Published in final edited form as:

Clin Exp Metastasis. 2011 August ; 28(6): 551–565. doi:10.1007/s10585-011-9391-y.

Differential Expression of FAK and Pyk2 in Metastatic and Non-metastatic EL4 Lymphoma Cell Lines

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Abstract

The murine EL4 lymphoma cell line exists in variants that are either sensitive or resistant to phorbol 12-myristate 13-acetate (PMA). In sensitive cells, PMA causes Erk MAPK activation and Erk-mediated growth arrest. In resistant cells, PMA induces a low level of Erk activation, without growth arrest. A relatively unexplored aspect of the phenotypes is that resistant cells are more adherent to culture substrate than are sensitive cells. In this study, the roles of the protein tyrosine kinases FAK and Pyk2 in EL4 phenotype were examined, with a particular emphasis on the role of these proteins in metastasis. FAK is expressed only in PMA-resistant (or intermediate phenotype) EL4 cells, correlating with enhanced cell-substrate adherence, while Pyk2 is more highly expressed in non-adherent PMA-sensitive cells. PMA treatment causes modulation of mRNA for FAK (up-regulation) and Pyk2 (down-regulation) in PMA-sensitive but not PMA-resistant EL4 cells. The increase in Pyk2 mRNA is correlated with an increase in Pyk2 protein expression. The roles of FAK in cell phenotype were further explored using transfection and knockdown experiments. The results showed that FAK does not play a major role in modulating PMA-induced Erk activation in EL4 cells. However, the knockdown studies demonstrated that FAK expression is required for proliferation and migration of PMA-resistant cells. In an experimental metastasis model using syngeneic mice, only FAK-expressing (PMA-resistant) EL4 cells form liver tumors. Taken together, these studies suggest that FAK expression promotes metastasis of EL4 lymphoma cells.

Keywords

cell-substrate adhesion; Erk mitogen-activated protein kinases; tyrosine kinases; T-lymphocytes; tumor promoters; metastasis

Introduction

EL4, a cell line originated from a carcinogen-induced murine lymphoma, provides a uniquely useful model system for studies of phorbol ester response and resistance. Responses of “wild-type” (WT) PMA-sensitive EL4 cells to phorbol 12-myristate 13-acetate (PMA) include PKC activation [1–4], tyrosine phosphorylation [5,6], Erk activation [2,4,7,8], adhesion [9], IL-2 production [10–14], and growth arrest [4,11,13,15]. To study the mechanisms by which PMA elicits these responses, we and other groups have characterized PMA-resistant EL4 cells that proliferate in the presence of PMA [4,9,17]. A high level of Erk activation is required for PMA-induced growth arrest in sensitive cells [4]; this response is conferred by expression of RasGRP [18], a phorbol ester receptor that directly activates Ras. Erks are activated by PMA, albeit to a lesser extent, in PMA-resistant cells; this response occurs via a Raf-1 mediated pathway that is modulated by Akt and RKIP [59]. Another intriguing and relatively unexplored aspect of the phenotypes is that PMA-resistant EL4 cells are much more adherent to substrate than are sensitive cells [4,17,19]. Although our group has previously shown that some PMA-resistant EL4 cell lines can form metastases in syngeneic mice [18], the metastatic capability of PMA-sensitive EL4 cells has not been previously reported. The formation of aggressive metastases by a lymphoma cell line is relatively unusual, and is likely to provide insight into the progression of human lymphomas. Therefore, the present study focuses on the factors contributing to adhesion and metastasis in EL4 lymphoma cells.

Adhesion plays a critical regulatory role in cells of hematopoietic origin, including EL4 cells [20]. For example, integrin-mediated adhesion of thymocytes to thymic stromal cells is important for thymocyte survival, migration, maturation, and repertoire selection [21]. Integrin signaling is mediated by proteins with cytoskeletal, docking, and/or catalytic activities. The latter include focal adhesion kinase (FAK) and the related kinase Pyk2 [22]. For adherent cells, disruption of integrin-mediated adhesion can result in apoptosis [23–25]. FAK, a tyrosine kinase, is involved in integrin signaling [26–28], is required for mitogen-induced motility [29], and protects against apoptosis [30–32]. Together, these effects are generally favorable for tumor cells, in which FAK is frequently over-expressed [33]. For example, extravasation of mouse mammary carcinoma cells to the lung is inhibited in the absence of FAK or integrin $\beta 1$ [34]. However, FAK can play both positive and negative roles in cell proliferation [35]. FAK is expressed in thymocytes [36], where it is involved in T-cell receptor signaling [37]. FAK levels are decreased in mature T-cells [35], and are increased in leukemias and lymphomas [38]. In acute myeloid leukemia, FAK expression is correlated with increased cell migration and poor prognosis [39]. Pyk2, also referred to as RAFTK, CAK β , and CadTK, is expressed in lymphocytes [40] and in the thymus [41]. In mature T-cells, Pyk2 participates in TCR-mediated signaling [42,43] and may be involved in directional killing [44]. Pyk2 is phosphorylated in response to many stimuli in hematopoietic cells [45–49]. For example, in the D1 thymocyte cell line, Pyk2 is activated by IL-7 and promotes survival [50]. In B-lymphocytes, Pyk2 is activated by IgG [51]. FAK and Pyk2 are potentially involved in PMA-induced responses. PMA can enhance tyrosine phosphorylation of both FAK [52] and Pyk2 [48]. Integrin-mediated adhesion activates Erks, and enhances mitogen-induced Erk activation [28,53]. Transfection of FAK into macrophages, which lack endogenous FAK, increases Erk activation [54]. In non-lymphocytic cells, Pyk2 mediates Erk activation by G protein-coupled receptors in some cases [55], but not all [56]. These studies prompted us to examine the roles of FAK, Pyk2, and adhesion in PMA-induced Erk activation in EL4 cells, initially addressing the hypothesis that FAK expression inhibits PMA response. The study progressed further to the goals of examining 1) the regulation of FAK and Pyk2 expression, 2) the role of FAK in cell migration, and 3) the metastatic capability of established EL4 cell lines. Taken together, the results support a role for FAK in contributing to the metastatic phenotype in EL4 lymphoma cells.

