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Opposing Roles of Zyxin/LPP ACTA Repeats and the LIM Domain Region in Cell-Cell Adhesion*

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Marc D. H. Hansen[1] **and Mary C. Beckerle**

From the Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah 84102

Cadherins mediate cell-cell adhesion by linking cell junctions to actin networks. Although several actin regulatory systems have been implicated in cell-cell adhesion, it remains unclear how such systems drive cadherin-actin network formation and how they are regulated to coincide with initiation of adhesion. Previous work implicated VASP in assembly of cell-cell junctions in keratinocytes and the VASP-binding protein zyxin colocalizes with VASP at cell-cell junctions. Here we examine how domains in zyxin and its relative LPP contribute to cell-cell junction assembly. Using a quantitative assay for cell-cell adhesion, we demonstrate that zyxin and LPP function to increase the rate of early cell-cell junction assembly through the VASP-binding ActA repeat region. We also identify the LIM region of zyxin and LPP to be a regulatory domain that blocks function of these proteins. Deletion of the LIM domains drives adhesion and increases VASP level in detergent insoluble cadherin-actin. Dominant-negative zyxin/LPP mutants reduce the rate of adhesion, lower VASP levels in detergent-insoluble cadherin-actin networks, and allow for the accumulation of capping protein at cell-cell contacts. These data implicate the LIM domains of zyxin and LPP in regulating cell-cell junction assembly through VASP.

Cadherin-mediated cell-cell adhesion integrates individual cells into tissues (1). Strong cell-cell adhesion is required to maintain cell morphology and tissue homeostasis (2). As individual cadherin-cadherin adhesion interactions are weak (3), strong adhesion is thought to require incorporation of cadherin into actin networks (4, 5). The cytoplasmic domain of cadherin binds either β-catenin or plakoglobin (6), which in turn binds α-catenin (7, 8). α-Catenin links cadherin to actin filaments either directly (8) or by binding the actin-binding proteins vinculin (9) or α-actinin (10). Cadherin anchors into detergent-insoluble actin networks concomitant with incorporation of α-catenin into cadherin complexes (4, 5). However, it remains unclear how binding of cadherin to actin filaments is regulated to coincide with cell-cell adhesion. Although cadherin has been demonstrated to induce dramatic changes in actin organization (11–13) and several actin regulatory programs have been generally implicated in cell-cell adhesion (13, 14), how actin rearrangements and cadherin engagement are coordinated remains poorly understood.

Understanding actin assembly mechanisms has been greatly improved by studies of the intracellular pathogen, *Listeria monocytogenes* (15). *Listeria* ActA protein hijacks host actin polymerization machinery, directing assembly of actin comet tails that propel the bacterium through the cytosol (16, 17). In addition to actin nucleation by the Arp2/3 complex, normal motility requires the activity of VASP family members (18) that are recruited via an array of proline-rich motifs termed ActA repeats (19). It has been proposed that VASP competes with capping protein (CapZ) for available barbed ends of actin filaments, protecting the barbed end and allowing continued polymerization (20). In such a model, local control of VASP concentration is critical in cellular regulation of actin organization. How cells control local VASP availability to regulate actin dynamics remains unclear.

ActA repeats are observed in several mammalian proteins, including zyxin (21) and its close relative, LPP (22). Interestingly, zyxin and LPP are both present at cadherin-based cell-cell contacts in epithelial cells and tissues (13, 22–24). Introduction of ActA repeats into cells displaces VASP, but not zyxin, from cell-substratum and cell-cell junctions (13, 21). Expression of the tetramerization domain of VASP, which has been postulated to act as a VASP dominant-negative, in mouse skin results in defects in cell-cell adhesion that generate a skin blistering phenotype (13). Based on these findings, it has been predicted that zyxin recruits VASP to drive actin rearrangements observed during cell-cell junction assembly. Consistent with a role for zyxin in actin dynamics, membrane targeting of zyxin results in local actin assembly (21). Likewise, expression of zyxin fragments that target to mitochondria in cells induces local actin polymerization (25). However, despite broad evidence for a role for zyxin in promoting actin dynamics, cell-cell junction organization appears normal in mice null for zyxin (24).

Here the role of zyxin and LPP in cell-cell junction assembly is examined in detail in MDCK[2] cells. Results demonstrate that zyxin and LPP drive cell-cell adhesion through their arrays of ActA repeats. Expressing constitutively active mutants of zyxin or LPP drives rapid cell-cell adhesion and induces VASP incorporation into actin networks. Conversely, expressing dominant-negative mutants of zyxin or LPP slows cell-cell adhesion and prevents displacement of capping protein from cell-cell junctions. Expression of full-length zyxin or LPP does not affect adhesion. Deletion of the LIM region is required to elicit constitutive activity of zyxin or LPP, demonstrating that the LIM region of zyxin and LPP acts as a regulatory domain that prevents uncontrolled activation of the zyxin/LPP pathway. Together these results demonstrate a role for zyxin/LPP and VASP in cell-cell junction formation and suggest a novel mechanism for controlling VASP activity in cellular processes.

EXPERIMENTAL PROCEDURES

Cell Culture—MDCK cells were cultured in Dulbecco's modified Eagle's medium + 10% fetal bovine serum. Synchronous initiation of adhesion by a calcium switch was performed as described (4). Quantitative measurement of the rate of cell-cell adhesion was performed as described (26). Briefly, cells were trypsinized and resuspended at 2.5×10^5 cells per ml. 20-μl drops of cell suspension were placed on the inner

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[1] To whom correspondence should be addressed: Physiology and Developmental Biology, Brigham Young University, 574 WIDB, Provo, UT 84602. Tel.: 801-422-4998; Fax: 801-422-0700; E-mail: marchansen@byu.edu.

[2] The abbreviations used are: MDCK, Madin-Darby canine kidney; GFP, green fluorescent protein; EGFP, enhanced GFP; PIPES, 1,4-piperazinediethanesulfonic acid.

