TYROSINE PHOSPHORYLATION OF PAXILLIN, FAK, AND p130CAS: EFFECTS ON CELL SPREADING AND MIGRATION

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1. ABSTRACT

Integrins are transmembrane receptors that mediate cell attachment to the substrate. At the cytoplasmic surface of the integrin, cytoskeletal proteins cluster into focal adhesions. The focal adhesions contain multiple proteins that provide a structural and signaling complex inside the cell. This review focuses on three of the cytoskeletal components of the focal adhesion, paxillin, FAK, and p130CAS, that are phosphorylated and play a regulatory role in cell spreading and cell migration. A brief discussion is included of tyrosine phosphorylation of the integrin in relation to localization and phosphorylation of these cytoskeletal proteins. The phosphorylation of integrins and cytoskeletal proteins regulates localization and downstream signaling with profound effects on cell movement.

2. INTRODUCTION

The cytoskeleton provides a framework within the cell that is critical for cell adhesion and cell movement. Upon cell attachment, the actin cytoskeleton may re-organize into stress fibers that terminate at the focal adhesion. The focal adhesion is the cellular interface between actin stress fiber and the integrin cell surface receptor. The "integrin" integrates information between the extracellular matrix on the extracellular surface and the actin cytoskeleton inside the cell through the recruitment and phosphorylation of specialized proteins into the focal contact (reviewed in (1; 2)). The dynamic nature of the focal contact allows cells to attach and detach during cell migration (3).   Our current understanding of the cytoskeleton shows that phosphorylation of the proteins in the focal contact provides one mechanism to remodel the focal contact. Live video microscopy shows that the focal contacts at the leading are forming and at the trailing edge are rapidly changing during cell migration compared to the stable focal contacts in the center of the cell (4). This review provides insight into three proteins that are dynamic components of the focal contact with information on their structure, localization to the focal contact, stimulation of phosphorylation, and role in cell spreading and cell migration.

3. DISCUSSION

3.1 Paxillin

Paxillin is a cytoskeletal protein that localizes to areas of cell/matrix contact called focal adhesions (reviewed in (5-7)). Paxillin is a member of a family of homologous proteins that include Hic-5, actopaxillin, and PaxB that have a limited tissue distribution (8-10). Smooth muscle cells express large amounts of paxillin with an overall wide tissue distribution. Platelets, notably, do not express paxillin, but express the paxillin family member Hic5 (5). Deletion of the paxillin gene in mice results in early embryonic lethality (day E7-E8) (5). Paxillin is a 68 kDa protein with no intrinsic enzymatic activity. Paxillin contains multiple domains that interact with cytoskeletal and signaling molecules leading to its classification as a scaffolding protein (11).   The amino terminal half of paxillin contains five LD repeats (Figure 1A). The novel LD repeats contain eight amino acids beginning with an invariant leucine-aspartate sequence for which they are named. The LD motifs mediate binding of focal adhesion
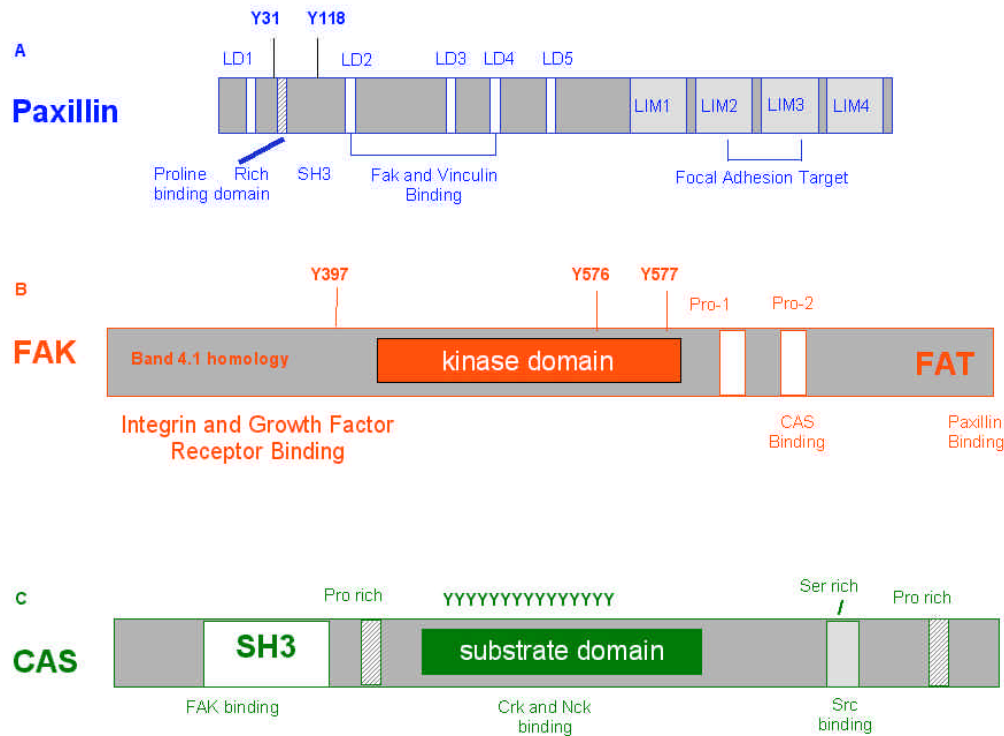
Figure 1. Schematic of the structure of A) paxillin, B) FAK, and C) p130CAS including structural motifs and critical tyrosine phosphorylation sites.

