Phosphorylation of Paxillin via the ERK Mitogen-activated Protein Kinase Cascade in EL4 Thymoma Cells*

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Intracellular signals can regulate cell adhesion via several mechanisms in a process referred to as “inside-out” signaling. In phorbol ester-sensitive EL4 thymoma cells, phorbol-12-myristate 13-acetate (PMA) induces activation of extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases and promotes cell adhesion. In this study, clonal EL4 cell lines with varying abilities to activate ERKs in response to PMA were used to examine signaling events occurring downstream of ERK activation. Paxillin, a multifunctional docking protein involved in cell adhesion, was phosphorylated on serine/threonine residues in response to PMA treatment. This response was correlated with the extent and time course of ERK activation. PMA-induced phosphorylation of paxillin was inhibited by compounds that block the ERK activation pathway in EL4 cells, primary murine thymocytes, and primary murine splenocytes. Paxillin was phosphorylated in vitro by purified active ERK2. Two-dimensional electrophoresis revealed that PMA treatment generated a complex pattern of phosphorylated paxillin species in intact cells, some of which were generated by ERK-mediated phosphorylation in vitro. An ERK pathway inhibitor interfered with PMA-induced adhesion of sensitive EL4 cells to substrate. These findings describe a novel inside-out signaling pathway by which the ERK cascade may regulate events involved in adhesion.

Cell adhesion is regulated via multiple mechanisms. Ligand of integrins on the cell surface initiates a cascade of events referred to as “outside-in” signaling. These events, initiated within the focal adhesion complex, include activation of focal adhesion kinase (FAK) and protein-protein interactions between focal adhesion components (1). Paxillin, a multifunctional docking protein, binds to FAK and is phosphorylated upon integrin ligation (2, 3). Additional steps downstream of integrin ligation include activation of the ERK mitogen-activated protein kinases (4).

Intracellular signaling events also regulate adhesion, in a process referred to as “inside-out” signaling (5). For example, receptor-mediated signals can “prime” integrins by increasing their affinity for ligand (6). Activation of protein kinase C (PKC) can enhance adhesion via a mechanism that does not require direct phosphorylation of integrin subunits (7). Thus, phosphorylation events involving multiple components of the focal adhesion complex may regulate adhesion (8).

Adhesion-mediated signaling has been most thoroughly studied in fibroblasts and epithelial cells but is also very important in lymphocytes. Paxillin is phosphorylated upon adhesion of mature T-cells to fibronectin (11) or P-selectin (12) and interacts with FAK in chemokine-treated T-cells (13). Although paxillin is a well characterized substrate for the tyrosine kinases FAK and Pyk2, it is also phosphorylated on serine/threonine residues in response to adhesion (3, 9, 10). Serine phosphorylation of paxillin can occur downstream of PKC activation (10). The functional roles of these phosphorylation events and the kinases involved have not been fully elucidated.

Our laboratory has utilized EL4, a murine thymoma cell line, as a model system in which to explore the relationship between activations of PKC isoforms and ERK mitogen-activated kinases. EL4 cell lines that are either sensitive or resistant to the effects of phorbol 12-myristate 13-acetate (PMA) have been described by our laboratory and others. In EL4 cells, PMA resistance is defined as a phenotype that confers resistance to the toxic effects of PMA. In PMA-sensitive EL4 cells, activation of PKC leads to the activation of ERKs (15) and to other downstream responses, including IL-2 expression (16–20) that culminate in growth arrest and death (14). PMA-induced cell death is blocked by PD98059, an inhibitor of the MEK/ERK pathway, in PMA-sensitive cells (18). The mechanism by which ERK activation results in growth arrest has not been delineated. In contrast, in PMA-resistant EL4 cells, PKC activates PKC isoforms but causes little or no ERK activation. The molecular mechanisms underlying this resistance to ERK activation appear to involve steps upstream of Raf-1 activation (22, 23). Several phenotypic differences have been noted between sensitive and resistant cells, including a higher level of expression of PKCε in sensitive cells (23, 24). None of these differences has been shown to be responsible for the defect in ERK activation in resistant cells. Regardless of the mechanisms involved in PMA resistance, EL4 cell lines can provide useful models in which to explore the roles of ERK activation in downstream responses. One of the responses of sensitive EL4 cells to PMA is enhanced adherence to tissue culture substrate (21). In this study, we utilized PMA-sensitive and -resistant EL4 cell lines to demonstrate a role for ERKs in regulating the phosphorylation state of paxillin.