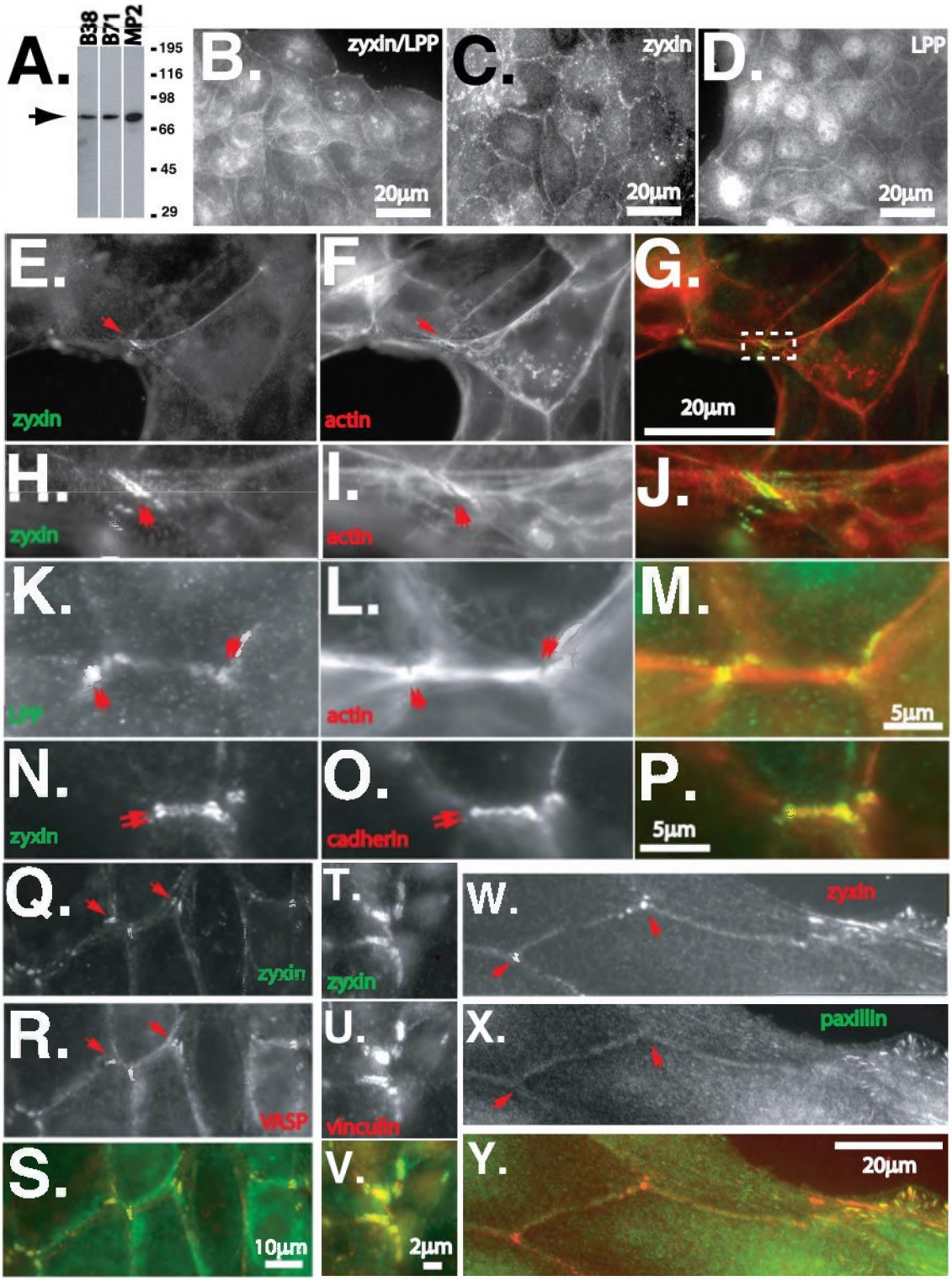

FIGURE 1. **Localization of zyxin and LPP at epithelial cell-cell junctions.** *A*, Western blot analysis of MDCK cell extract with antibodies recognizing both zyxin and LPP (B38) or with zyxin- (B71) or LPP-specific (MP2) antibodies. The *arrow* denotes the expected molecular weight of zyxin and LPP. *B–D*, Zyxin/LPP localization in MDCK cells was detected with anti-zyxin/LPP antibodies (*B*), zyxin-specific antibodies (*C*), or LPP-specific antibodies (*D*). *E–J*, zyxin (E and H, *green*) and phalloidin (*F* and *I*, *red*) staining in MDCK cells. The *boxed area* in G is shown at higher magnification in *H–J*. *K-M*, LPP (*K*, *green*) and phalloidin (*L*, *red*) staining of MDCK cells. *N–P*, relative distribution of zyxin (*N*, *green*) and cadherin (*O*, *red*) at cell-cell junction sites. *Q–S*, relative distribution of zyxin (*Q*, *red*) and VASP (R, *green*) at cell-cell contacts of MDCK cells. *T–V*, colocalization of zyxin (*T*, *red*) and vinculin (*U*, *green*) at cell-cell junctions of MDCK cells. *W–Y*, relative distribution of zyxin (*W*, *red*) and paxillin (*X*, *green*) at cell-cell junctions and at focal adhesions (*right*) of MDCK cells. *Red arrows* indicate plaques of intense zyxin or LPP staining at cell-cell junctions.

lid of a tissue culture plate, resulting in an inversion of the drop. Following 0–4-h incubation, cell aggregate sizes were determined either immediately or following trituration of the suspension. Cells were scored as part of small (1–10 cells), medium (11–50 cells), or large (>50 cells) aggregates of cells. Statistical analysis was performed using Statistica 6.0. To generate cell lines, MDCK cells were transfected using

effectene reagent (Stratagene) with pDSred2.C1 or pEGFP.N2 plasmids containing zyxin or LPP sequence inserts, as generated by PCR. Following 14 days of G418 selection, expression of fusion proteins in the resistant cells was determined by Western blot analysis. Cells were extracted with 1% SDS, then the extract separated in SDS-polyacrylamide gels. Gels were transferred to nitrocellulose and probed with anti-DSred2 or -GFP antibodies. Antibody binding was detected with the appropriate horseradish peroxidase-conjugated secondary antibody. A subpopulation of cells expressing DSred2 or GFP fusion protein was then isolated using a fluorescence-activated cell sorter. The same collection parameters were selected for all cell lines. Analysis of cell-cell adhesion in cell lines was performed within two passages of sorting.

Fluorescence—Cells grown on collagen-coated coverslips were washed with ice-cold Ringer's saline containing 1.8 mM $CaCl_2$. Cells were extracted on ice in CSK buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.5% v/v Triton X-100) for 10 min prior to fixation where appropriate. Cells were fixed on ice for 15 min with 4% paraformaldehyde in phosphate-buffered saline. Cells were blocked with Tris-buffered saline containing 0.4% bovine serum albumin and 0.075% saponin and then stained with the appropriate primary antibodies. Antibodies used were cadherin, vinculin (Sigma), VASP (Transduction), capping protein α subunit, zyxin/LPP (B38), zyxin (B71), and LPP (MP2). Antibody binding was detected with goat anti-mouse or -rabbit antibodies conjugated to Alexa-488 or -594. DSred2 or GFP fusion proteins were observed directly. All coverslips were mounted in VectaShield (Vector) and viewed under a Zeiss Axiophot microscope with 40× and 100× (both with a 1.30 numerical aperture) oil immersion objective lenses. Images were acquired using a Princeton Instruments RTE/CCD-1300-Y camera and OpenLab software. Pairs of extracted and whole cells were imaged with identical exposure settings.

RESULTS

Zyxin and LPP Localize to Cell-Cell Junctions in MDCK Cells—To explore the roles of zyxin and LPP in cell-cell junction assembly and strengthening we employed MDCK cells, a well established epithelial cell line that has been extensively used to study cadherin-based adhesion and is amenable to exogenous expression of recombinant proteins. To test whether antibodies raised against mammalian zyxin and/or LPP recognize the canine forms of these proteins, MDCK cell extracts were separated in SDS-polyacrylamide gels and subjected to Western blot analysis with these antibodies. A single band of the appropriate molecular weight was observed when B38 (anti-zyxin and -LPP), B71 (anti-zyxin), and MP2 (anti-LPP) antibodies were used (Fig. 1*A*). Zyxin/LPP have been previously observed at cell-cell junctions of primary mouse keratinocytes using the B38 antibody (13). In MDCK cells, B38 antibody, as well as antibodies specific for either zyxin or LPP, stain cell-cell junctions (Fig. 1, *B–D*). Staining was most commonly observed in small colonies or at the edges of large colonies, where cell-cell junctions are less mature (data not shown). Zyxin was observed in specific actin-rich structures at early cell-cell contacts (Fig. 1, *E–G*). At higher resolution, zyxin staining resolved into two distinct regions, one at the end of each bundle of actin filaments that terminate at the same position within the cell-cell contact (Fig. 1, *H–J*). LPP also localizes to the ends of bundles of actin filaments that terminate at cell-cell junctions (Fig. 1, *K–M*). These structures occur most commonly at the vertex, where multiple cells come into contact, and at sites at the edge of colonies where the cortical band of actin is joined at cell-cell contacts to form a continuous structure. Structures staining with zyxin and LPP antibodies are reminiscent of focal adhesions. However, radial bundles of actin that terminate in plaques of cadherin staining have also been described during the early stages of cell-cell contact formation in MDCK cells (11, 12). To distinguish between these two types of structures, we tested whether focal and cadherin-based adhesion components would colocalize with zyxin at these sites. An intensely staining plaque of cadherin staining was observed to localize precisely between the two regions of zyxin staining (Fig. 1, *N–P*). Components of focal adhesions were also observed to colocalize precisely with zyxin in these actin-rich structures, namely VASP (Fig. 1, *Q–S*) and vinculin (Fig. 1, *T–V*). It should be noted that VASP and vinculin are known to target to cadherin-based adhesions in addition to focal adhesions and that VASP is a known binding partner of zyxin and LPP. In contrast, paxillin did not colocalize with zyxin at these cell-cell contact sites, even though paxillin and zyxin colocalize precisely at focal adhesions at the edge of colonies of MDCK cells (Fig. 1, *W–Y*). We conclude that bundles of actin filaments terminate at cell-cell junctions in dense plaques of cadherin, as described previously (11, 12), and that zyxin, LPP, VASP, and vinculin localize to these structures. The dense cadherin plaque described by Adams *et al.* (11, 12) could be analogous to structures observed in primary keratinocytes, where zyxin, VASP, and vinculin colocalize with cadherin at the tips of actin rich filopodia between contacting cells (13).

