The vitronectin receptor associates with clathrin-coated membrane domains via the cytoplasmic domain of its β5 subunit

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SUMMARY

Rat myotubes cultured in fetal calf serum adhere to vitronectin-coated substrates through two distinct structures, focal contacts and clathrin-coated membrane domains. We studied the integrins in myotubes to learn how they associate with these two domains. Double label immunofluorescence studies with antibodies specific for clathrin, vinculin and several forms of integrin showed that focal contacts and clathrin-coated membrane domains contain both vitronectin receptors (VnR, containing β3- and β5-integrins) and fibronectin receptors (FnR, containing β1-integrin). VnR but not FnR associates tightly with the substrate in both domains, as the VnR alone remains attached to the coverslip when the lipid bilayer and other membrane proteins are removed by detergent. Ultrastructural studies confirmed the localization of the β5 subunit of the VnR at both domains.

We used intracellular injection and affinity chromatography to test the possibility that clathrin at coated membrane domains associates with the cytoplasmic sequence of the β5 subunit of the VnR.

Injection of a synthetic peptide containing the NPXY motif from the cytoplasmic domain of the human β5 subunit, SRAVYEMASNPLYRPST, depleted clathrin from coated membrane domains without affecting clathrin in perinuclear structures or vinculin at focal contacts. Injection of the homologous β1 peptide, MNAKWDTGPIYKTSST, also containing an NPXY motif, had no significant effect on any of these structures. Affinity matrices containing the β5 but not the β1 peptide selectively retained clathrin from myotube extract, and bound clathrin could be selectively eluted by soluble forms of the β5 but not the β1 peptide. Thus, a sequence including the NPXY motif in the integrin β5 subunit is involved in the specific anchoring of the VnR, but not the FnR, to clathrin-coated membrane.

Key words: Integrin, Vitronectin receptor, Fibronectin receptor, Vitronectin, Clathrin, Vinculin, Acetylcholine receptor, Muscle, Neuromuscular junction

INTRODUCTION

Rat myotubes grown in tissue culture adhere to a vitronectin-coated glass substrate through two distinct structures, focal contact domains and clathrin-coated membrane domains. Intracellularly, the focal adhesions contain vinculin, talin, bundles of actin microfilaments, and associated proteins (e.g. Bloch and Geiger, 1980; Bloch et al., 1989; Samuelsson et al., 1993; Plopper and Inger, 1993), and so are typical of focal adhesions in mononucleated cells. Clathrin-coated membrane domains are characterized by extensive arrays of clathrin that are too large to form as a result of individual coated vesicles fusing with the membrane (Pumplin and Bloch, 1990). Glycoproteins in these domains are so closely bound to the substrate that they block access of antibodies to the vitronectin coating the glass (Baetscher et al., 1986). The identity of these glycoproteins and their mode of adhesion to the tissue culture substrate have remained unknown.

Here we report that integrins, particularly vitronectin receptors (VnR), are present at the two types of contact domains, and so are likely to account for the close interactions with the vitronectin substrate. We focused our attention on the VnR at the clathrin-coated membrane domains, as an association between clathrin and this family of integrins has not been widely reported. We used intracellular injection and affinity chromatography to show that the association of clathrin with the VnR is mediated by a limited region of the short cytoplasmic sequence in the β5 subunit of the VnR. This region contains an NPXY motif that has been suggested to be a signal for assembly of clathrin complexes at the plasma membrane (Chen et al., 1990; Vaux, 1992).

MATERIALS AND METHODS

Culture of primary muscle cells

Primary cultures of rat myotubes were prepared as described (Bloch
and Geiger, 1980; Bloch, 1979). Briefly, muscles from neonatal rat hindlimb were dissociated enzymatically and suspended at 10^6 cells/ml in Dulbecco-Vogt modified Eagle’s medium containing 10% fetal calf serum (medium). Cultures were initiated by applying aliquots (0.4 ml) to glass coverslips (Vans Labs, Oxnard, CA) and were supplemented the next day with 1.5 ml medium. Medium was removed 3 days later and replaced with medium containing 2x10^{-5} M cytosine arabinoside to kill dividing cells. Cultures were used 6 days after initial plating for most immunolocalization experiments and 4-5 days after plating for intracellular injections.

**Isolation of substrate-attached material**

To isolate substrate-attached material (SAM), cultures were either extracted with saponin (Bloch, 1984) or treated briefly with ZnCl2 and sheared with a stream of ice cold buffer (Pumplin and Bloch, 1990). Saponin removes >99% of total cellular protein from cultures, leaving only SAM remaining on the coverslip (Bloch, 1984). Shearing after treatment with ZnCl2 removes most of the myotubes and contaminating fibroblasts, but leaves membrane and associated peripheral membrane proteins on the coverslips. Some cultures were first incubated with monotetramethylrhodamine-α-bungarotoxin (RT; 5 μg/ml in Hepes-buffered DMEM containing 5% fetal calf serum) to label acetylcholine receptors (AChR). Large clusters of AChR form preferentially at the myotube-substrate interface (e.g. Bloch and Geiger, 1980) and serve as a useful marker for SAM in these cultures.

