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Regulation of Vasodilator-stimulated Phosphoprotein Phosphorylation and Interaction with Abl by Protein Kinase A and Cell Adhesion*

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Members of the vasodilator-stimulated phosphoprotein (VASP) family are important regulators of actin cytoskeletal dynamics whose functions and protein-protein interactions are regulated by phosphorylation by the cAMP-dependent protein kinase (PKA). Herein, we show that phosphorylation of VASP is dynamically regulated by cellular adhesion to extracellular matrix. Detachment of cells stimulated PKA activity and induced PKA-dependent phosphorylation of VASP and the related murine-Enabled (Mena) protein. VASP and Mena were rapidly dephosphorylated immediately following reattachment but showed an intermediate level of phosphorylation during active cell spreading. This pattern correlated closely with adhesion-dependent changes in PKA activity. The *in vivo* interaction of VASP with the Abl tyrosine kinase, shown here for the first time, was readily apparent in adherent cells, lost following cellular detachment, and induced upon reattachment to matrix. Importantly, inhibition of PKA activity prevented phosphorylation of VASP and dissociation of VASP-Abl complexes after cellular detachment, whereas activation of PKA completely eliminated the co-immunoprecipitation of Abl activity with VASP. These data establish a new biochemical link between cell adhesion and regulation of VASP proteins and provide the first demonstration of a regulated interaction between VASP and Abl in mammalian cells.

Integrin-mediated cell adhesion to the extracellular matrix (ECM)[1] provides positional and morphological information crucial for temporal and spatial regulation of cellular events such as proliferation, differentiation, cell movement, and cell death (1). The influence of cell adhesion stems from both the physical anchoring afforded by integrin-ECM interaction and generation of signals that communicate the state of adhesion to the cell interior (1, 2). One of the most important responses to these signals is the assembly and organization of the cytoskeleton, particularly actin microfilaments. Integrity of the actin cytoskeleton establishes, maintains, and monitors cell shape and is required for efficient transduction of signals initiated by receptors at the cell surface, such as receptors for growth factors and G-protein agonists (1–3). Moreover, actin filament polymerization and tension against filaments drives leading edge protrusion and cell body translocation during cell movement (4). Thus, identifying targets of adhesion-dependent signaling that regulate the cytoskeleton is an important step toward understanding how adhesion influences cell behavior.

The cAMP-dependent protein kinase (PKA) is activated upon both cellular detachment (5) and, paradoxically, upon engagement of integrins (6, 7). Indeed, some facets of cytoskeletal assembly and integrity require PKA activity (*e.g.* activation of Rac (6), Cdc42 (8), and F-actin synthesis (7)), whereas others appear to be inhibited by it (*e.g.* activation of Rho (9) and p21-activated kinase (5) and Tyr phosphorylation of focal adhesion kinase and paxillin (10, 11)). Finally, both inhibition and hyper-activation of PKA inhibit cell migration (6, 12). These ostensibly disparate observations outline a complex role for PKA in cytoskeletal regulation and suggest that factors that regulate the actin cytoskeleton may be affected by anchorage-dependent changes in PKA activity.

As a major substrate for PKA and a crucial regulator of actin dynamics, the vasodilator-stimulated phosphoprotein (VASP) is an excellent candidate target for anchorage-dependent regulation by PKA. The VASP family of actin regulatory proteins (including the *Drosophila*-Enabled (Ena) protein, its mammalian homolog (Mena), and the Ena/VASP-like protein (Evl)) share a common, three-domain structure comprising an N-terminal Ena/VASP homology (EVH) type 1 domain, a central Pro-rich region, and a C-terminal EVH2 domain (13, 14). VASP family proteins localize to focal adhesions, largely through EVH1-mediated interaction with vinculin, zyxin, and palladin (14–16) and to filopodial tips and lamellipodial edges through a less clear mechanism that may involve the EVH2 domain (17, 18). The EVH2 domain mediates homo- and heterotypic oligomerization among VASP proteins (19), as well as the ability of the protein to bind, bundle, and nucleate actin filaments (19, 20). The Pro-rich region mediates interaction with the profilin family of monomeric actin-binding proteins (13, 14, 21), as well as with the SH3 domains of the Abl and Src-family tyrosine kinases (13, 21, 22).

The functions of VASP/Ena proteins are regulated by phosphorylation and all mammalian VASP family members (VASP, Mena, and Evl) are substrates for PKA (14). For murine VASP, phosphorylation occurs at three residues: Ser-153 near the

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[1] The abbreviations used are: ECM, extracellular matrix; PKA, protein kinase A/cAMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; VH, Ena/VASP homology; SH3, Src-homology type 3; MDCK, Madin-Darby canine kidney cells; VSV, vesicular stomatitis virus; FN, fibronectin; Fsk, forskolin; IBMX, isobutyl methylxanthine; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; GST, glutathione *S*-transferase; pSer, phosphoserine.

