A Requirement for Ankyrin Binding to Clathrin during Coated Pit Budding*

(Received for publication, June 22, 1999, and in revised form, September 9, 1999)

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Recent studies suggest that the mobility of clathrin-coated pits at the cell surface are restricted by an actin cytoskeleton and that there is an obligate reduction in the amount of spectrin on membranes during coated pit budding. The spectrin-actin cytoskeleton associates with membranes primarily through ankyrins, which interact with the cytoplasmic region of numerous integral membrane proteins. We now report that the fourth repeat domain (D4) of ankyrinRα binds to the N-terminal domain of clathrin heavy chain with high affinity. Addition of peptides containing the D4 region inhibited clathrin-coated pit budding in vitro. In addition, microinjection of D4 containing peptides blocked the endocytosis of fluorescent low density lipoprotein (LDL). AnkyrinRα peptides that contained repeat domains other than D4 had no effect on either in vitro budding or internalization of LDL. Finally, immunofluorescence shows that ankyrin is uniformly associated with endosomes that contain fluorescent LDL. These results suggest that ankyrin plays a role in the budding of clathrin-coated pits during endocytosis.

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Recent studies on the mechanism of coated pit budding in fibroblasts suggest that there is an obligate decline in plasma membrane spectrin prior to completing the final stages of the budding process (1). The decline in spectrin depends on the ability of annexin VI to bind spectrin and activate a calpain-like protease. When cells are grown in the presence of calpain inhibitor I (ALLN), annexin VI-dependent budding of clathrin-coated pits is arrested. The cells compensate by assembling a new population of coated pits that can bud in the absence of annexin VI and no longer require spectrin removal. The endosomes that form from these pits, however, have a distinctly different trafficking pattern in the cell.

The spectrin-actin cytoskeleton lines the cytoplasmic surface of a variety of cellular membranes including the endoplasmic reticulum, Golgi apparatus, lysosome, plasma membrane, and an unknown vesicular structure (2–5). At the plasma membrane, this cytoskeleton can control membrane topology, elasticity, and membrane protein composition (5, 6). Products of the three ankyrin genes (ankyrinR, B,G) provide the primary link between spectrin-actin networks and the plasma membrane. Ankyrins mediate this linkage through binding sites for both the β-subunit of spectrin and the cytoplasmic domains of several membrane proteins. Binding sites for these membrane proteins are largely localized to the membrane-binding domain at the N terminus of ankyrins. This highly conserved region of ankyrin is dominated by 24 tandem copies of a 33-amino acid motif that is commonly termed ANK repeats or ankyrin repeats (7–9).

Ankyrin repeats are a well characterized motif that provides sites of protein-protein interaction in numerous proteins (10). Nonankyrin molecules that contain ankyrin repeats typically have 4–7 tandem copies, and tandem arrays of repeats have been shown to fold cooperatively into stable structures (11, 12). The 24 tandem repeats found in ankyrins assemble into four folding domains of six repeats each (13) and are believed to associate with the cytoplasmic domains of at least seven unrelated membrane proteins (5). The binding sites for two of these membrane proteins, neurofascin and the anion exchanger, have been localized on the repeat domains. Surprisingly, both membrane proteins interact with ankyrin at discrete, noncompeting sites that require both repeat domains 3 and 4 (13, 14).

During a screen to identify new ankyrin-binding proteins, we discovered that the D4 region of ankyrinRα binds with high affinity to the N-terminal domain of clathrin heavy chain. Because of the role of spectrin in coated pit budding, we tested peptides containing D4 and found that they specifically block annexin VI-dependent budding in vitro and prevent the uptake of LDL when microinjected into cells.