Materials and Methods

Cell culture

EL4 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Atlanta Biologicals or Summit Biotechnology), non-essential amino acids, and penicillin/streptomycin. The original wild type (WT; PMA-sensitive) and variant (PMA-resistant) EL4 cell lines used in our lab were provided by Dr. David Morris (University of Washington). The NV cell line was developed from the WT cell line by selecting for proliferation in the presence of 100 nM PMA [4]. The PV cell line was derived from a flask of WT cells exhibiting increased adherence [17]. WT-derived clonal cell lines were maintained in suspension culture dishes (Corning) to discourage selection for adherent cells; PV-derived clones were maintained on standard tissue culture plastic. For some experiments, Petri (Sarstedt), Primaria® (Falcon), or fibronectin-coated (Collaborative Biomedical Products) dishes were used. Cells were photographed using a Nikon Diaphot microscope equipped with Hoffman interference optics.

Immunoblotting and immunoprecipitation

Antibodies were from the following sources: phospho-Erk, Promega or Cell Signaling; Erk, Transduction Labs and Cell Signaling; Erk-1, Santa Cruz; FAK, Santa Cruz and Transduction Labs; phospho-FAK, Transduction Labs; c-Cbl, Santa Cruz; Grb2, Transduction Labs; p38, Santa Cruz; MEK, Santa Cruz; PI3K, Transduction Labs; phospho-tyrosine, Transduction Labs (PY20) and Upstate (4G10); PTP1D, Transduction Labs; Pyk2, Upstate and Santa Cruz, RACK, Transduction Labs; Raf-1, Santa Cruz; Ras, Santa Cruz or Chemicon; RasGRP, Santa Cruz; RSK, Upstate; Shc, Transduction Labs; SOS, Santa Cruz.

For experiments using non-clonal WT and NV cells, whole cell extracts were prepared as previously described [4], but without sonication, in a lysis buffer containing 20 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM NaCl, 1 mM EGTA, 5 mM β -glycerophosphate, 30 mM sodium pyrophosphate, 100 μ M sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Extracts were sedimented at 100,000 $\times$ g for 10 minutes at 4°C to remove insoluble debris. Cytosol and membrane fractions were prepared without detergent, and with sonication, as described [4]. Proteins were separated by SDS-PAGE on 10 or 12.5% Laemmli gels, transferred to nitrocellulose or PVDF paper, and incubated with primary antibodies. Blots were developed using either gold-labeled secondary antibody with silver enhancement (Amersham), or chemiluminescent reagents (Amersham).

For experiments using clonal EL4 cell lines, whole cell extracts were prepared as described above, with minor modifications. After treatment, cells were collected by centrifugation at 1,200 $\times$ g. Adherent cells were harvested by scraping prior to the centrifugation. Cells were lysed in a lysis buffer containing 20 mM Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 30 mM sodium pyrophosphate, 100 μ M sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Extracts were sedimented at 10,000 $\times$ g for 10 min at 4°C to remove insoluble debris. Samples equalized for protein (100 μ g), as determined by Coomassie Protein Assay (Pierce), were separated by SDS-PAGE on 7.5% Laemmli gels, transferred to PVDF paper, incubated with antibodies, and developed using enhanced chemiluminescence (ECL) or ECL-Plus (Amersham). Blots were imaged by densitometry and quantified using NIH-Image software.

Transfections

Full-length mouse FAK cDNA, cloned into the mammalian expression vector pRc/CMV, was kindly provided by Dr. Steven Hanks (Vanderbilt University). Empty vector pcDNA3

(Invitrogen) was used as a negative control. Plasmid pGreen LanternTM-1, encoding GFP, was from GibcoBRL. Actively growing cells were washed once with RPMI 1640 medium. Cells (2×10^7) were mixed with 10 μ g of either pRc/CMV-FAK or pcDNA3 with or without 10 μ g of GFP plasmid. The cell suspension (0.4 ml) was electroporated using a BTX Electro Cell Manipulator with parameters of 140 V, 720 Ohms, and 3175 μ F across a BTX cuvette of 0.2 cm electrode gap. Pulse lengths varied between 25 and 28 ms. The electroporation conditions, optimized in our lab for EL4 cells, gave a transfection efficiency of ~10% as assessed by fluorescence microscopy of GFP-transfected live cells. Electroporated cells were incubated at room temperature for 15 minutes, then transferred to complete medium and returned to the cell culture incubator. Twenty-four hours later, cells were collected for further analysis. For experiments involving cell sorting, cells were washed twice with Dulbecco's modified phosphate-buffered saline containing 0.1% bovine serum albumin and once with 100 μ g/ml propidium iodide (PI) (Sigma). Cells were passed through 40- μ m nylon mesh and then sorted by flow cytometry using a FACStarplus (Becton Dickinson Immunocytometry Systems). To expedite sorting, GFP⁺PI⁻ cells were first collected by processing signals generated only by the FL1 channel. The cells were then sorted a second time, processing signals generated by the forward-light scatter channel. The purity of the final GFP⁺PI⁻ population was >95%. Cells were used for assay incubations within 1 hour after sorting.