EXPERIMENTAL PROCEDURES

Cell Culture—EL4 cells were maintained in RPMI 1640 with 10% fetal bovine serum (Summit Biotechnology), nonessential amino acids,
and penicillin/streptomycin. The clonal cell lines WT2 and V7, derived by limiting dilution of WT and PV cells, were maintained in suspension culture dishes (Corning) and tissue culture flasks (Falcon), respectively. V7 cells were seeded in 60-mm dishes (4 \times 10^6 cells/ml) 2 days before experiments, while WT2 cells were maintained at <2 \times 10^6 cells/ml. Cells were cultured without serum for 18 h before experiments. Thymocytes and splenocytes were prepared from thymi and spleens, respectively, of 6–8-week-old BDF1 mice (Jackson Laboratories), purified over a 1.080 g/ml OptiPrep gradient (Nycomed Pharma), and washed twice with RPMI. Reagent sources (and solvents) were as follows: PMA, Calbiochem (ethanol); PD98059, Calbiochem (Me_SO); U0126, Promega (Me_SO); 50 V and 250 V.

**Cell Proliferation Assays**—Growing cells (\approx 95% viability) were seeded in 24-well tissue culture plates at 2 \times 10^5 cells/well with 2 ml of complete medium. Cells were incubated with 100 nM PMA or 0.1% ethanol (vehicle) at 37 °C for varying times. Cell number was determined by counting trypan blue-excluding cells, using a hemacytometer.

**Immunoblotting and Immunoprecipitation**—Whole-cell extracts were prepared as described previously (20), with minor modifications. Cells were collected by centrifugation at 12000 \times g. Any adherent cells were collected by scraping. Cells were lysed in buffer containing 20 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 \muM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 \muM aprotinin, and 10 \muM leupeptin. Extracts were sedimented at 10000 \times g for 10 min at 4 °C to remove insoluble material. The insoluble pellet did not contain significant amounts of FAK or paxillin. Samples of 100 \mug of protein, as determined by a Coomassie protein assay (Pierce), were separated by SDS-PAGE on 7.5% Laemmli gels, transferred to polyvinylidene difluoride paper, incubated with antibodies, and developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Antibody sources were as follows: phospho-ERK, Promega; ERK1, Santa Cruz (recognizes ERK1 and ERK2); paxillin, Transduction Laboratories (recognizes paxillin and Hi-5) and Upstate Biotechnology, Inc. (Lake Placid, NY); phosphotyrosine, Upstate Biotechnology, Inc. (4G10). Blots were imaged by densitometry.

For immunoprecipitations, whole-cell extracts (1000 \mug in 1 ml) were precleared with protein A (Amersham Pharmacia Biotech) or protein G (Roche Molecular Biochemicals)-agarose beads for 1 h at 4 °C. Antibodies were added and incubated at 4 °C for 3 h with mixing. Protein A- or protein G-agarose was added and incubated overnight at 4 °C. For some experiments, immunoprecipitates from samples containing 250 \mug of protein were treated with or without 1 unit of calf intestine phosphatase (Calbiochem) or aliquots of anti-ERK2 (Upstate Biotechnology, Inc.) or active ERK2 (Calbiochem) in 20 mM HEPES (pH 7.5), 4 mM \beta-glycerophosphate, 2 mM EGTA, 0.4 mM EDTA, 10 \muM P3 inhibitor (KFARKGALKQKNV1), 1 \muM protein kinase inhibitor (TYYHDFIASGRTGRRNAD), 10 mM MgCl, 0.02% Triton X-100, and 50 mM ATP (with or without [γ-32P]ATP, NEN Life Science Products). Samples were subjected to SDS-PAGE, immunoblotting, or drying and autoradiography. Phosphoamino acid analysis was performed as described previously (25).

