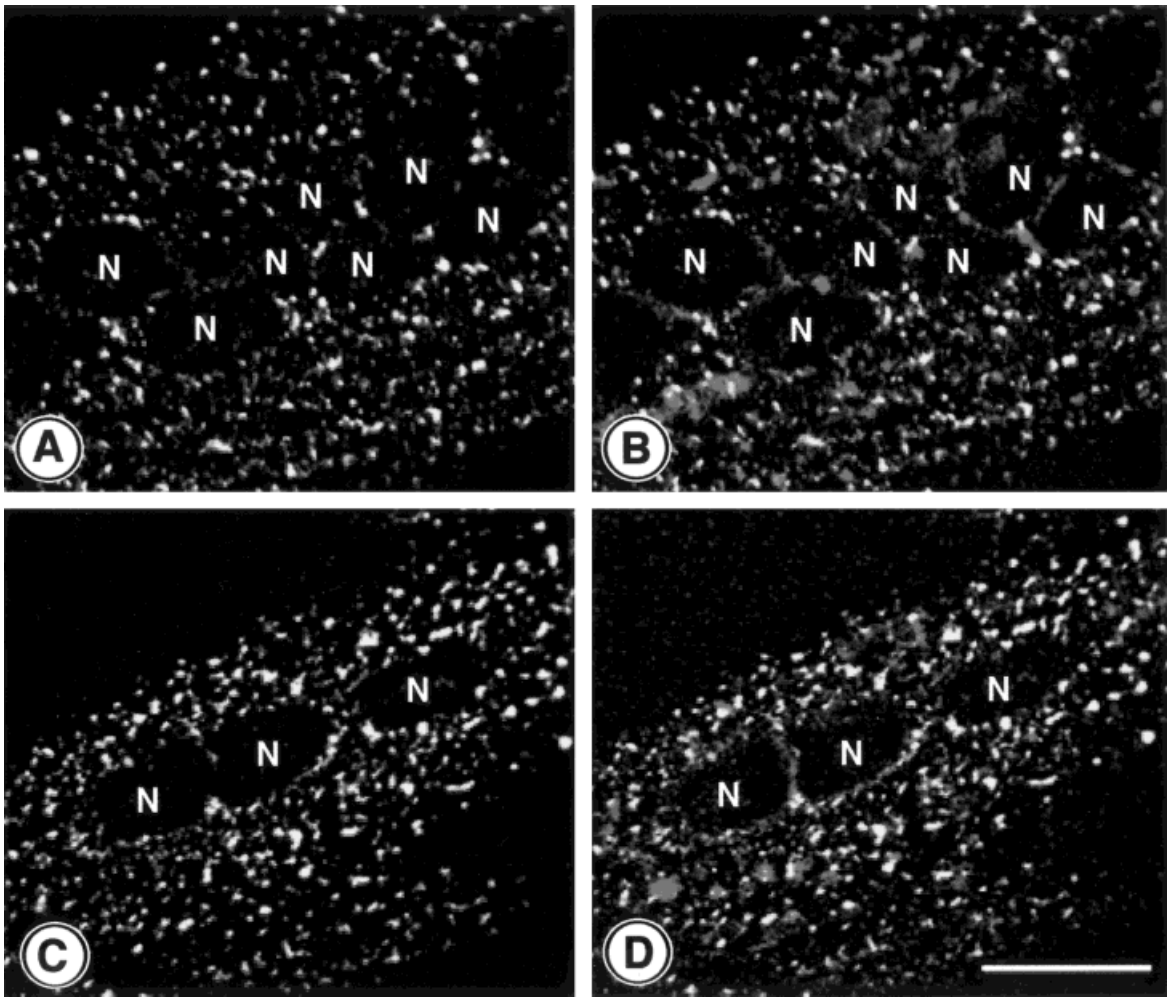

Fig. 7. Confocal fluorescent images of myotubes double labeled with the C172 vesicular epitopes and wheat germ agglutinin conjugated to FITC. **A,B**: Images taken through one myotube. **C,D**: Images taken through another myotube. The images of C172 vesicles were viewed with a rhodamine filter and WGA staining was viewed with a FITC filter. The images were merged together in register to form a single image shown in (B) and (D). Note that Golgi cisterna stained with the WGA do not appear to associate with the C172 vesicular epitope. N, Nuclei. Scale bar = 10 μm. (Color image available upon request to author.)

presented which suggest that actin filaments may play a role in endocytosis [Gottlieb et al., 1993]. In that respect it is of interest to note that C172 vesicles appear to track preferentially along the F actin filaments rather than microtubules.