Preparations of SAM were usually fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) immediately after saponin extraction or shearing. Samples were often treated briefly either before or after fixation with 0.05% or 0.5% Triton X-100 to expose extracellular determinants (Baetscher et al., 1986). Shearing and extraction with saponin yielded similar distributions of integrins, but FnR was only visualized in samples that were fixed before extraction with Triton X-100 (see Results).

**Immunolabeling**

Some samples, labeled with R-BT to visualize AChR clusters in SAM, were prepared as above and incubated with rab antibodies to integrins or with control antibodies, followed by fluoresceinated goat antibodies to rabbit IgG (FGAR). Samples that had not been prelabeled with R-BT were double-labeled with mouse monoclonal antibodies to vinculin or to the heavy chain of clathrin and with rabbit antibodies to the integrins. Bound antibodies were visualized with rhodaminylated goat antibodies to rabbit IgG (RGAR) and fluoresceinated antibodies to mouse IgG (FGAM). Appropriate controls demonstrated the specificity of the secondary antibodies.

Labeled coverslips were mounted in glycerol, 1 M Tris-HCl, pH 8.0 (9:1, v:v), supplemented with 1 mg/ml p-phenylenediamine to reduce photobleaching (Longin et al., 1993). Samples were observed with a Zeiss IM-35 microscope equipped for epifluorescence and were photographed using Kodak TMAX 3200 film that was exposed for 5-20 seconds and developed to an ASA of 1600 using Kodak TMAX developer (Eastman-Kodak, Rochester, NY). Alternatively, samples were observed with a Zeiss 410 confocal laser scanning microscope, with pinhole settings to yield the highest possible resolution. ‘Bleed-through’ of fluorescence from one channel to the other was minimal in both epifluorescence and confocal images.

**Antibodies**

Antibodies against vitronectin and the vitronectin and fibronectin receptors were obtained from Life Technologies (Gaithersburg, MD). Antibodies specific for the β1, β3 and β5 subunits of integrin were purchased from Chemicon (Temecula, CA). Monoclonal antibodies to vinculin (11.5) and to the heavy chain of clathrin (X-22) were kindly provided by Dr B. Geiger (Weizmann Institute, Rehovot, Israel), and Dr F. Brodsky (University of California, San Francisco, CA), respectively. An affinity-purified rabbit antibody to the COOH-terminal sequence of the β3 subunit of integrin (Ramassamy and Hemler, 1990) was also generously provided by Dr M. Hemler (Dana Farber Cancer Research Institute, Boston, MA) and affinity-purified rabbit antibodies to the COOH-terminal sequences of the β1-, and β3-integrin subunits were generously provided by Dr E. Ruoslahti (The Burnham Institute, La Jolla, CA). A non-immune mouse monoclonal IgG (MOPC-21) and normal rabbit serum were routinely used as controls in immunolabeling experiments. The specificity of the anti-integrin antibodies were tested by immunoblotting of proteins from myotube cultures, separated by SDS-PAGE and transferred electrophoretically to nitrocellulose (see below). The specificities of these and the other antibodies we have used have been established (Mahaffey et al., 1990; Pasqualini et al., 1993; Adams and Watt, 1991; Levesley et al., 1992; Ruoslahti and Pierschbacher, 1987). Secondary antibodies conjugated to fluorescein or rhodamine were obtained from Jackson Laboratories (West Chester, PA).

**Ultrastructural studies**

SAM was prelabeled with R-BT, isolated by shearing, fixed in paraformaldehyde and labeled for 1 hour at RT with affinity-purified rabbit antibody to the COOH-terminal region of the β3-integrin (diluted 1:100 in 1% bovine serum albumin in PBS), followed by fluoresceinated mouse anti-rabbit IgG (diluted similarly) for 1 hour at RT. After brief washing, samples were incubated overnight at 4°C with goat anti-mouse IgG adsorbed to 10 nm colloidal gold (Janssen Pharmaceuticals, Beerse, Belgium), diluted 1:10 in BANT (0.1% BSA, 0.5 M NaCl, 10 mM MgCl2, 20 mM Tris-HCl, 20 mM NaN3, pH 7.4; Luther and Bloch, 1989). AChR clusters labeled with the anti-integrin were chosen for the clarity of the fluorescence label and a minimum of material visible by phase contrast microscopy. Circles around these structures were scribed with a diamond marking objective and broken out of the coverslips. The SAM was subjected to quick-freezing and deep-etch rotary replication as described previously (Pumplin and Bloch, 1990). Electron micrographs of replicas were taken at low magnification (×1,400) to compare with corresponding fluorescence images and at high magnification (×30,000 or ×45,000) in stereo pairs with 6° of tilt.