EXPERIMENTAL PROCEDURES
Materials
Chromatography matrices were from Amersham Pharmacia Biotech. 0.4-μm size-selected, epoxy-activated, nonporous latex beads were custom synthesized by Bange, Inc. (Fishers, IN). pGEMEX plasmid was purchased from Promega (Madison, WI). 125I-Labeled Bolton-Hunter reagent was from ICN Radiochemicals (Costa Mesa, CA). 125I-Labeled streptavidin was from Amersham Pharmacia Biotech. ALLN (Calpain I inhibitor) was from CalBiochem (La Jolla, CA). Biotinylated horse anti-mouse IgG was from Vector Labs (Burlingame, CA). The anti-ankyrin mAb1 was raised against the spectrin-binding domain of ankyrinRα. The clathrin polyclonal antibody was raised against red cell clathrin. The anti-clathrin mAb was a gift from Francis Brodsky. The anti-spectrin mAb was from Sigma. Alexa 568 goat anti-mouse was from Molecular Probes (Eugene, OR). Aqua Poly/Mount was from Polysciences (Warrington, PA). All tissue culture reagents were from Life Technologies, Inc. All other chemicals were from Sigma. PMCA-LDL and HLPPS were prepared by standard methods (15, 16).

1 The abbreviations used are: mAb, monoclonal antibody; LDL, low density lipoprotein; DTT, dithiothreitol; BSA, bovine serum albumin; BIA, radioimmune assay; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PMCA, 3-pyrenemethyl-23,24-dinor-5-cholen-22-oate-3β-y1 oleate; ALLN, N-Ac-Leu-Leu-norleucinal; HLPPS, human lipoprotein poor serum.

* This work was supported by National Institutes of Health Grants DK29808 and HL20948 and the Perot Family Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1251-Labeled Bolton-Hunter reagent was from ICN Radiochemicals (Costa Mesa, CA). 1251-Labeled streptavidin was from Amersham Pharmacia Biotech. ALLN (Calpain I inhibitor) was from CalBiochem (La Jolla, CA). Biotinylated horse anti-mouse IgG was from Vector Labs (Burlingame, CA). The anti-ankyrin mAb1 was raised against the spectrin-binding domain of ankyrinRα. The clathrin polyclonal antibody was raised against red cell clathrin. The anti-clathrin mAb was a gift from Francis Brodsky. The anti-spectrin mAb was from Sigma. Alexa 568 goat anti-mouse was from Molecular Probes (Eugene, OR). Aqua Poly/Mount was from Polysciences (Warrington, PA). All tissue culture reagents were from Life Technologies, Inc. All other chemicals were from Sigma. PMCA-LDL and HLPPS were prepared by standard methods (15, 16).
Ankyrin Binding to Clathrin

Methods

General Methods—SDS-polyacrylamide gel electrophoresis was performed on 3.5–17% exponential gradient gels using the Fairbanks buffer system (17). Ankyrin repeat peptides were expressed from cDNAs derived from the ankyrinR gene using a T7 expression vector. Protein constructs were purified from expressing bacteria by gel filtration and ion exchange chromatography as detailed elsewhere (13).

Truncated annexin VI AnxVIΔ224 was prepared as described previously (1). The D4 peptide corresponds to a chymotryptic product of ankyrinR and encompasses amino acids 588–827. The D4 affinity column was made by covalently coupling 100 mg of D4 to 20 ml of CNBr-activated Sepharose according to the manufacturer’s protocol (Amersham Pharmacia Biotech). SV40 transformed human fibroblasts (designated SV589 cells) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% v/v foetal bovine serum and 20 mM Hepes, pH 7.4.

Brain Extract—12 adult rat brains (25 g) were homogenized in 100 ml of ice-cold lysis buffer containing 320 mM sucrose, 5 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1 mM DTT, 1 mM Na3VO4, and protease inhibitors (5 μg/ml leupeptin, 5 μg/ml pepstatin, 100 μg/ml phenylmethylsulfonyl fluoride, and 0.5 mM diisopropylphosphoramide). Nuclei were removed by centrifugation at 10,000 × g for 10 min at 4°C. Membranes were pelleted by centrifugation at 100,000 × g for 40 min. The supernatant from this spin was designated as cytosol. The membrane pellet was washed in lysis buffer and recentrifuged at 100,000 × g for 40 min. Membranes were resuspended in 100 ml of lysis buffer followed by the addition of 100 ml of ice-cold Triton X-100 buffer (5% v/v Triton X-100, 0.2% phosphatidylcholine, 200 mM KCl, 20 mM Hepes, pH 7.5, 4 mM DTT, 1 mM Na3VO4, and protease inhibitors) for 30 min on ice. The solution was then centrifuged for 45 min on ice at 100,000 × g to remove insoluble material.