**Intact Cell Phosphorylation Studies**—V7 cells (100-mm dish) were serum-starved for 18 h and then incubated in 5 ml of phosphate-free buffer (10 mM HEPES (pH 7.6), 136 mM NaCl, 4.9 mM KCl, 1.33 mM CaCl2, 1 glitfer glucose) for 1 h. [\textsuperscript{32}P]Orthophosphate (1 \muCi/ml; Amersham Pharmacia Biotech) was added. The cells were incubated at 37 °C for 4 h and then with or without 100 mM PMA for 15 min. Paxillin was immunoprecipitated as described above.

**Two-dimensional Electrophoresis**—Whole-cell lysates were prepared using lysis buffer containing 8 x urea, 2% Triton X-100, 1% dithiothreitol, 1.5% Pharmalyte (Amersham Pharmacia Biotech), and bromphenol blue. For some experiments, paxillin immunoprecipitates were resuspended in this lysis buffer. For the first dimension, lysates (400 \mug of protein) or immunoprecipitates were applied to Immobiline Dry Strips (pH 4–7) (Amersham Pharmacia Biotech) and subjected to flat bed electrophoresis at 500 V and 16 h at 3500 V. For the second dimension, proteins were separated by SDS-PAGE on 7.5% Laemmli gels. Proteins were transferred to polyvinylidene difluoride membrane for autoradiography and/or immunoblotting.

**Adhesion Assay**—WT2 cells were seeded at a density of 5 \times 10^5 cells/well in quadruplicate in 24-well tissue culture plates containing complete medium. PD98059 (50 \muM) or vehicle (0.2% Me_SO) was added for 90 min at 37 °C, followed by PMA (100 nM) or vehicle (0.2% ethanol) for 60 min. Cells were gently washed four times with 0.5 ml of medium. Attached cells were detached with 0.05% trypsin and counted using a hemacytometer. Photomicroscopy was performed using a Nikon Diaphot microscope equipped with Hoffman interference optics.

**Characterization of Clonal EL4 Cell Lines**—In our efforts to better define the basis of PMA sensitivity and resistance in EL4 cells, a series of clonal cell lines was developed. Cell lines derived from our original PMA-sensitive cell line were designated ‘WT’ (wild type). A flask of nonclonal WT cells maintained on standard tissue culture plastic underwent a spontaneous shift in phenotype, observed as increased adherence to substrate. This cell line, designated as ‘PV’, was retained and used to derive clonal lines designated as ‘V’ (variant). Clonal cell lines were incubated with and without 100 nM PMA to establish their phenotypes (Fig. 1). PMA inhibited proliferation of all WT-derived clones, indicating that they were PMA-sensitive. PV-derived clones V5, V7, V9, and V11 continued to proliferate in the presence of PMA, indicating that they were PMA-resistant. Clone V3 and V10 cells proliferated slowly and showed partial sensitivity to PMA. Subsequent studies examined the phenotypes of the clonal cell lines in more detail.

The ability of PMA to induce ERK activation was examined in clonal EL4 cell lines by immunoblotting (Fig. 2). Protein levels for ERK1 and ERK2 were similar between all cell lines tested, with ERK2 being the predominant isofrom (see legend to Fig. 2). PMA induced robust ERK activation in all of the WT-derived clones, as indicated by the appearance of phosphorylated active ERK. Clones V3 and V10 likewise responded to PMA with ERK activation. V3 and V10 thus exhibit an intermediate phenotype, since they survive in the presence of PMA (Fig. 1) despite ERK activation (Fig. 2). The other PMA-resistant clones (V5, V7, V9, and V11) showed little or no ERK activation when treated with 100 nM PMA for 15 min.