Pro-rich region and Ser-235 and Thr-274 in EVH2 (positions 157, 239, and 278 in human VASP) (14, 23). Although each site can be modified by both PKA and the cGMP-dependent protein kinase, Ser-153 is the preferred target for PKA (23, 24). Only two of these sites, corresponding to Ser-153 and Ser-235, are present in Mena (13), whereas Evl contains only a single, Ser-153-like site (22). PKA-mediated phosphorylation of VASP and Evl decreases but does not completely eliminate their ability to nucleate, bind, and bundle actin filaments (22, 25). Phosphorylation near the Pro-rich regions of VASP proteins also regulates interactions with SH3 domains (13, 21, 22, 25). For example, phosphorylation of Evl by PKA has been shown to abolish its interaction with Abl and neuronal Src SH3 domains (22). And although *Drosophila* Ena is not phosphorylated by PKA, it is Tyr-phosphorylated by Abl near the Pro-rich region (26, 27), which inhibits its interaction with Abl and Src (27). Interestingly, phosphorylation near the Pro-rich region of VASP proteins does not disrupt profilin binding and has no effect on EVH1-mediated interactions or EVH2-mediated oligomerization (13, 14, 22, 25). Thus, the interaction of VASP proteins with SH3 domains may represent a specific target for regulation by PKA. This report unites the concepts discussed above by demonstrating that cell adhesion dynamically regulates PKA activity, which in turn regulates VASP phosphorylation and interaction with Abl *in vivo*.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies against VASP include: a mouse monoclonal (Transduction Laboratories), diluted 1:1000 for immunoblotting REF52 and MDCK lysates; a rabbit polyclonal (Alexis Biochemicals), used for immunoblotting (at 1:2000) and immunoprecipitation (at 1 μl of antibody per 200 μg of cell lysate) from NIH3T3 cells; a phospho-specific rabbit polyclonal antibody, used at 1:1000 to visualize phosphorylation at Ser-153 (a generous gift of Dr. Qingyuan Ye (Cell Signaling)); and a phospho-specific polyclonal, used at 1:500 to visualize phosphorylation at Ser-235 (Upstate Biotechnology). A mouse monoclonal IgA antibody (Transduction Laboratories) was used at 1:500 to visualize Mena. Antibodies to Abl include a monoclonal (8E9; Transduction Laboratories), used at 1:250 for immunoblotting, and a rabbit polyclonal (K12; Santa Cruz Biotechnology), used for immunoprecipitation and kinase assays as described below and elsewhere (28). Epitope-tagged VASP was immunoprecipitated with a monoclonal antibody against a vesicular stomatitis virus (VSV) glycoprotein antigen (BD Biosciences). Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology or Calbiochem and routinely used at 1:5000.

Cell Culture—NIH3T3 fibroblasts were maintained in DMEM with 10% bovine calf serum. REF52 and WI38 fibroblasts and MDCK epithelial cells were maintained in DMEM with 10% fetal bovine serum. Neo and dnPKA cells were maintained in NIH3T3 medium supplemented with 1 mg/ml active G418. ECV304 endothelial cells were maintained in Medium 199 with 10% fetal bovine serum. For all cell lines and experiments, cells were grown to near-confluence, then serum-starved overnight (12–16 h) in media containing 0.1% serum. Forskolin (Fsk) and isobutyl methylxanthine (IBMX) (Sigma) were dissolved in Me_2SO and added to cells (at 25 and 50 μM, respectively) for 20 min. PDGF-BB (Upstate Biotechnology) was used at a final concentration of 20 ng/ml. Detachment and replating on fibronectin (FN)- or collagen-coated plates was performed as described (5). Briefly, cells were detached with trypsin-EDTA and collected into DMEM containing 1% bovine serum albumin (BSA) and 1 mg/ml soybean trypsin inhibitor. After centrifugation for 5 min at 500 $\times$ *g*, cells were washed once and resuspended in DMEM-BSA, then incubated in suspension with gentle rotation for the indicated period of time. For replating, 10-cm Petri dishes were coated with PBS containing 10 μg/ml FN (BD Life Sciences) or 20 μg/ml type-I collagen from rat tail (Biomedical Technologies Inc.) for 2 h at room temperature, then washed with PBS, and blocked for 30 min with DMEM + 2% BSA.

Plasmids and Transfections—A pcDNA3-based plasmid encoding VSV-tagged human VASP (24) and empty pCMV5 or pCMV5 encoding the Myc-tagged form of the PKA inhibitor, PKI, (5), were transfected into NIH3T3 cells using SuperFect (Qiagen) according to the manufacturer's protocol. Twenty-four h later, cells were serum-starved and cultured as described above.