kinase (FAK), vinculin, and E6 oncoprotein to paxillin and target FAK and vinculin to the focal contact. Paxillin has been demonstrated to bind directly to the alpha4 integrin cytoplasmic tail (12). The amino terminus of paxillin contains a proline rich region that binds SH3 domains of src and crk. The carboxy terminus of paxillin contains four LIM domains. The LIM domain is a double zinc finger motif that mediates binding to transcription factors, cytoskeletal proteins, and a tyrosine-phosphatase PTP-PEST. The LIM domain of paxillin mediates dimerization of paxillin. The assembly of a multi-protein complex by paxillin leads to multiple downstream signaling events. The signaling properties of paxillin have been reviewed elsewhere (5; 6; 13).

The localization of paxillin to focal contacts has been attributed to both the LIM domain and the amino terminal LD repeats. By expressing truncated proteins and deletion mutants, the third LIM domain was identified as critical for targeting of paxillin to focal adhesions (14). Additional studies show that PAG3 binds to the amino terminus of paxillin, and targets paxillin to the focal contact. PAG3, a paxillin binding protein, has GTPase-activating protein (GAP) activity, but does not localize to the focal contact itself (15). Therefore, both the amino and carboxy termini of paxillin may regulate localization to the focal contact. Because paxillin does not directly bind to most integrins and precedes FAK into the focal contact, the binding protein, that promotes localization to the focal contact, is not clear.