Recent evidence has implicated the dystrophin-glycoprotein complex (DGC) in AChR clustering. This oligomeric transmembrane complex is composed of transmembrane proteins thought to bind utrophin at the synapse [Ervasti et al., 1990; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Yoshida et al., 1994]. Another protein rapsyn may link AChRs to DGC contributing the unique structural features of the postsynaptic membrane [Apel et al., 1995]. Utrophin may bind to the F actin thereby facilitating the association of AChR clusters with the cytoskeleton [Apel et al., 1995]. The C172 vesicular protein may serve as a link between the plasma membrane and intracellular events that allows specialized membrane to be transported via actin filaments. Whether these vesicular proteins play a role in identifying postsynaptic membrane geared for endocytosis or other intracellular transport processes remains to be determined. The possibility that these vesicular proteins have a structural or regulatory function in linking postsynaptic membrane to the cytoskeleton is under investigation.

ACKNOWLEDGMENT

This research was supported by NIH grants NS24377 (to S.B.), GM38093 (to F.M.B.) MH00423 (to F.B.), and a senior postdoctoral fellowship from the California Affiliate of the American Cancer Society (to S.A.M.).

REFERENCES

Ahle S, Ungewickell E (1986): Purification and properties of a new clathrin assembly protein. EMBO J 5:3143–3149.

Ahle S, Mann A, Eichelsbacher U, Ungewickell E (1988): Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. EMBO J 4:919–929.

Ahle S, Ungewickell E (1990): Auxilin, a newly identified clathrin-associated protein in coated vesicles from bovine brain. J Cell Biol 111:19–29.

Apel ED, Roberds SL, Campbell KP, Merlie JP (1995): Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. Neuron 15:115–126.

Brodsky FM (1988): Living with clathrin: its role in intracellular membrane traffic. Science 242:1396–1402.

Burridge K, Fath K, Kelly T, Nuckolls G, Turner C (1988): Focal adhesions: transmembrane junctions between the extracellular matrix and the cytoskeleton. Annu Rev Cell Biol 4:487–525.

Bursztajn S (1984): Coated vesicles are associated with acetyl-choline receptors at nerve- muscle contacts. J Neurocytol 13:503–518.

Bursztajn S, Fischbach GD (1984): Evidence that coated vesicles transport acetylcholine receptors to the surface membrane of chick myotubes. J Cell Biol 98:498–506.

Bursztajn S, Nudelman HB, Berman SA (1987): Coated and smooth vesicles participate in acetylcholine receptor transport. Cell Tissue Res 248:535–540.

Bursztajn S, Schneider LW, Jong YJ, Berman SA (1991): Calcium and ionophore A23187 stimulates deposition of extracellular matrix and acetylcholinesterase release in cultured myotubes. Cell Tissue Res 265:95–103.

Bursztajn S, Yei YJ, Berman SA (1993): Differential distribution of vesicular carriers during differentiation and synapse formation. J Cell Biochem 53:251–264.

Ellman GL, Courtney KD, Andres V, Featherstone RM (1961): A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7: 88–95.

Ervasti JM, Ohlendieck K, Kahl SD, Gaver MG, Campbell KP (1990): Deficiency of glycoprotein component of the dystrophin complex in dystrophic muscle. Nature 345: 315–319.

Ervasti JM, Campbell KP (1991): Membrane organization of the dystrophin-glycoprotein complex. Cell 66:1121–1131.

Fausser JL, Ungewickell E, Ruch JV, Lesot H (1993): Interaction of vinculin with the clathrin heavy chain. J Biochem 114:498–503.

Glickman JN, Conibear E, Pearse BMF (1989): Specificity of binding of clathrin adaptors to signals on the Mannose-6-phosphate/insulin-like growth factor II receptor. EMBO J 8:1041–1047.

Goldstein JL, Brown MS, Anderson RGW, Russell DW, Schneider WJ (1985): Receptor-mediated endocytosis: Concepts emerging from the LDL receptor system. Ann Rev Cell Biol 1:1–39.

Gottlieb TA, Iavanov IE, Adesniki M, Sabatini DD (1993): Actin microfilaments play a critical role in endocytosis at the apical but not the basolateral surface of polarized epithelial cells. J Cell Biol 120:695–710.

Hall CW, Liebaers I, DiNatale P, Neufeld EF (1978): Enzymatic diagnosis of the genetic mucopolysaccharide storage disorders. Methods Enzymol 50:439–456.

Heuser JE, Reese TS (1973): Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315–344.

Heuser JE (1989): The role of coated vesicles in recycling of synaptic vesicle membrane. Cell Biol Int Rep 13:1063–1076.