Expression of Zyxin and LPP Deletion Mutants in MDCK Cells—To define zyxin and LPP function in cell-cell adhesion, DSred2-zyxin and -LPP fusion proteins were designed and expressed in MDCK cells. Zyxin and LPP share organization of several key protein domains that were targeted for deletion from zyxin or LPP fusion proteins (Fig. 2*A*). These sites include an α-actinin binding site in the N-terminal 50 amino acids (27), a series of ActA repeats (four in zyxin, two in LPP) that have been demonstrated to bind the EVH1 domains of VASP family members (21, 22), and a series of three C-terminal LIM domains. MDCK cells were transfected with constructs encoding the depicted zyxin or LPP fusion proteins. Expression of fusion proteins tagged with DSred2 or EGFP was confirmed by Western blot analysis. Cells were subjected to drug selection and a subpopulation of these cells was isolated using a fluorescence-activated cell sorter. Fluorescence-activated cell sorter sorting allowed isolation of cell populations that express different DSred2 fusion proteins within a specific range of fluorescence intensity, eliminating the possibility of different deletion mutants generating distinct effects due to a variation in fusion protein expression.

MDCK cells expressing zyxin or LPP fusion proteins were plated onto collagen-coated coverslips and the cell-cell junction localization of the protein fragments determined by direct DSred2 fluorescence. Importantly, DSred2 displayed a diffuse cytosolic distribution; specific targeting of the DSred2 protein to membrane sites, including cell-cell contacts, was not observed (Fig. 2*B*). In contrast, DSred2-zyxin (Fig. 2*C*) and -LPP (Fig. 2*D*) both exhibited some cell-cell junction localization when expressed in MDCK cells. When the α-actinin binding site, ActA repeats, or LIM domains were deleted from either zyxin or LPP, distinct localization patterns were observed (Fig. 2, *E–J*). Although strong localization in the cytosol and in the nucleus were observed for some fragments, it should be noted that varying levels of cell-cell junction staining was observed for every fragment tested. This junction staining did colocalize with the junction marker cadherin (data not shown).

Involvement of the LIM Domain Region of Zyxin and LPP in Cell-Cell Adhesion—Cells expressing zyxin or LPP fusion proteins were then analyzed in a quantitative assay for cell-cell junction assembly and strengthening (26, 28). Briefly, cells were placed in suspension culture and their ability to incorporate into aggregates measured as a function of time. Aggregates were also subjected to trituration forces that break weak

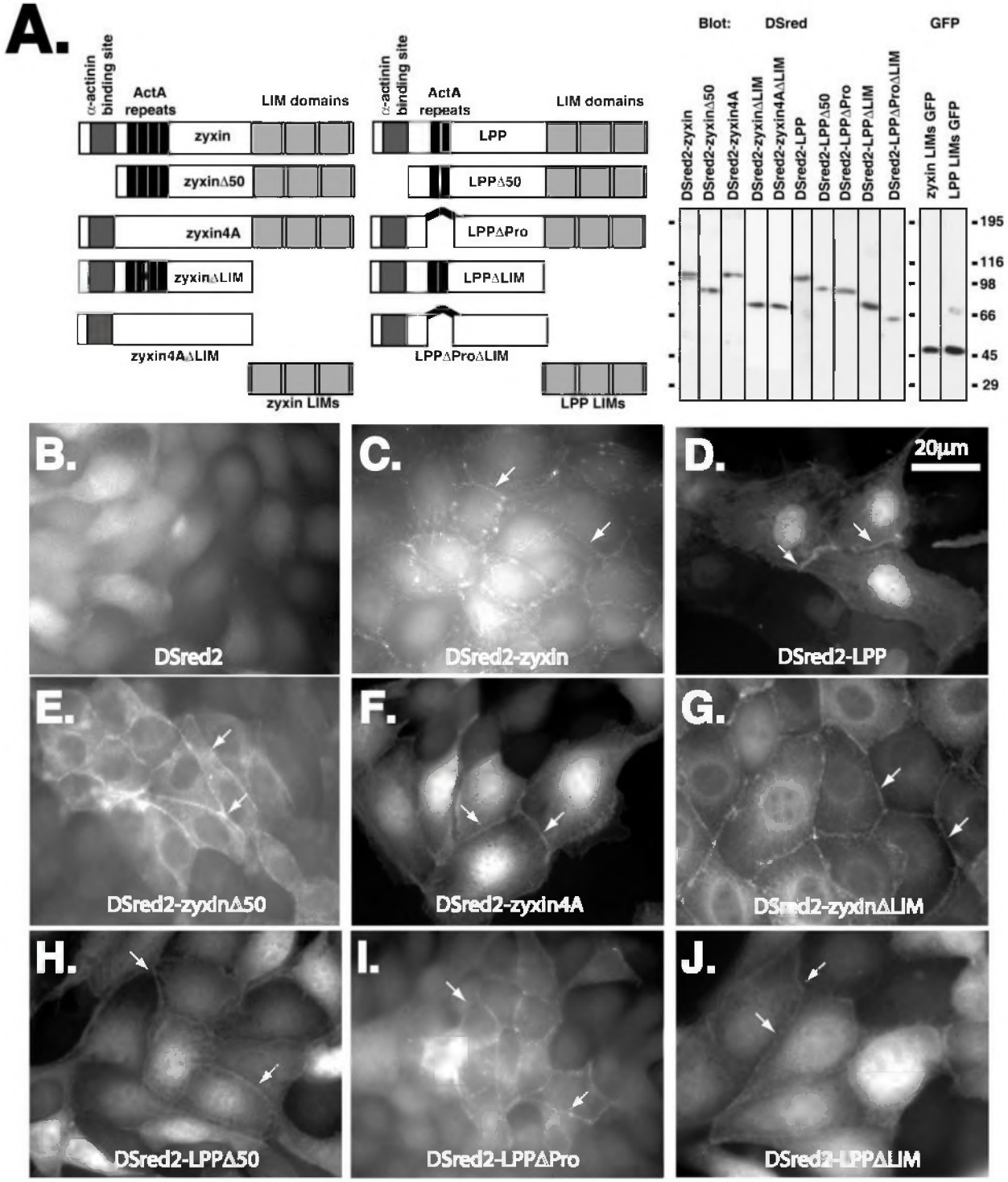

FIGURE 2. **Design and localization of zyxin and LPP deletion mutants in MDCK cells.** *A*, organization of α-actinin binding site, ActA repeats, and LiM domains of full-length and deletion mutants of zyxin and LPP. Western blot analysis of cell expressing zyxin or LPP mutants. *B–J*, direct fluorescence of DSred2 (*B*), DSred2-zyxin (*C*), DSred2-LPP (*D*), DSred2-zyxinΔ50 (*E*), DSred2-zyxin4A (*F*), DSred2-zyxinΔLiM (*G*), DSred2-LPPΔ50 (*H*), DSred2-LPPΔPro (*I*), and DSred2-LPPΔLiM (*J*) proteins expressed in MDCK cells. *Scale bar* = 25 μm. *Arrows* denote areas of cell-cell junction fluorescence.

cell-cell interactions, revealing the level of cell-cell junctions that have been strengthened. The average values from three experiments are presented here. To confirm that any observed differences in the rate of aggregate formation and strengthening were statistically relevant, multivariate logistical regression analysis was performed on the rate at which cells formed large aggregates. Based upon these data, a log-odds value (one half of the natural logarithm that a cell will be found in a large aggregate relative to the reference cell line, parental MDCK cells) was calculated for each cell line, both before and after trituration. A positive log-odds score indicates that it is more likely that a cell will be found in a large aggregate relative to the reference cell line, while a negative log-odds score indicates that it is less likely that a cell will be part of a large aggregate relative to the reference cell line. The log-odds value is computed with high fidelity, as indicated by *p* values that did not exceed

