Paxillin Associates with Poly(A)-binding Protein 1 at the Dense Endoplasmic Reticulum and the Leading Edge of Migrating Cells*

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Using mass spectrometry we have identified proteins which co-immunoprecipitate with paxillin, an adaptor protein implicated in the integrin-mediated signaling pathways of cell motility. A major component of paxillin immunoprecipitates was poly(A)-binding protein 1, a 70-kDa mRNA-binding protein. Poly(A)-binding protein 1 associated with both the α and β isoforms of paxillin, and this was unaffected by RNase treatment consistent with a protein-protein interaction. The NH2-terminal region of paxillin (residues 54–313) associated directly with poly(A)-binding protein 1 in cell lysates, and with His-poly(A)-binding protein 1 immobilized in microtiter wells. Binding was specific, saturable, and of high affinity (Kd of ~10 nM). Cell fractionation studies showed that at steady state, the bulk of paxillin and poly(A)-binding protein 1 was present in the “dense” polyribosome-associated endoplasmic reticulum. However, inhibition of nuclear export with leptomycin B caused paxillin and poly(A)-binding protein 1 to accumulate in the nucleus, indicating that they shuttle between the nuclear and cytoplasmic compartments. When cells migrate, poly(A)-binding protein 1 colocalized with paxillin-β at the tips of lamellipodia. Our results suggest a new mechanism whereby a paxillin-poly(A)-binding protein 1 complex facilitates transport of mRNA from the nucleus to sites of protein synthesis at the endoplasmic reticulum and the leading lamella during cell migration.

The development of cell polarity depends on an ability to organize components of the cytoskeleton with reference to the extracellular environment. In fibroblasts and other motile cells, polarity can be stimulated by chemotactic gradients, the extracellular environment. In fibroblasts and other motile cells, polarity can be stimulated by chemotactic gradients, the anterior portion of the cell forming a flattened, protrusive structure termed the lamellipodium. The leading edge or tip of this lamellipodium is the point at which members of the integrin family of adhesion receptors engage the extracellular matrix in an interaction that leads to the clustering of integrins into an array of small multiprotein focal complexes (1). These focal complexes contain a number of structural and signaling proteins capable of initiating the polymerization of actin within the lamellipodial tip (1), and this provides the driving force for cell migration.

The adaptor protein paxillin is recruited into focal complexes at an early stage following integrin engagement (2). Paxillin consists of an NH2-terminal region containing five leucine-rich motifs termed “LD domains” and a COOH-terminal region with four tandem LIM domains. Numerous proteins have been identified that bind to one or other of these domains, many of which are thought to participate in signaling pathways that regulate the dynamic properties of focal complexes and thereby cell motility (3). For instance, LD1 binds the integrin-linked kinase (ILK)1 (4) and the actin-binding proteins actopaxin (5) and vinculin (6). LD2 binds vinculin and the protein tyrosine kinase pp125FAK, while LD4 binds pp125FAK and the ARF-GAP protein, p95PKI, which in turn recruits PIX (6), a nucleotide exchange factor for Rac. The LIM domains contain binding sites for the protein tyrosine phosphatase PTP-PEST (7), and also for tubulin (8) suggesting that paxillin may provide links to both microtubules and actin microfilaments.

Mutations within the four major tyrosine phosphorylation sites of paxillin causes the loss of cell polarity in motile cells (2). Additionally, immunofluorescence and immunoelectron microscopy indicates that paxillin localizes to the perinuclear ER and Golgi membranes and binds to several proteins each exhibiting specific GAP activities toward ARF1 or ARF6 GTPases (9–12). Both these GTPases are essential in intracellular trafficking and the establishment of cell polarity. However, the precise roles and the mechanisms by which paxillin is involved in the establishment of cell polarity still largely remain to be established.