As discussed above, the PMA-resistant clones were derived from a variant cell line that showed increased adhesion. We therefore examined the expression of proteins involved in adhesion-mediated signal transduction. One of these proteins was paxillin. Paxillin and immunoprecipitated \textsuperscript{32}P-labeled protein were analyzed by SDS-PAGE at similar levels in PMA-sensitive and -resistant clones (Fig. 2). The calculated size of human paxillin (GenBank accession no. P49023) is 61 kDa. Phosphorylation of paxillin results in the appearance of multiple species of lower mobility on SDS-PAGE (i.e., "gel shift") (25). PMA induced shifts in paxillin mobility in all clones examined. However, the shifts were most
extensive (i.e. to ~72 kDa) in clones in which ERKs were activated. In clones V5, V7, V9, and V11, which were resistant to ERK activation, only partial shifts in paxillin mobility occurred in response to PMA. These data suggested that phosphorylation of paxillin was correlated with ERK activation.

A second protein of ~47 kDa was recognized in WT-derived cells by the anti-paxillin antibody. This protein also shifted in mobility in response to PMA (Fig. 2). Hic-5 (GenBank™ accession no. L22482), a paxillin homolog, has a calculated size of 48 kDa, is recognized by the anti-paxillin antibody used here, and undergoes mobility shifts upon phosphorylation (27). Leupaxin (GenBank™ accession no. AF062075), another paxillin homolog, is highly expressed in hematopoietic cells (28) but is 44 kDa in size. The 47-kDa protein may thus represent murine Hic-5 or a related protein.

WT2 and V7 were chosen as representative PMA-sensitive and -resistant cell lines, respectively, for further studies.

Correlation between ERK Activation and Paxillin Phosphorylation—Paxillin family members are substrates for the tyrosine kinases FAK and Pyk2. Additional work with EL4 clones has established that only PMA-resistant cells (e.g. V7) and not PMA-sensitive cells (e.g. WT2) express FAK. Thus, FAK is not required for PMA-induced phosphorylation of paxillin. Although both sensitive and resistant EL4 cells express Pyk2, PMA does not induce tyrosine phosphorylation of Pyk2 in either cell type. These observations prompted us to search for alternative pathways for the observed PMA-induced phosphorylation of paxillin.

In order to examine the relationship between ERK activation and paxillin phosphorylation, we compared PMA-induced ERK activation between WT2 and V7 clones in more detail (Fig. 3A). Immunoblotting of whole-cell extracts with anti-phosphotyrosine and anti-phospho-ERK antibodies revealed rapid and prolonged activation of ERKs in WT2 cells. ERK activation was greatly attenuated in V7 cells, consistent with Fig. 2 and with previous data for other PMA-resistant cell lines (19, 23). Interestingly, the time course of ERK activation differed between the two clonal cell lines. PMA (100 nM) induced maximal ERK phosphorylation within 1 min in WT2, while ERK activation was maximal, albeit weak, after 15 min in V7. Thus, V7 cells are not completely refractory to PMA. Rather, PMA-induced ERK activation is delayed and blunted in comparison with that seen in WT2 cells.

In the same experiment, paxillin phosphorylation was examined by immunoblotting (Fig. 3A). Partial shifts in paxillin mobility occurred in WT2 cells within 1 min after PMA addition. Additional species (~72 kDa) appeared by 30 min, with complete loss of the lower and presumably unphosphorylated 59-kDa band. Thus, PMA induced a rapid and progressive phosphorylation of paxillin in WT2 cells. In contrast, PMA-induced shifts were delayed (~15 min) in V7 cells. The extent of the shift was less complete in V7 than in WT2 cells, such that the unphosphorylated 59-kDa band was present in V7 cells throughout the incubation. These data show that paxillin phosphorylation is correlated with ERK activation in both EL4 clones but is much more extensive in WT2 than in V7 cells.

Treatment of paxillin immunoprecipitates from PMA-treated cells with calf intestinal phosphatase resulted in loss of the lower mobility bands from both WT2 and V7 cells (Fig. 3B), confirming that the gel shifts were due to phosphorylation.