D4 Column—The D4 column was loaded three times with the Triton X-100 extract and washed with 10 column volumes of wash buffer (2.5% v/v Triton X-100, 0.2% phosphatidylcholine, 100 mM KCl, 20 mM Hepes, pH 7.5, 2 mM DTT, and 1 mM Na3VO4). Bound proteins were eluted with elution buffer (wash buffer plus 1 M NaBr). Peak fractions (30 ml) containing the 170 kDa band (clathrin heavy chain) were pooled, dialyzed against lysis buffer and washed with 10 column volumes of wash buffer (2.5% v/v Triton X-100, 0.02% phosphatidylcholine, 100 mM KCl, 20 mM Hepes, pH 7.5, 2 mM DTT, and 1 mM Na3VO4) overnight, loaded on a MonoQ column, and eluted with a buffer containing 1 M NaBr gradient in column buffer. The peak fractions (6 ml) were pooled and used for the binding assays detailed below.

Binding Assay—Latex beads were coated with ankyrin peptides as described previously (18). The beads were then resuspended in assay buffer containing 10 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM Na3VO4, 100 mM NaCl, 10 mg/ml BSA, and 0.1% Tween 20. D4 affinity purified clathrin was labeled with 125I using the Bolton-Hunter method. Affinities were measured by incubating increasing concentrations of 125I-labeled clathrin with ankyrin peptide derivitized beads in assay buffer for 3 h on ice. 125I-labeled clathrin bound to the beads was separated from unbound by centrifugation through a cushion of 10% sucrose in assay buffer. Both bound and unbound fractions were counted and used to calculate saturation and Scatchard plots. Non-specific associations were assessed by adding the concentration of NaCl to 500 mM in parallel assays. Non-specific interactions represented less than 10% total binding and have been subtracted from the data presented. All measured values are the means of triplicate experiments with a standard deviation <5%.

Budding Assay—Clathrin-coated pit budding and spectrin removal were assessed in vitro as described previously (19). Briefly, SV589 fibroblasts grown as described previously (20) were attached to poly-l-lysine-coated coverslips by centrifugation (1800 × g for 10 min) followed by incubation with ice-cold Eagle’s medium’s minimum essential medium supplemented with 20 mM Hepes, pH 7.4, and 2% crystalline BSA for 1 h. The coverslips were then washed quickly with buffer A (50 mM Hepes, pH 7.4, 100 mM NaCl) and buffer B (25 mM Hepes-KOH, pH 7.0, 25 mM KCl, 1 M sucrose, 2.5 mM magnesium acetate, 0.2 mM DTT) and finally sonicated in buffer B on ice. The attached plasma membranes were washed three times with ice-cold buffer B. Budding was initiated by shifting to 37 °C for 10 min in the presence of budding mixture (buffer B with 1 mg/ml inactivated bovine brain cytosol, 1 mM ATP, 150 μM CNBr, and 1 mM EGTA) and the indicated concentration of ankyrin peptide. The membranes were then washed three times with ice-cold buffer B, fixed for 15 min with 3% paraformaldehyde in buffer B without DTT, and washed three times with buffer C (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, and 2 mM MgCl2). Coated pit budding was measured as the amount of clathrin lost from the membrane as determined using a radioimmune assay (RIA). Spectrin loss was also assessed by RIA. RIAS were performed using mAb against either clathrin or spectrin, a biotinylated horse anti-mouse IgG, and 125I-streptavidin as described previously (19). RIAS for clathrin and spectrin were also performed on membranes that were only washed and fixed. The counts from these RIAS were taken to be total starting values. The percentage of clathrin or spectrin loss is calculated as the ratio of scintillation counts from each experiment to the total starting values.

LDL Uptake Assay—SV589 fibroblasts were plated on coverslips in Dulbecco’s modified Eagle’s medium plus 10% foetal bovine serum and grown overnight. The medium was replaced with Dulbecco’s modified Eagle’s medium plus 10% lipoprotein-depleted human serum (HLPPS), 37 °C, for 4 h. LDL uptake was initiated by incubating cells in HLPPS medium containing 20 μg/ml PMCA-LDL at 37 °C in a CO2 incubator for 30 min. The coverslips were then washed twice with PBS, fixed with 3% paraformaldehyde in PBS on ice for 15 min, washed four times with PBS, and mounted with Aqua Poly/Mount.