**Intracellular injections**

Peptides were synthesized and purified by Dr N. Ambulos (Biopolymer Core Facility, University of Maryland at Baltimore). The lyophilized peptides were reconstituted in microinjection buffer (see below) and stored as a 10 mM solution at 4°C. One 19mer peptide, SRYERMASNPLYRPST, contained an ‘NPXY’ motif and flanking sequence of the cytoplasmic domain of the β3 subunit of human integrin (Williams et al., 1994). The other 19mer, MNAKWDTGENPILYKSVTT, contained the ‘NPXY’ motif and the flanking sequence from the homologous region of the β1 subunit (Williams et al., 1994). Peptides (5 μM) and fixable dextranrhodamine (0.75 mg/ml; Molecular Probes, Oregon) were dissolved in microinjection buffer (40.5 mM K2HPO4, 39 mM KH2PO4, 12 mM NaH2PO4, pH 7.25) for injection into cells.

Prior to injection, myotubes were incubated with R-BT to assist in identifying substrate-apposed membrane. Only unbranched myotubes of similar size (~400 μm in length, 15-20 μm in width) and showing large, well-defined clusters of AChR were selected for injection. Micropipettes with an inner diameter of 0.5 μm were made from borosilicate glass capillaries containing an internal filament (World Precision Instruments, Sarasota, FL) on a model P-87 Flaming/Brown microelectrode puller (Sutter Instrument Co., Novato, CA). Micropipettes were back-filled with the peptide-dextran solutions described above. For injection, the tip of the micropipette was pushed into the myotube by a smooth movement of the micromanipulator (MMO-203 Narashige, Tokyo, Japan). Approximately 3 nl of solution (an estimated 10% of the intra-cellular volume) was injected into the cell at a constant flow rate, and typically the microinjection took 1-2
seconds per myotube. The micropipette was then withdrawn and moved to the next cell. Microinjection was followed under fluorescent illumination to ensure that the injected fluorescent dextran marker was retained by the cell, consistent with the cell remaining intact and adhering to the substrate.

Following injection, cultures were incubated at 37°C for 1.5 hour, then fixed, permeabilized with 0.5% Triton X-100 for 5 minutes, and labeled with antibodies to clathrin or vinculin, followed by FGAM. Due to the presence of high concentrations of rhodamine-dextran in the myoplasm of injected cells, we usually used confocal laser scanning microscopy to examine these samples.

Affinity chromatography

Myotubes were collected at 4°C by scraping the cultures into 0.5% Triton X-100 in phosphate-buffered saline (PBS; pH 7.4) containing a cocktail of protease inhibitors (220 units/μl aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml antipain, 200 μg/ml soybean trypsin inhibitor, 100 μM phenylmethylsulfonyl fluoride). The cell extract was centrifuged at 3,000 rpm for 3 minutes at 4°C in a clinical centrifuge and the supernatant was subjected to affinity chromatography on matrices containing peptides from the cytoplasmic domains of the β1 or β5 subunits of integrin (see above), linked to a Multiple Antigenic Peptide (MAP) system (Tam, 1988).

The MAP-peptides were covalently bound to CNBr-activated Sepharose 4B (Pharmacia Biotech, Sweden), following the recommendations of the manufacturer. In addition to the β1- and β5-MAP conjugates, we also used an affinity matrix with an unrelated control peptide, SK-1, having the sequence, SEGLSDDEET. The columns were washed with PBS to elute unbound proteins; bound protein was then eluted in 0.5% SDS in PBS. Alternatively, material bound to the column was eluted with solutions containing 1 μg/ml of the β1 and β5 peptides dissolved in PBS. Fractions (1 ml) were collected, analyzed by SDS-PAGE in 7% acrylamide gels, and immunoblotted with antibodies to clathrin (X-22). Bound antibodies were visualized colorimetrically with alkaline phosphatase-linked secondary antibodies and a kit obtained from Kierkegaard and Perry (Gaithersburg, MD).

RESULTS

We isolated SAM from myotubes by extraction with saponin, to study AChR, or by shearing, to study structural proteins, and examined the distribution of the integrins in these preparations. We then focused on the interactions of integrin with clathrin-coated membrane, using intracellular injection techniques and affinity chromatography to identify a sequence on the cytoplasmic ‘tail’ of the integrin responsible for anchoring clathrin to coated membrane domains.

Antibodies to the integrins in muscle cultures

Rabbit antibodies to the β1, β3 and β5 subunits of integrin were obtained from two independent sources. We tested these antibodies by immunoblotting proteins isolated from cultures of rat myotubes that had been subjected to SDS-PAGE under reducing conditions (Fig. 1A). Antibodies specific for the β1 and β5 subunits both reacted with doublets, of 150 and 130 kDa, and 140 and 120 kDa, respectively, consistent with other reports (Damsky et al., 1985; Bozyczko et al., 1989). Antibodies to the β3 subunit also recognized a smaller band, at ~65 kDa (Fig. 1A). Antibodies to VnR and FnR labeled the same bands recognized by anti-β1 and anti-β3-integrin antibodies (Fig. 1B, arrows). In addition, anti-FnR also reacted with a pair of bands at 95 and 90 kDa (Fig. 1B, arrowheads) that are likely to represent the extracellular domains of α-integrins, expected under the reducing conditions we used. Only one band in the immunoblots was not accounted for by integrin subunits, but this was also present in the sample blotted with non-immune rabbit serum (Fig. 1, lane 4), indicating that it was due to non-specific binding of antibodies. Our results indicate that the subunit-specific antibodies and the antibodies to the heterodimeric integrins specifically recognize bands of the appropriate molecular masses in SDS-PAGE. Only antibodies that reacted with the integrins, and not non-immune