The targeting of certain mRNAs is emerging as a possible mechanism by which cell polarity can be generated and maintained. The mRNA for β-actin contains a specific sequence in its 3′-UTR termed the “ZIPcode” that directs it to the lamellipodium during cell migration (13). Introduction of ZIPcode antisense oligonucleotides into migrating cells delocalizes the β-actin mRNA from the lamellipodium and results in loss of polarity and directional movement (14). The mechanism for β-actin mRNA localization in fibroblasts is most likely medi-

1 The abbreviations used are: ILK, integrin linked kinase; aa, amino acids; AEBSF, 4-2-aminoethylbenzenesulfonyl fluoride; ARF, ADP-ribosylation factor; BSA, bovine serum albumin; CHC, clathrin heavy chain; RGFp, enhanced green fluorescent protein; eIF, elongation factor; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GAP, GTPase activating protein; GST, glutathione S-transferase; GFP, green fluorescent protein; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MOPS, 4-morpholinepropanesulfonic acid; PABP, poly(A)-binding protein; PBS, phosphate-buffered saline; PSF, PTB-associated splicing factor; PTB, polyypyrimidine tract-binding protein; RNP, ribonucleoprotein; RRm, RNA-recognition motif; UTR, untranslated region.

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ated by recruitment of a mRNA protein complex onto the actin cytoskeleton, and it is possible that this is mediated at least in part by the ZIPcode-binding protein (15). Recently, β-actin mRNA localization has been shown to require the activation of rho kinase (16), strongly implicating a role for myosin II in the translocation of ZIPcode bearing mRNAs to the lamellipodia. Indeed it has been shown that in yeast, Ash1 mRNA is translocated of an RNA-containing protein complex onto a myosin motor, Myo4p (17). In neuronal cells, β-actin mRNA is transported to the tips of neurites and advancing growth cones, most likely by virtue of the same ZIPcode. In contrast to fibroblasts, however, the acto-myosin system is not the main player in localizing β-actin mRNA to the neurite tip. β-actin mRNA is located in large macromolecular granules, and the transport of these along neuronal processes is dependent on microtubules (18). This suggests that, depending on the cell type, large ribonucleoprotein (RNP) complexes containing β-actin mRNA can be transported on both microfilamentous and microtubular cytoskeletal tracks.

In neurones, the mRNA for secreted neuropeptides such as vasopressin are transported to the tips of the dendrites (19). Recently, it has been shown that a satellite secretory pathway containing elements of the rough endoplasmic reticulum, the endosomal reticulum-Golgi interstitial compartment and Golgi that are necessary to locally translate and process these secretory proteins, is present in dendritic spines (20). The 3′-UTR of the vasopressin mRNA contains sequences responsible for localizing it to dendrites, termed the “dendritic localizer sequence” (19). Recently, the dendritic localizer sequence has been shown to bind to the multifunctional RNA-binding protein polyl(A)-binding protein 1 (PABP1), indicating that this protein may have a role in the polarized delivery of mRNA (21).

Using a proteomic approach, we have now shown that cellular paxillin is associated with PABP1 and that the two proteins bind directly to each other in vitro. Both paxillin and PABP1 shuttle between the cytosol and nucleus and localize to the leading edge of migrating cells and to the dense ER, the main site of secretory protein synthesis within the cell. Our findings raise the possibility that a paxillin-PABP1 complex plays a role in the cellular localization of certain mRNAs within the cell and that this may contribute to the development of cell polarity in migrating cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal mouse anti-paxillin, anti-clathrin heavy chain and anti-Rab11 were from Transduction Laboratories. Monoclonal mouse anti-PABP1 (clone 6B12) was a kind gift from Matthias Gorlach (22) (Jena, Germany). Rabbit polyclonal antibodies to PABP1 were a generous gift from Dan Schoenberg (Columbus, Ohio) and are described in (23) and to PABP2 was from Elmar Wahle (Halle, Germany). Rabbit polyclonal antibodies to PABP1 complex plays a role in vitro

**Cell Culture and Transfection**—Swiss and NIH 3T3 mouse fibroblasts were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and transfected as described previously (25). All experiments were carried out 24 h post-transfection. EGFP-paxillin-α and EGFP-paxillin-β cDNAs have been described previously (9).

**Expression and Purification of Paxillin and PABP1 Fusion Proteins**—The human sequence of PABP1 (residues 10–636) was cloned into the SacI and EcoRI sites of the His-tagged bacterial expression vector pQE-30 (Qiagen). PQE-30-PABP1 was transformed into E. coli strain BL-21, grown to a density of 0.5 (A600 nm) at 37 °C, and then induced with 0.5 isoamyl-1-thio-β-D-galactopyranoside for a further 2 h at 22 °C. E. coli were lysed in a French press in a buffer containing 20 mM MOPS buffer, pH 7.4, 0.5 mM NaCl, 20 mM imidazole, 2 mM benzamidine, 30 μg/ml leupeptin, 15 μg/ml aprotinin, and 1 mM PMSF.