Microinjection—SV589 fibroblasts were plated on coverslips as described above for the LDL uptake assay. The truncated annexin VI, AnxVIΔ224, (the first 192 residues of annexin VI) and ankyrin peptides D12 and D34 were dialedyzed against a buffer containing 10 mM sodium phosphate, pH 7.2, 100 mM KCl, and 1 mM DTT. The protein solutions were then diluted with buffer to 20 μM and combined with 20 μg/ml FITC-dextran to a final concentration of 10 μM protein and 10 μg/ml dextran. The solutions were then spun at 100,000 × g for 30 min to remove aggregates and diluted into 4 μg/ml in 1% BSA in PBS and incubated with the coverslips for 1 h at room temperature. The coverslips were then washed with PBS. Coverslips were mounted using Aqua Poly/Mount and viewed with a Zeiss Photomicroscope III. The images shown are representative of the results obtained in five separate trials.

Results

The N-terminal Domain of Clathrin Heavy Chain Associates with Repeat Domain 4 of Ankyrin—In an effort to identify novel ankyrin-binding proteins, we screened for proteins that could associate with an affinity column made from the fourth repeat domain (D4) of the membrane-binding domain of ankyrinR. The D4 affinity column was constructed using a recombinant peptide that encompasses amino acids 588–827 of ankyrinR. This region corresponds to a chymotryptic fragment of the membrane-binding domain of ankyrinR, which contains repeats 19–24 as well as some flanking sequences. A Triton X-100 extract of rat brain membranes was passed over the D4 column (Fig. 1, lanes 1 and 2), after which the column was extensively washed and eluted with a buffer containing 1 M NaBr (lane 3). Coomassie Blue staining showed that the eluate contained a prominent 170-kDa band along with several minor proteins, including a doublet of 30–55 kDa. A protein of similar molecular mass was retained on the D4 affinity column when cytosol was loaded in place of the Triton X-100 extract (lane 6). Further purification of the 170-kDa protein by MonoQ ion exchange removed most contaminating bands found in Triton X-100 eluate with the exception of the 30–35-kDa doublet (lane 7).

The identity of the 170-kDa protein was determined by microsequencing of proteolytic products. Chymotryptic proteolysis of the 170-kDa band generated two major products of 120 and 60 kDa. The 120- and 60-kDa proteins appeared to have blocked N termini; however, the 120-kDa product had an N-terminal sequence identical to an internal sequence of rat clathrin heavy chain (microsequencing data not shown). The
170-kDa protein was recognized by a clathrin-specific polyclonal antibody in immunoblots and displayed a typical triskelion appearance in rotary shadowing electron microscopic images (data not shown). The observed doublet of 30–35 kDa that co-eluted with the 170-kDa band (Fig. 1, lane 7) may correspond to the two light chains of the clathrin triskelion, which have apparent molecular masses of 33 and 36 kDa (21).

Previous studies have shown that clathrin heavy chain can be cleaved by proteolysis into two functionally distinct domains: a 120-kDa C-terminal region, which forms the polygonal lattice in clathrin coats, and a 60-kDa N-terminal domain that extends from the lattice toward the membrane and interacts with AP2 (22), β-arrestin (23), and the NPXV sequence of the LDL receptor (24). To determine which of these two regions contained the binding site for D4, D4 affinity purified clathrin was digested with V8 Staphylococcus protease at a 1:320 mass ratio for 16 h on ice. A D4 affinity column was loaded with 20 μl of the following: lane 1, Triton X-100 extract load; lane 2, Triton X-100 extract flow through; lane 3, eluted material from Triton X-100 extract load; lane 4, brain cytosol load; lane 5, brain cytosol flow through; and lane 6, eluted material from cytosol load. Lane 7 was run on a separate gel and contains 20 μl of the peak fraction off a MonoQ purification of the material shown in lane 3.