![Fig. 1. Specificity of antibodies to the β subunits of integrin in cultures of rat myotubes. Proteins extracted from cultures of rat myotubes were separated by SDS-PAGE under reducing conditions, transferred electrophoretically to nitrocellulose paper, and incubated with: (A) subunit specific rabbit antibodies to the β1, β3, and β5 subunits of integrin or with non-immune rabbit serum (NRS); and (B) rabbit antibodies to the heterodimeric integrins, FnR and VnR. Bound antibody was visualized chromogenically after labeling with a secondary antibody conjugated to alkaline phosphatase. (A) Each of the anti-β-subunit antibodies specifically labeled 1-3 bands with apparent molecular masses appropriate for their respective antigens. The antibodies to the β1 subunit labeled a doublet at 150 and 130 kDa, antibodies to the β3 subunit reacted with a single band at 135 kDa, and antibodies to the β5 subunit recognized three bands at 140, 120 and 65 kDa. The band visualized at lower molecular masses (around 40 kDa) associated with antibodies non-specifically, as indicated by the reaction with non-immune serum. The positions of the molecular mass standards are shown to the left. (B) Antibodies to the FnR specifically reacted with 4 bands, at 150, 130, 95 and 90 kDa. The former 2 bands (arrows) were also recognized by anti-β1-integrin (See A). The latter pair of bands (arrowheads) are likely to represent the extracellular domains of α-integrins. Antibodies to the VnR labeled bands at 140, 120 and 65 kDa (arrows), as also seen with the anti-β5-integrin (see A).]
serum or control antibodies, labeled SAM specifically (see below).

**Distribution of the integrins**

We used all the antibodies described above to localize integrins within AChR clusters. Neither the antibodies to the FnR and VnR, nor the subunit-specific antibodies to $\beta_1$ or $\beta_5$ integrin labeled the AChR-rich domains of clusters, but instead they all labeled the receptor-poor regions (e.g. Fig. 2). These regions have been previously shown to contain focal contacts and clathrin-coated domains (Bloch and Geiger, 1980; Pumplin and Bloch, 1990; see below). In general, antibodies to the FnR (not shown) or the $\beta_1$-integrin (Fig. 2A,B,C) labeled these domains of clusters less intensely than antibodies to the VnR (not shown) or to $\beta_5$-integrin (Fig. 2D,E,F). The clusters were not labeled by non-immune rabbit antibodies (not shown), and labeling by anti-$\beta_5$-integrin was inhibited by its peptide antigen (Fig. 2G,H,I). These results strongly suggest that labeling of AChR-poor domains by the anti-integrin antibodies was specific. Antibodies to the $\beta_3$ subunit of integrin did not label clusters to any significant extent, although they labeled focal contacts in fibroblasts brightly (not shown). These results suggest that the FnR and its $\beta_1$ subunit, and the $\beta_5$ but not the $\beta_3$ form of the VnR, are present in AChR clusters.

We compared the distribution of the FnR and VnR to vinculin and clathrin, which are markers for focal contacts and coated membrane domains in SAM derived from myotubes (Bloch and Geiger, 1980; Pumplin and Bloch, 1990). Anti-VnR and anti-FnR co-localized with anti-vinculin in focal contacts and with anti-clathrin in coated membrane domains (Fig. 3A-I). Similarly, antibodies to $\beta_1$-integrin (not shown) and $\beta_5$-integrin (e.g. Fig. 3J,K,L) colocalized with these markers. These results indicate that the FnR, VnR, and their constituent $\beta$ subunits are present in both focal contacts and clathrin-coated domains. In the following, we focus on the relationship between the clathrin-coated domains and the $\beta_1$- and the $\beta_5$-integrins.

As the antibody to the COOH-terminal sequence of $\beta_5$-integrin gave strong and specific labeling, we used it to confirm the localization of the VnR to focal contacts and coated membrane at the ultrastructural level. Colloidal gold particles, representing sites of antibody binding, appeared at both focal contact domains and at clathrin-coated membrane domains. Stereo views (Fig. 4) showed that gold particles at focal contacts were concentrated where actin filaments closely