Cell Lysis, Immunoblotting, Immunoprecipitation, and Abl Kinase Assays—Cells were washed twice with ice-cold phosphate buffered saline, then lysed in Triton lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 25 mM β-glycerophosphate, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol) containing protease and phosphatase inhibitors, using 750 μl per 10-cm dish. After 10 min on ice, lysates were scraped into microcentrifuge tubes and centrifuged at 14,000 rpm (in an Eppendorf microcentrifuge) for 15 min at 4 °C. Protein concentration of the supernatant lysate was determined by bicinchoninic acid assay (Pierce). For direct immunoblotting, aliquots of lysate were mixed with an equal volume of 2$\times$ Laemmli sample buffer and boiled for 5 min before loading on 10% (for VASP) or 7.5% (for Mena and Abl) SDS-PAGE gels. For VASP immunoprecipitation, 0.8–1.0 mg of protein was incubated with anti-VASP or anti-VSV antibody for 1.5 h at 4 °C. Immunocomplexes were collected by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology) for 30 min at 4 °C, washed four times with lysis buffer, then resuspended, and boiled in 1$\times$ Laemmli sample buffer. For some experiments, polyclonal VASP antibody was chemically crosslinked to beads using dimethyl pimelimidate (Pierce) according to the supplier's protocol. Abl immunoprecipitation kinase assays were performed essentially as described elsewhere (28). Briefly, c-Abl was immunoprecipitated from 200 μg of protein using 2 μg of K12 anti-Abl antibody. Immunocomplexes were washed twice with lysis buffer supplemented with 0.1% SDS and 1% sodium deoxycholate, twice with lysis buffer, twice with lysis buffer without NaCl, and twice with kinase buffer (20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol). VASP immunoprecipitates were prepared and processed for Abl kinase activity by immunoprecipitating VASP from 1 mg of protein and washing immunocomplexes six times with lysis buffer then twice with kinase buffer. Kinase reactions were carried out in 20 μl of kinase buffer containing 1 μM ATP, 5μCi [γ-^{32}P]ATP, and 0.5 μg of GST-Crk and were incubated for 40 min at 27 °C. Reactions were stopped by the addition of an equal volume of 2$\times$ Laemmli sample buffer and boiled for 5 min. Reaction products were separated on 10% SDS-PAGE gels, which were Coomassie-stained, dried, and exposed to a PhosphorImager plate (Molecular Dynamics). For kinase assays using a peptide substrate instead of GST-Crk, reactions were stopped by the addition of 5 μl of 100 mM EDTA and 20 μl of the stopped reaction was spotted onto phosphocellulose columns (Pierce), which were subsequently washed twice with 150 mM phosphoric acid and analyzed by liquid scintillation counting.

PKA Activity Assay—PKA activity was measured exactly as described previously (5). Briefly, cells in suspension or on FN-coated plates were rapidly washed twice with ice-cold PBS, then resuspended or covered in PKA assay buffer and snap frozen in liquid nitrogen. Lysates were thawed on ice, scraped into tubes (for adherent cells), then sonicated and clarified by centrifugation at 10,000 $\times$ *g* for 10 min at 4 °C. Supernatants were assayed for PKA activity by measuring transfer of radioactive phosphate from ATP into Kemptide (Sigma) in the presence or absence of purified PKI (Cell Signaling).

RESULTS

Cellular Detachment Promotes Phosphorylation of VASP and Mena—To investigate whether VASP phosphorylation is affected by changes in cell adhesion, NIH3T3 cells were serum-starved overnight, then left untreated, stimulated with Fsk + IBMX for 20 min or detached and incubated in suspension for 1 h. Extracts were then analyzed by immunoblotting with anti-VASP antibodies. Phosphorylation of VASP at Ser-153, the preferred PKA site, but not at Ser-235 or Thr-274, causes a significant electrophoretic mobility shift of VASP on SDS-PAGE gels (23, 24), and this shift is routinely used as a faithful indicator of Ser-153 phosphorylation (24, 25). In untreated, stably adherent cells, VASP was predominantly in the faster migrating, unphosphorylated form (Fig. 1*A*). Detachment of cells induced phosphorylation of VASP at Ser-153 almost as efficiently as treatment with Fsk + IBMX. (Fig. 1*A*). This effect was confirmed by immunoblotting with a phospho-specific antibody that recognizes VASP only when phosphorylated near the Pro-rich region (pSer-153 in murine and pSer-157 in human VASP; Fig. 1*B*). Detachment-induced phosphorylation of VASP at Ser-153 (or the analogous position) was observed in several cell lines, including rat embryo fibroblasts (Fig. 1*A*, *REF52*), MDCK epithelial cells (Fig. 1*C*), and in human fibroblast (WI38) and endothelial (ECV304) cell lines (data not shown).