![Fig. 3. Effects of PMA on ERK activation and paxillin phosphorylation in EL4 cells. A, WT2 and V7 cells were incubated with 100 nM PMA for the indicated times. Whole-cell extracts (100 μg of protein), were immunoblotted using antibodies against phosphotyrosine, phospho-ERK, and paxillin. Only the regions containing the proteins of interest are shown. B, cells were treated with 0.1% ethanol vehicle or 100 nM PMA for 60 min. Immunoprecipitates of paxillin were treated with or without calf intestine phosphatase. Samples were subsequently immunoblotted for paxillin. C, cells were treated with 100 nM PMA for the indicated times. Immunoprecipitates of paxillin, from samples containing 1000 μg of protein, were immunoblotted for phosphotyrosine followed by stripping and reprobing with anti-paxillin antibody. Normal mouse IgG (m; Chemicon) was used as an isotype control for the immunoprecipitating anti-paxillin antibody.](http://www.jbc.org/content/11335/Fig3)
The role of tyrosine phosphorylation of paxillin was examined (Fig. 3C). In WT2 cells, PMA treatment induced a weak and transient tyrosine phosphorylation of paxillin at 15 min. In contrast, paxillin gel shifts were sustained for at least 180 min after the addition of PMA to WT2 cells. In V7 cells, tyrosine phosphorylation of paxillin was seen in untreated cells but not in PMA-treated cells. The absence of significant PMA-induced tyrosine phosphorylation in either cell line suggested that the bulk of the observed phosphorylation occurred on serine/threonine residues.

Effects of Tyrosine Kinase and Phosphatase Inhibitors on PMA Response—The differences in the time course for PMA-induced ERK activation between WT2 and V7 cells (Fig. 3) suggested that the pathways mediating PMA response might differ between the two cell lines. The role of tyrosine phosphorylation in these pathways was therefore investigated. The dose response for PMA-induced tyrosine phosphorylation in WT2 and V7 cells is shown in Fig. 4. The basal level of tyrosine phosphorylation was markedly lower in V7 cells than in WT2 cells (Fig. 4A). This difference has been noted previously between other PMA-sensitive and -resistant cell lines (22). The factors responsible for this difference have not been elucidated. Notably, maximal phosphorylation of both ERKs and paxillin was achieved with lower doses of PMA in WT2 cells (1 nM PMA) than in V7 cells (0.1 μM PMA).

Protein-tyrosine kinase and phosphatase inhibitors were used to further examine the potential role of tyrosine phosphorylation in PMA response. In WT2 cells, genistein partially inhibited tyrosine phosphorylation of ERK in response to PMA treatment (Fig. 4A). As a specific MEK inhibitor (29), PD98059 reduced paxillin phosphorylation in WT2 cells and abolished this response in V7 cells. Interestingly, in WT2 cells, genistein reduced the extent of the paxillin phosphorylation induced by PMA (i.e., the ~72-kDa species was absent) but did not block the response. In WT2, phosphorylation of the 47-kDa paxillin homolog was also reduced by genistein. Dephostatin, a protein-tyrosine phosphatase inhibitor, had no effect on phosphorylation of ERK or paxillin in WT2 cells (Fig. 4B). In contrast, dephostatin (>10 μM) inhibited both events in V7 cells (Fig. 4B). Thus, although PMA can induce ERK activation in both cell lines, the response appears more dependent on tyrosine phosphorylation/dephosphorylation in V7 than in WT2 cells. More importantly, the data shown in Fig. 4 provide further evidence that ERK activation is correlated with paxillin phosphorylation in EL4 cells.

Effects of MEK Inhibitors on Paxillin Phosphorylation—We next examined whether paxillin phosphorylation occurred upstream or downstream of ERK activation. When WT2 and V7 cells were pretreated for 90 min with 0.1% Me2SO vehicle or 50 μM PD98059 prior to the addition of PMA for 15 min. Whole-cell extracts (100 μg of protein) were immunoblotted for phosphotyrosine and then reprobed for paxillin. B, cells were incubated for 90 min with 0.1% Me2SO vehicle (DMSO) or 50 μM PD98059 (PD) prior to the addition of 1 nM (WT2) or 100 nM (V7) PMA for 60 min. Immunoprecipitates of paxillin, from samples containing 1000 μg of protein, were immunoblotted for paxillin. Normal mouse IgG1 (m) was used as an isotype control for the immunoprecipitating antibody.
suggest that PMA-induced phosphorylation of paxillin involves kinase(s) in addition to MEK/ERK but that activation of the MEK/ERK pathway is required for maximal phosphorylation. Thus, paxillin phosphorylation occurs downstream of ERK activation.