FIG. 1. A 170-kDa band from rat brain membranes and cytosol binds repeat domain 4 of ankyrinR. A D4 affinity column was loaded with either 200 ml of Triton X-100 extract of rat brain membranes (lanes 1–3) or 100 ml of rat brain cytosol (lanes 4–6), washed, and eluted with 1 M NaBr. Shown is a Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis gel that was loaded with 20 μl of the following: lane 1, Triton X-100 extract load; lane 2, Triton X-100 extract flow through; lane 3, eluted material from Triton X-100 extract load; lane 4, brain cytosol load; lane 5, brain cytosol flow through; and lane 6, eluted material from cytosol load. Lane 7 was run on a separate gel and contains 20 μl of the peak fraction off a MonoQ purification of the material shown in lane 3.

D4 Inhibits Annexin VI-dependent Coated Pit Budding in Vitro—Previous work suggests that the spectrin cytoskeleton may be involved in annexin VI-dependent coated pit budding. Spectrin is removed from immobilized plasma membranes concomitant with clathrin-coated pit budding, and the presence of either an anti-spectrin IgG or the actin-binding domain of spectrin in the assay blocks annexin VI-dependent coated pit budding (1). Because spectrin binds to ankyrin (25) and the D4 region of ankyrin binds to clathrin (Fig. 3), we tested whether D4 affected coated pit budding using a radioimmune assay that measures the amount of clathrin on immobilized plasma membrane (Fig. 4). In this assay, addition of cytosol, ATP, and Ca2+ at 37°C causes about a 25% decline in the amount of clathrin on immobilized fibroblast membranes. An additional 40–45% of the coated pits bud when annexin VI is present in the budding reaction (19). The presence of increasing amounts of either D4 (Fig. 4A, □) or D34 (Fig. 4A, ■) in the budding assays containing cytosol, ATP, Ca2+, and annexin VI caused a progressive reduction in the amount of clathrin lost. Inhibition plateaued at ~25% loss with a half-maximal inhibition occurring at 30 and 20 nM, respectively. D4 (Fig. 4B, □) and D34 (■) also blocked spectrin removal with half-maximal inhibition at 15 and 10 nM, respectively. By contrast, addition of up to 320 nM of D3 (○) or D12 (●) had no effect on either clathrin loss (A) or spectrin removal (B). D2 and D23 could not be tested in this assay because they were not soluble in low salt buffers.

ALLN, a short peptide that inhibits cysteine proteases, blocks annexin VI-dependent clathrin-coated pit budding both in vivo and in vitro. Fibroblasts treated with ALLN for at least 30 min assemble a new population of coated pits that bud independently of annexin VI (1). We compared the effects of D4 on annexin VI-dependent and independent budding using membranes prepared from fibroblasts treated without or with ALLN for 1 h, respectively (Fig. 4). The addition of 50 nM D4 or D34 to the budding assay caused greater than 50% inhibition of both clathrin loss and spectrin removal from membranes of untreated fibroblasts (Fig. 4, C and D, solid bars). By contrast, the addition of these same peptides had no effect on clathrin loss from membranes of fibroblasts that had been treated with ALLN for 1 h (Fig. 4C, gray bars). As shown previously (1), coated pit budding from membranes of ALLN-treated fibroblasts was not accompanied by spectrin removal (Fig. 4D, compare solid bars with gray bars). Peptides D3 and D12, which did not bind clathrin, had no effect on clathrin loss or spectrin removal from membranes isolated from either treated (gray bars) or untreated cells (solid bars). These findings suggest that the D4 region specifically blocked annexin VI-dependent
D34 Inhibits Internalization of PMCA-LDL—Previously we identified a dominate-negative acting annexin VI (AnxVI\textsubscript{D192}) corresponding to the first 192 amino acids of the protein that inhibited coated pit budding \textit{in vitro} (1). Microinjection of this peptide into fibroblasts markedly reduced the internalization of fluorescent LDL. We used microinjection to see whether peptides containing the D4 repeat would also inhibit LDL uptake (Fig. 5). A buffer containing 10 mg/ml FITC-labeled dextran, either with no additions (Buffer) or with 10 mM peptide D12 (D12), D34 (D34) or peptide AnxVI\textsubscript{D192} (Δ192) added, was microinjected into SV589. Cells were allowed to recover for 30 min before 20 μg/ml PMCA-LDL was added, and the cells were further incubated for 30 min in HLPPS. The FITC-labeled cells were picked out, and the amount of PMCA-LDL internalized was compared with surrounding noninjected cells. The uptake of PMCA-LDL by cells injected with the D12 peptide was normal (D12) and did not differ from cells that received buffer alone (Buffer). As shown previously (1), uptake of LDL in cells injected with AnxVI\textsubscript{D192} was markedly inhibited (Δ192). The D34 peptide (D34) was nearly as effective as AnxVI\textsubscript{D192} at inhibiting LDL internalization.