Northern blotting

Total RNA was recovered and purified using RNeasy mini kits according to the manufacturer's directions (Qiagen). After quantification by A260, samples (5 μ g) were denatured and fractionated by electrophoresis on 1.2% agarose gels containing 250 mM formaldehyde. RNA was transferred to Duralon-UV nylon membranes (Stratagene) in 10X SSC buffer by capillary action. RNA was cross-linked using UV light (Stratalinker, 120 mJ); hybridization was carried out at 68°C in Quickhybe (Stratagene) for 1–2 hours. Probes corresponding to ~600 bp non-coding regions near the 3' terminus of either FAK or Pyk2 were prepared via reverse transcriptase polymerase chain reaction (RT-PCR) using EL4 RNA. Oligonucleotide primers (20–22mers) corresponding to 5' position 3430 (upper) and 3' position 4107 (lower) of murine FAK (GenBank accession no. M95408), and 5' position 2951 (upper) and 3' position 3564 (lower) of murine Pyk2 (GenBank accession no. D45854) were synthesized by the MUSC oligonucleotide synthesis facility. The resulting PCR products were cloned utilizing TA cloning kit and propagated according to the manufacturer's directions (Invitrogen). Plasmids purified using mini-prep kits (Qiagen) were verified by restriction enzyme digest and by sequencing (MUSC sequencing facility). Inserts were excised via restriction digest and purified from agarose gels using Qiagen spin columns. The resulting probes were radiolabeled with [³²P]-dCTP using Prime-It Kit II (Stratagene) and separated from unincorporated nucleotide on Sephadex G-50 in TE buffer. A ~1000 bp probe specific for GAPDH, kindly provided by Dr. Gian Re (Dept. of Pathology, MUSC), was used to normalize for loading. After hybridization, membranes were washed for 30 minutes in 2x SSC, 0.1% SDS followed by two 15-minute washes in 0.1xSSC/0.1% SDS, and exposed overnight at –80°C to Kodak XOMat/AR film. To strip for re-probing, membranes were placed in boiling 0.1X SSC containing 0.1% SDS and incubated at 25°C for 15 minutes; this procedure was repeated twice. Blots were imaged by densitometry and quantified using NIH-Image software.

Microarray analysis

Total RNA was extracted from duplicate samples of WT2 and V7 cells using the RNeasy Mini kit from Qiagen. Genomic DNA was removed from total RNA by treating with DNase 1 from Invitrogen. Concentration and purity of total RNA was determined by absorbance at 260 nm, 280 nm, and by OD_{260/280} ratio. Integrity of RNA was assessed with gel

electrophoresis. RNA (5 µg per sample) was used for Affymetrix microarray at the Center for Integrated Biotechnology, WSU Genomics Core Laboratory, as previously described [57]. Normalized and log transformed microarray data were analyzed using Genesifter software. This program identifies differentially expressed genes based on Gene Ontology (GO) Consortium (<http://www.geneontology.org/GO.doc.html>) and KEGG public pathway resource (<http://www.systems-biology.org/001/001.html>). Differential expression of genes from duplicate samples was determined by using pairwise analysis. Statistical significance was determined using Student's t-test and corrected using the method of Benjamini and Hochberg. Genes differentially expressed by 2-fold or more were included in the analysis.

siRNA Experiments

FAK siRNA was obtained from Invitrogen (PTK2 Validated Stealth RNAi DuoPak, catalog number 47595). Cells were treated with this reagent in the presence of 1% Lipofectamine RNAiMAX (Invitrogen) in opti-MEM I medium. After treatment with and without 50nM FAK siRNA for 72 hrs, V7 cells were treated with 100 nM PMA or 10 nM EGF for 10 mins. Whole-cell extracts were prepared for immunoblotting as described above. For cell proliferation assays, V7 cells were seeded in 24-well plates at a density of 2×10^4 cells/well in triplicate. After serum-starvation for 18 hrs, the cells were incubated with 10% FBS, lipofectamine alone, or 10 or 50 nM FAK siRNA for 48 hrs. Live cells (excluding trypan blue) were then counted using a hemacytometer. For cell migration assays, V7 cells ($2 \times 10^5/0.5\text{ml}$), treated with or without 50nM siRNA for 72 hrs, were plated on uncoated culture inserts with 8mm diameter pore size membranes (BD Falcon™ Cell Culture Inserts, BD Biosciences) in 24-transwell cell culture dishes. The bottom chamber was filled with 0.75 ml serum-free RPMI medium with or without 10% FBS. After incubation for 24 hrs at 37°C in a humidified 5% CO₂ incubator, cells remaining in the insert were removed with a cotton swab. Migrated cells on the bottom of the filters were stained with crystal violet and counted by light microscopy.

Mouse tumorigenesis studies

These experiments were carried out using a protocol described previously by others [58] and approved by the IUCAC at MUSC. Male C57BL/6 mice (NCI) were used at 4 weeks of age, with 6–7 animals in each group. EL4 cell lines were collected by centrifugation and re-suspended in sterile phosphate-buffered saline (PBS). An aliquot of 0.1 ml, containing 3×10^4 cells, was injected into the tail vein of each animal. PBS alone was injected into control animals. All animals were sacrificed on 20 days after injection. Necropsies were performed to quantify liver weight; the location of tumors, number of tumors, and liver appearance were also recorded. Statistical significance was determined by unpaired t-test using InStat software (GraphPad, San Diego, CA).