Phosphorylation of Paxillin in Thymocytes and Splenocytes—EL4 is a transformed murine cell line that retains characteristics typical of T lymphocytes. We therefore asked whether ERK-mediated phosphorylation of paxillin also occurs in primary lymphocytes. In murine thymic lymphocytes (Fig. 6A), PMA-induced ERK2 activation was maximal by 1 min and then gradually declined. Paxillin mobility shifts were observed by 1 min but became more extensive after 15–30 min. Interestingly, the uppermost bands seen at 15–30 min in thymocytes were not observed in PMA-treated WT2 EL4 cells.

Thymocytes were next incubated with PMA in the absence and presence of two MEK inhibitors. U0126 is an alternative inhibitor of MEK activation (30). Both U0126 and PD98059 inhibited ERK activation and paxillin phosphorylation (Fig. 6B). U0126 was more potent than PD98059, in accordance with published data (30).

Paxillin phosphorylation was also examined in murine splenocytes (Fig. 6B). PMA-induced phosphorylation of paxillin was inhibited by PD98059 and U0126 in splenocytes, as was the case in thymocytes. These data indicate that ERK-mediated phosphorylation of paxillin occurs in at least two types of primary lymphocytes.

Phosphorylation of Paxillin by ERK2 in Vitro—Paxillin and its homologs are proline-rich proteins that contain numerous consensus sites, (S/T)P (31), for potential phosphorylation by ERKs. The possibility that paxillin served as an ERK substrate was therefore examined. Paxillin was immunoprecipitated from untreated WT2 cells, incubated with recombinant active GST-ERK2 in the absence and presence of Mg-ATP, and subjected to immunoblotting (Fig. 7A). In the presence of Mg-ATP and active GST-ERK2, paxillin and the 47-kDa protein were phosphorylated, as assessed by gel shifts. Paxillin from V7 cells was also phosphorylated by ERK2 in vitro, indicating that similar phosphorylation sites exist in both cell types. The reagent concentrations used here were optimized for maximal phosphorylation (data not shown). Nonetheless, phosphorylation of paxillin in vitro did not replicate the maximal shift seen in intact PMA-treated WT2 cells.

Further verification of ERK-mediated phosphorylation was obtained from an in vitro reaction utilizing [32P]ATP and active ERK2. Phosphate was incorporated into paxillin in the presence of active ERK2 and ATP (Fig. 7B). The [32P]-labeled band co-migrated with the major phosphorylated bands detected by immunoblotting, confirming that the gel shift was due to phosphorylation by ERK2. Phosphoamino acid analysis results (data not shown) showed phosphorylation of paxillin on serine/threonine, with no detectable tyrosine phosphorylation.

Two-dimensional Analysis of Paxillin Phosphorylation in Intact Cells and in Vitro—We next asked whether paxillin species that were phosphorylated by ERK in vitro were also phosphorylated in intact cells. Due to the complexity of the phosphorylation pattern observed for paxillin on one-dimensional gels,
we utilized two-dimensional PAGE to visualize the full spectrum of paxillin species with high resolution (Fig. 8, A–D). An extremely complex pattern of paxillin species was seen on two-dimensional gels, even in untreated WT2 cells (Fig. 8A). PMA treatment of WT2 cells caused a shift in the pattern of paxillin to include new species with more acidic PI values (Fig. 8B), consistent with phosphorylation. The most acidic species had the lowest mobility in the second dimension, as expected from our one-dimensional gel results. In untreated V7 cells (Fig. 8C), the more basic species predominated, suggesting that the extent of basal phosphorylation is lower in V7 than in WT2. Following PMA treatment of V7 cells (Fig. 8D), a shift to more acidic species occurred but was not as extensive as in WT2 cells.