Localization of Ankyrin to Endosomes Containing LDL—Because both the \textit{in vitro} and \textit{in vivo} assays for coated pit budding indicated a function for the ankyrin repeat domain, we used immunofluorescence to determine whether ankyrin was associated with any membranes in the endocytic pathway. Cells that had been grown in the absence of lipoproteins were incubated in the presence of fluorescent LDL for 30 min before fixation and processing for immunofluorescence using an antibody that recognizes the spectrin-binding domain of ankyrinB (Fig. 6). The mAb ankyrin had a punctate staining pattern in coated pit budding.

\textit{D34 Inhibits Internalization of PMCA-LDL}—Previously we identified a dominate-negative acting annexin VI (AnxVI\textsubscript{D192}) corresponding to the first 192 amino acids of the protein that inhibited coated pit budding \textit{in vitro} (1). Microinjection of this peptide into fibroblasts markedly reduced the internalization of fluorescent LDL. We used microinjection to see whether peptides containing the D4 repeat would also inhibit LDL uptake (Fig. 5). A buffer containing 10 mg/ml FITC-labeled dextrans, either with no additions (Buffer) or with 10 mM peptide D12 (D12), D34 (D34) or peptide AnxVI\textsubscript{D192} (Δ192) added, was microinjected into SV589. Cells were allowed to recover for 30 min before 20 μg/ml PMCA-LDL was added, and the cells were further incubated for 30 min in HLPPS. The FITC-labeled cells were picked out, and the amount of PMCA-LDL internalized was compared with surrounding noninjected cells. The uptake of PMCA-LDL by cells injected with the D12 peptide was normal (D12) and did not differ from cells that received buffer alone (Buffer). As shown previously (1), uptake of LDL in cells injected with AnxVI\textsubscript{D192} was markedly inhibited (Δ192). The D34 peptide (D34) was nearly as effective as AnxVI\textsubscript{D192} at inhibiting LDL internalization.
LDL. SV589 cells grown on coverslips in HLPPS were incubated in the buffer no additions (Buffer) or 10 μM peptide D12 (D12), D34 (D34), or AnxVIΔ192 (Δ192) was microinjected into SV589. Cells were allowed to recover for 30 min before further incubation in the presence of 20 μg/ml PMCA-LDL for 30 min. Cells were then fixed and processed for microscopy. Microinjected cells were identified by FITC fluorescence (left panels). Internalized PMCA-LDL appeared as large vesicles scattered through out the cell (right panels). Bar, 10 μm.

FIG. 5. Microinjected ankyrin repeat D34 blocks LDL internalization. A buffer containing 10 mg/ml FITC-labeled dextran with either no additions (Buffer) or 10 μM peptide D12 (D12), D34 (D34), or AnxVIΔ192 (Δ192) was microinjected into SV589. Cells were allowed to recover for 30 min before further incubation in the presence of 20 μg/ml PMCA-LDL for 30 min. Cells were then fixed and processed for microscopy. Microinjected cells were identified by FITC fluorescence (left panels). Internalized PMCA-LDL appeared as large vesicles scattered through out the cell (right panels). Bar, 10 μm.

FIG. 6. Co-localization of ankyrin and internalized fluorescent LDL. SV589 cells grown on coverslips in HLPPS were incubated in the presence of 20 μg/ml PMCA-LDL for 30 min. Cells were then processed for immunofluorescence localization of ankyrin. The left panel shows the distribution of ankyrin, and the right panel shows the distribution of PMCA-LDL. Bar, 10 μm.