Results

Adhesion phenotype in EL4 cells

Differences between EL4 cells lines with respect to adhesion are readily apparent in the course of culturing the cells. PMA-sensitive (“WT”) EL4 cells grow in suspension, with multicellular aggregates present to variable extents. PMA-resistant (“NV”) cells can also proliferate in suspension, but adhere well to standard tissue culture surfaces [4]. NV cells attach to tissue culture, fibronectin-coated, or Primaria® dishes, but form aggregates in Petri or suspension tissue culture dishes (Figure 1A). WT cells have similar non-adherent morphologies on all substrates tested (data not shown). To discourage unintentional selection of adherent cells, WT cell lines are routinely maintained in suspension culture dishes. Experimental incubations were conducted on standard tissue culture plastic unless otherwise noted.

Expression of FAK in WT and NV EL4 cells

We previously established that PMA-resistant EL4 cells are deficient in PMA-induced Erk activation, as compared to PMA-sensitive EL4 cells [2,4]. The cell lines used in some of our previous studies were termed “wild-type” (WT) for PMA-sensitive lines, and “variant” [2,7] or “NV” [4] for PMA-resistant lines. The expression levels of signal transduction proteins potentially involved in the PMA-sensitive phenotype were examined by immunoblotting. Proteins expressed at similar levels between WT and NV cells included Raf-1, Ras, MEK, Rsk, PI3K, JNKs, p38/HOG, RACK, Grb-2, SOS, PTP1D, Shc, and c-Cbl (59, 60, and data not shown). Differences in expression levels of PKC isoforms [4], paxillin [17], and RasGRP [18] between sensitive and resistant cells were addressed in previous publications.

Of the proteins initially tested, FAK showed the most profound difference in expression levels between PMA-sensitive and -resistant cells. Initial experiments indicated that FAK is expressed only in PMA-resistant (“V”) and not in PMA-sensitive (“WT”) cells; an example of this result is shown in Figure 1. In the experiments shown in panels B–D, cells were treated for 10 minutes with or without 100 nM PMA. This treatment induces maximal Erk activation in WT cells [2,4,7], with slight activation in NV cells, as demonstrated in panels B and C. Erk activation was confirmed by gel shifts (Figure 1B), and by immunoblotting using anti-phospho-Erk antibody (Figure 1B) and anti-phosphotyrosine (Figure 1C). The immunoblot for FAK (Figure 1C) shows that FAK is expressed only in PMA-resistant cells. Immunoblots of archival extracts from the sensitive and resistant EL4 cell lines originally used in our lab [2] likewise showed that FAK is expressed only in PMA-resistant (“variant”) cells (data not shown). PMA treatment had no effect on FAK protein levels in NV cell lines within 10 minutes (Figure 1C).

In view of the observed differences in expression of FAK between sensitive and resistant EL4 cells, we asked whether adhesion itself could modify PMA-induced Erk activation. PMA-sensitive (WT) and -resistant (NV) cells were grown overnight in standard, Petri, suspension, Primaria®, or fibronectin-coated tissue culture dishes prior to incubation with and without PMA. In some experiments cells were grown in tissue culture dishes, dislodged, and then incubated in suspension. In all cases, Erks were fully activated in response to PMA in PMA-sensitive cells, and were only partially activated in PMA-resistant cells (data not shown). FAK protein levels did not vary significantly between PMA-resistant cells grown on different substrates (data not shown). When NV cells grown in suspension culture dishes were transferred to Primaria or fibronectin-coated dishes, they attached within 20–40 minutes, but Erks were not activated during this time [19]. In summary, these experiments established that PMA-induced Erk activation, a key variable between sensitive and resistant cells, is independent of cell-substrate interaction.

In situations where FAK or Pyk2 mediates Erk activation, disruption of the actin cytoskeleton can inhibit the response [55,61]. Incubation of EL4 cells for 30 minutes with 1 μ M cytochalasin D inhibited subsequent PMA-induced Erk activation by approximately 25% in both WT and NV cells, as assessed by *in vitro* Erk activity assays and gel mobility shifts (data not shown). Since similar effects were seen in cells expressing or not expressing FAK, we concluded that any role of the cytoskeleton in Erk activation is independent of FAK.

Expression of FAK and Pyk2 in clonal EL4 cell lines

The heterogeneous nature of the WT and NV cell lines prompted us to examine FAK expression in more detail, using clonal EL4 cell lines developed in our lab. The derivation of these cell lines has been reported previously [17]. All clones designate “V” adhere readily to tissue culture plastic, while WT clones do not. Erks are robustly activated by PMA in all

WT-derived clones, but are activated to only a minor extent in most V-derive clones. Two clones of “intermediate” phenotype, V3 and V10, are exceptions in that they show moderate Erk activation when treated with PMA. Clones WT2 and V7 are used by our lab as representative PMA-sensitive and -resistant cell lines, respectively [17,18].

Immunoblots were performed to show the levels of several signaling proteins in clonal EL4 cells (Figure 2). Enhanced expression of RasGRP in PMA-resistant cells, described in detail previously [18], is confirmed in this blot. The extent of PMA-induced Erk activation is shown by immunoblotting for phospho-Erk, with immunoblotting for total Erk used to confirm equal loading. The FAK immunoblot revealed that all V-derived clones, and no WT-derived clones, express FAK (Figure 2). Intermediate clones V3 and V10, which are partially sensitive to PMA-induced Erk activation [17,18] (the response is relatively high in this particular experiment), also express FAK. These data indicate that FAK is not solely responsible for PMA resistance. Since FAK and Pyk2 can, in some cases, play reciprocal or overlapping cellular roles [62,63], we examined Pyk2 expression in EL4 cell lines (Figure 2). WT-derived clones express Pyk2, while most PV-derived clones do not. Interestingly, clones with the intermediate phenotype (V3 and V10), in which PMA induces a moderate level of Erk activation [17,18], consistently express more Pyk2 than other PV-derived clones. Treatment of cells for 15 minutes with 100 nM PMA does not alter FAK or Pyk2 protein levels (e.g., Figure 2). In summary, PMA-sensitive EL4 cells (e.g., WT2) express only Pyk2 and not FAK, while PMA-resistant cells (e.g., V7) express FAK and very low levels of Pyk2. EL4 cell lines with an intermediate phenotype (V3 and V10) express FAK as well as moderate levels of Pyk2.