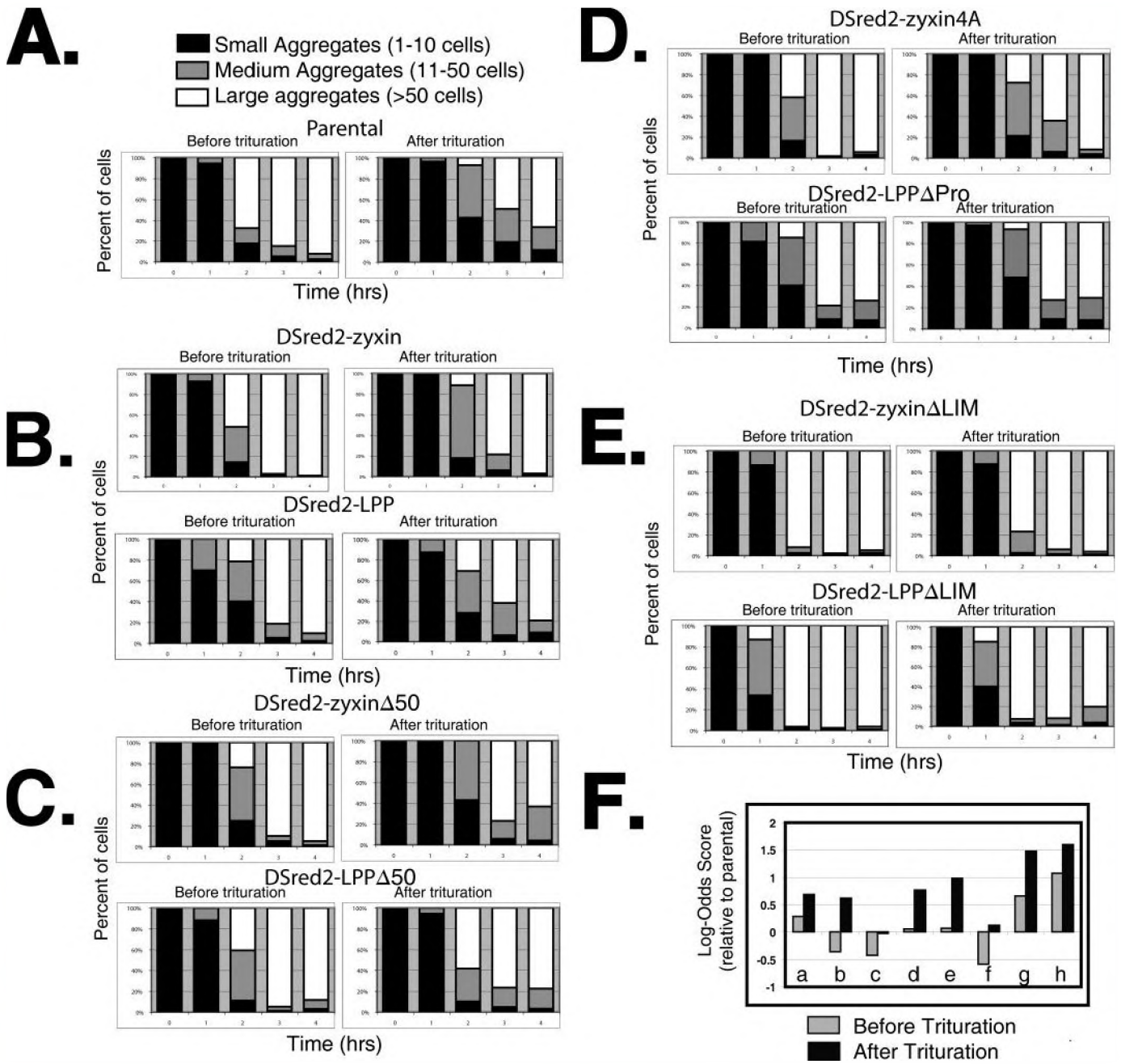

FIGURE 3. **Deletion of zyxin or LPP LIM domains accelerates cell-cell adhesion.** *A*, rate of cell aggregation in suspension of parental MDCK cells. *B*, rate of aggregation of MDCK cells expressing either DSred2-zyxin or -LPP. *C*, rate of aggregation of cells expressing DSred2-zyxinΔ50 or -LPPΔ50 mutants. *D*, rate of aggregation of cells expressing DSred2-zyxin4A or -LPPΔPro mutants. *E*, rate of aggregation of cells expressing DSred2-zyxinΔLIM or -LPPΔLIM mutants. *F*, statistical comparison of rates at which cell lines aggregate into large aggregates (>50 cells) in suspension as a function of time, before or after trituration. The log-odds scores were computed relative to the reference cell line, parental MDCK cells. The designation of the *x* axis is as follows: *a*, DSred2-zyxin; *b*, DSred2-LPP; *c*, DSred2-zyxin$^{\Delta 50}$; *d*, DSred2-LPP$^{\Delta 50}$; *e*, DSred2-zyxin^{4A}; *f*, DSred2-LPP$^{\Delta Pro}$; *g*, DSred2-zyxin$^{\Delta LIM}$; and *h*, DSred2-LPP$^{\Delta LIM}$.

10^{-10} for any calculation. We established the following criteria for determining that a cell line demonstrates a statistically relevant difference in the rate of cell-cell junction formation. First, the two log-odds scores (for before and after trituration) must both be either positive or negative to be considered biologically relevant. Second, both log-odds scores for the cell line must exceed the error range (either positively or negatively), which we compute as double the standard error of the mean for all experiments. These values are 0.346763 and 0.358388 for before and after trituration scores, respectively. Using such stringent statistical criteria, any experimental variation is eliminated from consideration. Thus, for the adhesion assays, we present the raw count data for each cell line (Fig. 3, *A–E*), as well as log-odds calculations for cell lines in reference to parental MDCK cells (Fig. 3*F*).

Parental MDCK cells (Fig. 3*A*) assemble into large aggregates (>50 cells) by 2 h, accounting for 68.1% of cells. After 3 and 4 h, large aggregates account for 85.2 and 92.2% of cells, respectively. Aggregation approaches maximum after 3 h in suspension, indicating that a plateau phase is reached. When cells are triturated prior to scoring, thus disrupting any weak cell-cell interactions, cells found in large aggregates after 2 h is reduced to 7.2%. Similarly, only 49.0 and 66.8% of parental cells are found in large aggregates after 3 and 4 h in suspension, respectively. Plateau phase for aggregation, as measured following trituration,

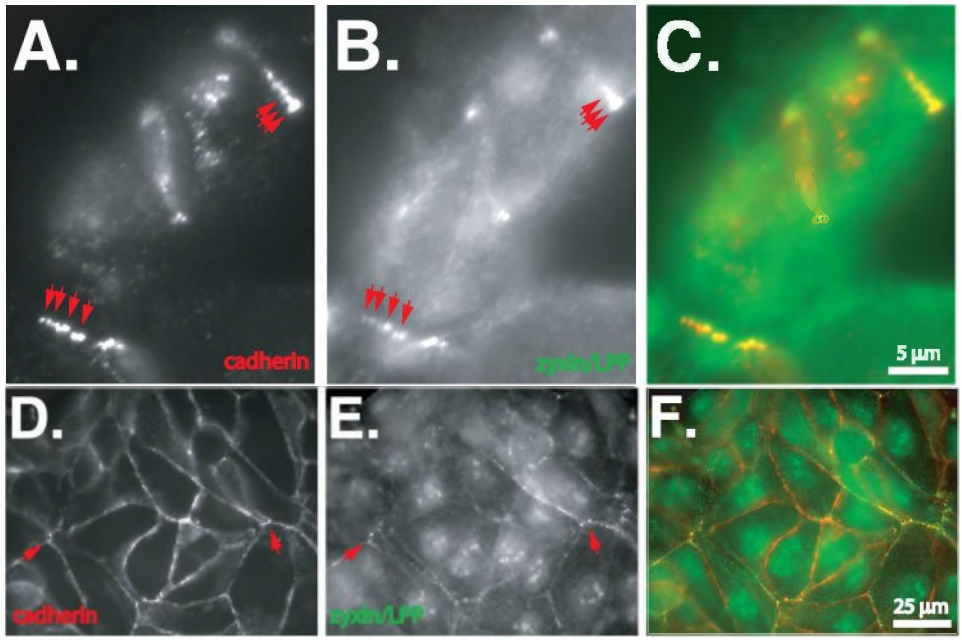

FIGURE 4. **Zyxin/LPP localization during early cell-cell junction formation.** Cadherin (*red*) and zyxin/LPP (*green*) antibody staining of MDCK cells synchronously induced to form cell-cell junctions for 2 (*A–C*) or 4 h (*D–F*) using a calcium switch is shown.

is reached only by 4 h or later, demonstrating the occurrence of a lag between initial contact events and junction strengthening.