**Coomassie staining, excised from the gel, and destained completely with anhydrous EtOH after 2 h at 22 °C.**
GST followed by horseradish peroxidase-conjugated anti-rabbit and visualized by chromogenic reaction with ortho-phenylenediamine as described previously (25). In parallel with this, nonspecific binding of GST fusion proteins to BSA-coated wells was determined, and these values subtracted from those obtained using His-PABP1 coated wells. 

Isolated Gradient Centrifugation—Cells were grown to 60% confluence, and the monolayers scraped into cell fractionation buffer containing 20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.25 mM sucrose, 1 mM EDTA, 1 mM Na2VO4, 1 mM AEBFS, 20 \mu M leupeptin, 25 \mu g/ml aprotinin, and 3 \mu g/ml pepstatin A. The cells were lysed by 10 passes of a Dounce homogenizer, and nuclei removed by centrifugation at 700 \times g for 10 min. Post-nuclear supernatants were layered onto an linear gradient of 10–40% iodixanol in cell fractionation buffer and centrifuged at 48,000 \times g for 18 h at 4°C. Gradients were eluted and linearity of the gradient confirmed by refractometry. Proteins were precipitated from the gradient fractions with 20% trichloroacetic acid, and the migration positions of paxillin and intracellular compartments determined by Western blotting with antibodies against the following marker proteins; calreticulin (ER), rab11 (endosomes), and clathrin heavy chain (clathrin-coated vesicles).

Immunofluorescence—For immunofluorescence analysis, cells were fixed in 2% (w/v) paraformaldehyde in PBS for 20 min at room temperature. Following this, they were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 min, and the paraformaldehyde quenched with 50 mM NH4Cl for 10 min. Nonspecific binding sites were blocked with PBS containing 1% (w/v) BSA for 1 h, and cells incubated with the primary antibodies at room temperature for 1 h. Detection was by FITC or Texas Red-conjugated secondary antibodies. The actin cytoskeleton was visualized by counterstaining with FITC or Texas Red-conjugated phalloidin in PBS for 10 min at room temperature. Coverslips were mounted in Proflade antifade mountant (Molecular Probes) and viewed on a Leica confocal laser scanning microscope with EGFP fluorescence being collected into the fluorescence channel.

RESULTS

Identification of Proteins Co-immunoprecipitating with Paxillin—Numerous paxillin-binding proteins have been identified using GST-paxillin pull down assays and yeast two hydrid screens. However, it is unclear how many of these proteins form stable complexes with paxillin in the cell. To approach this question, magnetic beads conjugated to anti-paxillin monoclonal antibodies were incubated with Triton X-100 lysates of subconfluent 3T3 fibroblasts labeled to steady state with [35S]methionine/cysteine. Following extensive washing in a buffer containing 1.5% Triton X-100 and 0.75% Igepal CA-630, co-precipitating proteins were eluted from the beads with a buffer containing 0.5% SDS for 10 min on ice. Under these elution conditions, paxillin itself remained associated with the beads (data not shown), thus allowing the identification of co-precipitating proteins in the same molecular weight range as paxillin. Analysis of the eluted proteins by SDS-PAGE and fluorography identified several components that were specifically found in the paxillin but not in the control immunoprecipitates, most notably proteins of 170 kDa, 105 kDa and 70 kDa (Fig. 1A). To identify these proteins, paxillin was immunoprecipitated from lysates made from 10^9 cells, and the co-precipitating components visualized by staining the gels with colloidal Coomassie. Bands corresponding to the 170-kDa, 105-kDa and 70-kDa proteins (Fig. 1B) were excised from the gel and digested with trypsin, and the resulting peptides analyzed by MALDI-TOF mass spectrometry. The 170-kDa protein, p170, yielded 13 tryptic peptides corresponding to those predicted to be obtained from clathrin heavy chain-1 (CHC-1) (Table I). CHC has previously been identified as a binding partner for paxillin (6), although the significance of this is not yet known. Interestingly, the other two paxillin-associated proteins, p105 and p70, are both proteins known to bind mRNA. From p105, we identified 16 peptides that matched polyuridine tract binding (PTB) protein-associated splicing factor (PSF) (Table I), a protein known to bind to PTB protein and proposed to play a role in the regulation of pre-mRNA splicing. From p70, we identified six peptides from PABP1 (Table I) that associate with the 3’-polyA tail of mRNAs.