Phosphorylation of FAK and Pyk2 in EL4 cell lines

Tyrosine phosphorylation of FAK and Pyk2 reflects the activation state of these kinases. The effects of PMA on phosphorylation of FAK and Pyk2 were tested by immunoprecipitation followed by immunoblotting (Figure 3A). FAK is constitutively phosphorylated in both V7 and V3 cells; phosphorylation increases ~2-fold when cells are treated with PMA for 15 minutes (Figure 3B). A similar response was seen in NV cells (data not shown). Pyk2 is constitutively phosphorylated in WT2 and V7 cells. This phosphorylation is decreased in response to PMA in both cell lines (Figure 3B). The results shown in Figure 3C, using a phospho-FAK specific antibody, confirm that PMA increases FAK phosphorylation on Y397, consistent with FAK activation. This figure also demonstrates that EGF increases FAK phosphorylation in V7 cells. The effect of PMA was consistently greater than that of EGF, when both were added for 10 minutes.

Expression of mRNA for FAK and Pyk2 in EL4 cells

We next tested whether the differences in levels of FAK and Pyk2 proteins between sensitive and resistant EL4 cells reflected differences in mRNA levels. Oligonucleotide primers were used to amplify mRNA sequences encoding FAK and Pyk2 by RT-PCR from V7 cells and WT cells, respectively. PCR products were used to generate oligonucleotide probes for analysis of expression of FAK and Pyk2 by Northern blotting (Figure 4). The normalized results showed that PMA-resistant cells express a much higher level of FAK mRNA than PMA-sensitive cells. Most of the resistant clones express Pyk2 mRNA at lower levels than are seen in sensitive cells, except for V3, which expresses levels similar to those in WT. The three WT cell lines tested express little or no FAK mRNA; levels of Pyk2 mRNA were variable but lower than in V7. These data suggest that differences in expression of FAK and Pyk2 proteins are mediated largely at the mRNA level.

As an alternative method for assessing transcript levels, Affymetrix microarray analysis was performed for RNA isolated from WT2 and V7 cells. Based on this analysis, V7 cells were

found to express 11.6-fold higher levels of FAK mRNA than WT2 cells. In contrast, WT2 cells express 3-fold higher levels of Pyk2 mRNA than V7 cells. These data confirm the differences observed in the Northern blot analysis.

Expression of mRNA for FAK and Pyk2 in EL4 cells

The studies discussed above prompted us to examine whether expression levels of FAK and/or Pyk2 are regulated in response to PMA treatment. Such modulation could be relevant to the series of events that eventually lead to growth arrest in PMA-sensitive cells. WT2 cells were incubated with 100 nM PMA for times up to 48 hours. Northern blots were performed to assess levels of FAK and Pyk2 mRNA in the treated cells. As shown in Figure 5, Pyk2 mRNA is markedly up-regulated in WT2 cells after 4 hours of PMA treatment. The increase is maximal by 8 hours, and then gradually declines. FAK mRNA, which is undetectable in untreated cells, increases progressively from 8 to 48 hours.

Immunoblotting experiments were performed to determine whether up-regulation of mRNA for Pyk2 and FAK results in increased protein levels. As shown in Figure 6, Pyk2 protein is transiently decreased in PMA-treated WT2 cells at 2 hours, increases above control levels by 6 hours, and remains at an elevated level for 10–24 hours. Pyk2 protein levels decline by 2 hours in V7 cells following PMA addition, and do not recover subsequently. Erk activation persists for at least two hours after PMA addition in both WT2 and V7 cells. Thus, down-regulation of Pyk2 in both cell lines occurs when Erk activity is still elevated. For FAK, the situation is quite different. FAK protein could not be detected in WT2 cells at times up to 24 hours after PMA addition (Figure 6), despite induction of FAK mRNA (Figure 5). FAK protein levels are not altered by PMA in V7 cells. In summary, PMA induces complex changes in both message and protein levels for FAK and Pyk2 in EL4 cells. However, based on the kinetics, these changes do not appear to influence early PMA-induced events such as Erk activation.

Effects of FAK expression on PMA-sensitive EL4 cells

Since the EL4 cell lines were originally characterized with respect to their PMA sensitivity, we asked whether FAK expression affects PMA-induced Erk activation. In other words, expression of FAK might be one of the factors contributing to the suppression of PMA response in PMA-resistant cells. To test whether FAK expression has a negative effect on Erk activation, we transiently transfected WT2 EL4 cells with cDNA encoding human FAK. The transient transfection was successful, as indicated by immunoblotting for FAK (Figure 7A). PMA-induced Erk activation is slightly lower in the FAK-transfected cell population than in the vector-transfected control. Repeated attempts to stably over-express FAK in PMA-sensitive EL4 cells were unsuccessful. Since transfection efficiency is quite low (~10%) in WT EL4 cells, these cells were co-transfected with separate plasmids encoding FAK and green fluorescent protein (GFP) so that transiently-transfected cells could be selected by flow cytometry. Sorted cells are enriched in FAK as compared to unsorted cells (Figure 7B). PMA-induced Erk activation is decreased by 17 and 24%, respectively, in unsorted and sorted GFP/FAK-transfected cells. Since the effect was not significantly enhanced in the sorted cells, we conclude that FAK does not substantially alter Erk activation in PMA-sensitive EL4 cells. However, since PMA-sensitive cells express RasGRP [18], which greatly enhances PMA-induced Erk activation, the role of FAK in PMA-resistant cells (which lack RasGRP) may not be completely analogous.