Ankyrin

PMCA-LDL

the cytoplasm of the cell in addition to staining on the margins of the cell (Ankyrin). PMCA-LDL was seen in numerous vesicular structures (PMCA-LDL). A comparison of the two images (arrows) showed that nearly all of the vesicular structures containing fluorescent LDL were positive for ankyrin.

DISCUSSION

This study provides the first evidence that ankyrins can associate directly with clathrin and participate in annexin VI-dependent, clathrin-mediated endocytosis. The N-terminal domain of clathrin heavy chain contained the binding site for ankyrin. The membrane-binding domain of ankyrinG contained two binding sites for clathrin: a site on the second repeat domain (D2) and a site on the fourth repeat domain (D4). The functional significance of the site on D2 is unclear because clathrin was unable to bind peptides containing D2 in combination with either D1 (D12) or D3 (D23). The inability of clathrin to associate with D12 or D23 suggests that D1 and D3 may sterically hinder the ability of clathrin to associate with D2. By contrast, domain 3 (D3) may strengthen the clathrin association with D4 because the binding of clathrin to D34 had a 2-fold higher affinity than to D4 alone. The D4 and D34 peptides inhibited budding from membranes prepared from untreated but not ALLN-treated fibroblasts. One possibility is that D4 competes with an endogenous ankyrin that normally must bind to clathrin during annexin VI-dependent, coated pit budding. This would explain why LDL uptake in vivo was inhibited by microinjected D34 peptides and endosomes containing fluorescent LDL are positive for ankyrin.

Coated pits are found in regions of the membrane rich in membrane cytoskeletal elements (26–28), and rapid-freeze, deep-etch electron microscopy shows actin-like filaments near clathrin lattices (29). Recent studies using clathrin tagged with green fluorescent protein indicate that the mobility of coated pits is restricted by an actin cytoskeleton (30). This suggests there is a functional linkage between coated pits and the cytoskeleton and annexin VI functions to disconnect this linkage during budding. Ankyrin D4 peptides most likely block budding by preventing the release of spectrin from the membrane. Exactly how an interaction between ankyrin and clathrin regulates annexin VI-dependent release of spectrin remains to be determined. We were unable to detect ankyrin in coated pits, although isolated brain-coated vesicles were positive for ankyrin (data not shown). Nevertheless, nearly every endosome that contained fluorescent LDL also was positive for ankyrin. It is possible that ankyrin binding to clathrin delivers ankyrin to endosomes during the final phases of the budding step.

We may not have detected ankyrin in coated pits by immunofluorescence because only specific isoforms interact with clathrin, the reactive epitope in ankyrin was masked, or the association of ankyrin with clathrin is regulated. The C-terminal portion of ankyrin is highly variable among its various isoforms, and this region appears to control both the localization and binding specificity of individual ankyrin isoforms. We have tested whether the two most prevalent ankyrin isoforms in erythrocytes (band 2.1 and 2.2) can associate with clathrin, and neither displays clathrin binding activity (data not shown). Several additional ankyrin gene products are potential candidates for an in vivo association with clathrin. AnkyrinG119 contains the D34 region but only a 5-kDa regulatory domain, suggesting that this C-terminal region may not regulate the binding activities of this ankyrin (2). AnkyrinG480 contains a complete membrane-binding domain and a very large C-terminal region (9). Interestingly, indirect immunofluorescence has co-localized ankyrinG480 with amphiphysin II at axonal initial segments and nodes of Ranvier, which are rich in clathrin-coated pits (9, 27, 28, 31). Additional ankyrins may associate with clathrin as a result of specific regulatory events. A variety of kinases can modulate the binding activity of ankyrins by phosphorylation (5). Proteolytic processing may also play a role because the C-terminal region is highly sensitive to proteolysis and a calpain-like protease is required for annexin VI-dependent clathrin-coated pit budding in fibroblasts (1). Future work will examine which ankyrin isoform is the in vivo partner for clathrin.

Acknowledgments—We gratefully acknowledge Judy Phelps for performing the microsequencing, Jonathon Davis for expertise in the rotary shadowing experiment, and Amanda McDaniel for assistance with the microinjection experiments.

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