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RAPID INDUCTION AND ISOLATION OF FOCAL ADHESION COMPLEXES

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Summary: Focal adhesion complexes (FACs) containing integrin β_1, talin, vinculin, talin, α-actinin, and paxillin formed within 15 min when round cells bound magnetic microbeads coated with integrin ligands, such as fibronectin or RGD-containing peptide, but not when coated with acetylated-low density lipoprotein. Newly formed FACs were isolated and collected for biochemical analysis using a combination of detergent extraction, sonication, dounce homogenization, and magnetic pelleting. Isolated bead complexes were greatly enriched for all FAC proteins when compared with either the whole cytoskeleton or basal cell membranes whereas actin (a general cytoskeletal marker) was relatively depleted. This method which permits isolation of intact FACs within minutes following integrin ligation should facilitate analysis of both FAC assembly and the molecular basis of integrin signaling.

Cell adhesion to extracellular matrix (ECM) is required for maintenance of cell growth and function (1,2). Cells attach to ECM molecules, such as fibronectin (FN), through binding of cell surface integrin receptors that cluster in localized attachment domains or "focal adhesions". Focal adhesions were originally defined morphologically as regions of the ventral cell membrane that came in closest contact with the underlying ECM and in which actin stress fibers terminated (3-5). However, more recent studies demonstrate that cell adhesion is mediated through formation of a focal adhesion complex (FAC) which contains actin-associated proteins, such as talin, vinculin, paxillin, and α-actinin (6). FAC proteins interact with the cytoplasmic portion of integrins (6) and thus, physically interconnect ECM with the actin cytoskeleton (CSK). This molecular bridge provides a

Abbreviations: FAC, focal adhesion complex; RGD, arg-gly-asp; ECM, extracellular matrix; FN, fibronectin; CSK, cytoskeleton; AcLDL, acetylated-low density lipoprotein; CSK-EB, cytoskeletal extraction buffer; PBS, phosphate buffered saline; TBS, tris-buffered saline; PIPES, 1,4 piperazinediethanesulfonyic acid; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethyl sulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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transmembrane path for transfer of CSK tension to the ECM and hence, is critical for cell spreading, migration, and tissue remodeling (1). Structural interconnections between integrins and FAC proteins also mediate mechanosensation, the process by which cells sense and respond to external mechanical signals (1,7). In addition, the FAC appears to function as a site for local chemical signaling by integrins (8-10).

The mechanism of FAC assembly and organization is poorly understood. Morphological approaches have localized numerous proteins to the FAC, but provide no information on their functional properties or the mechanism of protein-protein interaction. Gel filtration (11,12), modified gel overlay (13), recombinant DNA (14), and microinjection (13,15-17) techniques have been used to identify specific binding interactions between individual FAC proteins, but they fail to address higher order structural interactions within the complex that may be critical for chemical and mechanical signaling. Analysis of FAC assembly, structure, and function therefore would be greatly facilitated if intact FACs could be isolated in a form amenable to biochemical analysis.

Recently, methods have been described for isolation of ventral cell membranes which retain FAC proteins in relatively high concentrations (18-20). However, these extracts also contain many other CSK and basal cell surface proteins that are not relevant to FAC structure or function. We now describe a procedure using magnetic microbeads coated with specific integrin ligands which permits rapid induction of FAC formation as well as isolation of newly formed FACs from the remainder of the cell surface and CSK. Isolated FACs are enriched for integrin β_1, talin, vinculin, paxillin, and α-actinin, but not actin, when compared with either whole CSK or a preparation of basal cell membranes. This ability to isolate intact FACs within minutes after integrin ligation should greatly facilitate analysis of the molecular basis of integrin signaling.