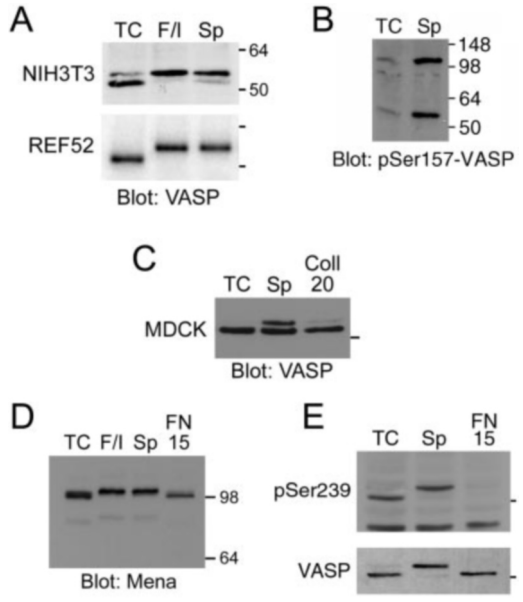

FIG. 1. **Cell adhesion controls phosphorylation of VASP and Mena.** Serum-starved cells (NIH3T3 (*A*, *B*, *D*, *E*), REF52 (*A*), or MDCK (*C*)) were left adherent to tissue culture plates (*TC*), treated for 20 min with 25 μM Fsk and 50 μM IBMX (*F/I*), detached, and cultured in suspension for 1 h (*Sp*) or replated for 15 or 20 min onto dishes coated with fibronectin (*FN 15*) or collagen (*Coll 20*) as indicated. Lysates were analyzed with antibodies against total VASP, the phosphorylated form of Ser-153 (Ser-157 in human VASP; *pSer157-VASP*) or Ser-235 (*pSer239*), or Mena, as indicated. Lines and numbers beside the panels indicate the position of molecular mass markers (50 kDa for *C* and *E*).

As apparent in Fig. 1*B*, a higher molecular weight band was routinely detected in immunoblots using the phospho-Ser 153 antibody and behaved similarly to VASP in response to various experimental treatments, suggesting that it may be a VASP family member. Mena has at least three splice variants, generating proteins of 80, 88, and 140 kDa (13), and thus represented a likely candidate for this protein. Moreover, because Mena, like VASP, is expressed in fibroblasts and epithelial cells, is phosphorylated by PKA, and exhibits altered mobility when phosphorylated (13), the effect of detachment on Mena phosphorylation was directly investigated. Like VASP, Mena was phosphorylated both in cells treated with Fsk + IBMX and, significantly, in cells placed in suspension (Fig. 1*D*). The mobility shift of VASP proteins is due to phosphorylation near the Pro-rich region. To determine whether detachment also affected phosphorylation near the EVH2 domain, immunoblots were probed with a phospho-Ser-239 specific antibody. The basal level of Ser-239 phosphorylation in quiescent, adherent cells did not increase following detachment (Fig. 1*E*). In total, these data demonstrate that cellular detachment induces phosphorylation of VASP family proteins specifically near the Pro-rich region at sites preferred by PKA.

Dynamic Regulation of VASP Phosphorylation by Cell Adhesion and during Cell Spreading—To determine if the detachment-induced phosphorylation of VASP and Mena was reversible, detached cells were replated onto dishes coated with FN (for fibroblasts) or type-I collagen (for epithelial cells) and harvested for immunoblot analysis. Both Ser-153 of VASP and the equivalent residue near the Pro-rich region of Mena were rapidly dephosphorylated following adhesion to ECM as judged by the increased electrophoretic mobility of the proteins (Fig. 1, *C–E*). Interestingly, although detachment did not promote significant phosphorylation of VASP at Ser-235, this site, like Ser-153, was rapidly dephosphorylated upon adhesion to ECM (Fig. 1*E*).

To further investigate the dynamics of regulation of VASP phosphorylation by adhesion, a detailed time course was performed (Fig. 2*A*). Conversion of VASP to its phospho-Ser 153 form was more than 60% complete within 10 min after NIH3T3 cell detachment and more than 95% complete after 1 h (Fig. 2*A*, *left panel*). Interestingly, the rapid and complete dephosphorylation of Ser-153 seen early after adhesion to FN was followed by a period of intermediate phosphorylation as cells continued to spread (Fig. 2*A*, *right panel* and *B*). By 1 h after plating, VASP phosphorylation returned to a low baseline level equivalent to that seen in stably adherent cells (Fig. 2*A*, *right panel* and *B*). A similar bi-phasic course of phosphorylation, with slightly different kinetics, was observed in epithelial cells that had been detached and replated onto collagen (Fig. 2*C*). Light microscopy showed both cell types to be firmly attached but rounded at the early time points during which Ser-153 was dephosphorylated (data not shown). During the later time points at which VASP phosphorylation was intermediate, cells were actively spreading and exhibited multiple lamellipodia and extensive membrane ruffling, while the later decrease in phosphorylation corresponded with a contractile, polygonal morphology. These data suggest that phosphorylation of VASP at Ser-153 changes dynamically in response to changes in cell adhesion and also during cell spreading.