Protein bands observable at 50 and 97 kDa were not analyzed by mass spectrometry. We detected proteins of these sizes associated with the control IgG (Fig. 1A), thus it was difficult to ensure the specificity of their association with paxillin immunoprecipitates. In addition, we were unable to identify some of the less abundant components of the paxillin immunoprecipi-
Paxillin Binds to PABP1

Proteins co-immunoprecipitating with paxillin were resolved in SDS-PAGE and visualized by colloidal Coomassie staining, and bands corresponding to proteins of 170 kDa, 105 kDa, and 70 kDa excised from the gel displayed in Fig. 1B. Proteins were digested with trypsin, and the resulting peptides analyzed by MALDI-TOF mass spectrometry. Peptides derived from p170 correspond to clathrin heavy chain, from p105 to PTB-associated splicing factor (PSF) and those from p70 to poly(A)-binding protein (PABP1).

| Proteins                  | Peptide bands | Total coverage |
|---------------------------|---------------|----------------|
| p170 Clathrin HC-1        | 164 LLLIGASIQR | 176            |
|                           | 316 LFQGVLGNYR | 320            |
|                           | 441 YGEPGFMAER | 447            |
|                           | 332 GHFPGKE   | 338            |
|                           | 286 PTIHNAYG  | 292            |
| p105 PTB-associated splicing factor | 33 SPPPMGLNQNR | 44 222/707 = 31% |
|                           | 280 ANLSLR    | 286            |
|                           | 299 LFQGVLGNYR | 315            |
|                           | 320 YGEPGFMAER | 330            |
|                           | 332 GHFPGKE   | 338            |
|                           | 341 GFFIFK    | 347            |
|                           | 357 PTHAALNG  | 363            |
| p70 Poly(A)-binding protein 1 | 31 FSPAGPILSIR | 41 77/522 = 15% |
|                           | 32 GLGAYYNFQP | 367            |
|                           | 35 GPPGVQG    | 361            |
|                           | 369 GREEEYPKNKPR | 706            |
|                           | 354 IVATKLVLAL | 382            |
|                           | 372 QAHLTNQQMQR | 382            |

GFP-paxillin-α and GFP-paxillin-β were expressed at similar levels and immunoprecipitates of GFP-paxillin-α and GFP-paxillin-β contained similar amounts of PABP1, suggesting that both paxillin isoforms bind to PABP1 in vivo (Fig. 2B).

In the translation initiation complex, PABP1 binds directly to elf4G (23), and it is possible that paxillin is part of the translation complex. However, both elf4G and elf4E were absent from the paxillin immunoprecipitates (Fig. 2A), indicating that the association of PABP1 with paxillin and elf4G are likely to be mutually exclusive.

PABP1 Binds Directly to Paxillin—The NH₂-terminal region of paxillin contains five LD domains, and the COOH-terminal region comprises four LIM domains. The LD domains are involved in interaction with other focal adhesion proteins, such as pp125FAK, vinculin, p21-activated kinases, actopaxin, p56LCK, Git-2, and ILK, while the LIM domains bind PTP-PEST and are necessary for focal adhesion targeting and the recruitment of paxillin to the microtubular cytoskeleton (3, 12, 26). To determine the ability of paxillin to bind PABP1, lysates from [35S]methionine/cysteine labeled cells were incubated with a GST fusion spanning paxillin residues 54–313 that contains LD domains 2 through to 5. This fusion protein precipitated bands of 125 kDa and a prominent band of 70 kDa (Fig. 3A). Western blotting confirmed the identity of the 70-kDa band as PABP1, and PABP2 was not recruited to GST-paxillin-α or -β (Fig. 3A).
To establish if PABP1 binds directly to paxillin, His-tagged PABP1 was expressed in *E. coli* and purified on a nickel-agarose column. GST-paxillin-(54–313) coupled to magnetic beads was then incubated with His-PABP1, and binding determined by Western blotting. The results clearly demonstrate that GST-paxillin-(54–313), but not GST alone, binds to purified His-PABP1 (Fig. 3C), indicating that the two proteins interact directly. To obtain an estimate of the affinity of this interaction, microtiter wells coated with recombinant His-PABP1 were incubated with various concentrations of either GST or GST-paxillin-(54–313), and bound fusion proteins were detected using horseradish peroxidase-labeled anti-GST antibody, followed by visualization with o-phenylenediamine. Using this approach, we were able to show that GST-paxillin bound specifically to His-PABP1, and this binding was saturating.