Paxillin contains two critical tyrosine phosphorylation sites at Tyr31 and Tyr118 (16). Several mechanisms exist to phosphorylate paxillin on tyrosine in cells. Adhesion to extracellular matrix proteins, fibronectin, vitronectin, or laminin, through integrin receptors stimulates the phosphorylation of paxillin in fibroblasts (17). Additional studies have showed that adhesion of corneal epithelial cells to collagen, laminin, and fibronectin promotes paxillin phosphorylation (18). Abl kinase localizes to the focal contact and may phosphorylate paxillin (19). Growth factor stimulation enhances phosphorylation of paxillin in quiescent cells. Platelet-derived growth factor (PDGF), upon binding its tyrosine kinase receptor, stimulates paxillin phosphorylation in vascular smooth muscle cells (20), and vascular endothelial cell growth factor (VEGF) stimulates paxillin phosphorylation in endothelial cells (21). Agonists for seven transmembrane, G-protein coupled receptors including bombesin, vasopressin, endothelin, lysophosphatidic acid (LPA), and sphingosine 1-phosphate (S1P) stimulate paxillin phosphorylation in fibroblasts (22; 23; 24). LPA and S1P stimulate paxillin localization to the focal contact in endothelial cells (25). The growth factors act through FAK in association with Src to phosphorylate paxillin (16). Cyclic strain induces phosphorylation of paxillin in aortic endothelial cells (26). Therefore, phosphorylation of paxillin is at the downstream convergence of integrin ligation, growth factor stimulation, and biomechanical manipulation of the cells.
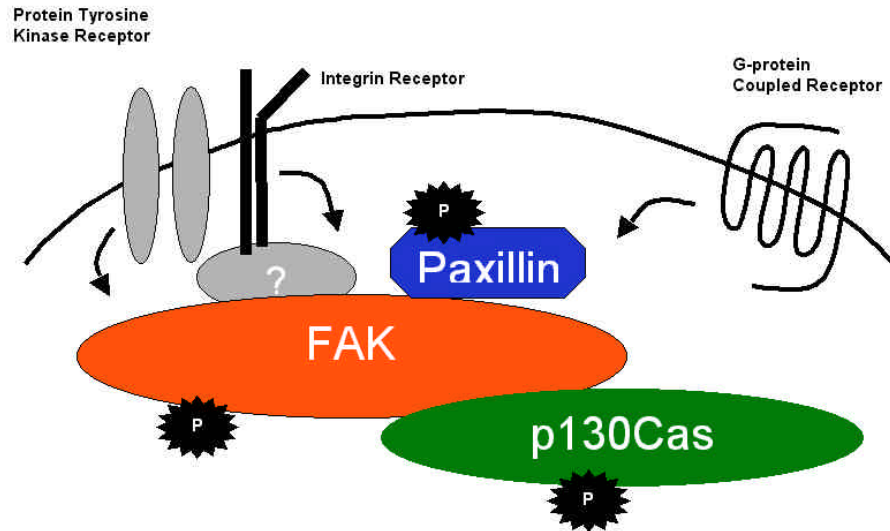
Figure 2. Schematic depicting phosphorylation of cytoskeletal proteins in the focal contact by integrins, tyrosine kinase growth factor receptors, and G-protein coupled receptors.

Paxillin phosphorylation regulates cell spreading and cell migration (16). Tyrosine phosphorylation of paxillin at Tyr31/118 is not required for localization to the focal contact, however serine/threonine phosphorylation of the LIM domain is important for localization (27). Richardson et al. (28) showed that paxillin tyrosine phosphorylation plays a role in chicken embryo cell spreading probably through Src phosphorylation of paxillin. One result of tyrosine phosphorylation is the generation of binding sites for FAK and vinculin and the subsequent localization of these proteins to the focal contact. Paxillin phosphorylation is an early and requisite step in cell spreading as demonstrated by paxillin preceding FAK to the focal contact (28). In live video microscopy of a fusion protein of paxillin with green fluorescent protein, paxillin is recruited early during lamellipodia formation consistent with paxillin recruiting other proteins to the focal contact (29).

Phosphorylation of paxillin has been correlated with alterations in cell migration. Nara bladder tumor II (NBT-II) cells preferentially phosphorylate paxillin after attachment to collagen compared to fibronectin or laminin (30). The phosphorylation of paxillin correlated with cell migration in these cells, i.e., NBT-II cells showed enhanced migration on fibrillar collagen compared to fibronectin, vitronectin or laminin. Mutation of Tyr31 and Tyr118 in paxillin permitted adhesion of CHO cells or NBT-II cells while mutant paxillin inhibited cell migration on fibrillar collagen suggesting a role for paxillin phosphorylation in NBT-II cell migration In contrast, transfection of wild type paxillin inhibits cell migration of COS7 cells, NMuMG cells, and MM-1 cells while transfection of paxillin with mutations in Tyr31/118 promotes migration (31). In a model of NMuMG cell differentiation and migration induced by TGFbeta, paxillin phosphorylation is increased compared to other proteins. A paxillin double mutant at Tyr31/118 to Phe, Y31/118F, localized to focal adhesions at the cell periphery that were larger than focal adhesions in cells containing wild type paxillin. Circumferential actin at the leading edge was lost in the Y31/118F paxillin mutant compared to the wild type control (32). Therefore, a loss of paxillin phosphorylation leads to an increase in NMuMG cell migration and to an altered localization of the paxillin into larger focal contacts at the cell edge. Tyrosine phosphorylation of paxillin may regulate the focal contact turnover and in turn the actin cytoskeleton (5). In two different cell types, the paxillin Y31/118F mutant either promotes or inhibits cell migration. Therefore the role of paxillin in cell spreading is clear while the role in cell migration is complex and merits further study.