Activation of Protein Kinase A during Cell Spreading—The increase in Ser-153 phosphorylation of VASP during cell spreading suggested that PKA might become activated over the same time course. To address this, PKA activity was measured in NIH3T3 cells at various times after detachment and replating onto FN (Fig. 2*D*). As reported previously (5), PKA activity was high in cells soon after detachment but decreased as cells remained in suspension. The observed decrease in PKA activity with prolonged incubation in suspension is in contrast to the sustained phosphorylation of VASP at Ser-153 (compare Fig. 2*A*, *left panel* with *D*). The reasons for this disparity are currently unknown. However, following adhesion to FN, PKA activity decreased initially but increased during cell spreading and returned to baseline at later time points, a time course that closely paralleled Ser-153 phosphorylation. These data correlate changes in PKA activity with changes in VASP phosphorylation at Ser-153 during cell adhesion and cell spreading.

Regulation of VASP-Abl Interaction by Cell Adhesion and Cell Spreading—Adhesion-dependent changes in VASP phosphorylation near the Pro-rich region at Ser-153 may affect VASP interaction with SH3 domains *in vivo*. To investigate this, VASP immunoprecipitates from stably adherent and detached cells were analyzed for the presence of Abl and Src. For unknown reasons, attempts to co-immunoprecipitate Src with VASP under a variety of conditions proved unsuccessful (data not shown). However, Abl was routinely detected in VASP immunoprecipitates from stably adherent cells (Fig. 3) and was therefore chosen for further investigation. The readily detectable interaction between VASP and Abl observed in stably adherent cells was ablated by treatment with Fsk + IBMX and, importantly, by cellular detachment (Fig. 3, *A–C*), in a manner paralleling VASP phosphorylation (Fig. 3, *A–C*). A time course showed that the level of Abl that co-precipitated with VASP increased significantly upon initial cellular adhesion to FN, fell slightly as VASP phosphorylation increased during cell spreading, and settled over time to levels comparable to those seen in stably adherent cells (Fig. 3*C*). These data represent the first demonstration of interaction between VASP and full-length Abl *in vivo* and demonstrate dynamic regulation of this interaction by cell adhesion and spreading.

PKA Regulates Anchorage-dependent VASP-Abl Interaction—Data thus far suggest that adhesion-dependent changes in PKA activity regulate VASP phosphorylation, which in turn

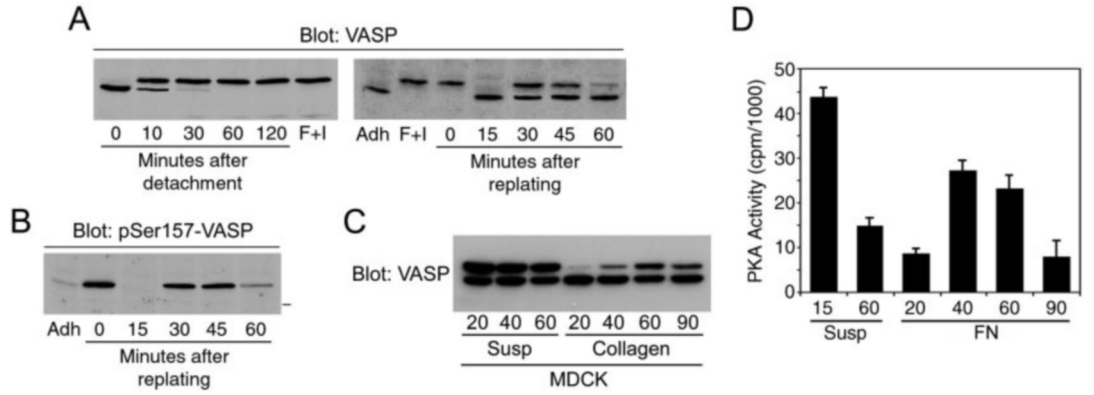

FIG. 2. **VASP phosphorylation is dynamically regulated by cell adhesion and during cell spreading and correlates with PKA activity.** *A* and *B*, NIH3T3 cells were detached from tissue culture plates and incubated in suspension or held in suspension for 1 h and replated on FN for the indicated times before harvesting and analysis by immunoblotting with antibodies against total VASP (*A*) or against pSer157-VASP (*B*). Lysates from stably adherent (*Adh*) cells and cells treated with Fsk + IBMX (*F*+*I*) were included as references for unphosphorylated and fully phosphorylated VASP, respectively. 0 min after detachment indicates stably adherent cells, whereas 0 min after replating indicates cells held in suspension for 1 h. *C*, MDCK cells were detached and held in suspension (*Susp*) or replated on collagen for the indicated number of minutes, then harvested, and analyzed by VASP immunoblotting. *D*, NIH3T3 cells were detached and held in suspension (*Susp*) or replated on FN for the indicated number of minutes and then harvested for PKA activity assay. The average and standard error from three separate experiments are shown.