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**Fig. 2.** PABP1 associates with paxillin immunoprecipitates. A, NIH 3T3 fibroblasts were grown to 60% confluence, lysed, and immunoprecipitated with control IgG1 (MOPC) or an anti-paxillin monoclonal antibody (anti-Pax) as for Fig. 1. The immunoprecipitating proteins were dissolved by boiling in SDS-sample buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were probed with antibodies against PABP1, PABP2, paxillin (PAX), elF4E, and elF4G, and detection was by a second antibody followed by enhanced chemiluminescence. B, NIH 3T3 fibroblasts were transfected with EGFP (GFP), EGFP-paxillin-α (GFP-Pax-α) and EGFP-paxillin-β (GFP-Pax-β). Cells were then grown to 60% confluence over 24 h and lysed as for Fig. 1. The lysates were immunoprecipitated with anti-GFP monoclonal antibody, and immunoprecipitating proteins analyzed by Western blotting as for A.

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**Fig. 3.** Association of PABP1 with GST-paxillin-(54–313). A, cells were metabolically labeled with [35S]methionine/cysteine and lysed as for Fig. 1A. Lysates were incubated with magnetic beads conjugated to GST or GST-paxillin-(54–313) (GST-Pax), and coprecipitating proteins were resolved by SDS-PAGE and visualized by autoradiography. B, lysates from unlabeled cells were incubated with magnetic beads conjugated to GST, GST-paxillin-(54–313) (GST-Pax), or GST-paxillin LD domains 1, 2, 4, or 5 (GST-LD1, 2, 4, 5). Coprecipitating proteins were resolved by SDS-PAGE and analyzed by Western blotting for PABP1 and PABP2. C, purified, *E. coli*-expressed His-PABP1 was incubated with magnetic beads conjugated to GST, GST-paxillin-(54–313) (GST-Pax), or GST-paxillin LD motifs 1, 2, 4, or 5 (GST-LD1, 2, 4, 5). Coprecipitating proteins were resolved by SDS-PAGE and analyzed by Western blotting for GST, PABP1, and PABP2. D, the loading of the GST-fusion proteins was confirmed by Western blotting for GST.
A logarithmic plot revealed that 50% saturation of binding was obtained at 10 \text{nM GST-paxillin} (Fig. 4B). The recruitment of protein complexes to paxillin can be ascribed, in certain cases, to the association of individual LD domains with specific sequences in the protein ligand, termed the paxillin binding subdomain (31). To determine whether individual LD domains were capable of interacting with PABP1, LDs 1, 2, 4, and 5 were expressed as GST fusion proteins and incubated with either cell lysates or recombinant His-PABP1. GST-LD4 was able to precipitate PABP1 from cell lysates and to bind to purified His-PABP1, but the interaction was weak compared with that with GST-paxillin-(54–313) (Fig. 3, B and C). Consistent with this, binding of GST-LD4 to His-PABP1 immobilized in microtiter wells was not significantly greater than GST over a range of fusion protein concentrations, which gave saturable binding with GST-paxillin-(54–313) (Fig. 4A and B). These data indicate that the NH$_2$-terminal region of paxillin binds with high affinity to

Fig. 5. Association of paxillin with the dense ER. A, Swiss 3T3 fibroblasts were grown to 60% confluence, and the monolayers scraped into a buffer containing 0.25 M sucrose. The cells were then lysed by 10 passes of a Dounce homogenizer, and the nuclei removed by centrifugation at 700 × g for 10 min. The postnuclear supernatant was layered onto a 10–40% (v/v) iodixanol gradient and centrifuged at 48,000 × g for 18 h at 4°C. Gradients were eluted and linearity of the gradient confirmed by refractometry. Proteins were precipitated from the gradient fractions with 20% (w/v) trichloroacetic acid, and the migration positions of paxillin (PAX), the ER (calreticulin; CalR), endosomes (Rab11), and clathrin-coated vesicles (clathrin heavy chain; CHC) determined by Western blotting. B–D, cells were double stained for paxillin (B; green) and calreticulin (C; CalR, red) and viewed by confocal microscopy. Colocalization of the fluorophores is shown by yellow in the merged images (D). The plane of focus is centered at 2.5 μm above the coverslip. Bar, 5 μm.
Paxillin binds to PABP1 in an interaction that may involve LD4. However, the affinity of the single LD domain for PABP1 was insufficient to support efficient complex formation.