Jackson AP, Seow HF, Holmes N, Drickamer K, Parham P (1987): Clathrin light chains contain brain-specific insertion sequences and a region of homology with intermediate filaments. Nature 326:154–159.

Jasmin BJ, Cartaud J, Bornens M, Changeux JP (1989): Golgi apparatus in chick skeletal muscle: changes in distribution during endplate development and after denervation. Proc Natl Acad Sci USA 86:7218–7222.

Jasmin BJ, Changeux JP, Cartaub J (1991): Organization and dynamics of microtubules in torpedo marmorata electrocyte: selective association with specialized domains of the postsynaptic membrane. Neuroscience 43: 151–162.

Keen JH, Black MM (1986): The phosphorylation of coated membrane proteins in intact neurons. J Cell Biol 102: 1325–1333.

Keen JH (1987): Clathrin assembly proteins: affinity purification and a model for coat assembly. J Cell Biol 105:1989–1998.

Keen JH (1990): Clathrin and associated assembly and disassembly proteins. Annu Rev Biochem 59:415–438.

Keen JH (1993): Clathrin and associated assembly and disassembly proteins. Annu Rev Biochem 59:415–439.

Kirchhausen T, Harrison SC, Chow EP, Mattaliano RJ, Ramachandran KL, Smart J, Brosius J (1987): Clathrin heavy chain: molecular cloning and complete primary structure. Proc Natl Acad Sci USA 84:8805–8809.

Kirchhausen T, Nathanson KL, Matsui W, Vaisberg A, Chow EP, Burne C, Keen JH, Davis AE (1989): Structural and functional division into two domains of the large (100- to 115-kda) chains of the clathrin-associated protein complex AP-2. Proc Natl Acad Sci USA 86:2612–2616.

Kirchhausen T, Davis AC, Frucht S, Greco BO, Payne G, Tubb B (1991): AP17 and AP19, the mammalian small chains of the clathrin-associated protein complexes show homology to Yap17p, their putative homolog in yeast. J Biol Chem 266:11153–11157.

Kohtz DS, Puszkin S (1988): A neuronal protein (NP185) associated with clathrin-coated vesicles. Characterization of NP185 with monoclonal antibodies. J Biol Chem 263:7418–7425.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lindner R, Ungewickell E (1992): Clathrin-associated proteins of bovine brain coated vesicles. An analysis of their number and assembly-promoting activity. J Biol Chem 267:16567–16573.

Mahler H (1955): DPNH cytochrome c reductase (animal). In Colowick SP, Kaplan NO (eds): "Enzymology." Vol II. New York: Academic Press, pp. 688–693.

Maecker HT, Kitamura K, Brenner MB, Levy R (1987): Isolation of anti-idiotypic antibodies to T cells using an anti-framework determinant antibody. J Immunol. Methods 92:219–226.

Maycox PR, Egenhard L, Rectz A, Morris SA, Reinhardt J (1992): Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. J Cell Biol 118:1379–1388.

Morris SA, Ahle S, Ungewickell E (1989): Clathrin coated vesicles [review]. Curr Opin Cell Biol 1:684–690.

Morris SA, Mann A, Ungewickell E (1990): Analysis of 100-180-kda phosphoproteins in clathrin-coated vesicles from bovine brain. J Biol Chem 265:3354–3357.

Morris SA, Schroder S, Plessman U, Weber K, Ungewickell E. (1993): Clathrin assembly protein AP180: primary structure, domain organization and identification of a clathrin binding site. EMBO J 12:667–675.

Murphy J-E, Pleasure IT, Puszkin S, Keen JH (1991): Clathrin assembly protein AP-3. The identity of the 155K protein, AP 180, and NP185 and demonstration of a clathrin binding domain. J Biol Chem 266:4401–4408.

Murphy J-E, Hanover JA, Froehlich M, DuBois G, Keen JH (1994): Clathrin assembly protein AP-3 is phosphorylated and glycosylated on the 50-kda structural domain. J Biol Chem 269:21346–21352.

Nishikawa S, Kitamura H (1987): Microtubules, intermediate filaments, and actin filaments in the odontoblast of rat incisor. Anat Rec 219:144–151.

Park J, Bursztajn S (1990): The role of pH in intracellular acetylcholine receptor ligand processing. J Cell Biol 70: 177–188.

Pearse BM (1987): Clathrin and coated vesicles. EMBO J 6:2507–2512.

Pearse BM (1988): Receptors compete for adaptors found in plasma membrane coated pits. EMBO J 7:3331.