MATERIALS AND METHODS

Experimental System. Bovine capillary endothelial cells were isolated, cultured, and serum-deprived prior to experimental manipulation, as described previously (21). Quiescent cell monolayers were dissociated by brief exposure to trypsin-EDTA and plated (7.5×10^3 cells/cm^2) in defined medium (21) on 8-well glass culture slides (Labtek) coated with a low density (25 ng/ml) of FN (Cappel) that promotes cell attachment but not cell spreading (21). Tosyl-activated magnetic microbeads (4.5 μm diameter; Dynal Inc.) were coated (all at 50 μg/ml in 0.1 M carbonate buffer, pH 9.4; 22) with FN, acetylated-low density lipoprotein (AcLDL; Biomedical Technologies Inc.), or RGD-containing peptide (Peptite 2000; Telios Pharmaceuticals). After 18 hr, the round adherent cells were incubated with microbeads (10 beads/cell) for 15 or 30 min, washed in cold PBS, and placed on ice. To identify CSK-associated FAC proteins, cells were detergent-extracted for 1 min in ice cold CSK extraction buffer (CSK-EB; 0.5% Triton-X-100, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, 10 mM

PIPES, pH 6.8) that maintains the integrity of the CSK, as previously described (23). The same method was used to isolate the whole CSK fraction from confluent monolayers for Western blot analysis. To isolate basal cell surfaces, nitrocellulose paper (0.45 μm pore; Schleicher and Schuell) was wetted in water, placed on top of a cell monolayer for 15 sec, and then quickly removed with forceps, thereby "wet cleaving" the cells and removing the apical cell surfaces (19). The remaining adherent basal cell surfaces were scraped into PBS containing 1% aprotinin and pelleted by centrifugation.

Immunofluorescence Microscopy. Cells were fixed for 30 min in 4% paraformaldehyde/PBS after detergent extraction, washed in PBS, and incubated for 1 hr with primary antibodies (all diluted 1:50 in 0.2% Triton-X-100/0.1% BSA in PBS). Rabbit antisera against talin, paxillin, and α-actinin were generously provided by Dr. Keith Burridge. Rabbit anti-serum against integrin β_1 was a gift from Dr. R. Hynes and mouse monoclonal antibodies against actin were obtained from Boehringer Mannheim. Primary antibodies were visualized using rhodaminated goat-anti-rabbit or sheep-anti-mouse immunoglobulins (diluted 1:250 and 1:50 in the same buffer, respectively; Cappel).

Isolation of FACs. Cells dispersed with trypsin-EDTA were washed twice in 1% BSA/DMEM, placed in polypropylene tubes (Costar), suspended (1 x 10^6 cells/ml) in defined medium (21) containing magnetic RGD-beads (2 x 10^7/ml), and rotated for 30 min at 37°C. RGD-coated beads were chosen for large-scale isolation of FACs because they exhibited less non-specific clumping during magnetic pelleting. Microbeads and bound cells were collected by placing a magnet at the side of the tubes, suspended in ice cold CSK-EB without detergent, and transferred to 5 ml polystyrene tubes (VWR). All subsequent procedures were carried out at 4°C using a side pull magnetic separation unit (Advanced Magnetics). The magnetic bead pellet was then transferred into complete CSK-EB, sonicated for 10 sec (output setting, 4; output power, 10%; XL2005 cell disruptor, Heat Systems), and homogenized in a 2 ml dounce homogenizer (20 strokes). The microbeads were magnetically pelleted and washed 5 times with CSK-EB prior to use; 1 x 10^8 cells yielded 1 mg FAC protein.

Western Blot Analysis. Proteins in the bead complexes, whole CSK pellet, and basal membrane preparations were dissolved in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1 % SDS, 150 mM NaCl, 50 mM Tris pH 7.2, 1% PMSF), separated by SDS-PAGE (7.5% acrylamide), and transferred to nitrocellulose (0.45 μm pore) using a semi-dry blotting system (Polyblot SBD-1000; American Bionetics). Equal protein (50 μg), as determined using a BCA protein microassay (Pierce), was loaded in each gel lane. Nitrocellulose was incubated sequentially with 0.5% casein (Sigma) in TBS to block non-specific binding sites, primary rabbit or mouse antibodies (all diluted 1:500 in TBS containing 0.05% Tween 20), and affinity-purified horseradish peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse immunoglobulins (both 1:6000 dilution). Labelled proteins were visualized using an ECL chemiluminescence kit (Amersham). Densitometric quantitation was carried out using a Datacopy GS Plus optical scanner and NIH public domain Image software (24).