3.2 Focal Adhesion Kinase

Focal adhesion kinase (FAK) is a critical component of the focal adhesion and provides both structural and kinase activity to the focal contact. FAK is a 120kDa protein that is present in most tissues and is conserved across species (reviewed in (33; 34)). Disruption of the FAK gene in mice results in embryonic lethality due to a defect in migration in mesodermal cells (35). The FAK family of proteins includes FAK and Pyk2. Both proteins contain a central kinase domain that acts to phosphorylate other proteins (36). The central kinase domain of FAK has two sites of tyrosine phosphorylation at Tyr576 and Tyr577 (Figure 1B). The amino terminus of FAK contains a Band 4.1 homology domain as well as binding sites for the integrin cytoplasmic domains and growth factor receptors. After auto-phosphorylation, Tyr397 binds to PI3-kinase and Src. The carboxy terminus of FAK binds several proteins including p130CAS, Grb2, talin, paxillin, and Graf. The focal adhesion targeting (FAT) sequence is in the carboxy terminus and binds paxillin suggesting that paxillin promotes localization of FAK to the focal contact. Because the carboxy terminus of FAK is critical for localization to the focal contact, the

importance of the direct interaction with the integrin at the amino-terminus is not clear. FRNK, a protein corresponding to the carboxy terminus of FAK, is expressed autonomously in some cell types possibly through alternative splicing. FRNK lacks the kinase domain, binds paxillin, and negatively regulates FAK activity through disruption of FAK localization to the focal contact.

FAK was identified initially as a 120kDa protein that was tyrosine phosphorylated in NIH 3T3 cells in response to adhesion to fibronectin, but not laminin or Type IV collagen (37). Subsequent studies showed that integrin clustering either with antibodies or adhesion to fibronectin, laminin, and collagen stimulated FAK phosphorylation in KB cells (38). In Caco-2 intestinal epithelial cells, adhesion to collagen I, collagen IV, and laminin stimulated tyrosine phosphorylation of FAK, while adhesion to poly L-lysine and fibronectin did not stimulate tyrosine phosphorylation of FAK (39). Therefore, substrate specificity of FAK phosphorylation does exist in some cell types. Phosphorylation of FAK at Tyr397 occurs through autophosphorylation and is integrin-dependent (40). Additional sites for both tyrosine and serine/threonine phosphorylation of FAK have been demonstrated. Both LPA and S1P stimulate FAK phosphorylation in Swiss 3T3 fibroblasts through G-protein coupled receptors (23; 24; 41). LPA stimulates phosphorylation of FAK in EAhy endothelial cell line (42). PDGF, acting through its tyrosine kinase receptor, stimulates phosphorylation of FAK in quiescent cells (23). Therefore, both cell adhesion and stimulation by growth factors triggers phosphorylation of FAK. Phosphorylation of FAK produces binding sites and permits interactions with multiple downstream signaling molecules including Src and p130CAS. The interaction with signaling molecules has been discussed in detail elsewhere (13; 34).

FAK phosphorylation is not required for cell attachment, but is important for cell spreading and cell migration. Using a tetracycline-inducible promoter for wild-type and mutant FAK expression in FAK-deficient cells, Y397F FAK expressing and FAK-deficient cells, showed delayed cell spreading compared to cells expressing wild type FAK (43). Over-expression of FAK stimulates cell migration in Chinese Hamster ovary cells (44). In FAK-deficient cells, cell migration is impaired and can be restored by expression of FAK (35). The FAK autophosphorylation site, catalytic activity, and binding of p130CAS are required to restore cell migration in FAK deficient cells (45). FAK plays a role in the inhibition of cell migration in S1P-stimulated breast cancer cells (46). Over expression of mutant FAK at the Tyr397 autophosphorylation site (Y397F), prevented the S1P inhibition of cell migration. EGF and PDGF do not stimulate cell migration in FAK-deficient fibroblasts (47). Kinase dead FAK, but not Y397F FAK, is phosphorylated at Y397 by EGF and PDGF, and restores growth factor-stimulated cell migration in FAK-deficient cells. Together these studies demonstrate a critical role for tyrosine phosphorylation of FAK in regulating cell migration and integrating adhesion and growth factor signals. FAK may

be critical for the turnover of the focal complex that regulates cell migration because 1)FAK-/- cells have large stable focal contacts, 2) Src is not properly targeted to the focal adhesion in the absence of FAK, and 3) the src-family of protein tyrosine kinases promotes focal contact turnover (47).