**Fig. 2.** Integrins in regions of AChR clusters poor in AChR. Cultures of myotubes were labeled with R-BT (A,D,G), and clusters were isolated by extraction with saponin (see Materials and Methods). Samples were fixed, permeabilized briefly with Triton X-100, and labeled with antibodies to the $\beta_1$ (B) or $\beta_5$ (E) subunits of integrins, followed by FGAR. The results show that both the $\beta_1$- and $\beta_5$-integrin subunits are present in dart-like structures resembling focal contacts (arrows in B,C and E,F), and in small spots resembling clathrin-coated domains (arrowheads in B,C and E,F). Double color overlay (C and F) shows that neither protein codistributes with AChR (AChR is shown in red and the integrin subunits are shown in green in C,F). The binding of antibodies to the $\beta_5$-integrin subunit was blocked in a 10 $\mu$M solution of the immunogenic peptide (G,H, and overlay in I), suggesting that the labeling shown in E is specific. Bar, 10 $\mu$m.
approached the inner membrane surface, consistent with the location of the cytoplasmic tail of β5-integrin. Clathrin-coated domains had more label around their edges, suggesting that the clathrin coat limits access of gold-adsorbed antibodies to the cytoplasmic tails of β5-integrin molecules. Alternatively, the β5 subunit of integrin may be concentrated in these regions, while more central areas may be enriched in other integrins.

**VnR, but not FnR, blocks access to the substrate**

We showed previously that vitronectin coats the coverslip glass on which myotubes are grown, and that extraction of AChR clusters with Triton X-100 leaves material remaining on the cover glass that binds concanavalin A (Dmytrenko et al., 1990). This material is so tightly attached to the substrate that it blocks access of antibodies to the vitronectin beneath it (Baetscher et al., 1986). We examined SAM further to learn if this material contains integrins.

Even after extraction of unfixed, isolated membrane fragments with Triton X-100, we detected significant amounts of VnR attached to the substrate (Fig. 5A,B). The labeling pattern was consistent with the presence of the VnR in both focal contact and clathrin-coated membrane domains.

**Fig. 3. VnR and FnR codistributes with vinculin and clathrin.** Substrate-attached membrane (SAM) from cultures of rat myotubes was isolated by treatment with ZnCl₂ followed by shearing, fixed, permeabilized with Triton X-100, and labeled with rabbit antibodies to the FnR (B,E), VnR (H), or β5-integrin subunit (K), together with mouse monoclonal antibodies to either vinculin (D,J) or the heavy chain of clathrin (A,G). The bound antibodies were visualized with RGAM and FGAR. Double color overlay images (C,F,I,L) show that labeling of the VnR and the FnR overlap (yellow) with labeling of both vinculin (at focal contacts, arrows) and clathrin (at coated membrane domains, arrowheads).

Similar results to those shown in B and E were obtained with the antibodies specific for the β1 subunit of integrin (Fig. 2). Bar, 20 μm.
Similarly treated samples were not labeled by a fluorescent lipid probe, C18-dil (not shown), indicating that the lipid bilayer had indeed been removed by the detergents. AChR had also been extracted from these samples, as R-BT labeling was absent. Despite strong labeling by antibodies to the VnR, little FnR labeling remained in these samples (Fig. 5C,D), suggesting that most of the FnR had also been extracted by detergent. Thus, the binding of VnR to the substrate is not dependent on FnR. Furthermore, the VnR probably associates more stably with the substrate than the FnR and is therefore more likely to block access of anti-vitronectin antibodies to the vitronectin coating the coverslip.

To test this idea we compared labeling by anti-VnR and anti-vitronectin antibodies. We observed a complementary staining pattern (Fig. 5E,F): where VnR was present, vitronectin could not be immunolabeled, and where vitronectin was brightly immunolabeled, VnR was not detectable. The VnR-rich domains were also stained with fluoresceinated concanavalin A (e.g. Fig. 5A,B), which we had previously used to identify glycoproteins that were closely apposed to vitronectin on the glass coverslip (Baetscher et al., 1986). These results suggest that the tight adhesion of myotubes to the substrate is mediated by VnR rather than by FnR.

Perturbing clathrin-coated domains by microinjection

Clathrin is bound to integral membrane proteins via the adaptin complex (reviewed by Robinson, 1994; Schmid, 1997). During endocytosis from the surface, AP-2 binds to integral membrane proteins at or near sequences of amino acids, such as FRXY and NPXY, that are thought to form tight turns that serve as signals for assembly of coated membrane (Chen et al., 1990; Collawn et al., 1990; Vaux, 1992). We therefore studied the role of the NPXY motif common to both integrin subunits in anchoring clathrin complexes at coated membrane domains.

We synthesized oligopeptides containing these NPXY motifs (see Materials and Methods) and injected them into myotubes. After injection, cells were incubated briefly, then fixed, permeabilized and immunolabeled. Given that the VnR is more stably associated with clathrin-coated membrane domains than the FnR, we anticipated that the oligopeptide containing the NPXY motif from the β5 subunit would have a more potent effect than the homologous β1 oligopeptide.