Pearse BM (1989): Characterization of coated-vesicle adaptors:their reassembly with clathrin and with recycling receptors. Methods Cell Biol 31:229–246.

Pearse BM, Robinson MS (1990): Clathrin, adaptors and sorting. Annu Rev Cell Biol 6:151–171.

Perry DG, Hanson V, Benuck ML, Puszkin S (1991): Neuronal protein NP185 in avian and murine cerebellum: expression during development and evidence for its presence in nerve endings. J Histochem Cytochem 39:1461–1470.

Perry DG, Li S, Hanson V, Puszkin S (1992): Neuromuscular junctions contain NP185: the multifunctional protein is located at the presynaptic site. J Neurosci Res 33:408–417.

Pley U, Parham P (1993): Clathrin: its role in receptor-mediated vesicular transport and specialized functions in neurons [review]. Crit Rev Biochem Mol Biol 28:431–464.

Porter-Jordon K, Benson RJ, Buoniconti P, Fine RE (1986): An acetylcholinesterase- mediated density shift technique demonstrates that coated vesicles from chick myotubes nay contain both newly synthesized acetylcholinesterase and acetylcholine receptors. J Neurosci 6:3112–3119.

Prasad K, Lippoldt RE (1988): Molecular characterization of the AP180 coated vesicles assembly protein. Biochemistry 27:6098–10855.

Pumplin DW, Bloch RJ (1990): Clathrin-coated membrane: A distinct membrane domain in acetylcholine receptor clusters of rat myotubes. Cell Motil Cytoskeleton 15:121–134.

Robinson MS (1987): Vesicle proteins: Molecular heterogeneity and intracellular distribution studied with monoclonal antibodies. J Cell Biol 104:887–895.

Robinson MS (1989): Cloning of cDNAs encoding two related 100-kd coated vesicle proteins (alpha-adaptins). J Cell Biol 108:833–842.

Robinson MS (1990): Cloning and expression of gamma-adaptin, a component of clathrin-coated vesicles associated with the Golgi apparatus. J Cell Biol 111:2319–2326.

Robinson MS, Kreis TE (1992): Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. Cell 69:129–138.

Solca FF, Lurio DI, Diltz CD, Johnson RS, Kumar S, Rubel EW, Fischer EH (1994): Identification and purification of a chicken brain neuroglia-associated protein. J Biol Chem 269:27559–27565.

Sorkin A, Carpenter G (1993): Interaction of activated EGF receptors with coated pit adaptins. Science 261:612–615.

Sorkin A, McKinsey T, Shih W, Kirchhausen T, Carpenter G (1995): Stoichiometric interaction of the epidermal growth factor receptor with the clathrin-associated protein complex AP-2. J Biol Chem 270:619–625.

Sousa R, Hrinya Tannery N, Lafer EM (1990): Two new monoclonal antibodies provide immunohistochemical evidence for the unique biochemical similarity of the mouse globus pallidus, entopeduncular nucleus and substantia nigra pars reticulata. Neuroscience 34:403–410.

Sousa R, Tannery NH, Zhou S, Lafer EM (1992): Characterization of a novel synapse-specific protein. I. Developmental expression and cellular localization of the F1-20 protein and mRNA. J Neurosci 12:2130–2143.

Su B, Hanson V, Perry D, Puszkin S (1991): Neuronal specific protein NP185 is enriched in nerve endings: binding characteristics for clathrin light chains, synaptic vesicles, and synaptosomal plasma membrane. J Neurosci Res 29:461–473.

Ungewickell E, Branton D (1981): Assembly units of clathrin coats. Nature 289:420–422.

Vigers GP, Crowther RA, Pearse BM (1986): Location of the 100 kd-50 kd accessory protein in clathrin coats. EMBO J 5:2079–2085.

Ye W, Lafer EM (1995): Clathrin binding and assembly activities of expressed domains of the synapse-specific clathrin assembly protein AP-3. J Biol Chem 270:10933–10939.

Yoshida M, Suzuki A, Yamamoto H, Noguchi S, Mizuno Y, Ozawa E (1994): Dissociation of the complex of dystrophin and its associated proteins into several unique groups by n-octyl β-D-glucoside. Eur J Biochem 222:1055–1061.

Zhou S, Sousa R, Tannery NH, Lafer EM (1992): Characterization of a novel synapse specific protein. II. cDNA cloning and sequence analysis of the F1-20 protein. J Neurosci 12:2144–2155.

Zhou S, Tannery NH, Yang J, Puszkin S, Lafer EM (1993): The synapse-specific phosphoprotein F1-20 is identical to the clathrin assembly protein AP-3. J Biol Chem 268: 12655–12662.