Paxillin and PABP1 Are Associated with the Dense ER—It is possible that the paxillin-PABP1 complex may be involved in delivery of mRNA to the translation machinery and so be located at sites of protein synthesis. We therefore analyzed the subcellular distribution of paxillin using density gradient centrifugation and immunofluorescence. On iodixanol gradients, paxillin resolved into two components; a light fraction, unlikely to be associated with macromolecular structures, and a fraction buoyant at ~30% iodixanol (density \( \approx 1.16 \) g/ml) that co-sedimented with a dense element of calreticulin-containing ER (Fig. 5A) (32). Immunofluorescence indicated that although paxillin was present in focal adhesions, a substantial amount of immunoreactive paxillin resided in the perinuclear region of subconfluent fibroblasts (Fig. 5B). This perinuclear staining colocalized with calreticulin, consistent with paxillin being associated with the ER (Fig. 5, B–D). A similar analysis of cells expressing GFP-paxillin-\( \alpha \) or \( \beta \) showed that both paxillin isoforms co-sedimented with PABP1 in the dense ER on iodixanol gradients (Fig. 6A) and colocalized in the perinuclear region (Fig. 6, B–E). Taken together, the gradient fractionation and immunofluorescence data indicate that the paxillin-\( \alpha \)-PABP1 and paxillin-\( \beta \)-PABP1 complexes are localized to the dense ER, where the translation machinery is positioned for synthesis of membrane and secretory proteins.

Paxillin and PABP1 Shuttle between the Nuclear and Cytoplasmic Compartments—Many proteins involved in the processing and trafficking of mRNA, including PABP1, shuttle in and out of the nucleus (33). Nucleo-cytoplasmic shuttling can be revealed by blocking export of proteins from the nucleus with leptomycin B, which inhibits the CRM1 transporter (34). Leptomycin B caused both isoforms of paxillin and PABP1 to move from the perinuclear region to the nucleus (Fig. 6, F–I). Analysis of endogenous paxillin distribution by indirect immunofluorescence clearly shows that it is the perinuclear pool of paxillin that moves to the nucleus following leptomycin B treatment (Fig. 6, J–M). Inhibition of nuclear export has little effect on the paxillin content of the focal adhesions. This indicates that the ER-pool of paxillin/PABP1 complex undergoes continuous nucleo-cytoplasmic shuttling and is consistent with a role for this complex in the trafficking of mRNA from the nucleus to sites of translation in the cytoplasmic compartment.

Paxillin and PABP1 Are Localized to the Lamellipodium—Certain mRNAs are targeted to the leading edge of migrating cells (16), and ribosomes have been observed at integrin-containing focal complexes (35). We therefore investigated the...
localization of paxillin and PABP1 in migrating fibroblasts. Cells were transfected with either GFP-paxillin-α or -β and grown to confluence. The monolayers were then wounded, and cells stimulated to migrate into the wound by addition of platelet-derived growth factor. PABP1 localized to the tips of F-actin-rich lamellipodia, which extended into the wound (Fig. 7, A and B). In these migrating cells, GFP-paxillin-α was present in focal adhesions under the main body of the cell, and there was no indication that PABP1 was present in these structures (Fig. 7, C and D). Strikingly, however, paxillin-β was closely colocalized with PABP1 at the leading edge of the lamellipodium, as well as in the perinuclear region (Fig. 7, E and F). These data indicate that, in addition to trafficking between the nucleus and the dense ER, the paxillin-β-PABP1 complex is targeted to the leading edge of migrating cells and may be involved in the delivery of mRNA to this region of the cell.