MDCK cells expressing DSred2 fusion protein containing either full-length zyxin or LPP assemble and strengthen cell-cell junctions at a rate that does not significantly differ from parental MDCK cells (Fig. 3, *B* and *F*). Similarly, deletion of either the α-actinin binding sites or the ActA repeats from either full-length zyxin or full-length LPP resulted in no striking effect on the rate of cell-cell junction formation and strengthening according to our criteria (Fig. 3, *C*, *D*, and *F*). In contrast, expression of DSred2 fusion proteins containing either zyxin or LPP that lack the LIM domain region alters the rate of cell-cell contact assembly and strengthening to a degree that satisfies all statistical criteria (Fig. 3, *E* and *F*). Aggregation of both DSred2-zyxin$^{\Delta LIM}$ and DSred2-LPP$^{\Delta LIM}$ cells approaches maximum much earlier than the parental MDCK cells (Fig. 3*E*). After 2 h, large aggregates account for 92.2% of DSred2-zyxin$^{\Delta LIM}$ cells, compared with 68.1% of parental MDCK cells. Only 2.4% of DSred2-zyxin$^{\Delta LIM}$ cells occur in small aggregates (10 or fewer cells), compared with 17.4% of the parental cell line. Large aggregates account for 96.6% of DSred2-LPP$^{\Delta LIM}$ cells after 2 h, while small aggregates account for only 2.0%. The rate of strengthening initial cell-cell contacts is also very markedly increased in cell expressing DSred2-zyxin$^{\Delta LIM}$ or -LPP$^{\Delta LIM}$. After 2 h in suspension, 77.1% of DSred2-zyxin$^{\Delta LIM}$ cells remain in large aggregates after trituration, compared with only 7.2% of parental MDCK cells. After 4 h, 96.3% of DSred2-zyxin$^{\Delta LIM}$ cells remain in large aggregates following trituration, compared with 66.8% of MDCK cells. Following trituration, large aggregates account for 92.8 and 80.5% of DSred2-LPP$^{\Delta LIM}$ cells after 2 and 4 h, respectively. Importantly, the rate of aggregation as measured before and after trituration is reached nearly simultaneously in these cell lines. Thus, a large percentage of cells in large aggregates have strengthened their cell-cell interactions sufficiently enough to withstand disruption by trituration. After 2 h in suspension, 83.6% of DSred2-zyxin$^{\Delta LIM}$ cells remain in large aggregates following trituration, compared with only 10.6% of parental MDCK cells. 96.1% of DSred2-LPP$^{\Delta LIM}$ cells remain in large aggregates following trituration after 2 h in suspension. Clearly the LIM region is critical for zyxin and LPP function in cell-cell adhesion.

That the results observed can be accounted for by the DSred2 tag is unlikely. All of the fusion proteins contain the DSred2 tag and it is only by deletion of the LIM region that an effect is observed. Clearly the LIM region, and not the DSred2 tag, accounts for changes in cell-cell adhesion rates observed. We also observed that MDCK cells expressing GFP fusion proteins containing LIM domain deleted zyxin at high levels displayed an aberrant morphology, while those expressing a GFP fusion protein containing full-length zyxin at high levels did not.[3] This is consistent with the results observed with DSred2 fusion proteins: that deletion of the LIM domain unmasks the function of zyxin and LPP in cell-cell adhesion.

Zyxin/LPP Localize to Sites of Early Cadherin-based Adhesion—Expression of either zyxin or LPP mutants lacking LIM domains results in faster aggregate formation and more immediate strengthening of cell-cell interactions within aggregates. Thus, both zyxin and LPP appear to function in early cell-cell contact formation. Given that zyxin and LPP mutants drive changes in cell-cell junction formation after 2 h of cell-cell contact in the adhesion assay, we sought to define how endogenous zyxin/LPP was distributed in MDCK cells relative to cadherin when cell-cell adhesion had been synchronously induced and allowed to proceed for 2 h. MDCK cells were maintained in culture at single cell density for 48 h and then seeded onto collagen-coated coverslips at confluent density in low calcium medium. Under this condition, cells are unable to convert cell-cell interactions into cadherin-based adhesions. Normal calcium levels are restored 3 h following plating in low calcium medium, resulting in synchronous induction of cadherin-based adhesion. Two hours after the initiation of cell-cell adhesion, cells were extracted and then fixed, allowing the visualization of the newly forming detergent insoluble cadherin-actin network. In these cells, cadherin staining occurs as arrays of bright puncta or plaques along cell-cell contacts that shows striking coincidence with zyxin/LPP (Fig. 4, *A–C*). After 4 h of cell-cell contact formation, fewer cadherin plaques are observed and cadherin staining becomes more uniform along the cell-cell junction; many areas of cadherin staining in these cells are devoid of zyxin/LPP staining (Fig. 4, *D–F*). At this stage, zyxin/LPP antibody labels only a subset of the remaining cadherin plaques. Taken with the time-dependent effect of the zyxin and LPP mutants, these data suggest that

[3] M. D. H. Hansen, unpublished results.