Shortly after injection of the β5 peptide, the labeling of clathrin-coated membrane domains by anti-clathrin was significantly reduced (Fig. 6B). This was not due to direct effects of the β5 peptide on the anti-clathrin antibody, as immunoblotting by the antibody was unaffected in the presence of the peptide (Fig. 8, lanes 1,2), even at concentrations much higher (μM) than those achieved intracellularly following injection. Injection of the β1 peptide had no detectable effects on clathrin labeling and, indeed, cells injected with the β1 peptide could usually not be distinguished from uninjected controls (Fig. 6D,F). Double-blind evaluation revealed that a significant reduction of labeling by anti-clathrin occurred in 21 of the 27 cells (77%) injected with the β5 peptide, but only in 9 of the 33 cells (27%) injected with the β1 peptide (n=33). This difference was highly significant by Chi² analysis (P<0.0001).

We also examined cells injected with the β5 peptide to learn if clathrin was altered elsewhere in the cell. Clathrin labeling at perinuclear structures seemed unaffected by the β5 peptide,
suggesting that this sequence was not involved in the binding of clathrin to Golgi membranes (Fig. 7D). Vinculin at focal contacts was also not affected by the injected β5 peptide (Fig. 7F), consistent with evidence that other integrin sequences help to mediate the binding of cytoskeletal proteins at focal contacts (Reszka et al., 1992; Otey et al., 1993; Lewis and Schwartz, 1995). These results, together with the lack of effect of the β1 peptide, show that when myotubes are grown on a vitronectin substrate, the clathrin-AP-2 complex associates with membrane domains that are enriched in the β5 subunit of integrin by virtue of its ability to recognize the cytoplasmic sequence containing the NPXY motif and adjacent amino acids.

**Selective association with the clathrin complex with β5-integrin demonstrated by affinity chromatography**

The effect of the injected β5 peptide could be due to interaction of this peptide with the clathrin-AP-2 complex, or it could be indirect. For example, proteins with NPXY motifs are known to be involved in downstream signaling initiated by the activation of receptor tyrosine kinases (reviewed by Fantl et al., 1993). Injection of the β5 oligopeptide might therefore have pleiotropic effects on cells that result in the selective disaggregation of clathrin-coated membrane domains. In this case, we would not expect to see binding of the clathrin complex to the β5 peptide in biochemical assays. If, however, the injected oligopeptide directly inhibits the ability of the clathrin complex to associate with integrins at coated membrane domains, the oligopeptide should associate with clathrin in vitro.

We performed affinity chromatography on resins containing the β5 and β1 peptides to try to distinguish between these possibilities. We prepared the oligopeptides as MAP
conjugates, to facilitate coupling to CNBr-activated Sepharose and to increase the binding capacities of the affinity resins. Myotube extracts were prepared in PBS containing 0.5% Triton X-100 and passed over the affinity columns; bound protein was eluted with SDS and analyzed by SDS-PAGE and immunoblotting. The affinity column carrying the $\beta_5$ peptide was considerably more effective in retaining clathrin than the column carrying the $\beta_1$ peptide (Fig. 8A, lanes 4, 6). An unrelated sequence, SK-1, prepared as a MAP peptide and conjugated to Sepharose identically, was unable to retain any detectable clathrin (Fig. 8A, lanes 8). The uninjected control is shown in Fig. 8A, lanes 4, 6. The $\beta_5$ but not the $\beta_1$ peptide perturbed clathrin organization at the substrate-attached membrane. Bar, 20 $\mu$m.

DISCUSSION

Rat myotubes in tissue culture attach to the substrate through two distinct membrane domains. One domain resembles focal contacts in mononucleate cells (for a review see Kinch and Burridge, 1995). The other is composed of clathrin-coated membrane plaques that are flat and several times larger than individual clathrin-coated vesicles (Pumplin and Bloch, 1990). Our results indicate that the clathrin complex associates with a specific cytoplasmic sequence of $\beta_5$-integrin, consistent with the idea that this sequence selectively anchors clathrin at coated membrane domains.
The identity of VnR as the molecule that mediates substrate adhesion at the clathrin-coated membrane domains could not have been predicted easily from earlier studies. Smaller clathrin-coated structures have been reported to mediate cell-substrate adhesion (Maupin and Pollard, 1983; Nicol and Nermut, 1987; Nermut et al., 1991; Wayner et al., 1991; Tawil et al., 1993) but, with the exception of podosomes or 'point contacts', the adhesive molecules that anchor them to the substrate have not been identified. Podosomes and point contacts are small, punctate structures that mediate adhesion through integrins and that associate with vinculin and actin (Marchisio et al., 1988; Nermut et al., 1991; Tawil et al., 1993; Arregui et al., 1994). Although the much larger clathrin-coated membrane domains of rat myotubes lack vinculin and actin (Figs 3 and 4; see also Pumplin and Bloch, 1990), they are also anchored to the substrate by integrins and, in particular, by the VnR.