**DISCUSSION**

Using MALDI-TOF mass spectrometry, we have identified the mRNA-binding protein PABP1 as a particularly abundant component of paxillin immunoprecipitates. *In vitro* studies show that PABP1, but not PABP2, binds directly to the NH₂-terminal region of paxillin, and with high affinity (Kₐ of ~10 nM). Cell fractionation and immunolocalization studies show that both paxillin and PABP1 are found in the ER, the site at which both membrane and secreted proteins are synthesized. Addition of leptomycin B reveals the nucleocytoplasmic shuttling of paxillin and PABP1, but at steady state the complex resides primarily in the dense ER. Interestingly, a proportion of paxillin-β and PABP1 relocalizes to the tips of lamellipodia when cells are induced to migrate.

Hitherto, the search for paxillin-binding proteins has involved either yeast 2-hybrid screens (8) or GST-fusion protein pull-down assays (6, 10, 12, 26, 30). This has led to an understanding of how paxillin functions as a modular adaptor protein, recruiting a host of cell signaling and actin-binding proteins that are important in regulating the dynamic properties of focal complexes and focal adhesions. To determine the complement of cellular proteins most stably associated with endogenous paxillin, we initially screened paxillin immunoprecipitates by Western blotting for known focal adhesion proteins. Surprisingly, established paxillin ligands such as pp125FAK were present at rather low levels and others, such as vinculin, were almost undetectable, as previously reported (30). This result suggests that paxillin might be associated with proteins that are distinct from these components of focal adhesions.

**Paxillin Binds PABP1**—The most abundant protein co-immunoprecipitating with paxillin is the mRNA-binding protein, PABP1. The NH₂ terminus of paxillin associates directly with PABP1 with an approximate Kₐ of 10 nM. The high affinity of this interaction, combined with the abundance of PABP1 in the cytoplasm (22), is consistent with the notion that the majority of cellular paxillin is associated with PABP1 most of the time. Interestingly, PABP1 contains two regions of sequence with similarity to the paxillin binding subdomains of actopaxin, vinculin, and pp125FAK (5) (Fig. 8A). One of these is in the NH₂ terminus (residues 17–30), and the other is nearer to the COOH-terminal end of the protein (residues 345–358) (Fig. 8, A and B). PABP2, by contrast, contains no motif recognizable as a paxillin binding subdomain. Inspection of the domain structure of PABP1 (Fig. 8B) shows that residues 17–30 are located within the first of the four RRMs (RRMs 1–4) (36). The three-dimensional structure of RRMs 1 and 2 of PABP1 co-crystallized with poly(A)⁺ RNA reveals that the paxillin binding subdomain motif corresponds exactly to a surface-exposed loop connecting the first β-sheet to the first α-helix of PABP1 (37).

None of the residues in this loop are directly involved with the coordination of mRNA, making it an excellent candidate for a paxillin binding site.

Although the paxillin LD4 domain binds a small amount of PABP1 in pull-down assays, its affinity for PABP1 was very much lower than that of a paxillin polypeptide (residues 54–313) spanning LD2-LD5. It is possible, therefore, that more than a single LD domain is required for efficient binding to PABP1. Indeed both FAK and vinculin interact with more than one paxillin LD domain and, like PABP1, each contains two paxillin binding subdomains. Mutation of either of these sites in FAK abolishes paxillin binding (38). A further possibility that we cannot exclude is that paxillin LD3 is involved in binding PABP1, and no protein ligands for this LD domain have been identified to date. However, we have been unable to investigate this properly as our attempts to synthesize LD3 as a stable GST fusion have been unsuccessful.