3.3. p130CAS

p130CAS is a scaffolding protein that is recruited to the focal adhesion upon integrin ligation (48). The 130 kDa protein was named for its association with and phosphorylation by Crk (Crk-Associated Substrate), p130CAS (49). p130CAS is widely distributed in human tissues and cells and is a member of a family of proteins that includes HEF1 and Efs. HEF1 and Efs have limited tissue distribution, but similar binding domains. Deletion of the p130CAS gene in mice results in embryonic lethality due to deformation of the heart and blood vessels (50). From the amino to carboxy terminus, p130CAS contains an SH3 domain, a substrate binding domain, a serine-rich region, and a hetero-/homo-dimerization domain (Fig 1C). The SH3 domain regulates localization to the focal contact through interaction with FAK. The substrate-binding domain contains up to 15 potential phosphorylation sites and mediates interaction with SH2 domains in Crk (51). There are two poly proline rich regions between the SH3 and substrate domain and the serine-rich region and the C-terminus. These poly proline rich regions of p130CAS mediate interaction with non-receptor tyrosine kinases including Lyn, Fyn, and Src.

The phosphorylation of p130CAS in fibroblast cells is stimulated by adhesion via integrins to multiple substrates including vitronectin, laminin, collagen, and fibronectin, but not poly L-lysine (52). Cytochalasin D inhibits p130CAS phosphorylation demonstrating a requirement for cytoskeletal organization. However, in Src- and Crk-transformed cells, phosphorylation is independent of adhesion consistent with the loss of anchorage dependent growth. Both Src and Crk promote phosphorylation of p130CAS in response to adhesion in normal cells (49) and in the absence of adhesion in the transformed cells (52). In normal fibroblast cells, phosphorylation promotes localization to the focal contact while non-phosphorylated p130CAS remains diffusely distributed in the cytoplasm (49). In quiescent Swiss 3T3 cells, stimulation with LPA or bombesin promotes tyrosine phosphorylation of p130CAS (23). Similar to other focal adhesion proteins, both integrin-mediated adhesion and growth factors promote p130CAS phosphorylation.

p130CAS is not required for cell adhesion; however, p130CAS -deficient fibroblasts show a delay in cell spreading compared to p130CAS re-expressing cells (53). p130CAS is necessary for cell migration in a wound healing assay (53). p130CAS acts downstream of FAK to promote cell migration in CHO cells (54). The FAK/p130CAS interaction promoted cell migration of CHO cells, but did not require Erk phosphorylation (54). In FG human pancreatic carcinoma cells, an interaction between p130CAS and Crk activates Rac and Rac activation is necessary for cell migration. Therefore, the FAK/p130CAS interaction has been deemed a "molecular

Table 1. Role of tyrosine phosphorylation in the adhesion, spreading and migration of three focal adhesion components

Protein	Critical residues for tyrosine phosphorylation	Signaling function	Role in adhesion	Role in cell spreading	Role in cell migration
Paxillin	Tyr 31/118	Adaptor	No	Yes/P-Tyr	Yes/ P-Tyr
FAK	Tyr 397	Kinase	No	Yes/P-Tyr	Yes/ P-Tyr
p130CAS	Multiple Tyr in substrate domain	Adaptor	No	Yes/P-Tyr	Yes/ P-Tyr

switch" for cell migration (55). The FG human pancreatic carcinoma cells with increased phosphorylation of p130CAS also promoted tumor cell metastasis in a model system, i.e, chick chorioallantoic membrane. In Cos7 cells and NMuMG cells, over-expression of p130CAS -GFP stimulated cell migration compared to vector controls (31). Deletion of the substrate domain, containing the tyrosine phosphorylation sites, reduced cell migration to vector control or below (31). Therefore the tyrosine phosphorylation of p130CAS and subsequent association with Crk regulates cell migration.