RESULTS AND DISCUSSION

Rapid Induction of FAC Formation. FN-coated magnetic microbeads bind tightly to endothelial cells and activate intracellular chemical signaling pathways (e.g., Na^+/H^+ antiporter) within 10 to 30 min following bead binding (21,22). We used these beads in conjunction with immunofluorescence microscopy to analyze FAC assembly at similar early times. The FAC proteins, integrin β_1, talin, vinculin, paxillin, and α-actinin were recruited to the cell surface directly beneath the site of bead binding within 15 min after bead

addition (Fig. 1). FAC formation first appeared in a crescent along the bead-cell surface interface which then progressed to encircle the entire bead surface by approximately 30 min. FAC formation was also accompanied by increased cortical actin staining in the region of the bead (Fig. 1). In contrast, beads coated with AcLDL, which binds to transmembrane "scavenger" receptors, did not induce recruitment of any FAC protein although increased cortical actin staining was also observed (Fig. 2). This rapid appearance of actin under both conditions may represent a change in the local distribution of microfilaments due to mechanical deformation of the cell cortex (7) rather than actual recruitment of new protein to the FAC. Similar staining patterns were obtained using RGD-beads and neither bead exhibited staining for myosin, ankyrin, or tubulin (data not shown). Importantly, use of microbeads coated with integrin ligands also permitted morphological analysis of the process of FAC assembly. For example, quantitation of bound beads that exhibited staining at 15 and 30 min revealed that different proteins were recruited to the bead-associated FAC at different rates (Fig. 3; integrin β_1 = actin $\geq$ talin > α-actinin $\geq$ paxillin >> vinculin). The slow recruitment of vinculin may explain why this FAC component was not detected in previous immunolocalization studies using ECM-coated microbeads (25,26).

Isolation of the FAC. The magnetic properties of the microbeads also provided a means to physically isolate these newly formed FACs from the remainder of the cell. After incubation with RGD-beads in suspension for 30 min, cells with bound beads were

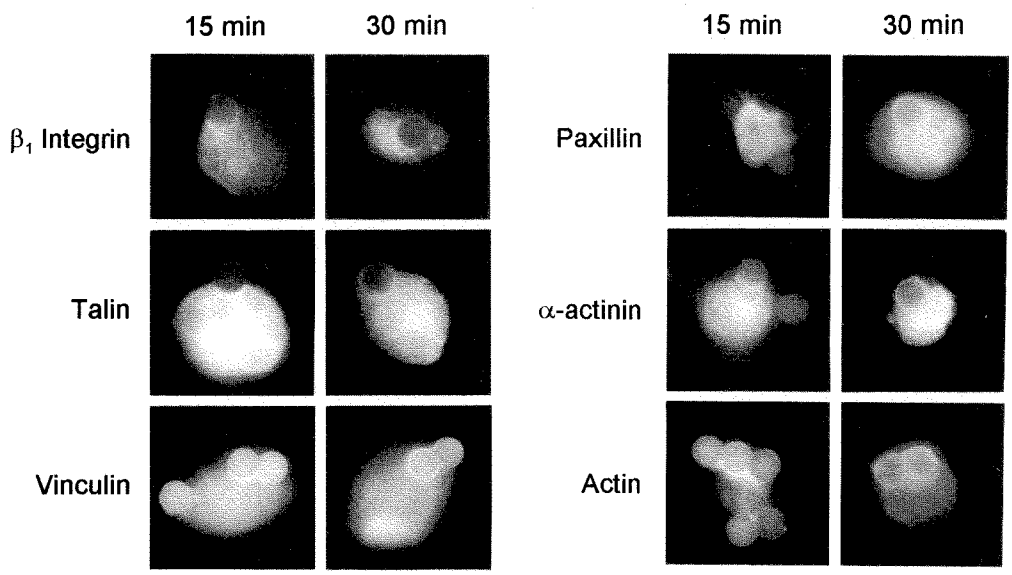

Fig. 1. Cell surface binding to FN-beads induced rapid recruitment of integrin β_1, talin, vinculin, paxillin, α-actinin, and actin to the site of bead binding within 15 and 30 min (left and right, respectively) after bead addition.