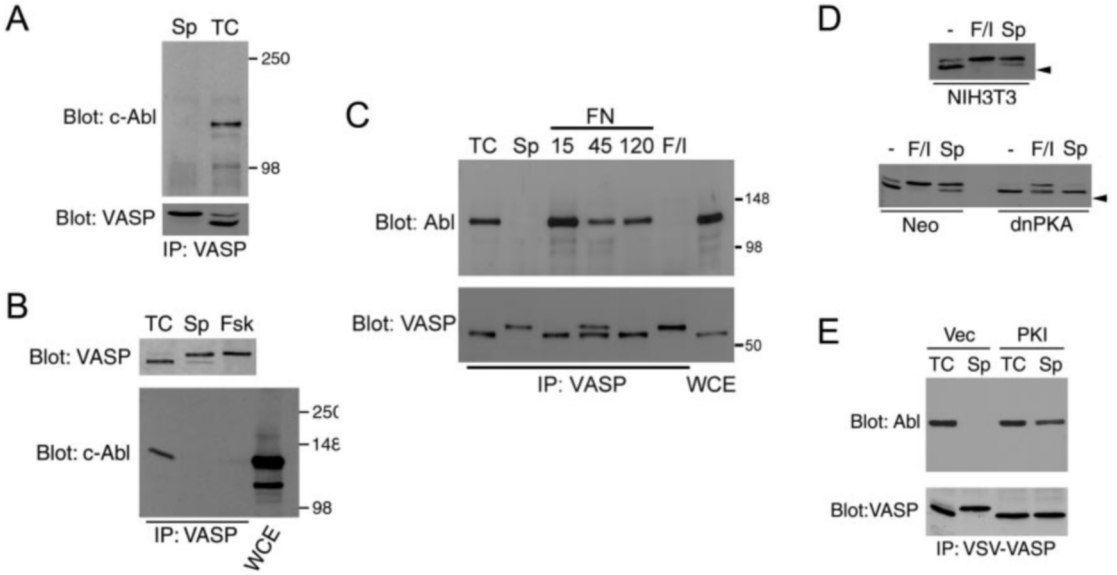

FIG. 3. **The interaction of VASP with Abl is anchorage-dependent and controlled by PKA.** *A*, VASP immunoprecipitates (*IP*) from NIH3T3 cells stably adherent to tissue culture plates (*TC*) or cultured in suspension for 1 h (*Sp*) were analyzed by immunoblotting with c-Abl antibody. *B*, an experiment similar to *A* with the inclusion of an IP from Fsk-treated cells and 40 μg of whole cell extract (*WCE*) for control and reference. For both *A* and *B*, a portion of each extract was reserved prior to immunoprecipitation and analyzed by VASP immunoblot as indicated. *C*, chemically cross-linked anti-VASP beads were used to immunoprecipitate VASP from lysates from stably adherent cells (*TC*), Fsk + IBMX (*F*/*I*)-treated cells, and cells incubated in suspension for 1 h (*Sp*) or re-plated on FN for the indicated number of minutes. Eighty and 20% of the immunoprecipitates were analyzed by anti-Abl and anti-VASP immunoblotting, respectively. Ten μg of whole cell extract (*WCE*) were run for reference. *D*, adherent NIH3T3, Neo, or dnPKA cells were left untreated (−), treated with Fsk + IBMX (*F*/*I*), or detached and cultured in suspension for 1 h (Sp). Lysates were analyzed by VASP immunoblotting. The *arrowhead* indicates the position of a 50-kDa marker. *E*, NIH3T3 cells were transiently transfected with a plasmid encoding VSV-tagged VASP and either an empty control vector (*Vec*) or one encoding the PKA inhibitor protein (*PKI*). After recovery, cells were serum-starved and left attached to tissue culture plates (*TC*) or detached and cultured in suspension for 1 h (Sp) and then harvested for immunoprecipitation with anti-VSV antibody and analysis by VASP and Abl immunoblotting.