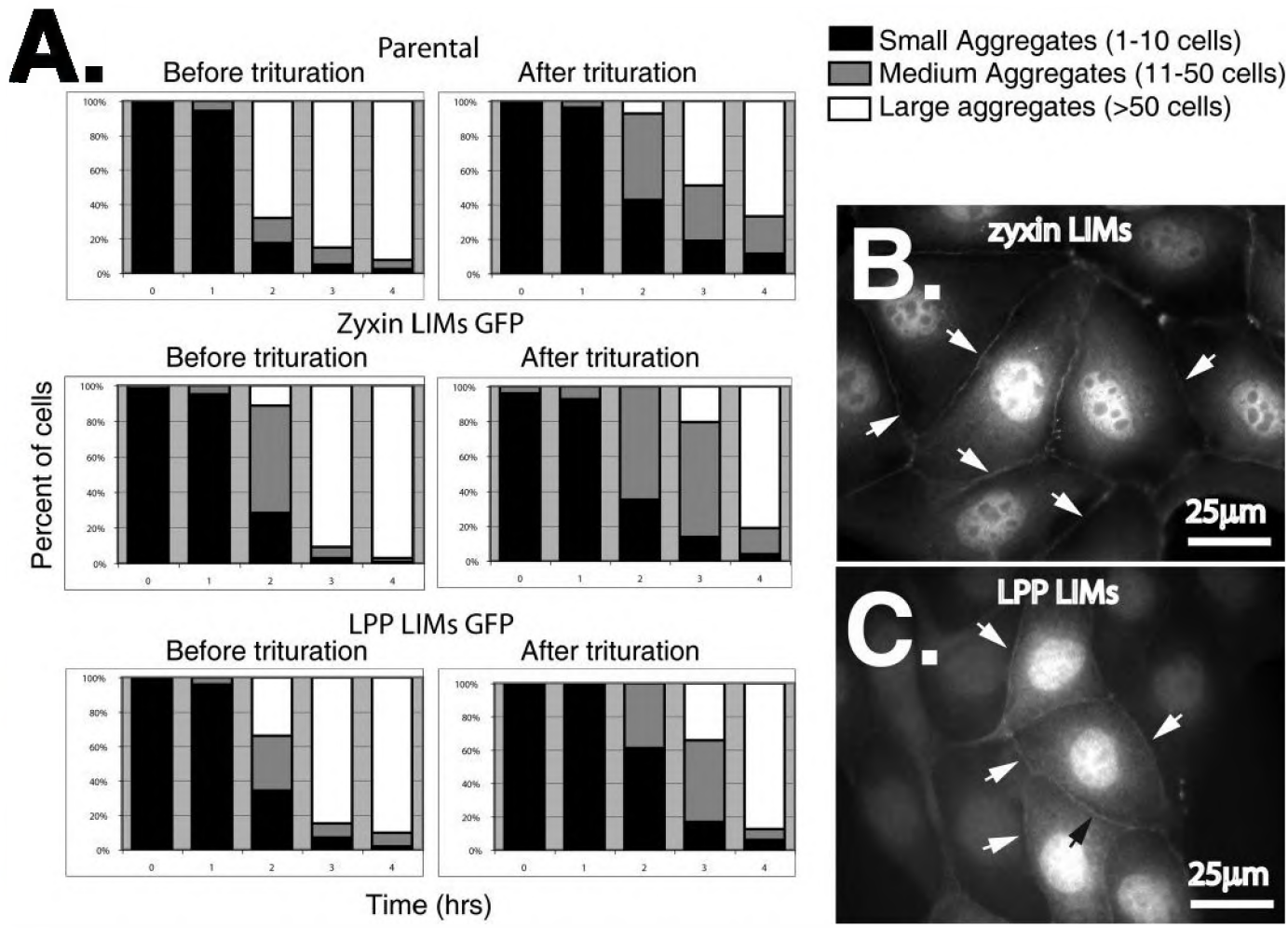

FIGURE 5. **Zyxin and LPP LIM region is a regulatory domain.** *A*, rate of aggregation of parental MDCK cells (reproduced from Fig. 3) and of cells expressing either zyxin or LPP LiM domains fused to GFP. *B* and *C*, direct fluorescence of GFP fusion proteins containing the LiM domain region of either zyxin (*B*) or LPP (*C*) expressed in MDCK cells. The *arrows* in *B* and *C* denote localization to cell-cell junctions.

zyxin and LPP function early and transiently in the formation and strengthening of cadherin-based cell-cell junctions. Furthermore, the localization of zyxin/LPP at the stage of adhesion examined indicates that the relevant site of action for these proteins in cell-cell junction formation is at cadherin plaques.

Zyxin and LPP Drive Cell-Cell Contact Formation through ActA Repeats—One possible explanation for the finding that deletion of the zyxin or LPP LIM regions enhances adhesion strength is that the LIM region itself exhibits an activity that negatively regulates cell-cell adhesion. If this were the case, one would predict that expression of the zyxin or LPP LIM domains would slow the rate of adhesion strengthening. However, as can be seen in Fig. 5*A*, we observe no striking change in adhesion strengthening when either the zyxin or LPP LIM region is expressed. Statistical analysis confirmed this conclusion; the log-odds scores for these cell lines, relative to parental MDCK cells, do not exceed the error range. The zyxin or LPP LIM region did display cell-cell junction localization, although the most prominent localization is in the nucleus (Fig. 5, *B* and *C*).

An alternative mechanism by which zyxin and LPP mutants lacking LIM domains could drive rapid strengthening of nascent cell-cell contacts would be if the function of the LIM domains is to negatively regulate an activity that resides outside the LIM region. If such were the case, removal of the LIM region could unmask an activity that enhances cell-cell adhesion. Since cytoskeletal anchorage is linked to strong cell-cell adhesion, the N-terminal protein of both zyxin and LPP contain functional ActA repeats, and zyxin and LPP both colocalize strongly with VASP at cell-cell junctions, we postulated that zyxin and LPP drive changes in cell-cell adhesion through their ActA repeats. If the LIM domains of zyxin and LPP act as a regulatory domain that prevents normal operation of the ActA repeats, then deletion of the ActA repeats in addition to the LIM domains would generate zyxin and LPP mutants that are unable to drive cell-cell contact formation and strengthening. Consistent with this hypothesis, DSred2-zyxin$^{4A\Delta LIM}$ cells exhibit a significant reduction in the rate of cell-cell junction assembly when compared with DSred2-zyxin$^{\Delta LIM}$ cells (Fig. 6*A*). Large aggregates account for 34.1% of DSred2-zyxin$^{4A\Delta LIM}$ cells after 2 h of cell-cell contact formation, 73.3% of cells after 3 h, and 56.0% of cells after 4 h. DSred2-LPP$^{\Delta Pro\Delta LIM}$ cells also exhibit a decreased rate of cell-cell contact formation when compared with DSred2-LPP$^{\Delta LIM}$ cells (Fig. 6*B*). Large aggregates were not observed in DSred2-LPP$^{\Delta Pro\Delta LIM}$ cells after 2 h in suspension, while only 49.5 of these cells were observed in large aggregates after 3 h and only 64.4% were in large aggregates after 4 h. Furthermore, disruption of ActA repeats causes a significant reduction in the rate at which cell-cell adhesion is strengthened (Fig. 6, *A* and *B*, after trituration). After 2 h of contact formation only 9.1% of DSred2-zyxin$^{4A\Delta LIM}$ cells remain in large aggregates when the aggregates are subjected to trituration. Even after 3 and 4 h in suspension, large aggregates account for only 48.6 and 27.0% of cells, respectively,