Next, we used radioactive phosphate to trace the phosphorylation events occurring after phosphorylation of paxillin in intact cells and in vitro. Since PMA-induced paxillin phosphorylation was strictly dependent on ERK activation in V7 cells, we used these cells for this series of experiments. Paxillin was immunoprecipitated from 32P-labeled V7 cells and run on two-dimensional gels (Fig. 9, A–D). The immunoblot pattern for immunoprecipitated paxillin was somewhat different from that seen in whole-cell lysates (compare Figs. 8C and 9A). This may reflect the selectivity of the immunoprecipitating antibody toward different species of paxillin. In control cells, all but the most basic species observed (Fig. 9A) were phosphorylated to some extent (Fig. 9B). When V7 cells were treated with PMA for 15 min, new species of paxillin appeared (Fig. 9C, bar) that were phosphorylated (Fig. 9D, bar). We next tested whether these new species appeared after phosphorylation of paxillin in vitro by activated ERK. The results show that co-migrating species were generated in vitro (Fig. 9E), and were phosphorylated (Fig. 9F). Together, these data demonstrate that paxillin can be phosphorylated both in intact cells and in vitro by activated ERK.

Effects of PMA on Cell Adhesion—As mentioned earlier, PMA has been shown to enhance substrate adhesion of PMA-sensitive EL4 cells (21). We asked whether this response was ERK-dependent. WT2 cells were incubated for 60 min on standard tissue culture plastic in the absence and presence of PMA and/or PD98059 (23). Adherent cells were photographed (Fig. 10A) and quantitated (Fig. 10B). Only 4% of untreated cells adhered to the plate. PMA caused 75% of cells to adhere, consistent with a previous report (21). PD98059 had no effect when added alone but significantly decreased PMA-induced adherence by 40%. V7 cells adhered to tissue culture plastic in the absence of PMA. PMA did not enhance adhesion, nor did PMA-induced adherence in V7 cells (data not shown). These results indicate that the MEK/ERK pathway contributes to PMA-induced adherence of sensitive EL4 cells.

Fig. 8. Two-dimensional electrophoresis of paxillin. WT2 and V7 EL4 cells were treated with and without 100 nM PMA for 15 min. Whole-cell extracts were subjected to two-dimensional electrophoresis (pH 4–7) and then immunoblotted for paxillin. The lanes on the left contain molecular size markers for calibration purposes.

DISCUSSION

In this study, we found that paxillin is phosphorylated downstream of MEK activation in intact cells and can be phosphorylated by ERK2 in vitro. ERK-mediated phosphorylation of paxillin occurs not only in EL4 cells but also in thymocytes and splenocytes. Adhesion of PMA-sensitive EL4 cells is partially ERK-dependent, suggesting that phosphorylation of paxillin may play a role in regulating adhesion.

A critical feature of this study was the use of PMA-sensitive and -resistant strains of the EL4 cell line to examine events occurring downstream of ERK activation. Notably, the PMA-resistant cells were isolated without utilizing PMA to exert selective pressure. The pathways mediating PMA-induced ERK activation in EL4 cells have been extensively examined but have not been completely established. Although the identification of these pathways was not a major goal in this work, our results nonetheless provide additional insight into the mechanisms utilized by PMA-sensitive and -resistant cells. In particular, the results presented in Figs. 3 and 4 indicate that the two cell types utilize different routes to activate ERKs. The
ERK activation seen in PMA-sensitive cells is rapid, complete, requires nanomolar doses of PMA, and is largely independent of tyrosine phosphorylation. In PMA-resistant cells, ERK activation is slow in onset, incomplete, requires high doses of PMA, and is dependent on tyrosine phosphorylation. The similar effects of a tyrosine kinase inhibitor and a tyrosine phosphatase inhibitor in resistant EL4 cells may reflect involvement of a cytosolic tyrosine kinase that must be dephosphorylated prior to docking of paxillin to FAK or other proteins. That such regulatory phosphorylations can potentially occur in the cytosol prior to docking of paxillin to FAK or other proteins. That such “priming” steps may occur is suggested by the data reported here. First, phosphorylation of paxillin in response to ERK activation is most extensive in WT2 cells, in which paxillin is prephosphorylated to a greater extent (Fig. 8). Second, a tyrosine kinase inhibitor reduces the extent of paxillin phosphorylation in WT2 cells without blocking ERK activation (Fig. 4A), indicating that additional kinases must be involved in phosphorylating paxillin in intact cells. Third, the two-dimensional electrophoresis results (Fig. 9) show that paxillin is phosphorylated to a greater extent in PMA-treated EL4 cells. The complex pattern of serine/threonine phosphorylation observed in these cells, in which tyrosine phosphorylation is not prominent, indicates that a complete description of the sites involved will be an important long-term goal.