**The Potential Role of Paxillin-PABP1 Interaction in mRNA Trafficking**—mRNA in the nucleus is tightly associated with several proteins collectively known as the nuclear ribonucleoprotein complex (hnRNP), and it is proposed that nuclear export is mediated by signals present in these RNA-binding proteins. An emerging feature of many nuclear RNA-binding
proteins is that they also have a role in the cytoplasm (39). Many of these first complex with mRNA in the nucleus and therein are involved in the regulation of transcription and splicing. They then accompany the processed mRNA into the cytoplasm where the same proteins have been shown to regulate processes such as translation and the tethering of mRNA to the cytoskeleton. PABP1 has been identified as a component of the cleavage factor I complex responsible for mRNA 3′-end formation and length regulation (40) in the nucleus, but also shuttles to the cytoplasm (33) where it is known to be a component of the translation initiation complex (41). Here we confirm the notion that PABP1 shuttles in and out of the nucleus, and additionally show that paxillin accompanies it on this particular leg of its journey. The focal adhesion protein zyxin has recently been shown to accumulate in the nucleus following treatment with leptomycin B (42). Neither zyxin nor paxillin contains a consensus nuclear localization signal, thus it is possible that these proteins may be transported by an unconventional import mechanism. It has been shown that both the proline-rich regions and LIM domains of zyxin contribute to nuclear import (42) and, as paxillin also contains these types of motif, these may contribute to the nuclear-cytoplasmic shuttling of the paxillin-PABP1 complex. The presence of PSF in the paxillin-PABP1 complex is also consistent with its being part of an hnRNP complex. PSF (43) was first identified as a binding partner for PTB protein, which is involved in the regulation of mRNA splicing (44). PTB has recently been shown to bind to a novel protein called raver1 that has homology with the RNA-binding motifs of PTB and PSF, and is a ligand for the focal adhesion proteins vinculin and α-actinin (47). Like raver1, PSF contains two nuclear localization signals (45), and it is therefore possible that PSF contains the information necessary for nuclear import of paxillin. PSF does not contain sequences recognizable as a paxillin binding subdomain and, yet, we have not investigated whether PSF interacts directly with paxillin, but present evidence suggests that vinculin, α-actinin, and paxillin may all be components of RNP complexes that shuttle in and out of the nucleus.

PABP1 binds with high affinity to the dendritic localizer sequence in the 3′UTR of the vasopressin mRNA (21) and may be involved in targeting mRNA to polyribosomes on peripherally located ER elements (20). On iodixanol gradients, paxillin and PABP1 are present in substantial quantities in the dense, polyribosomal ER. Such a localization raises the possibility that the paxillin-PABP1 complex recruits mRNAs encoding those proteins destined for secretion.

Although RRM 1 and 2 contain the minimal determinant for high affinity PABP1 binding to poly(A) (Fig. 8B) (36), PABP1 function is likely to involve binding to other mRNA sequences (22). RRM4, for instance, which actually binds poly(U) or poly(G) better than poly(A), is essential for maintenance of growth in yeast (36); so the possibility that this domain contributes to the selection of 3′UTR targeting motifs in mRNA must be considered. The ability of mRNA-binding proteins to select particular message subsets is well known. The mRNA profiles detected in HuB, elF4E, and PABP1-mRNA complexes are distinct from one another and from those of the transcriptosome (46). It is possible that the association of paxillin with PABP1 is further able to refine the mRNA subset associated with PABP1, and it will be interesting to determine, whether any mRNAs associated with paxillin immunoprecipitates are particularly enriched in those encoding proteins involved in cell adhesion and cell motility.

Here, we report that paxillin-β and PABP1 are localized to the tips of the lamellipodium, suggesting that the paxillin-β-PABP1 complex may also contribute to mRNA localization in this region. Paxillin-β may act as an adaptor to recruit mRNA to the actin cytoskeleton, via its interaction with actopaxin or...
vinculin, and the presence of the 34-amino acid insert in paxillin-β may favor this association. Following arrival at the lamellipodium it is believed that β-actin mRNA is translated (14), and the clustering of integrins causes the movement of ribosomes to focal complexes (35) indicating that these structures may be an important site of protein synthesis. PABP1 is established to associate with elF4G (Fig. 8A), and this complex increases the efficiency of translation by appropriately restraining the 5′- and 3′-ends of the mRNA (41). It is clear from our data, however, that paxillin is not part of this complex. Indeed the complete absence of elF4G from paxillin immunoprecipitates indicates that association of paxillin and elF4G with PABP1 may be mutually exclusive. This is consistent with paxillin being involved in the trafficking and localization of mRNA prior to translation. Upon arrival at sites designated for protein synthesis, for instance the ER and the lamellipodium, PABP1 could switch allegiance from paxillin to elF4G thus transferring its cargo, the mRNA, into the translation complex.

Our results describe a high affinity interaction of the adaptor protein paxillin with the mRNA-binding protein, PABP1. Together they form part of a complex that shuttles in and out of the nucleus and which localizes to sites of protein synthesis in the ER and the lamellipodium. We propose that paxillin may contribute to the generation of cell polarity by mediating the localization of mRNA to the actin and microtubular cytoskeletons.

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