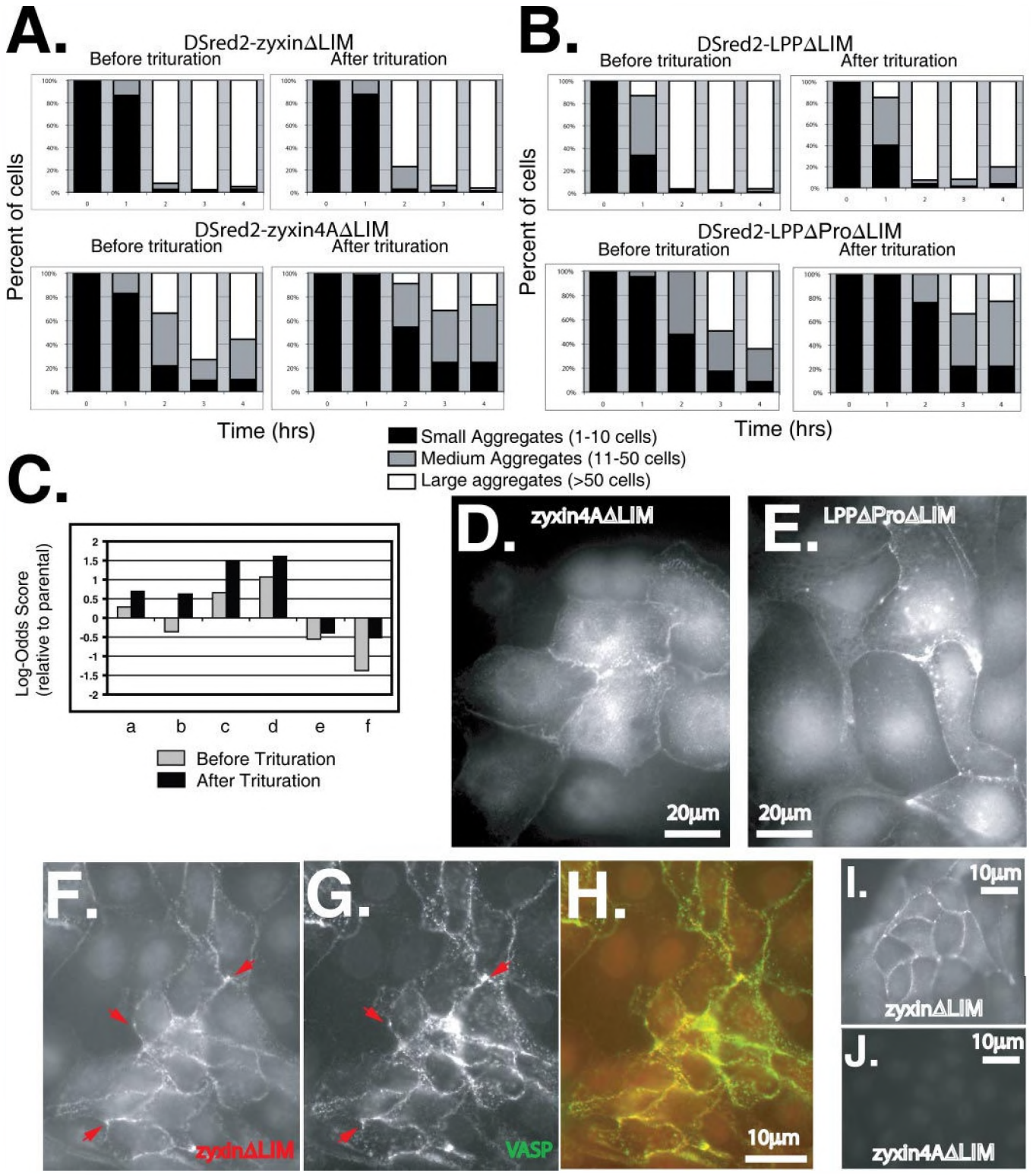

FIGURE 6. **Zyxin and LPP function in cell-cell adhesion requires ActA repeats.** *A*, rate of aggregation of cells expressing DSred2-zyxinΔLiM (reproduced from Fig. 3) or DSred2-zyxin4AΔLiM. *B*, rate of aggregation of cells expressing DSred2-LPPΔLiM (reproduced from Fig. 4) or DSred2-LPPΔProΔLiM. *C*, statistical comparison of rates at which cell lines aggregate into large aggregates (>50 cells) in suspension as a function of time, before or after trituration. The log-odds scores are calculated relative to the reference cell line, parental MDCK cells. The designation of the *x* axis is as follows: *a*, DSred2-zyxin; *b*, DSred2-LPP; *c*, DSred2-zyxin$^{\Delta LIM}$; *d*, DSred2-LPP$^{\Delta LIM}$; *e*, DSred2-zyxin$^{4A\Delta LIM}$; and *f*, DSred2-LPP$^{\Delta Pro\Delta LIM}$. *D*, direct fluorescence of DSred2-zyxin$^{4A\Delta LIM}$ expressed in MDCK cells. *E*, direct fluorescence of DSred2-LPP$^{\Delta Pro\Delta LIM}$ expressed in MDCK cells. *F–H*, colocalization of DSred2-zyxin$^{\Delta LIM}$ (*red*), as viewed by direct fluorescence, with VASP (*green*) antibody staining in extracted MDCK cells. *I–J*, direct DSred2 fluorescence in extracted zyxin$^{\Delta LIM}$ (*I*) or zyxin$^{4A\Delta LIM}$ (*J*) cells.

when aggregates are subjected to trituration. Large negative log-odds scores were calculated for both DSred2-zyxin$^{4A\Delta LIM}$ and -LPP$^{\Delta Pro\Delta LIM}$ cells (Fig. 6*C*). These results indicate that the ActA repeats are essential for the increased rate of cell aggregation and strengthening of cell-cell interactions observed when the DSred2-zyxin$^{\Delta LIM}$ or -LPP$^{\Delta LIM}$ constructs are expressed. Based upon our statistical criteria, we can con-

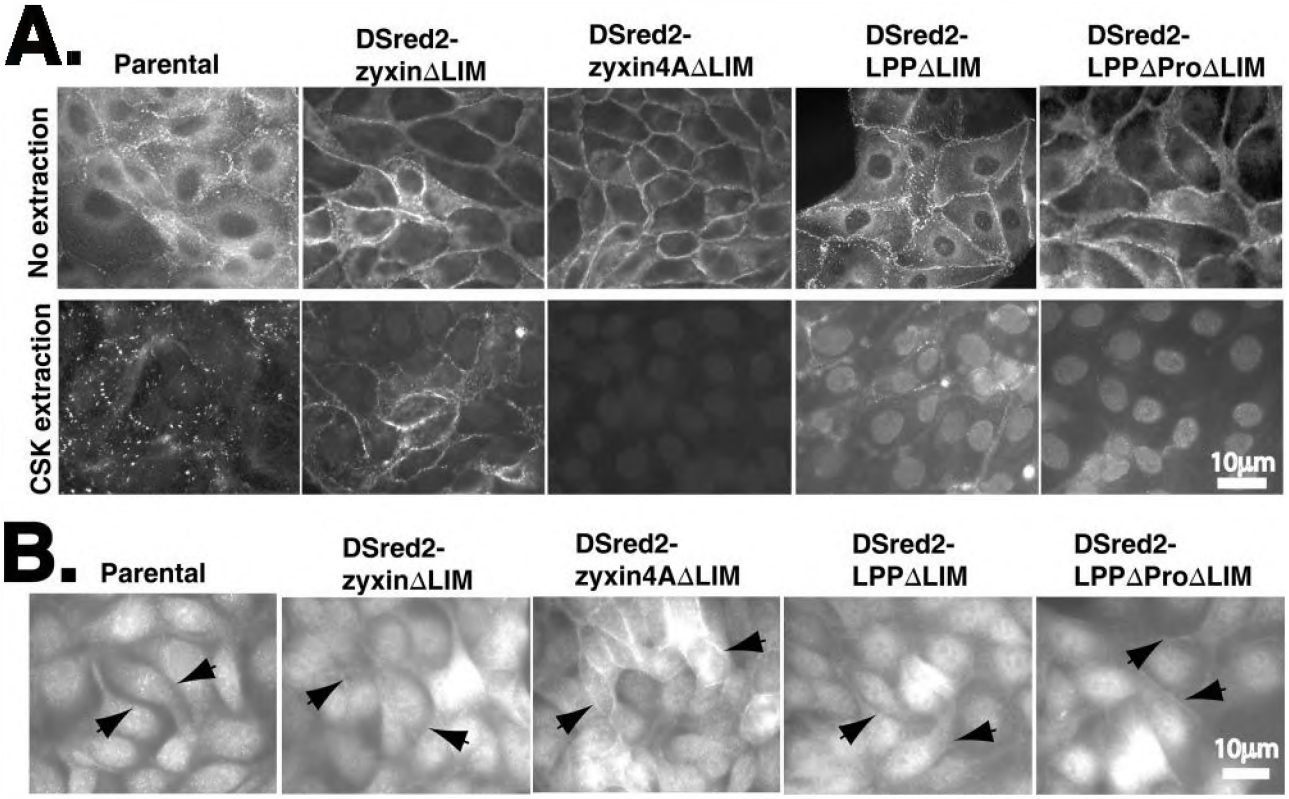

FIGURE 7. **Zyxin and LPP drive VASP into cadherin-actin networks.** *A*, VASP localization in parental MDCK cells and in cells expressing zyxin or LPP mutants. Cells were fixed directly to observe total protein distribution or extracted prior to fixation to observe protein organization in detergent-insoluble cytoskeletal networks. *B*, capping protein α subunit localization in whole cells expressing zyxin or LPP mutants.

clude that both DSred2-zyxin$^{4A\Delta LIM}$ and LPP$^{\Delta Pro\Delta LIM}$ cells form cell-cell junctions at a rate that is significantly slower than the parental cell line.

We then examined whether perturbation of both the ActA repeats and LIM domains of zyxin or LPP prevented the localization of these mutant proteins to cell-cell contacts in MDCK cells. Direct fluorescence of either zyxin$^{4A\Delta LIM}$ (Fig. 6*D*) or LPP$^{\Delta Pro\Delta LIM}$ (Fig. 6*E*) revealed that these mutant proteins target to cell-cell contacts of MDCK cells. DSred2-zyxin$^{\Delta LIM}$ and VASP colocalize precisely at cell-cell junctions, where they are resistant to detergent extraction (Fig. 6*F*-H). Interestingly, compared with DSred2-zyxin$^{\Delta LIM}$ (Fig. 6*I*), DSred2-zyxin$^{4A\Delta LIM}$ (Fig. 6*J*) fails to accumulate at detergent insoluble cadherin-actin networks. These observations reveal that the stable incorporation of zyxin into cadherin-actin networks is dependent on VASP. VASP could be required to dock zyxin into the cadherin-actin network, although an ActA repeat peptide displaced VASP, but not zyxin, from cell-substratum or cell-cell adhesions (13, 21). More likely, then, is the possibility that VASP activity is required for the stabilization of zyxin into detergent insoluble cadherin-actin networks.