3.4. Integrins

Adhesion of cells to the extracellular matrix is dependent upon the integrin family of cell adhesion molecules (1). Integrin receptors are comprised of a heterodimer of non-covalently associated alpha and beta subunits. The cytoplasmic tail of the integrin family of beta subunits contains one or two NPXY motifs that encode for a tight turn. The phosphorylation of these tyrosine(s) regulates integrin function. Using a model system of GD25 fibroblast-like cells that are deficient in beta1 integrin subunit, both wild type beta1 integrin subunits (beta1-GD25) and Y783,795F beta1 integrin mutants were compared for function. Cells expressing the Y783,795F mutant beta1 integrin subunits adhered to laminin, similar to the beta1-GD25 cells, but unlike the beta1-deficient GD25 cells (56; 57). The beta1-GD25/Y783,795F cells showed a delayed spreading response (58) similar to tyrosine phosphorylation-deficient cytoskeletal proteins. The Y783,795F expressing cells developed numerous, fine focal contacts compared to the large focal contacts of the paxillin and FAK tyrosine phosphorylation mutants. The Y783,795F beta1 mutant cells had decreased cell migration across filters similar to the tyrosine phosphorylation mutants of the paxillin, FAK, and p130CAS. Sakai et al.(56) propose that the integrin cycles between a phosphorylated and de-phosphorylated state in the wild type, but in the Y783,795F mutant, the integrin cannot cycle resulting in an altered focal contact that does not remodel to permit cell migration. Additional studies showed that adhesion via the Y783,795F mutant beta1 integrin did not phosphorylate FAK or paxillin, although FAK and paxillin localized to the focal contact (58). In contrast, the Y783,795F mutant beta1 integrin phosphorylated p130CAS under the same conditions demonstrating a difference between the three focal contact proteins. A chimeric beta3 integrin tail with a mutant tyrosine in the NPXY sequence showed defective phosphorylation of FAK while the wild type receptor chimera phosphorylated FAK (59). Therefore, these studies suggest that integrin ligation regulates cell adhesion, localization of the cytoskeletal proteins to the focal contact, and phosphorylation of some focal contact components. Whereas integrin phosphorylation regulates the rate of cell spreading and phosphorylation of other focal adhesion components. Integrin independent mechanisms, like growth factor receptors, also promote phosphorylation of the focal adhesion components as described previously.

4. SUMMARY AND PERSPECTIVE

Cell adhesion via integrins occurs in the absence of phosphorylation of the cytoskeletal proteins paxillin, FAK, and p130CAS (Table 1). In contrast, the subsequent spreading of the cells is regulated by tyrosine phosphorylation of these proteins. The cycling between phosphorylated and de-phosphorylated states may be a critical regulatory mechanism of these proteins. Absence of the cytoskeletal proteins and mutations in the tyrosine residues show dramatic effects on cell migration. Cell migration is a tightly regulated process that requires a fine balance between adhesion, as the leading edge moves forward, and de-adhesion, as the trailing edge is detached. The assembly of signaling complexes downstream from the integrin is presumably also tightly regulated by phosphorylation. Growth factors play an important role in protein phosphorylation and cell migration. The role of focal contact proteins as integrators of the integrin and growth factor pathway is just beginning to be elucidated. Therefore, the tyrosine phosphorylation of paxillin, FAK, p130CAS, and beta1 integrins are examples of the role of phosphorylation in regulating spreading and migration of adherent cells from multiple extracellular inputs.

The knowledge of focal adhesion assembly and tyrosine phosphorylation has been greatly expanded using cells derived from knockout mice. The ability to introduce both wild type and mutant proteins into null cells has provided information on the role of tyrosine phosphorylation in regulating protein localization and function at the cellular level. Gaps remain in understanding the sequential events that occur to assemble a focal contact upon cell adhesion and spreading. The recruitment of proteins, the binding interactions, and the regulation of phosphorylation are being addressed independently, but gaps remain in the synthesis of the information for a cohesive picture of focal contact assembly. The disassembly of the focal contact is critical for cell movement. Live video microscopy of wild type and designer proteins holds great promise as a tool to contribute to our understanding of the assembly and disassembly of the focal adhesion during cell adhesion, spreading, and migration.

5. ACKNOWLEDGEMENTS

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Abbreviations: FAK, focal adhesion kinase; p130CAS, crk associated substrate; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; PDGF, platelet-derived growth factor; VEGF, vascular endothelial cell growth factor; Y397F FAK, Tyr 397 substituted by Phe

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