The presence of extracellular ligands is known to influence the accumulation and organization of integrins in the plasma membrane (e.g. Singer et al., 1988; Miyamoto et al., 1995). Consistent with this, the presence of vitronectin on the glass substrate (Baetscher et al., 1986) is probably the single most important determinant of integrin localization in cultured myotubes. The vitronectin-coated glass coverslip binds cellular material tightly enough to resist extraction by neutral detergent and to inhibit access of antibodies to the vitronectin on the substratum (Baetscher et al., 1986). Although myotubes express both VnR and FnR, and both integrins associate with substrate-attached membrane (SAM), the FnR binds to the substrate only weakly (see below). By contrast the VnR binds very tightly indeed, so that it remains attached to the substrate even after many other integral and peripheral membrane proteins have been extracted by detergent (Fig. 5B). This tight binding is almost certainly the result of the specificity of VnR for vitronectin and the high local concentrations of both ligand and receptor achieved at the myotube-substrate interface.

A vitronectin substrate cannot explain the presence of VnR at two different membrane-cytoskeletal domains, however. Why should VnR in one domain associate with vinculin and proteins of focal contacts, but at the other associate only with clathrin, adaptin, and other proteins of coated membrane? One possible explanation is that different forms of the VnR (e.g. containing β3 or β5 subunits) tend to associate preferentially with different structural proteins. Although the inconsistent labeling by anti-β3-integrin antibodies has made this difficult to confirm in myotube membranes, this

Fig. 7. Microinjection of the β5 peptide selectively displaces clathrin from coated membrane domains at the plasma membrane. Myotubes were labeled with R-BT and then injected with a solution containing the β5 or β1 oligopeptides (0.5 μM) together with rhodamine-dextran (0.75 mg/ml), as outlined in the legend to Fig. 6. Samples were then fixed, permeabilized, and labeled with anti-clathrin (B,D) or anti-vinculin antibodies (F), followed by secondary antibodies (FGAM). Injected cells, identified from their intracellular content of rhodamine-dextran, were observed and photographed under confocal laser optics. Fluorescently labeled clusters of AChR marked the substrate-attached membrane in A and E; the AChR cluster shown in A is not in the focal plane in C. Microinjection of the β5 peptide perturbed the clathrin-coated domain at the substratum (B). The perinuclear staining of clathrin in the same cell was unaffected (D, arrow). Injection of the β5 peptide did not affect vinculin at focal contacts (E,F). Bar, 20 μm.
after unbound material was washed free of the columns, bound material was eluted with a solution containing SDS (lanes 4-6). Lane 4: MAP-β5-Sepharose; lane 5: MAP-β1-Sepharose; lane 6: MAP-SK1-Sepharose, which serves as an additional control. Clathrin, detected in immunoblots using monoclonal antibody X-22, was selectively eluted by the β5 oligopeptide.

Fig. 8. Affinity chromatography using the β1- and β5-integrin oligopeptides. (A) Lanes 1 and 2 show rat hippocampal homogenates that were subjected to SDS-PAGE, transferred electrophoretically to nitrocellulose paper, and immunoblotted with X-22 anti-clathrin in the absence (lane 1) or the presence (lane 2) of the soluble β5 peptide (1 μM). The peptide did not inhibit the reaction of anti-clathrin with its antigen. Lane 3 shows an immunoblot of myotube extracts probed with a non-specific mouse IgG, MOPC-21. No clathrin was detected. Lanes 4-6 show the results of immunoblots using the fractions from myotube extracts that were eluted from affinity columns linked to MAP-peptides. Synthetic MAP-peptides, with sequences identical to the sequences containing and flanking the NPXY motifs of the β5 and β1 subunits of human integrins, or a control MAP-peptide, were covalently coupled to Sepharose beads. Extracts of myotubes prepared with non-denaturing detergent were applied to the columns. After unbound material was washed free of the columns, bound material was eluted with a solution containing SDS (lanes 4-6). Lane 4: MAP-β5-Sepharose; lane 5: MAP-β1-Sepharose; lane 6: MAP-SK1-Sepharose, which serves as an additional control. Clathrin, detected in immunoblots using monoclonal antibody X-22, was retained specifically by the MAP-β5-Sepharose, which serves as an additional control. Clathrin, detected in immunoblots using monoclonal antibody X-22, was selectively eluted by the β5 oligopeptide.

Explanation would be consistent with our results with fibroblasts (P. De Deyne and R. J. Bloch, unpublished), and with the results reported by others in carcinoma cells and astrocytes (Wayner et al., 1991; Tawil et al., 1993). In the former, αβ3 partitioned preferentially into focal adhesions and αβ5 partitioned into distinctive structures at substrate-associated membrane that appeared as small spots or irregular lines (see also Nicol and Nerum, 1987). From our results, it is likely that the regions enriched in αβ5 in carcinoma cells and astrocytes are clathrin-coated membrane domains. Thus, these structures are probably more widespread than previously recognized and may play an important role in adhesion in many cells other than myotubes.