While tyrosine-phosphorylated paxillin localizes preferentially to focal adhesions, most paxillin is cytosolic (45). Paxillin can localize to focal adhesions in the absence of FAK (10). Pyk2, which does not localize to focal adhesions, also binds to and phosphorylates paxillin in lymphoid cells (46, 47). Thus, regulatory phosphorylations can potentially occur in the cytosol prior to docking of paxillin to FAK or other proteins. That such “priming” steps may occur is suggested by the data reported here. First, phosphorylation of paxillin in response to ERK activation is most extensive in WT2 cells, in which paxillin is prephosphorylated to a greater extent (Fig. 8). Second, a tyrosine kinase inhibitor reduces the extent of paxillin phosphorylation in WT2 cells without blocking ERK activation (Fig. 4A), indicating that additional kinases must be involved in phosphorylating paxillin in intact cells. Third, the two-dimensional electrophoresis results (Fig. 9) show that paxillin is phosphorylated to a greater extent in PMA-treated EL4 cells. The complex pattern of serine/threonine phosphorylation observed in these cells, in which tyrosine phosphorylation is not prominent, indicates that a complete description of the sites involved will be an important long-term goal.

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ERK activation seen in PMA-sensitive cells is rapid, complete, requires nanomolar doses of PMA, and is largely independent of tyrosine phosphorylation. In PMA-resistant cells, ERK activation is slow in onset, incomplete, requires high doses of PMA, and is dependent on tyrosine phosphorylation. The similar effects of a tyrosine kinase inhibitor and a tyrosine phosphatase inhibitor in resistant EL4 cells may reflect involvement of a cytosolic tyrosine kinase that must be dephosphorylated prior to activation. The slow onset observed in resistant cells could reflect a requirement for production of an autocrine factor. Further studies will be needed to address these possibilities.

ERKs have previously been implicated in “inside-out” signaling (32–36). For example, one-third of the total ERK2 associates with the cytoskeleton in fibroblasts (37). Activation of ERK2 results in locomotion of CHO cells (34). ERKs phosphorylate neurofilament proteins involved in neurite outgrowth (38). However, to our knowledge, this is the first report to link ERK2 activation to paxillin phosphorylation.

The presence of multiple bands of phosphorylated paxillin on one- and two-dimensional SDS-PAGE indicates multiple phosphorylation sites. ERKs may phosphorylate more than one site on paxillin. In addition, two alternatively spliced isoforms of paxillin, α and β, are expressed in mouse thymus (39). Both isoforms are multiply phosphorylated (40), are of similar size, and are probably recognized by the anti-paxillin antibody used here. Thus, both multiple phosphorylations and multiple paxillin isoforms may contribute to the complex pattern of paxillin species observed in our study.

It has been previously reported that paxillin is phosphorylated largely on serine residues in response to treatment of cells with PMA (10). Paxillin is maximally phosphorylated on serine/threonine in mitotic cells (41). The association of paxillin with unknown serine/threonine kinases has also been reported (9, 42). While some serine/threonine phosphorylation sites in paxillin have been identified (3, 9), these are not ERK consensus sites. The kinases responsible for these previously reported phosphorylation events remain to be established. Our data indicate that phosphorylation of paxillin involves multiple kinases, only one of which is ERK. Nonetheless, activation of ERK is required for PMA-induced phosphorylation of paxillin in resistant EL4 cells, thymocytes, and splenocytes.
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