Zyxin/LPP LIM Domain Deletion Mutants Alter Balance of VASP and Capping Protein at Cell-Cell Junctions—To determine whether zyxin or LPP alter the incorporation of actin regulatory proteins into the cadherin-actin network at cell-cell junctions, we examined how the balance of VASP and capping protein was affected by alterations in the activity of either zyxin or LPP. Unextracted DSred2-zyxin$^{\Delta LIM}$ and DSred2-LPP$^{\Delta LIM}$ cells display similar levels of VASP antibody staining at cell-cell junctions when compared with DSred2-zyxin$^{4A\Delta LIM}$ or DSred2-LPP$^{\Delta Pro\Delta LIM}$ cells (Fig. 7*A*). However, when cells are extracted prior to fixation, thus leaving only proteins incorporated into detergent insoluble cytoskeletal elements, a striking alteration in VASP staining is observed. DSred2-zyxin$^{\Delta LIM}$ cells retain significant cell-cell junction labeling with VASP antibodies even when extracted, while cell-cell junctions of extracted DSred2-zyxin$^{4A\Delta LIM}$ cells do not stain with VASP antibodies. The amount of VASP that resists detergent extraction is much higher than that of parental MDCK cells (Fig. 7*A*) or cells expressing full-length zyxin or LPP (data not shown). A similar, but less robust, trend is observed in cells expressing LPP mutants. It should be noted that LPP contains two ActA repeats, of which only one binds VASP *in vitro* (22), compared with four functional ActA repeats in zyxin (15, 21). This stoichiometric difference could account for the difference in VASP staining intensity in extracted DSred2-zyxin$^{\Delta LIM}$ cells as compared with DSred2-LPP$^{\Delta LIM}$ cells (Fig. 7*A*).

VASP has been proposed to increase actin polymerization by binding actin barbed ends and shielding them from capping protein (20). VASP binding is proposed to allow barbed ends to continue polymerization (20), while capping protein prevents extension of the actin barbed end (29). Thus the balance between VASP and capping protein availability at actin barbed ends must be carefully regulated to control actin filament length in cells. To test whether zyxin and LPP function to regulate the balance of VASP and capping protein activity at cell-cell contacts, the staining pattern of capping protein in parental MDCK cells and cells expressing zyxin or LPP mutants was determined (Fig. 7*B*). Parental MDCK cells displayed no specific localization of capping protein at cell-cell junctions nor did cells expressing full-length zyxin or LPP (data not shown). In contrats, DSred2-zyxin$^{4A\Delta LIM}$ and -LPP$^{\Delta Pro\Delta LIM}$ cells display an increased level of capping protein at cell-cell junctions when compared with DSred2-zyxin$^{\Delta LIM}$ or -LPP$^{\Delta LIM}$ cells or to the parental MDCK cell line. DSred2-zyxin$^{4A\Delta LIM}$ retained significantly more capping protein staining at cell-cell junctions than DSred2-zyxin$^{\Delta LIM}$ cells. Although a similar effect was observed in cells expressing LPP mutants,

DSred2-LPP$^{\Delta Pro\Delta LIM}$ cells displayed only slightly more capping protein staining at cell-cell junctions when compared with DSred2-LPP$^{\Delta LIM}$ cells. Again, given the relative number of VASP binding ActA repeats in zyxin (four repeats) and LPP (one or two repeats), expression of zyxin mutants would likely result in larger effects on VASP and capping protein distribution at steady state than would expression of LPP mutants. The effect of zyxin and LPP mutants on the distribution of VASP and capping protein at cell-cell junctions is consistent with the conclusion that zyxin and LPP drive cell-cell adhesion through VASP, perhaps by regulating polymerization rates at actin barbed ends.

DISCUSSION

Actin rearrangements induced by cadherin engagement have been carefully documented in several epithelial cell types (11–13). Intense research focus has been applied to characterizing the role of actin nucleation in cell-cell adhesion (30, 31). Far less is known about how other actin regulatory pathways might also contribute to adhesion and how these pathways might be activated by cadherin engagement. Bershadsky and Geiger (32) proposed that zyxin could recruit VASP into cadherin complexes by binding α-actinin. VASP could then regulate local changes in actin dynamics. In support of this idea, VASP localizes to cell-cell junctions of keratinocytes, where it is coincident with zyxin staining (13). Furthermore, expression of the VASP tetramerization domain, predicted to act as a dominant-negative, in the skin of mice resulted in a blistering phenotype, and keratinocytes derived from those mice displayed aberrant cell-cell junction morphology (13). In such a model, it remains unclear what biochemical function is played by VASP in cadherin-actin network formation. Furthermore, it is unclear how the recruitment of zyxin and VASP to cell-cell junctions is coordinated to coincide with cell-cell adhesion. The results outlined in this report provide a new level of detail in how zyxin functions through VASP and perhaps VASP family members that also bind zyxin or LPP ActA repeats, in cell-cell adhesion.

Our results provide important evidence that the ability of zyxin to recruit and/or activate VASP is controlled by an autoregulatory mechanism. It is the LIM region of zyxin and LPP that acts as a regulatory domain: zyxin/LPP mutants containing the LIM region do not affect cell-cell adhesion, while deletion of the LIM region results in accelerated cell-cell junction formation (Fig. 3). The ability of VASP to become incorporated into the actin cytoskeleton at cell-cell contacts is increased in cells expressing constitutively active, LIM region deleted forms of zyxin or LPP (Fig. 7*A*). In contrast, incorporation of VASP into detergent-resistant cytoskeletal actin networks at cell-cell junctions is blocked in cells expressing zyxin or LPP mutants that lack both the regulatory LIM region and ActA repeats, as demonstrated by increased capping protein staining of cell-cell junctions (Fig. 7*B*). These "dominant-negative" mutants are expected to prevent increases of VASP activity in response to cadherin engagement. Although we do not measure VASP activity directly, the ability of VASP to become associated with the detergent insoluble actin cytoskeleton is certainly consistent with a model whereby the LIM region of zyxin and LPP could alter VASP activity. The observed accumulation of capping protein at cell-cell contacts in cells expressing dominant-negative zyxin/LPP, as well as the lack of VASP incorporation into detergent insoluble cadherin-actin networks, certainly suggests that VASP activity is diminished. Since VASP localization at cell-cell junctions and its activity, albeit detected indirectly, can be uncoupled, we favor the hypothesis that the LIM region regulates VASP activity and not VASP recruitment to cell-cell junctions. It is critical that the precise mechanism by which the LIM region regulates the function of these proteins in cell-cell adhesion be more fully explored.

Zyxin and LPP colocalize with VASP at cell-cell junctions and, more specifically, at actin-rich bundles that terminate at cadherin plaques within the cell-cell contact (Fig. 1). Importantly, at the times when zyxin and LPP mutants most noticeably affect cell-cell adhesion (2 h), coincident staining of zyxin/LPP and cadherin is frequently observed (Fig. 4). The possibility that zyxin and LPP function redundantly in cell-cell adhesion is supported by several observations. The same deletion mutants of each protein generate very similar phenotypes when expressed in cells. Furthermore, mutants of these proteins affect cell-cell adhesion at precisely the same time. The similar localization of zyxin and LPP at cell-cell junction structures also suggests that these proteins share function. This might explain why zyxin null mice display a normal skin phenotype (24). It will be important in future work to define the distinct roles of these proteins in more detail using the mouse model system.

The identification of ActA repeats in zyxin led to speculation that zyxin might regulate actin assembly. In support of this, zyxin localizes to actin rich sites at the plasma membrane (23) and binds VASP via its ActA repeats (21). The current thinking is that zyxin directs actin assembly by recruiting VASP family members to cellular sites. Ectopic expression of zyxin fragments has been shown to drive local actin assembly, and it is of interest, given the results reported here, that these studies employ zyxin fragments that lack the LIM domain region (21, 25). Our findings suggest that such zyxin fragments would activate VASP in an unregulated manner.

It has been shown previously that interference with the ability of VASP family members to interact with ActA repeat-containing proteins aversely affects cell-cell adhesion (13). Here we expand on these observations and show that zyxin and LPP act as VASP ligands to play a role in the strengthening of cadherin-based cell-cell adhesions. This activity is regulated by the LIM regions of these proteins and depends on the VASP binding ActA repeats present in the N-terminal halves of zyxin and LPP. Our findings provide new insight into the function of zyxin and LPP, expanding the view of their roles as proteins that dock VASP family members to regulators of VASP family member activity.

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