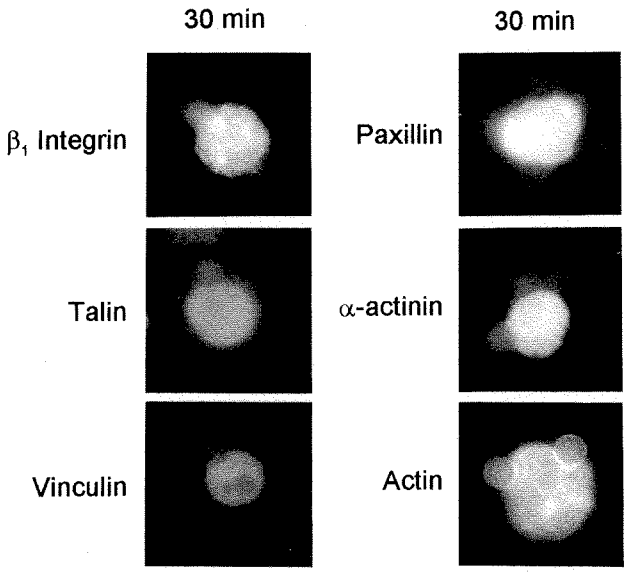

Fig. 2. Cell surface binding to AcLDL-beads did not induce FAC formation. Only increased cortical actin staining was observed at the site of AcLDL-bead binding 30 min after bead addition.

magnetically pelleted, detergent extracted in a buffer that maintains CSK integrity, sonicated to physically disrupt the CSK, dounce homogenized to remove nuclei, and extensively washed to remove cellular structures not intimately associated with the beads. Light microscopic analysis confirmed that this procedure resulted in isolation of individual beads which lacked any visible cellular extensions (not shown).

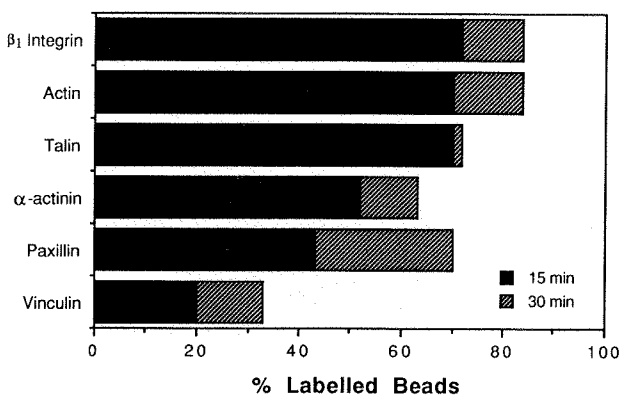

Fig. 3. Time course of FAC assembly. The relative rate of recruitment of the different FAC proteins to the surface of FN-beads was determined by scoring all cell bound beads in at least two different experimental wells and measuring the percentage of beads that exhibited fluorescent staining for the different antigens at 15 and 30 min.

Western blotting revealed that all of the FAC components that were visualized along the cell-bead interface in living cells (Fig. 1) were retained in the isolated bead complexes. Isolated FACs (Fig. 4, lane 3) were greatly enriched for integrin β_1, vinculin, paxillin, talin, and α-actinin when compared with either extracts of the whole CSK (Fig. 4, lane 1) or a crude preparation of basal cell membranes (Fig. 4, lane 2). In contrast, the bead complexes were not enriched for actin (Fig. 4). Densitometric quantitation of these results (Fig. 5) revealed that vinculin, paxillin, talin, and α-actinin were concentrated approximately 53, 11, 1.5, and 2 fold, respectively, in the isolated FAC relative to the whole CSK and 5, 7, 2, and 10 fold relative to basal cell membranes. Under these conditions in which equal protein was loaded, integrin β_1 could not be visualized in the whole CSK fraction although it was a major component of the isolated FAC. Furthermore, the efficiency of our isolation method was also much greater than that obtained using past approaches (18-20). Just as an example: integrin β_1 was enriched over 50 fold in the FAC compared to the basal cell membrane fraction obtained by the wet-cleaved method (Fig. 5). On the other hand, approximately twice the amount of actin was found in the whole CSK fraction compared with the isolated FAC (Fig. 5), thus confirming that the intact FAC was physically removed from the remainder of the CSK during magnetic isolation. Protein complexes that were isolated from cells bound to AcLDL beads using identical separation methods in the same experiment did not contain sufficient protein for biochemical analysis.