Although clathrin associates with VnR at coated membrane domains, it is still not clear if this interaction is direct or indirect. The oligopeptides we used for microinjection and affinity chromatography contain an NPXY motif, a conserved sequence present in the cytoplasmic tails of the β1, β3, and β5 subunits. In other molecules this sequence is believed to form a tight turn that interacts with the adaptin complex at the plasma membrane (Chen et al., 1990; Collawn et al., 1990; Chang et al., 1993; for reviews, see Robinson, 1994; Schmid, 1997). It therefore seems likely that the β5 oligopeptide interacts directly with the adaptin complex and only indirectly with clathrin. Preferential binding of the β5 oligopeptide to adaptins at the plasma membrane would also explain its inability to disturb clathrin binding to Golgi membrane, which is mediated by a different set of adaptins (reviewed by Sandoval and Bakke, 1994; Robinson, 1994; Schmid, 1997). We have not been able to determine if the β5 peptide does indeed bind to the adaptins, because these proteins have not yet been characterized in developing skeletal muscle. In preliminary experiments, antibodies to α1-adaptin (kindly provided by Dr F. Brodsky) labeled the clathrin-coated membrane domains of myotubes and also recognized several proteins that bound preferentially to the affinity matrix coupled to the β5 oligopeptide (not shown). The apparent molecular masses of these proteins were significantly greater than 100,000, the molecular mass of α1-adaptin, suggesting that muscle, like nerve (Zhou et al., 1993; Newman et al., 1995) may have predominantly large isoforms of this molecule.

Cytoplasmic tails of β subunits of integrins have many binding activities that have been ascribed to particular sequences (Reszka et al., 1992; Otey et al., 1993; Cone et al., 1994; Filardo et al., 1995; Lewis and Schwartz, 1995; reviewed by Williams et al., 1994). Our results strongly suggest that the ability of VnR to concentrate clathrin at coated membrane domains depends largely on the NPXY sequence and surrounding amino acids in the cytoplasmic tail of the β5 subunit. The homologous peptide of the β1-integrin, which helps to mediate the association of FnR with cytoskeletal proteins at focal contacts (Tapley et al., 1989; Reszka et al., 1992; Lewis and Schwartz, 1995), contains an NPXY sequence that is almost identical to that of the β5 peptide, differing only in the substitution of isoleucine for valine. Clathrin therefore seems likely that the ability of the β5 subunit to interact preferentially with clathrin relies in large part on amino acids that surround the NPXY motif, rather than on the NPXY signal itself. This is consistent with the fact that mutation of the NPXY sequence of β1-integrin to NPXS did not affect the clathrin-mediated internalization of the FnR in Chinese hamster ovary cells (Vignoud et al., 1994;
but see Vaux, 1992). Our results suggest that the specificity of association of clathrin with β5-integrin can be studied effectively using homologous oligopeptides in which selected amino acids have been altered.

Although association of the β5 oligopeptide with the clathrin complex is sequence-specific, it is probably not modulated by phosphorylation. Our affinity chromatography protocols used synthetic peptides that were not phosphorylated and that were unlikely to be modified to any significant extent during the course of the experiment. This suggests that, unlike the interaction of β1-integrin with talin (Tapley et al., 1989), phosphorylation of the tyrosine in the NPXY sequence does not significantly alter the ability of β5-integrin to interact with the clathrin-adaptin complex. Phosphorylation of the NPXY motif of β5-integrin probably has other important effects, however. Following ligand-integrin binding and activation of soluble tyrosine kinases (Blystone et al., 1996; Miyamoto et al., 1995; Law et al., 1996), phosphorylation of the NPXY sequence of integrin might lead to the binding of proteins containing SH2 or PTB domains, with subsequent activation of several signaling cascades (Kuriyan and Cowburn, 1997; Law et al., 1996). This raises the interesting possibility that the NPXY motif of β5-integrin serves as an intermediate in intracellular signaling when phosphorylated, but as a structural element when unphosphorylated.

A reciprocal relationship between the activation of a tyrosine kinase cascade and the association of clathrin with large plaques of VnR, while of potential interest in a wide variety of cells, may be especially relevant in embryonic muscle cells at the time of innervation. Recent evidence indicates that agrin, a macromolecule deposited on muscle cells by motor neurons, can both bind to integrins (Martin and Sanes, 1997) and activate a muscle-specific receptor tyrosine kinase (MuSK: De Chiara et al., 1996) to initiate the process of AChR clustering (for reviews, see Glass and Yancopoulos, 1997; Slater, 1997; Wells and Fallon, 1997). Simultaneously, neuregulin released by the motor neuron activates erbB receptors in the muscle membrane that in turn modulate the synthesis of new AChR (Francoeur et al., 1995). These new AChR are processed through clathrin-coated vesicles (Bursztajn and Fischbach, 1984; Porter-Jordan et al., 1986; see also Matthews-Bellinger and Salpeter, 1983) and may be inserted preferentially into the postsynaptic membrane as it is assembled (Campanelli et al., 1992). The association of integrins with both agrin in the synaptic cleft and large arrays of clathrin on the cytoplasmic surface of the sarcocellma suggests that clathrin-coated membrane domains may help both to direct the local insertion of AChR and to organize the signal transducing mechanisms responsible for postsynaptic differentiation of the neuromuscular junction.

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