Effects of FAK knockdown on PMA-resistant cells

We next approached the role of FAK from the opposite direction, using FAK siRNA to knock down expression of FAK in PMA-resistant V7 cells. As shown in Figure 8A, this strategy was successful in decreasing FAK protein levels. In Figure 8B, the proliferation of

V7 cells treated with and without FAK siRNA was assessed. The growth rate is significantly reduced in the FAK knockdown cells, with almost no proliferation occurring at the higher dose of siRNA. These data indicate that FAK is critical for the proliferation of PMA-resistant cells that express endogenous FAK.

Cell migration requires a series of regulated events involving cell adhesion. We have previously assessed cell migration and invasion in clonal EL4 cells, showing that serum and EGF enhance migration of V7 cells [59]. In Figure 8C, the effects of FAK knockdown on V7 cell migration are shown. Incubation with FAK siRNA significantly inhibited migration of V7 cells in response to serum. Notably, migration in the FAK knockdown cells is significantly lower than that observed in the absence of serum, indicating severe interference with cell motility.

Experimental metastasis studies

The metastasis of sensitive and resistant EL4 cells was examined in an animal model. Although such cell lines have existed for many years, their relative abilities to form metastases had not previously been reported. Cells were injected into syngeneic mice via the tail vein according to a published protocol [58]. Mice were sacrificed after 20 days; previous survival experiments using PMA-resistant cell lines indicated that morbidity and mortality were observed subsequent to this time [64]. As previously noted [58], EL4-derived tumors generated in this manner metastasize predominantly to the liver, as evidenced by increased liver size and visible tumor nodules. Mice injected with PMA-resistant V7 cells exhibited an increase in liver weight (Figure 9B) that was accompanied by visible tumor nodules in the liver (Figure 9A) and kidneys (data not shown). Injection of V5, another PMA-resistant strain, also resulted in a significant increase in liver mass (Figure 9B), and in a smaller number of tumors in the liver and kidneys (data not shown). In contrast, no significant increase in liver mass was observed in mice injected with PMA-sensitive WT2 cells (Figure 9B). No visible tumors were observed in mice injected with phosphate-buffered saline (vehicle) (Figure 9A), or with WT2 or WT3 cells (data not shown). Mice injected with V10 cells (intermediate phenotype) likewise did not develop visible tumors (data not shown) or exhibit increased liver weight (Figure 9B). In a separate survival experiment (data not shown), 100% of the PBS-injected control mice, and 75% of the WT2-injected mice, survived for 31 days after injection, whereas 100% of V7-injected mice died by 26 days (n=6 animals/group). In summary, these data establish that sensitive and intermediate phenotype EL4 cells are not metastatic in the animal model tested, and that only PMA-resistant cells generate metastases.

Discussion

In this study, we have further characterized the difference in phenotype between PMA-sensitive and -resistant EL4 cell lines. The data presented here establish that FAK is expressed in resistant cells, but not in sensitive EL4 cells. Pyk2, a FAK-related kinase, is expressed at higher levels in sensitive cells than in resistant cells. FAK expression is correlated with the enhanced substrate adhesion and enhanced metastatic potential that is seen in PMA-resistant cells. More specifically, FAK is required for serum-induced proliferation and migration of these cells. This result is consistent with the known roles of FAK in cell motility and proliferation, which were recently reviewed [65].

We first explored the hypothesis that FAK can suppress PMA-induced Erk activation. Our accumulated data indicate that FAK is not solely responsible for the PMA-resistant phenotype with respect to Erk activation. However, it remains possible that FAK protects resistant cells against PMA-induced growth arrest, or interferes with the roles of other proteins (e.g., Pyk2 or RasGRP) in these cells. It is unlikely that Pyk2, a FAK-related kinase

expressed in sensitive cells, plays a major positive role in PMA-induced Erk activation in sensitive cells, since we have already shown a critical role for RasGRP in this regard [18]. However, it is likely that Pyk2 contributes to adhesion signaling pathways in EL4 cells.

The roles of FAK and Pyk2 in regulating proliferation, differentiation, and activation of T-lymphocytes remain to be fully explored. It is interesting to note that expression of FAK is augmented in leukemia/lymphoma cell lines [40]. FAK has also been implicated in the regulation of T-cell locomotion [66]. Our data show that both FAK and Pyk2 are subject to modulation in response to signal transduction events in EL4 cells. Work by others has shown that PMA can induce dephosphorylation of FAK and Pyk2 in Jurkat T-cells [67]. In EL4 cells, we found that PMA induced dephosphorylation of Pyk2 (WT2 and V7 cells), and phosphorylation of FAK (V7 cells) (Figure 4). A study of primary human T-cells showed that PMA reverses phosphorylation of FAK and Pyk2 induced by ligation of the T-cell receptor or CD28 [67]. Thus, the roles of adhesion proteins in phorbol ester and diglyceride response are worthy of further consideration.