regulates VASP-Abl interaction. To reinforce these observations, the effect of cell adhesion on VASP phosphorylation and VASP-Abl interaction was examined in cells in which PKA was inhibited. dnPKA cells are a stable NIH3T3 cell line in which activation of PKA, including that elicited by cellular detachment (5) and cell spreading,[2] is blocked by expression of a dominant negative regulatory subunit of PKA (5). The basal level of VASP phosphorylation in stably adherent cells and the increased phosphorylation induced by Fsk + IBMX were significantly inhibited in dnPKA cells (Fig. 3*D*). More importantly, the detachment-induced phosphorylation of VASP, readily detected in NIH3T3 cells and a control stable cell line (Neo), was completely absent in dnPKA cells (Fig. 3*D*). A similar effect was seen when PKA was inhibited by the PKA inhibitor protein, PKI; transient expression of PKI inhibited phosphorylation of epitope-tagged VASP in non-adherent cells (Fig 3*E*, *lower panel*). Significantly, inhibition of VASP phosphorylation in non-adherent cells promoted anchorage-*independent* interaction of VASP with Abl (Fig. 3*E*, *upper panel*). These data demonstrate that adhesion-dependent changes in PKA activity regulate VASP phosphorylation and the adhesion-dependent interaction between VASP and Abl.

[2] A. Howe, unpublished observations.

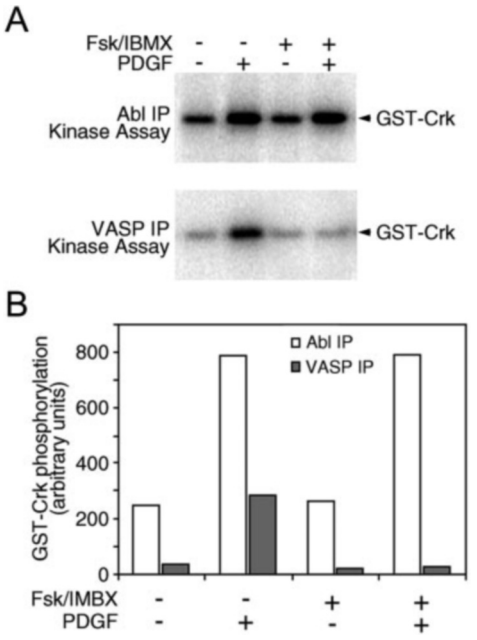

FIG. 4. **PKA regulates the association of Abl tyrosine kinase activity with VASP.** *A*, NIH3T3 cells were treated for 20 min with Me_2SO (0.2% v/v) or Fsk + IBMX and then left untreated or stimulated for 5 min with PDGF as indicated. Cells were lysed, and immunoprecipitations using antibodies against Abl or VASP were processed for Abl kinase assay using [^{32}P]ATP and GST-Crk as substrates. Reaction products were either separated by SDS-PAGE and visualized (*A*) or quantitated (*B*) by PhosphorImager analysis.

Association of Abl Kinase Activity with VASP—The association of Abl with VASP prompted investigation of whether Abl kinase activity was also associated with VASP. To address this, Abl and VASP immunoprecipitates were isolated from quiescent, stably adherent NIH3T3 cells and processed for Abl tyrosine kinase assay using GST-Crk as a substrate. Phosphorylation was assessed by SDS-PAGE separation of reaction products and PhosphorImager analysis. Specific modification of tyrosine residues in GST-Crk was confirmed by immunoblotting with anti-phosphotyrosine antibodies (data not shown). Only basal levels of kinase activity were present in Abl immunoprecipitates (Fig. 4*A*, *upper panel*), and little significant activity was detected in VASP immunoprecipitates (Fig. 4*A*, *lower panel*). Stimulation of cells with PDGF (28) stimulated Abl activity nearly 4-fold in Abl immunoprecipitates (Fig. 4, *A* and *B*) and, importantly, nearly 6-fold in VASP immunoprecipitates (Fig. 4, *A* and *B*). Pretreatment of cells with Fsk + IBMX had no effect on the ability of PDGF to stimulate Abl activity or to activate Abl but completely eliminated the coprecipitation of PDGF-stimulated Abl activity with VASP (Fig. 4, *A* and *B*). Similar results were also seen using a commercially available, optimized peptide as the substrate for Abl (data not shown). These data demonstrate that PKA can regulate the association of Abl activity with VASP *in vivo*.

DISCUSSION

This work describes the dynamic, multiphasic activation of PKA by cellular detachment, adhesion, and spreading and links this activity to regulation of VASP phosphorylation and interaction with the Abl proto-oncogene product. This is the first demonstration of a biochemical link between cell-matrix adhesion and regulation of the VASP family of cytoskeletal regulatory proteins, as well as the first demonstration of the interaction of VASP with full-length Abl protein *in vivo*.