In summary, we have demonstrated that binding of microbeads coated with integrin ligands rapidly induces formation of intact FACs containing integrin β_1, talin, vinculin, paxillin, and α-actinin at the bead-cell interface in round cells. We also showed that these magnetic microbeads can be used to isolate intact FACs in sufficient quantity to allow

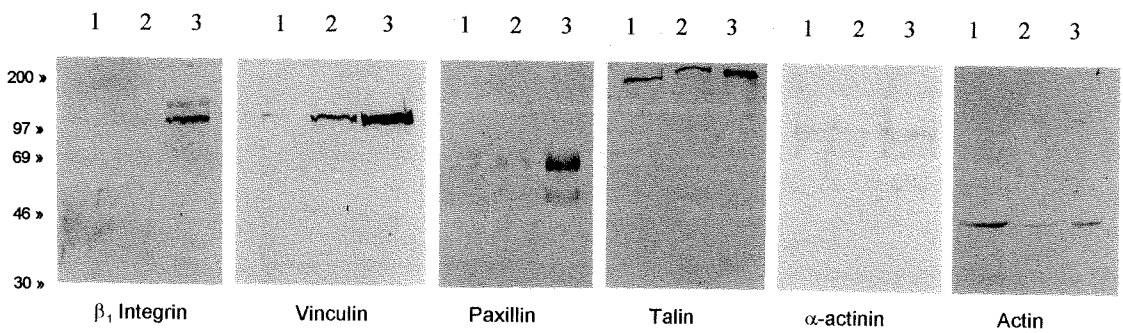

Fig. 4. Enrichment of FAC proteins during the FAC isolation procedure. Western blot analysis of equal proteins from the whole CSK (lane 1), wet-cleaved basal cell membrane preparation (lane 2), or bead-isolated FACs (lane 3). All of the blots are aligned; molecular weights (Kda) for protein standards are indicated at the left.

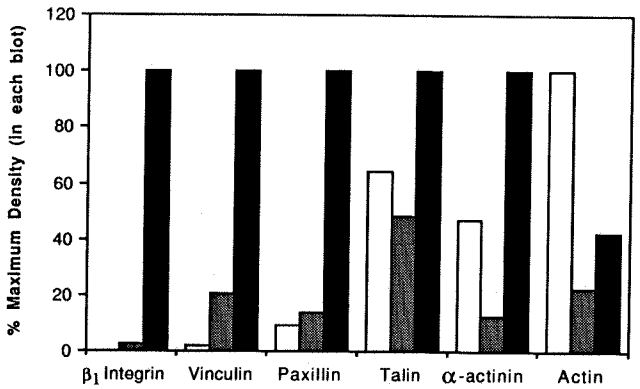

Fig. 5. Densitometric quantitation of Western blot data shown in Fig. 4. Densities are expressed as percent of the maximum density recorded for each antigen. Integrin β_1 was not detectable in the whole CSK fraction under these conditions in which equal protein (50μg) was loaded per lane. Open bars, whole CSK; stippled bars, wet-cleaved basal cell membrane preparation; black bars, isolated FAC.

biochemical characterization of their constituent proteins. Furthermore, the isolated FACs exhibited a molecular composition similar to that observed *in situ*. Importantly, this method provides many advantages when compared to those used in the past to enrich for FAC proteins: 1) the isolated FAC is physically separated from contaminating cell surface proteins and the remainder of the actin CSK, 2) the efficiency of FAC protein enrichment is much greater, 3) FAC assembly can be separated from the CSK changes that accompany cell spreading, and 4) newly forming FACs can be isolated within minutes after integrin ligation. This last advantage is perhaps most critical. Most past studies analyzed FACs many hours after they have formed and second messengers have been released. In contrast, our method offers a unique opportunity to study and isolate FACs at a time when molecular linkages between ECM and the CSK are just forming and integrin signaling pathways are first activated (21,22,27-29). Thus, this simple method may be used to examine the sequential biochemical changes that lead to FAC assembly and to analyze FAC structure-function relations at critical times during cell activation *in situ* and after isolation under defined conditions *in vitro*.

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