The factors regulating transcription of FAK mRNA have been examined to some extent. FAK and Pyk2 are both subject to complex regulation that involves alternative splicing in various species, including the mouse [68]. A study characterizing the promoter region of human FAK identified NF κ B and p53 as positive and negative regulators of FAK transcription, respectively [69]. In addition, an Ets family member has been identified as critical to FAK transcription in the mouse [70]. It is not yet clear how PMA might regulate FAK transcription in EL4 cells. One possible functional explanation for the differential expression of FAK and Pyk2 in EL4 cells is that these cells require either FAK or Pyk2 for viability, and that resistant cells express FAK to compensate for decreased expression of Pyk2 (or vice versa). However, although FAK and Pyk2 can complement each other in some respects, there are major differences between these kinases. The two kinases are differently localized, perhaps due to the ability of FAK, but not Pyk2, to bind talin [71]. Pyk2 does not entirely compensate for FAK in fibroblasts from FAK-knockout mice [62]. Interactions between the two kinases have been observed [63], and Pyk2 can facilitate activation of FAK [72]. In two studies, FAK was found to inhibit tyrosine phosphorylation of Pyk2 [73,74]. In fibroblasts, over-expression of Pyk2 causes cell rounding [75]. This effect was antagonized by FAK, again indicating that the balance between FAK and Pyk2 is likely important. The non-redundant roles of FAK and Pyk2 in regulating cell motility were recently reviewed [65]. Our results show that PMA treatment can up-regulate expression of FAK and Pyk2 mRNAs. Although expression of FAK could be a protective response, sensitive EL4 cells undergo growth arrest despite the observed induction of FAK expression. Interestingly, Pyk2 protein is up-regulated by PMA in sensitive EL4 cells, but down-regulated in resistant cells. Further studies will be needed to fully address the mechanisms regulating expression of FAK and Pyk2 in EL4 cells.

The data obtained to date with the EL4 model suggest that PMA resistance is a multifactorial phenotype. The current study has identified FAK and Pyk2 as potential participants in the phenotypic differences between EL4 cell lines. Based on these findings, we postulate that multiple alterations in a signal transduction “axis” may work together to confer the PMA resistant phenotype to EL4 cells.

Importantly, the results of the experimental metastasis studies suggest that FAK expression may contribute to tumorigenesis of EL4 cells. This is consistent with the results of our FAK knockdown studies, which showed that FAK is required for proliferation and migration of PMA-resistant EL4 cells. These studies demonstrate for the first time that the PMA-sensitive phenotype, which is characterized by high levels of RasGRP and Pyk2 and the absence of FAK, does not support metastasis. In contrast, the PMA-resistant phenotype, characterized

by low levels of RasGRP and Pyk2 and the presence of FAK, confers metastasis. These findings are consistent with previous work examining the role of FAK in metastatic capability of mammary cells both *in vitro* and *in vivo* [34]. However, the existence of intermediate phenotypes illustrates the complexities of the EL4 cellular variants. Indeed, our microarray results indicate that PMA-sensitive and -resistant cells vary with respect to the expression of many transcripts (data not shown). The functional relationships between the differentially expressed proteins are of interest, and remain to be fully explored. Nonetheless, it is clear that FAK plays a critical role in supporting the proliferation and migration of EL4 lymphoma cells that are capable of forming aggressive tumors in an animal model.

Acknowledgments

This work was supported by the National Institutes of Health (CA58640 and CA94144), National Science Foundation (EPS-9630167), U.S. Department of Defense (DAMD17-98-8524 and DAMD17-01-1-0730), the University Research Committee of the Medical University of South Carolina, and the Medical Scientist Training Program at the Medical University of South Carolina. We thank David Morris for supplying EL4 cells, Steven Hanks for supplying the FAK expression vector, David Cole for the Pyk2 expression vector, April Wisheart-Johnson for technical assistance, and Derek J. Pouchnik for assistance with the microarray analysis performed in the core facility supported by the Center for Biotechnology at WSU.

Abbreviations

EGF	epidermal growth factor
FAK	focal adhesion kinase
PMA	phorbol 12-myristate 13-acetate

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Figure 1. Erk activation and FAK expression in EL4 cell lines

In Panel A, NV cells were grown overnight in either a Petri dish (non-adherent) or a Primaria® (adherent) culture dish. Cells were photographed using interference contrast microscopy. In Panel B, whole-cell extracts from WT and NV cells, treated with or without 100 nM PMA for 15 minutes, were immunoblotted in parallel using anti-Erk1 (left) and anti-phospho-Erk (right) antibodies, respectively. In Panel C, whole-cell extracts were prepared from WT and NV cells treated with or without 100 nM PMA for 10 minutes. Three separately maintained stocks of NV cells, labeled NV, NV1, and NV2, were tested. Immunoblotting was carried out using anti-phosphotyrosine (PY20) and anti-FAK antibodies. The positions of the molecular size markers are shown on the right.

Figure 2. Characterization of protein expression in clonal EL4 cell lines
Cells were treated with or without 100 nM PMA for 15 minutes. Whole-cell extracts, equalized for protein content, were immunoblotted using antibodies against FAK, Pyk2, RasGRP, phospho-Erk, and total Erk. The phenotype with respect to “PMA sensitivity” refers to PMA-induced growth arrest and Erk activation, as traditionally defined in EL4 cell lines.