Like VASP/Ena proteins, Abl is an important regulator of cytoskeletal dynamics. Abl is activated and transiently localized to focal adhesions upon adhesion to ECM (29), which may be mediated by several proteins known to bind Abl (*e.g.* paxillin (30), Abl-interacting (Abi) proteins (31), Crk (32), VASP (Ref. 14 and this study). Furthermore, domains in the C terminus of Abl direct binding to both monomeric actin and actin filaments (33); binding to F-actin inhibits Abl and appears to mediate the anchorage dependence of Abl activity (34). Physiological activation of Abl is required for actin cytoskeletal remodeling in response to growth factors (28), whereas oncogenic activation of Abl induces transformation characterized by anchorage-independent cell division and dramatic changes in cell morphology (35). A recent report has shown that Abl drives the formation of filopodia-like microspikes (34). Importantly, VASP proteins are also known to localize to and promote the formation of these structures (17, 18, 36). These observations, plus genetic and biochemical evidence from *Drosophila* (37), emphasize the potential importance of VASP-Abl interaction and its regulation by PKA.

The direct effect of VASP-Abl interaction on the activity of either protein is unknown. Except for a neural-specific variant of Mena, mammalian VASP proteins do not appear to be Tyr-phosphorylated (13), so their regulation by Abl must occur through a mechanism other than direct phosphorylation. One possibility is given by the fact that Abl can compete with profilin for binding to Mena and Evl (13, 22) and, therefore, perhaps also to VASP. As profilin binding is not inhibited by phosphorylation, PKA-mediated phosphorylation of VASP proteins would promote dissociation of Abl, which may facilitate formation of profilin-containing complexes. Finally, Abl may also modulate VASP function by phosphorylating VASP effectors or binding partners; of note, both vinculin and zyxin are phosphoproteins and contain sequences that resemble known consensus sites for Abl phosphorylation (38).

VASP may also directly alter Abl activity. The Abl SH3 domain is crucial for the intramolecular folding that holds Abl in a catalytically inactive conformation (39). Moreover, the kinase activity of Abl is increased by the interaction of its SH3 domain with many, but not all, Abl-interacting proteins (40). Thus, VASP may either directly activate Abl or may preferentially interact with unfolded, activated Abl. Of course, interaction with VASP would also presumably bring Abl into close proximity to actin filaments, which may inhibit Abl activity by engaging its F-actin binding domain (34). The balance of these effects *in vivo* is currently unknown.

VASP-Abl interaction may help localize Abl activity to adhesive or cytoskeletal structures (*e.g.* focal adhesions, filopodia) and thereby facilitate modification of Abl targets within those structures. Regulating this localization via phosphorylation of VASP is an attractive function for adhesion-dependent changes in PKA activity. Indeed, altered regulation of VASP-Abl interaction may contribute to the anchorage-independent growth factor signaling and blunted cell migration observed in dnPKA-expressing cells (5, 12). Phosphorylation of VASP proteins does not affect EVH1-mediated interactions (15, 25). Consistent with this, VASP-vinculin complexes are not disrupted in detached cells.[3] Thus, activation of PKA may selectively displace Abl without affecting EVH1-mediated VASP localization. While global changes in cell adhesion (*e.g.* detachment and reattachment) and PKA activity have sweeping effects on VASP-Abl interaction, localized changes in adhesion and PKA activity may affect distinct, localized VASP-Abl complexes. The intricate anchoring of PKA to various subcellular locales via protein kinase A anchoring proteins provides a conduit for this type of spatial control (41). Of particular interest in this regard

[3] K. DeMali and K. Burridge, manuscript in preparation.

is the interaction of PKA with WAVE, a member of the Wiskott-Aldrich Syndrome protein family, that localizes to lamellipodia and other actin-rich structures and also interacts with Abl *in vivo* (18, 42). As both WAVE and VASP bind to the Abl SH3 domain, their binding would be mutually exclusive. It is therefore tempting to posit that WAVE-mediated recruitment of PKA may serve to regulate the balance of Abl-WAVE and Abl-VASP complexes (and perhaps VASP-profilin, as mentioned above). This putative equilibrium and its functional consequences have yet to be identified, but efforts are underway.

Cell adhesion may affect other aspects of VASP function in addition to VASP-Abl interaction. As phosphorylation has been shown to inhibit the ability of VASP proteins to nucleate, bind, and bundle F-actin, dephosphorylation of VASP upon adhesion may promote microfilament nucleation and bundling, which may facilitate initial cell spreading. Interestingly, dephosphorylation of VASP is mediated in part by protein phosphatase 2A (14, 43), which has recently been shown to be activated by integrin engagement (44). Equilibrium between phosphorylated and non-phosphorylated forms later during cell spreading may be required for dynamic association of VASP with remodeling microfilaments. The return to a predominantly dephosphorylated form of VASP as cells become polygonal may reflect an increase in VASP F-actin bundling, which may enhance stress fiber formation.

The present work places phosphorylation of VASP proteins by PKA and the subsequent regulation of VASP-Abl interaction at the nexus of cell adhesion and cytoskeletal dynamics and may have broad implications for regulation of cell motility and anchorage-dependent cell growth.

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