Figure 3. Effects of PMA on tyrosine phosphorylation of FAK and Pyk2 in clonal EL4 cell lines
 In Panel A, WT2 and V7 cells were treated with 100 nM PMA for the indicated times. Immunoprecipitates of Pyk2 or FAK from WT2 or V7 cells, respectively, were immunoblotted with anti-phosphotyrosine antibody (4G10), followed by stripping and re-probing with anti-Pyk2 or FAK antibodies. Normal goat IgG (g) or rabbit IgG (r) were used as isotype controls for the immunoprecipitating anti-Pyk2 or anti-FAK antibodies, respectively. In Panel B, cells were incubated with 0.1% ethanol vehicle or 100 nM PMA for 15 minutes. Immunoprecipitates of Pyk2 or FAK from samples containing 1 mg protein were immunoblotted using anti-phosphotyrosine antibody (4G10), followed by stripping and re-probing with anti-Pyk2 or FAK antibodies, respectively. Tyrosine phosphorylation was quantified by densitometry and normalized to immunoprecipitated Pyk2 or FAK. For purposes of comparison, the values for tyrosine phosphorylation obtained from individual vehicle controls were defined as 100%; data for PMA-treated cells are relative to these

values. Data represent mean $\pm$ S.D. of values from triplicate immunoprecipitations. Statistical significance (PMA vs. control) was analyzed by unpaired two-tailed t-test; the only significant value was for Pyk2 in WT2, $p < 0.01$. In Panel C, serum-starved V7 cells were incubated without additions (control), with 100 nM PMA, or with 100 nM EGF for 10 minutes. Whole-cell extracts were immunoblotted for phospho-FAK (Y397) and for actin as loading control. Results are representative of three separate experiments.

Figure 4. Expression of FAK and Pyk2 mRNA in clonal EL4 cell lines

Messenger RNA levels for Pyk2 and FAK were assessed in wild-type and variant EL4 cell lines by Northern blotting. "WT" refers to the non-clonal parental cell line; all other cell lines shown are clonal. Total RNA was resolved on a 1.2% agarose gel under formaldehyde denaturing conditions. RNA was transferred to nylon membranes and probed with ~0.6 kb radiolabeled fragments specific for either Pyk2 or FAK. In Panel A, the autoradiogram is shown. All blots were also probed for GAPDH (not shown), which was used to normalize for loading. In Panel B, the data were quantified by densitometry and normalized to GAPDH. For purposes of comparison, the normalized values for Pyk2 in WT cells and for FAK in V7 cells were defined as 100%; other data are expressed relative to these values.

Figure 5. Effects of PMA on levels of mRNA for FAK and Pyk2 in clonal EL4 cells
 WT2 cells were incubated with and without 100 nM PMA as indicated. Total RNA was probed for FAK, Pyk2, and GAPDH by Northern blotting. In Panel A, the autoradiogram of the blot is shown. In Panel B, results were quantified by densitometry as described for Figure 4.

Figure 6. Effects of PMA on levels of FAK and Pyk2 protein in clonal EL4 cell lines
WT2 and V7 cells were incubated with 100 nM PMA for the indicated times. In Panel A, whole-cell lysates were immunoblotted for FAK, Pyk2, and phospho-Erk. The lane marked “s” contains molecular size markers. In Panel B, results were quantified by densitometry and are expressed in arbitrary relative units (p-Erk in untreated V7 cells is defined as “1”).

Figure 7. Effects of FAK transfection on PMA response in clonal EL4 cell lines

In Panel A, WT2 cells were transfected with either pRc/CMV-FAK (“FAK”) or pcDNA3 (“Vector”), incubated for 24 hours, and then treated with or without 100 nM PMA for 15 minutes. Whole-cell lysates were prepared from 5×10^6 cells. NV cells were similarly treated, but without transfection. Immunoblots were performed for FAK, phospho-Erk, and total Erk. In Panel B, WT2 cells were co-transfected with plasmids pGreen LanternTM-1 and either pRc/CMV-FAK or pcDNA3. Cells were then subjected to fluorescence-activated cell sorting. Cells that were alive and expressed GFP were sorted, and were then treated with or without 100 nM PMA for 15 minutes. Whole-cell lysates isolated from 5×10^4 untransfected or sorted cells were immunoblotted using antibodies to FAK, phospho-Erk, and total Erk. Phospho-Erk was quantified by densitometry, with correction for background and normalization for total ERK expression. For both sorted and non-sorted cells, Erk activation was expressed relative to that seen in PMA-treated vector controls.

Figure 8. Effects of FAK siRNA on EL4 cell proliferation and migration

V7 EL4 cells were incubated with and without 10 nM or 50 nM FAK siRNA for 72 hrs, serum-starved, and then incubated as described. In *Panel A*, cells were incubated with or without 100 nM PMA or 100 nM EGF for 10 minutes. Immunoblots were performed for FAK and phospho-Erk. Actin was used as a loading control. All blots were from the same membrane, imaged in a single image. In *Panel B*, 2×10^4 cells were incubated with 10% fetal calf serum; live cells were counted after 48 hrs. Each value represents the mean $\pm$ S.D. of values from triplicate wells of cells. In *Panel C*, cells were allowed to migrate across a transwell insert with 10% serum as the attractant. Migrated cells were counted after 24 hrs. Each value represents mean $\pm$ S.D. of values from quadruplicate wells of cells. For panels B and C, statistical significance was analyzed by unpaired two-tailed t-test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

A **B**

Figure 9. Experimental metastasis studies using EL4 cell lines

In Panel A, mice were injected with either PBS (vehicle control) or EL4 V7 cells as described in methods. After 25 days, the mice were sacrificed and the excised livers were photographed. In Panel B, mice were injected with PBS (vehicle control) or the indicated EL4 cell lines. After 20 days, the mice were sacrificed and liver weight was quantified. Six or seven animals were used in each experimental group; error bars indicate S.E.M., and an asterisk indicates statistical significance ($p < 0.01$) by unpaired t-test as compared to the vehicle control.